JIMMA UNIVERSITY
SCHOOL OF GRADUATE STUDIES

CHARACTERIZATION OF FUNGAL PATHOGENS ASSOCIATED WITH LEAF AND FRUIT SPOT DISEASE OF CITRUS IN ETHIOPIA

Ph.D. Dissertation

By

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March, 2016

Jimma, Ethiopia

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A Dissertation Submitted to the School of Graduate Studies,

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By

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Jimma, Ethiopia

DEDICATION

I dedicate this dissertation manuscript to my lovely daughters Yeabsira and Tsion; to my beloved wife, Tigist Yeshitila, for understanding me and taking care of the life issues at home; to my holy father, the late Priest Armimo Misikir who passed away at the age of 133 during my study; to my parents, Dagnew Moges and Bosena Eskeziaw; to my sisters and brothers; to my father- and mother-in-law; to my brothers- and sisters-in-law; and to my special friends, Yitayal Abebe and Alemshet Lemma whose encouragement kept me going.

STATEMENT OF THE AUTHOR

First, I declare that this dissertation is my original work and that all sources of materials used for this dissertation have been duly acknowledged. This dissertation has been submitted in partial fulfillment of the requirements for Ph.D. degree at Jimma University and is deposited at the University library to make available to borrowers under rules of the library. I solemnly declare that this dissertation is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Place: Jimma University, Jimma, Ethiopia

Date of Submission: _____

BIOGRAPHICAL SKETCH

Asmare Dagnew Moges was born on February 4, 1975 in Durbette, West Gojjam, Ethiopia. He attended elementary and secondary schools education from 1981 to 1992 at Ayalew Mekonnen Elementary School in Durbette, and Merawi Senior Secondary School at Merawi, respectively. Asmare joined the then Alemaya University of Agriculture (now Haramaya University), Haramaya, Ethiopia in November 1992 and graduated with Bachelor of Science degree in Plant Sciences in July 1996.

Upon graduation, Asmare was recruited as Junior Researcher in the then Ethiopian Agricultural Research Organization, now Ethiopian Institute of Agricultural Research, at Melkassa Agricultural Research Center (MARC) in February 1997. After two years and nine months of service at MARC, he joined Jordan University of Science and Technology, Irbid, Jordan in October 2000 to pursue graduate study and earned Master of Science degree in Plant Production (Horticulture specialization) in December 2002. After graduation, he returned to Ethiopia and worked at MARC where he served at Assistant Researcher, Associate Researcher and Researcher positions for seven years. He was the coordinator of the National Fruit Research Program (10/2007 to 12/2008) and National Tropical Fruits Project (10/2007 to 10/2010) at a national level, and Tropical Fruits and Plant Tissue Culture Research Team (9/2003 to 1/2009) and Crops Research Team (1/2009 to 10/2010) at MARC.

He is married to Tigist Yeshitila; and has two daughters, Yeabsira and Tsion. Asmare joined Jimma University in October 2010 for postgraduate study. He studied at Dalhousie University in Canada for one academic term (Fall 2012/13) as student intern. He carried out his laboratory research works (9/2014 to 7/2015) at Biosciences eastern and central Africa, International Livestock Research Institute (BecA-ILRI), Nairobi, Kenya. He is getting his Ph.D. degree from the Department of Horticulture and Plant Sciences, Jimma University under the supervisions of Dr. Derbew Belew, Dr. Belayneh Admassu, and Dr. Mohammed Yesuf. After graduation, he will return to MARC to resume his position as researcher in the Horticulture and Agricultural Biotechnology Departments. His research works will include breeding, tissue culture and pathology on horticultural crops that will feature techniques he has learned during his Ph.D. program.

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ACRONYMS AND ABBRIVATIONS

ACT	actin
AFLP	Amplified Fragment Length Polymorphism
AMOVA	Analysis of Molecular Variance
BecA-ILRI	Biosciences eastern and central Africa - International Livestock Research Institute
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CSA	Central Statistical Agency of Ethiopia
CTAB	Cetyltrimethylammonium Bromide
DAP	Diammonium Phosphate
DNA	Deoxyribonucleic Acid
EDTA	Ethylene diaminetetraacetate acid
EPPO	European and Mediterranean Plant Protection Organization
FAO	Food and Agriculture Organization of the United Nations
F_{ST}	Wright's fixation index
GPS	Global Positioning System
ha	hectare
HWE	Hardy-Weinberg Equilibrium
ICRISAT	International Crops Research Institute for Semi-Arid Tropics
ITS	Internal Transcribed Spacers
LSU	Long Subunit
MARC	Melkassa Agricultural Research Center
Mbp	Mega base pairs
MCMC	Markov Chain Monte Carlo Algorithm
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimal Inhibitory Concentration
MISA	MIcroSAtellite Identification Tool

ML	Maximum Likelihood
MP	Maximum Parsimony
NJ	Neighbor-Joining
N_m	number of migrants
PCoA	Principal Coordinate Analysis
PCR	Polymorphic Chain Reaction
PDA	Potato Dextrose Agar
PIC	Polymorphic Information Content
RAPD	Random Amplified Polymorphic DNA
rpm	rotation per minute
SDS	Sodium Dodecyl Sulfate
SNNP	South Nations, Nationalities and People
SNP	Single Nucleotide Polymorphism
spp.	species
SSR	Simple Sequence Repeats
T_a	annealing temperature
T_m	melting temperature
USDA-ARS	United States Department of Agriculture-Agricultural Research Service

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CHARACTERIZATION OF FUNGAL PATHOGENS ASSOCIATED WITH LEAF AND FRUIT SPOT DISEASE OF CITRUS IN ETHIOPIA

ABSTRACT

Citrus is economically important fruit crop grown throughout Ethiopia at small and commercial scales. However, citrus production and productivity in many parts of Ethiopia is seriously threatened by leaf and fruit spot disease. This disease has been reported to cause high yield and quality losses on citrus. Nevertheless, limited information have been available on the etiology of the causal pathogen and epidemiology of the disease. Therefore, surveys and laboratory works were conducted to assess the distribution, incidence and severity of leaf and fruit spot disease of citrus, and to characterize the causal pathogen under laboratory conditions using cultural, morphological, pathogenicity and molecular features. Surveys were conducted on forty-nine citrus orchards in twenty-eight districts in the major citrus growing areas of Ethiopia. Disease prevalence, incidence and severity on citrus leaves and intact fruits were determined using random sampling techniques. During the surveys, the status of citrus production and management practices were also assessed using questionnaire and field observations. Infected citrus leaves and fruits samples collected from various orchards were surface sterilized and isolated on water agar and potato dextrose agar media. Pure cultures were prepared using single spore or hyphal tip for each fungal isolate. The daily colony growth was measured and the growth rate at daily basis was calculated, while colony color and density were assessed by visual observation. Each fungal isolate culture was also evaluated for conidial and mycelial morphology by the help of stereomicroscope. Pathogenicity test for each isolate was conducted using citrus detached leaves following the standard procedure. The identity and phylogenetic relationships of the fungal isolates were analyzed using three sets of universal primers that span internal transcribed spacers, portion of long subunit region of the nuclear ribosomal DNA and partial actin gene sequences. The distribution and frequency of microsatellite loci in C. gloeosporioides *genome were analyzed by generating pair-end reads from* C. gloeosporioides *isolate using the high-throughput Illumina sequencing platform, and reads were de novo assembled into a draft genome. Simple*

sequence repeat markers were developed from the draft assembled whole genome sequences of C. gloeosporioides. *Fifty simple sequence repeat markers across the genome were screened using thirteen geographically representative* C. gloeosporioides *isolates. The polymorphic simple sequence repeat markers were used to analyze the genetic diversity and population structure of* C. gloeosporioides *isolates from different geographical regions of Ethiopia. The survey results showed that leaf and fruit spot disease of citrus has widely distributed in the wet humid areas of the south, southwest, central and northwest parts of Ethiopia. However, the disease was not recorded in the low moisture areas of the southeast, the central rift valley and the eastern parts of the country. The disease prevalence in the districts surveyed ranged from zero to 100%. The damage of leaf and fruit spot disease varied with citrus species and locations. The overall mean incidences and severities of the disease were highest on sweet oranges followed by mandarins. Lemons and limes were the least affected citrus species. Disease incidences and severities were higher in Jimma town, Abeshege, Aleta Wendo, Kebena, Mana, Gomma, Ginbo and Debre Werk districts. Disease incidences in the different orchards ranged from zero to 81.7% on leaves and from zero to 100% on fruits. Disease severity also varied from zero to 75% on leaves and from zero to 100% on fruits. The questionnaire assessment and field observations indicated that various citrus species (sweet orange being the major species) and varieties with two to seventy years of age were produced by smallholder and commercial farmers. The assessment also showed that diseases and insect pests, poor agronomic and irrigation practices, and shortage of adapted high yielding varieties were the major citrus production constraints in the country. In the present surveys, it has been observed that commercial citrus orchards practice field sanitation, pruning, irrigation, and fertilizer and pesticide applications. However, most of the orchards of the smallholders were not well managed. The colony characters such as color, density and daily growth rate varied among fungal isolates. The colonies varied from white to dark gray in color. The majority of the isolates produced circular, wooly or cottony colonies with pale brown or grayish white color. The fungal isolates produced colonies with compact, medium or sparse density. The average daily colony growth rate ranged from 0.04 to 2.3 cm. Some isolates were very slow-growing, whereas most cultures had characteristic fast-growing compact aerial mycelia. Majority of the fungal isolates did sporulate, but the type of conidia*

they produced were not similar. These isolates produces hyaline, ovoid to oblong, slightly curved or dumbbell shaped conidia. Pathogenicity tests on detached leaf assays also revealed the association of most of the fungal isolates with foliar disease symptoms of the test citrus cultivars. Based on the multilocus analyses, more than 85% of the fungal isolates were belonged to Colletotrichum *species complex (81% were* C. gloeosporioides*). The phylogenetic analysis of the isolates based on multilocus sequences delineated them as* C. gloeosporioides *sensu lato (broad sense) and* C. boninense *spp. complexes. Each single locus sequence analysis also identified 163 isolates as* C. gloeosporioides *or its teleomorph* Glomerella cingulata*. These findings provide information on the causal pathogen of leaf and fruit spot disease of citrus in Ethiopia and suggest the need for in-depth studies to determine the role of* C. gloeosporioides *species complex in leaf and fruit spot disease epidemiology. The results also demonstrated that multilocus sequences are reliable methods for phylogenetic analysis of species within the genus* Colletotrichum*. These findings provide baseline information for further population genetic studies of the pathogen. The information will also be useful in developing effective disease management practices against* C. gloeosporioides*. A genome-wide microsatellite database of 5030 microsatellite motifs were identified in* C. gloeosporioides *genome. Of these, 94.6% were perfect motifs. Trinucleotide repeats were the most frequent; whereas penta- and hexanucleotide motifs were the least abundant. The number of motifs decreased as the number of the repeats increased. A/T repeats were more abundant than G/C repeats in the* C. gloeosporioides *genome. In penta- and hexanucleotide repeats, GC-rich motifs were predominant. Twenty-one simple sequence repeat markers showed polymorphism and demonstrated allele diversity among the thirteen test isolates of* C. gloeosporioides*. This small-scale population study could serve as a proof-of-concept showing that the genome sequencing approach was successfully applied for microsatellite discovery and development of simple sequence repeat markers. Twenty-three polymorphic simple sequence repeat markers produced a total of 118 alleles among the 163* C. gloeosporioides *isolates. The polymorphic information content values ranged from slightly to highly informative. The gene diversity among the loci ranged from 0.106 to 0.664. Analysis of molecular variance showed that 85% of the total variation was due to the differences of isolates within a population. The genetic differentiation in the total populations was low as*

evidenced by high level of gene flow estimate (Nm=4.8) between populations. Populations of Ethiopian C. gloeosporioides *from citrus were generally characterized by a low level of genetic diversity. Unweighted Neighbor-joining and population structure analyses grouped the isolates into three major clusters regardless of their geographic origins. The microsatellite markers developed and used in this study were useful to comprehend the genetic diversity and population structure of* C. gloeosporioides *isolates from main citrus growing regions of Ethiopia. Despite regional differences, the observed genetic diversity in all four populations was lower than expected suggesting inter-regional exchanges of planting materials and/or fruits, and dispersal of inoculum among the regions. Information generated in this study were useful in understanding the pathogen biology and provided basis for other studies on disease development, host-pathogen interaction, and developing disease management strategies for the control of leaf and fruit spot disease of citrus in Ethiopia. The SSR markers developed in this study could be used to characterize* C. gloeosporioides *isolates that infect other fruit crops. In conclusion, the disease should be managed at any cost. Frequent disease monitoring and precautions are essential. Care should be taken during transporting the planting materials and fruits from affected areas to locations where the disease is not recorded. Citrus growers should apply soil fertility management practices in their orchards, and practice general hygiene and sanitary measures. Application of relatively safe fungicides could reduce the disease damage. It is also necessary to investigate the reactions of the available citrus cultivars to the pathogen and select disease tolerant/resistant scions. In the long term, integrated disease management approaches including biocontrol need to be in place in the country.*

1. GENERAL INTRODUCTION

Citrus (*Citrus* species), which originated in Southeast Asia, is economically important fruit crop worldwide (Davies and Albrigo, 1994; Timmer *et al*., 2003; Manner *et al*., 2006). The major commercial species: oranges, tangerines, lemons, limes, and grapefruit belong to the genus *Citrus* of the family Rutaceae. To date, citrus is cultivated in more than 140 countries, mainly located between 35° south and north latitudes. However, the major commercial production areas are in the subtropics where the finest quality citrus fruits are produced (Manner *et al.*, 2006; Liu *et al.*, 2012).

Citrus is one of the major fruit crops in international trade (Liu *et al.*, 2012) with more than 130 million metric tons (FAO, 2014). According to FAO's report, Brazil, China, United States, Spain, Mexico and Italy are the leading citrus producing countries, representing about two-thirds of the global production. On the other hand, Spain, South Africa, Turkey, United States and Egypt are the largest exporters (FAO, 2014).

Citrus is also a major source of cash and nutrition in many developing countries (Timmer *et al*., 2003). In Africa, the citrus industry is relatively a recent development (Seif and Hillocks, 1993; Mohammed, 2002) where the lead producing countries at a commercial scale are Egypt, South Africa, Morocco, Algeria, and Tunisia (FAO, 2014). However, in tropical Africa, citrus has been grown largely by smallholder farmers for domestic consumption and export (Mohammed, 2002).

Although the exact time of citrus introduction to Ethiopia is not known, citrus production started seven decades ago by expatriates and some government officials (Mohammed, 2002; Seifu, 2003). Since then, its economic importance is on the rise. Oranges, mandarins, limes, lemons and grapefruits are the major commercial citrus species that are cultivated by both smallholder and commercial farmers in Ethiopia (Seifu, 2003; Mohammed, 2007). The current total acreage and annual production of citrus in Ethiopia are estimated at 7,040

hectares and 72,459 tons, respectively. The national average yields of citrus are also estimated 9.65 tons/ha for smallholders and 13.35 tons/ha for commercial scale (CSA, 2015a, b). Commercial farms are mainly located in the central rift valley and the eastern parts of Ethiopia contributing about 45.7% to the total production (CSA, 2015a); whereas small-scale production is scattered throughout the country contributing the rest of the production (CSA, 2015b). Large portion of citrus fruits produced are consumed locally as fresh fruit, juice and marmalade (Seifu, 2003). Some citrus fruits such as sweet orange and lime are exported to Djibouti, Europe and the Middle East (Joosten *et al.*, 2011).

Citrus production and productivity in Ethiopia is seriously threatened by various biotic diseases resulting in the declining of citrus groves (Mohammed, 2007; Mohammed *et al.*, 2009). Citrus producers reported a 50% incidence of various diseases in the major citrus orchards (Sisay, 2007). Virus and virus-like diseases such as citrus tristeza, greening and psorosis (Tesfaye and Habtu, 1985), occurrence of citrus canker on Mexican lime and sour orange trees (Eshetu and Sijam, 2007), and various fungal diseases such as foot rot and gummosis (*Phytophthora* spp.), anthracnose (*Colletotrichum gloeosporioides* [Penz.] Penz. & Sacc.), Phaeoramularia leaf and fruit spot (*Pseudocercospora angolensis* [T. Carvalho & O. Mendes] Crous & U. Braun), Alternaria leaf spot (*Alternaria citri* Ellis and Pierce), melanose (*Diaporthe* spp.), and fruit rots such as blue mold (*Penicillium italicum*) and green mold (*Penicillium digitatum*) (Tesfaye and Habtu, 1985; Eshetu, 1999; Mohammed, 2007; Sisay, 2007; Mohammed *et al.*, 2009) were reported in many citrus orchards. Some of these diseases have been reported responsible for high yield and quality losses on citrus in Ethiopia (Seifu, 2003; Mohammed, 2007; Sisay, 2007).

Leaf and fruit spot is among the major diseases causing serious damage in many citrus producing areas of Ethiopia (Eshetu, 1999; Yigzaw and Gelelbelu, 2002; Mohammed, 2007; Sisay, 2007). The symptoms of leaf and fruit spot disease of citrus were first observed in Aleta Wendo and Dale areas in the southern Ethiopia in 1988 (Yimenu, 1993). In 1990, similar disease symptoms were evident at Bebeka farm in the southwest Ethiopia (Eshetu, 1999). A similar disease was also reported in other parts of the country (Yigzaw and

Gelelbelu, 2002; Mohammed, 2007; Sisay, 2007). Leaf and fruit spot disease of citrus is a critical threat to citrus production in Ethiopia due to its impact on yield, exchange of materials and citrus trade (Mohammed, 2013). In some of the affected areas, farmers reported entire fruit loss and were compelled to abandon their citrus trees (Eshetu, 1999; Mohammed, 2007). In 1993, the causal pathogen of leaf and fruit spot was reported as *Phaeoramularia angolensis* based on cultural and morphological characteristics (Eshetu, 1999). However, several pathogens could be associated with the leaf and fruit spot disease of citrus (Seif and Kungu, 1990; Kuate, 1998). There is no comprehensive information on the current status of leaf and fruit spot disease of citrus in Ethiopia. Detailed information on the etiology of the causal pathogen and the epidemiology of the disease is also lacking (Mohammed, 2013). The population genetic diversity of the causal pathogen of this disease has not also been studied. These shortcomings have prompted the need for further in-depth studies to examine and understand the causal pathogen and to assess the disease distribution and the level of damage due to leaf and fruit spot of citrus in Ethiopia. Diagnosis and regular monitoring of the disease are required for devising sustainable disease management practices thereby to reduce the yield loss and improve the quality of citrus fruits (Mohammed, 2013).

Morphological and cultural features as well as host-preference criteria have been used for species identification and delimitation of pathogens (Silva *et al.*, 2012). However, morphological characters are not considered reliable because they are instable and usually vary with the environment and the growth stage (Atkins and Clark, 2004; Bhat *et al.*, 2010). These limitations led to the development of DNA based techniques that are reliable methods, and have been extensively employed in plant pathology without the confounding effects of the environment (Bridge *et al*., 2003; Schaad *et al.*, 2003; Choi *et al.*, 2012; Weir *et al.*, 2012; Aiello *et al.*, 2015). The majority of the studies which employed phylogenetic analysis for species identification have relied on the internal transcribed spacer (ITS) region of the nuclear ribosomal DNA (Peres *et al.*, 2002; Cai *et al.*, 2009). However, the resolution provided by the ITS barcode may not be adequate to discriminate among individuals of a species complex, and effectively differentiate physiological races (Weir *et al.*, 2012). The ITS sequence data available in public domains can cause considerable confusion as sequence data are often

entered and named incorrectly, and identical sequences could entered under different names (Crouch *et al*., 2009). To overcome these problems, the use of multilocus sequences has been recommended to analyze pathogen species (Farr *et al*., 2006; Crouch *et al*., 2009).

Genetic diversity refers to the genetic variation among individuals or groups of individuals in populations of the same species. In an organism, more diversity means more adaptation to the environment (Kesawat and Das, 2009). The polymorphic diversity could also provide significant information relating to the pathosystem (Benali *et al.*, 2011). The advent of molecular markers made possible to make direct inferences about genetic diversity of organisms at the DNA level (Agarwal *et al.*, 2008; Benali *et al*., 2011). Genetic diversity is analyzed by a specific method or a combination of methods (Kesawat and Das, 2009). In genetic diversity studies, the most frequently used DNA based markers are simple sequence repeats (SSR), also known as microsatellites. These markers are one of the powerful genetic markers that are widely exploited for the genetic study of plant pathogens (Benali *et al*., 2011). They have been used in population genetic diversity studies of various fungi (Berbegal *et al.*, 2011; Cai *et al.*, 2013; Marulanda *et al.*, 2014; Cabral *et al.*, 2016). These markers are readily inherited, co-dominant, ubiquitous and abundance, and they have extensive genome coverage (Tautz, 1989; Morgante *et al.*, 2002). Microsatellites are commonly used to assess genetic relationships between pathogen populations and individuals through the estimation of genetic distances and based on their allelic frequencies (Villareal *et al.*, 2002).

Therefore, the general objective of this study was to generate information on the current status of leaf and fruit spot disease of citrus in Ethiopia and characterize the causal pathogen of the disease to devise and recommend appropriate disease management strategies.

The specific objectives were to:

1. assess the distribution, incidence and severity of leaf and fruit spot disease of citrus in Ethiopia,
2. isolate and identify the causal pathogen of leaf and fruit spot disease of citrus using conventional and molecular methods,

3. construct the whole genome sequence, identify microsatellite loci and develop polymorphic SSR markers for *Colletotrichum gloeosporioides*, and
4. characterize the genetic diversity and population structure of *C. gloeosporioides* from citrus using the newly developed SSR markers.

REFERENCES

Agarwal, M., N. Shrivastava and H. Padh. 2008. Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Report*, 27:617-631.

Aiello, D., R. Carrieri, V. Guarnaccia, A. Vitale, E. Lahoz and G. Polizzi. 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology*, 163:168-177.

Atkins, S.D. and I.M. Clark. 2004. Fungal molecular diagnostics: a mini review. *Journal of Applied Genetics*, 45:3-15.

Benali, S., B. Mohamed, H.J. Eddine and C. Neema. 2011. Advances of molecular markers application in plant pathology research. *European Journal of Scientific Research*, 50:110-123.

Berbegal, M., C.D. Garzon, A. Ortega, J. Armengol, R.M. Jimenez-Diaz and M.M. Jimenez-Gasco. 2011. Development and application of new molecular markers for analysis of genetic diversity in *Verticillium dahliae* populations. *Plant Pathology*, 60:866-877.

Bhat, Z.A., W.S. Dhillon, R. Rashid, J.A. Bhat, W.A. Dar and M.Y. Ganaie. 2010. The role of molecular markers in improvement of fruit crops. *Notulae Scientia Biologicae*, 2:22-30.

Bridge, P.D., T. Singh and D.K. Arora. 2003. The application of molecular markers in the epidemiology of plant pathogenic fungi. pp. 93-113. In: D.K. Arora, P.D. Bridge and D. Bhatnagar (eds.), Fungal Biotechnology in Agricultural, Food, and Environmental Applications. Mycology Series, Vol. 21, Marcel Dekker Inc.

Cabral, P.G.C., E. Maciel-Zambolim, S.A.S. Oliveira, E.T. Caixeta and L. Zambolim. 2016. Genetic diversity and structure of *Hemileia vastatrix* populations on *Coffea* spp. *Plant Pathology*, 65(2):196-204.

Cai, L., K.D. Hyde, P.W.J. Taylor, B.S. Weir, J. Waller, M.M. Abang, J.Z. Zhang, Y.L. Yang, S. Phoulivong, Z.Y. Liu, H. Prihastuti, R.G. Shivas, E.H.C. McKenzie and P.R. Johnston. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Diversity*, 39:183-204.

Cai, G., C.W. Leadbetter, M.F. Muehlbauer, T.J. Molnar and B.I. Hillman. 2013. Genome-wide microsatellite identification in the fungus *Anisogramma anomala* using illumina sequencing and genome assembly. *PLOS ONE*, 8(11):e82408.

Choi, O., O. Choi, Y-S. Kwak, J. Kim and J-H. Kwon. 2012. Spot anthracnose disease caused by *Colletotrichum gloeosporioides* on tulip tree in Korea. *Mycobiology*, 40:82-84.

Crouch, J.A., B.B. Clarke and B.I. Hillman. 2009. What is the value of ITS sequence data in *Colletotrichum* systematics and species diagnosis? A case study using the falcate-spored graminicolous *Colletotrichum* group. *Mycologia*, 101:648-656.

CSA (Central Statistical Agency). 2015a. Large and medium scale commercial farms sample survey 2014/25, Vol. 8. Statistical Bulletin. CSA, Addis Ababa, Ethiopia.

CSA (Central Statistical Agency). 2015b. Agricultural sample survey 2014/2015: report on area and production of major crops for private peasant holding, Vol. 1. Statistical Bulletin 578. CSA, Addis Ababa, Ethiopia.

Davies, F.S. and L.G. Albrigo. 1994. Citrus. Wallingford, UK: CAB International. 254p.

Eshetu Derso. 1999. Occurrence, prevalence and control methods of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 54:225-232.

Eshetu Derso and K. Sijam. 2007. Citrus canker: a new disease of Mexican lime (*Citrus aurantifolia*) and sour orange (*C. aurantium*) in Ethiopia. *Fruits*, 62:89-98.

FAO (Food and Agricultural Organization of the United Nations). 2014. Citrus fruit: Fresh and processed - annual statistics. Online: http://faostat3.fao.org/download/Q/QC/E. Retrieved March 20, 2016.

Farr, D.F., M.C. Aime, A.Y. Rossman and M.E. Palm. 2006. Species of *Colletotrichum* on *Agavaceae*. *Mycological Research*, 110:1395-1408.

Joosten, F., D. Boselie, Bekele Wolde and Lemma Dessalegn. 2011. Exporting fruit and vegetables from Ethiopia: assessment of development potentials and investment options in the export-oriented fruit and vegetable sector. Ethiopia-Netherlands Horticulture Partnership Programme.

Kesawat, M.S. and B.K. Das. 2009. Molecular markers: it's application in crop improvement. *Journal of Crop Science and Biotechnology*, 12(4):169-181.

Kuate, J. 1998. Citrus leaf and fruit spot disease caused by *Phaeoramularia angolensis*. *Cahiers Agricultures*, 7:121-129.

Liu, Y., E. Heying and S.A. Tanumihardjo. 2012. History, global distribution, and nutritional importance of citrus fruits. *Comprehensive Reviews in Food Science and Food Safety*, 11:530-545.

Manner, H.I., R.S. Buker, V.E. Smith, D. Ward and C.R. Elevitch. 2006. *Citrus* (citrus) and *Fortunella* (kumquat). Species Profiles for Pacific Island Agroforestry. 35p. Online: www.traditionaltree.org. Retrieved May 10, 2011.

Marulanda, M.L., A.M. Lopez, L. Isaza and P. Lopez. 2014. Microsatellite isolation and characterization for *Colletotrichum* spp., causal agent of anthracnose in Andean blackberry. *Genetics and Molecular Research*, 13:7673-7685.

Mohammed Yesuf. 2002. *Phaeoramularia* leaf and fruit spot of citrus: a major threat to citrus production in tropical Africa, present status and future perspectives. pp. 18-25. In: J.M. Wesonga, T. Losenge, C.K. Ndungu, K. Ngamau, F.K. Ombwara, S.G. Agong, A. Fricke, B. Hau and H. Stutzel (eds.), Proceedings of the Horticulture Seminar on Sustainable Horticultural Production in the Tropics, 3-6 October 2001, Department of Horticulture, Jomo Kenyatta University of Agriculture and Technology, Kenya.

Mohammed Yesuf. 2007. Distribution and management of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 62:99-106.

Mohammed Yesuf, Wondirad Mandefro, Eshetu Ahmed, Girma Adugna, Dereje Tadesse, Temam Hussen and Meki Shehabu. 2009. Review of pathological research on fruit crops in Ethiopia. pp. 231-252. In: Abraham Tadesse (ed.), Increasing Crop Production through Improved Plant Protection, Vol. II. Plant Protection Society of Ethiopia (PPSE). EIAR, Addis Ababa, Ethiopia.

Mohammed Yesuf. 2013. Pseudocercospora leaf and fruit spot disease of citrus: achievements and challenges in the citrus industry: a review. *Agricultural Sciences*, 4:324-328.

Morgante, M., H. Hanafey and W. Powell. 2002. Microsatellites are preferentially associated with non-repetitive DNA in plant genome. *Nature Genetics*, 30:194-200.

Peres, N.A.R.. E.E. Kuramae, M.S.C. Dias and N.L. de Sousa. 2002. Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil. *Journal of Phytopathology*, 150:128-134.

Schaad, N.W., R.D. Frederick, J. Shaw, W.L. Schneider, R. Hickson, M.D. Petrillo and D.G. Luster. 2003. Advances in molecular based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annual Review of Phytopathology*, 41:305-324.

Seif, A.A and R.J. Hillocks. 1993. *Phaeoramularia* fruit and leaf spot of citrus with special reference to Kenya. *International Journal of Pest Management*, 39:44-50.

Seif, A.A. and J.N. Kungu. 1990. Association of *Colletotrichum* species with the fruit and leaf spot of citrus caused by *Phaeoramularia angolensis*. National Agricultural Research Laboratories, Annual Report of Plant Pathology Department for 1990.

Seifu Gebre-Mariam. 2003. Status of commercial fruit production in Ethiopia. Ethiopian Agricultural Research Organization, Addis Ababa, Ethiopia. 91p.

Silva, D.N., P. Talhinhas, V. Varzea, L. Cai, O.S. Paulo and D. Batista. 2012. Application of the Apn2/MAT locus to improve the systematics of the *Colletotrichum gloeosporioides* complex: an example from coffee (*Coffea* spp.) hosts. *Mycologia*, 104:396-409.

Sisay Bekele Mekbib. 2007. Identification of citrus (*Citrus sinensis*) postharvest pathogens from Ethiopia and their control. Ph.D. Dissertation, University of Pretoria, Pretoria, South Africa. 225p.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, 17:6463-6471.

Tesfaye Tedilla and Habtu Assefa. 1985. A review of research activities on fruit crop diseases in Ethiopia. pp. 263-276. In: Tsedeke Abate (ed.), Proceedings of the First Ethiopian Crop Protection Symposium, 4-7 February 1985, Addis Ababa, Ethiopia.

Timmer, L.W., S.M. Garnsey and P. Broadbent. 2003. Diseases of citrus. pp. 163-195. In: R.C. Ploetz (ed.), Diseases of Tropical Fruit Crops. CABI, Wallingford, UK.

Villareal, L.M.M.A., C. Lannou, C. de Vallavieille-Pope and C. Neema. 2002. Genetic variability in *Puccinia striiformis* f.sp. *tritici* populations sampled on a local scale during natural epidemics. *Applied and Environmental Microbiology*, 68:6138-6145.

Weir, B.S., P.R. Johnston and U. Damm. 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*, 73:115-180.

Yigzaw Dessalegn and Gelelbelu Girma. 2002. *Phaeoramularia angolensis*: a citrus disease in Northwest Ethiopia. *AgriTopia*, 17(1):12-13.

Yimenu J. 1993. Preliminary survey report on leaf spot disease of citrus in Sidamo. Coffee and Tea Authority, Addis Ababa, Ethiopia.

2. LITERATURE REVIEW

2.1. CITRUS DISEASES

Citrus is prone to many diseases affecting the leaf, fruit, stem and root (Manner *et al.*, 2006). Preharvest infections on citrus are mainly caused by fungal pathogens such as *Phytophthora* spp., *Colletotrichum gloeosporioides*, *C. acutatum*, *Alternaria citri* Ellis and Pierce, *Mycosphaerella citri*, *Elsinoe fawcetti*, *Botrytis cinerea* Pers ex Fr, *Diplodia natalensis* Pole-Evans, and *Phomopsis citri* Faw (Browning *et al.*, 1995; El-Ghaouth *et al.*, 2002; Naqvi, 2004; Manner *et al.*, 2006; Lima *et al.*, 2011). The recently described *Colletotrichum boninense* is also well established as anthracnose pathogen of a diverse range of host plants including citrus (Moriwaki *et al.*, 2003; Johnston *et al.*, 2005; Damm *et al.*, 2012). The common postharvest pathogenic fungi infecting citrus fruits worldwide are *Penicillium digitatum*, *P. italicum*, *Geotrichum candidum*, *Diaporthe citri*, *C. gloeosporioides* and *Trichoderma viride* (Naqvi, 2004; Manner *et al.*, 2006; Sisay, 2007).

In Ethiopia, the major fungal diseases that have been reported to affect citrus are foot rot and gummosis, anthracnose, Phaeoramularia leaf and fruit spot, Alternaria leaf spot, melanose, and fruit rots such as blue and green molds (Tesfaye and Habtu, 1985; Eshetu, 1999; Sisay, 2007; Mohammed *et al.*, 2009). Among fungal diseases, citrus leaf and fruit spot disease has been reported to cause significant yield and quality losses on citrus (Eshetu, 1999; Seifu, 2003; Mohammed, 2007; Sisay, 2007). Several fungal pathogens can be associated with leaf and fruit spot diseases on citrus (Seif and Kungu, 1990; Kohmoto *et al.*, 1993; Peres *et al.*, 2005; Dean *et al.*, 2012; Schultz *et al.*, 2013; Pegg *et al.*, 2014). The details of the major fungal pathogens that may be associated with leaf and fruit spot diseases on citrus are described as follows.

2.1.1. *Colletotrichum gloeosporioides*

2.1.1.1. Geographical distribution and economic importance

The generic name *Colletotrichum* was first introduced by Corda in 1831 for *Colletotrichum lineola*, a species found associated with a member of the Apiaceae in the Czech Republic (Cannon et al., 2012). The name *C. gloeosporioides* was first proposed in Penzig in 1882, based on *Vermicularia gloeosporioides*, the type specimen of which was collected from *Citrus* in Italy (Weir *et al.*, 2012). *Colletotrichum* spp. have mainly tropical and subtropical distribution, but there are some high-profile species affecting temperate crops (Cannon *et al.*, 2012). The genus *Colletotrichum* comprises important plant pathogens, causing significant crop losses in a wide range of plant species worldwide (Sutton, 1980; Hyde *et al.*, 2009; Da Silva and Michereff, 2013; Gautam, 2014). *Colletotrichum* was ranked as eighth economically important plant pathogenic fungi (Dean *et al*., 2012).

Members of the genus *Colletotrichum* cause devastating preharvest and postharvest diseases in many economically important fruits including citrus. The damage caused by *Colletotrichum* spp. extends to important staple food crops, vegetables, and ornamentals grown throughout the tropics and subtropics (Dean *et al.*, 2012). They are primarily responsible for anthracnose disease on leaves, stems, flowers and fruits of young and mature tissues of numerous economically important crops (Agrios, 2005; Cai *et al.*, 2009; Dean *et al.*, 2012). Other diseases associated with *Colletotrichum* are twig dieback, fruit and leaf spots, flower abortion, immature fruit drop, rotting of fruits, seedling blight and root rots (Agrios, 2005; Peres *et al.*, 2005; Da Silva and Michereff, 2013). They are also important postharvest pathogens because latent infections, which are initiated before harvest and become active after the fruit has been stored or appear on the market shelf (Prusky, 1996).

2.1.1.2. Symptoms and damage

Colletotrichum spp. cause anthracnose spots and blights of aerial plant parts and postharvest rots on a variety of hosts (Dean et *al.*, 2012). Typical symptoms of anthracnose disease appear as sunken, water-soaked dark brown spots or lesions, that are semi-circular or angular shaped, surrounded by yellow halo on leaves, and often coalesce to form larger lesions (Fig 2.1) (Agrios, 2005; Peres *et al.*, 2005; Cai *et al.*, 2009; Dean *et al.*, 2012). These spots enlarge, and lead to wilting, withering, and dying of infected plant tissues (Hiremath *et al.*, 1993). However, the symptoms greatly vary from host to host (Gautam, 2014). A fruit loss of up to 100% can occur as a result of *Colletotrichum* spp. (Prusky, 1996).

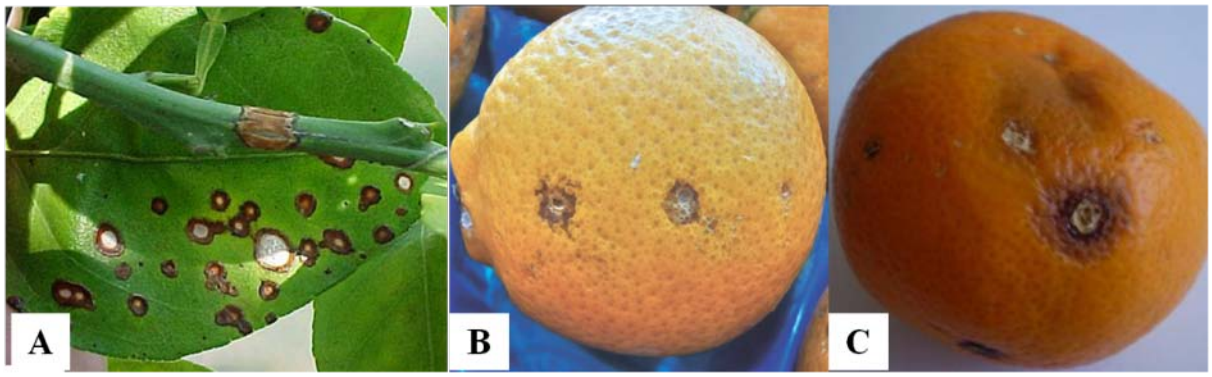

Fig 2.1. Symptoms of anthracnose spots caused by *Colletotrichum gloeosporioides* on citrus. Lesions on (A) leaf and twig, and (B, C) fruits. Adopted from the web (http://itp.lucidcentral.org/id/citrus/diseases/key/Diseases_of_Citrus/Media/Html/Anthracnose.htm), Zivkovic *et al*. (2012) and Aiello *et al*. (2015).

2.1.1.3. Taxonomy and biology

Colletotrichum spp. are widely distributed worldwide as plant pathogenic, saprobic, and endophytic lifestyles (Nicholson and Moraes, 1980; Sutton, 1992; Joshee *et al.*, 2009; Rojas *et al.*, 2010; Yuan *et al.*, 2011). Several pathogenic species of *Colletotrichum* can cause anthracnose diseases on tropical fruit trees including *Citrus* spp. (Peres *et al.*, 2005; Da Silva and Michereff, 2013; Aiello *et al.*, 2015). *Colletotrichum* is an asexual genus, classified

within the Fungi imperfecti. It belongs to the morphological classification of the phylum Ascomycota (Sutton, 1992; Cannon *et al.*, 2012; Dean *et al.*, 2012; Gautam, 2014). The fungus *Colletotrichum* comprises *Colletotrichum* as anamorph or asexual state while *Glomerella* as sexual or teleomorph state (Cannon *et al.*, 2012; Damm *et al.*, 2012). Phylogenetic analysis of the genus *Colletotrichum* reveals that it comprises nine major clades, and a number of small clusters and isolated species (Cannon *et al.*, 2012). However, the taxonomy and phylogeny of the genus *Colletotrichum* remains in a state of flux because many uncertainties exist with regard to the systematics of fungal pathogens from this genus (Sutton, 1992; Hyde *et al.*, 2009; Cai *et al.*, 2009; Dean *et al.*, 2012).

The fungus *C. gloeosporioides* is one of the frequently reported plant pathogens in the genus *Colletotrichum* (Gautam, 2014). Many researchers use the name *C. gloeosporioides* in both broad and strict senses. When used in a broad sense, it refers to the taxon as the *C. gloeosporioides* species complex. This species complex was recently investigated by Weir (2012). The multi-gene phylogeny analysis defined 22 species and one subspecies within the *C. gloeosporioides* complex. These include *C. aenigma*, *C. aeschynomenes*, *C. alatae*, *C. alienum*, *C. aotearoa*, *C. asianum*, *C. clidemiae*, *C. cordylinicola*, *C. fructicola*, *C. gloeosporioides*, *C. horii*, *C. kahawae* sub sp. *ciggaro*, *C. kahawae* sub sp. *kahawae*, *C. musae*, *C. nupharicola*, *C. psidii*, *C. queenslandicum*, *C. salsolae*, *C. siamense*, *C. theobromicola*, *C. ti*, *C. tropicale*, and *C. xanthorrhoeae* (Weir, 2012).

The fungus produces various specialized structures such as conidia, acervulli, setae and appressoria during infection process (Gautam, 2014). Conidia are formed in acervulli within the host tissue (Sutton, 1992; Cannon *et al.*, 2012; Dean *et al.*, 2012), and escape through an opening at the top of acervulli, spread over distances and infect other health plant tissues. Acervulli are the asexual fruiting bodies produced during the infection process in the tissue of infected host as small, flask shaped structure with a small cushion at the bottom, of which short crowded conidiophores are formed and can be observed on the surface of diseased plants. Setae are also emerged from acervulli. The appressoria allow the fungus to penetrate the host cuticle and epidermal cell wall directly. The whole infection process, including the

formation of the various specialized structures and infection results into tissue necrosis (Gautam, 2014).

C. gloeosporioides produces circular, wooly or cottony colonies on culture media with characteristic pale brown or grayish white color (Hiremath *et al.*, 1993). Pathogen isolates of *C. gloeosporioides* grown on potato dextrose agar medium formed gray to dark gray colonies (Zivkovic *et al.*, 2012). The mycelium of growing culture is hyaline, septet and branched. The conidiomata are acervular, separate, and composed of hyaline to dark brown septet hyphae. In culture, the fungus produces sclerotia, which are dark brown, occasionally setose. The setae are usually long, brown and septet. The appressoria are smooth, simple, clavate or irregular and varied from light to dark brown. The conidiogenous cells are enteroblastic, phialidic and hyaline. The dimension of conidiogenous cells tend to vary. The fungus produces hyaline, one-celled, ovoid to oblong, slightly curved or dumbbell shaped conidia, average of 10 to 15 µm in length and 5 to 7 µm in width (Fig 2.2). There is a great variation in size and shape of conidia depending upon the host from which the pathogen is isolated and its area of origin. Normally the conidia may be oblong with obtuse ends (Freeman *et al.*, 1998; Zivkovic *et al.*, 2012; Gautam, 2014). The waxy acervulli produced in infected tissues are subepidermal, typically with setae, and simple, short and erect conidiophores (Gautam, 2014).

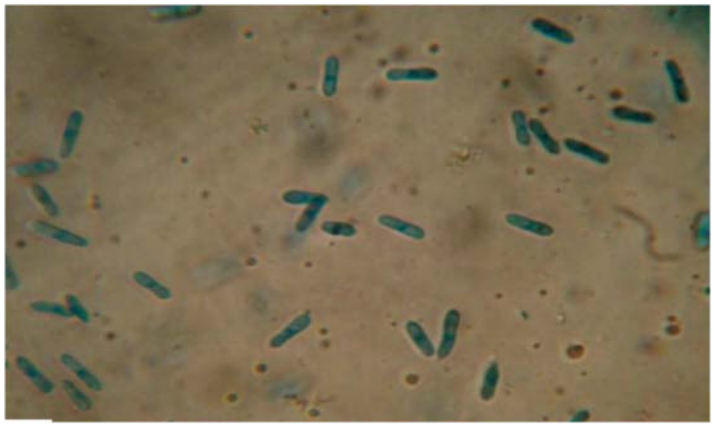

Fig 2.2. Conidia of *C. gloeosporioides* stained with cotton blue.
Adopted from Gautam (2014).

It was demonstrated that growth parameters such as temperature, moisture and pH could affect the growth and sporulation of *Colletotrichum* on culture media (Agostini *et al.*, 1992; Hubballi *et al.*, 2011). *Colletotrichum gloeosporioides* show maximum growth at pH range of 6.5 to 7 and temperature of 25 to 30ºC whereas, exposure of the fungus to alternate cycles of 12 h light and 12 h darkness resulted in the maximum mycelial growth in comparison to 24 h exposure to continuous light and 24 h exposure to continuous dark (Hubballi *et al.*, 2011).

2.1.1.4. Epidemiology and disease cycle

The pathogen *Colletotrichum* is cosmopolitan in distribution. Conidia are the primary inoculum, and spread over short distances by rain-splash or overhead irrigation and infect other healthy plant tissues (Gautam, 2014). Conidia are disseminated long distances by wind (Nicholson and Moraes, 1980; Farr *et al.*, 2006). Long-distance transmission of the fungus can occur via the movement of infected plant materials such as fruits (Freeman *et al.*, 2013).

Colletotrichum requires warm and humid conditions to infect different plant hosts (Farr *et al.*, 2006). Identifying and targeting the source of inoculum is necessary as a management strategy (Abang *et al.*, 2003). The fungus can survive as quiescent inside host tissues during unfavorable conditions, or conidia remain dormant for long periods in dead plant materials or in the soil (Freeman *et al.*, 2013). Therefore, the most important sources of *Colletotrichum* inoculum are infested plant debris, infected plant tissues and alternate hosts, and infected soil (Nicholson and Moraes, 1980; Farr *et al.*, 2006; Gautam, 2014).

Colletotrichum spp. initially establish infection through a brief biotrophic phase, associated with large intracellular primary hyphae, although some species are described as subcuticular. The fungus later switches to a destructive, necrotrophic phase, associated with narrower secondary hyphae which ramify throughout the host tissue (Dean *et al.*, 2012). Infection occurs via an appressorium that develops from the germinating spore on the plant surface, followed by turgor-driven penetration of the cuticle (Deising *et al*., 2000) and in some cases of epidermal cells by infective hyphae (Bailey *et al.*, 1992).

2.1.1.5. Host range and susceptibility

Pathogenic *Colletotrichum* spp. cause diseases in a wide range of hosts including cereals and grasses, legumes, fruits, vegetables, other perennial crops and trees (Sharma *et al.*, 2005; MacKenzie *et al.*, 2007; Dean *et al.*, 2012). Most crops are susceptible to one or multiple species of *Colletotrichum* (Freeman *et al.*, 1998; Dean *et al.*, 2012; Weir *et al.*, 2012).

C. gloeosporioides causes preharvest diseases and postharvest decay on many tropical, subtropical and temperate fruits such as citrus (Agostini *et al.*, 1992; Zivkovic *et al.*, 2012; Aiello *et al.*, 2015), avocado and almond (Freeman *et al.*, 1996), mango (Afanador-Kafuri *et al.*, 2003; Adhikary *et al.*, 2013), apple (Johnston *et al.*, 2005), olive (Schena *et al.*, 2014), strawberry (Smith and Black, 1990; Freeman *et al.*, 2013), cranberry (Doyle *et al.*, 2013), and soursop (Alvarez *et al.*, 2014). The fungus has been commonly associated with Key Lime anthracnose and postharvest diseases on citrus (Weir *et al.*, 2012). It has been reported as a causal agent of post-bloom fruit drop on sweet orange in Brazil (Lima *et al.*, 2011). The ability of *C. gloeosporioides* to cause anthracnose on citrus fruits has been demonstrated in China (Huang *et al.*, 2013). In the tropical Asia, *C. siamense* has been identified as a pathogenic species causing anthracnose disease on a wide range of tropical fruits (Udayanga *et al.*, 2013). Recently, *C. gloeosporioides* has been reported to cause severe lesions on fruits of sweet orange at preharvest stage in Italy (Aiello *et al.*, 2015). The virulence and pathogenicity of *C. asianum*, and *C. fructicola* have also been demonstrated on various fruit species in Brazil (Lima *et al.*, 2015).

2.1.1.6. Disease management

Anthracnose caused by *Colletotrichum* spp. is a serious problem which results in considerable losses in various fruits including citrus. Some of the management practices used to control anthracnose include sorting of infected plant parts to avoid further dispersal of the inoculum, avoiding mechanical injury, and the use of some registered fungicides (Gautam, 2014).

Due to the toxic and harmful effects of chemical fungicides, the use of biopesticides has been preferred to control anthracnose (Gautam, 2014). Anthracnose management on the basis of radiation induced biochemical mutation in *C. gloeosporioides* and the use of chemicals were suggested (Naqvi, 2004; Patel, 2009). Phyto-extracts as biopesticides to control anthracnose was practiced (Rawal, 1998; Singh *et al.*, 2009). Different plant species were suggested for their antifungal activity against anthracnose pathogen, *C. gloeosporioides* (Deshmukh *et al.*, 2010; Gautam *et al.*, 2013). However, the effectiveness of phyto-extracts to inhibit the *C. gloeosporioides* growth was observed to vary with plant species (Kuberan *et al.*, 2012).

Several essential oils from clove, cedar wood, lemon grass, peppermint, eucalyptus, citronella, castor oil and neem oils were tested for their inhibitory effect on conidia germination, growth of germ tube and mycelial growth of *C. gloeosporioides* isolates (Chauhan and Joshi, 1990; Padman and Janardhana, 2012). Secondary metabolites of endophytic fungi such as *Aspergillus flavus* and *Aspergillus niger* against *C. gloeosporioides* were reported in India (Gautam *et al.*, 2013; Ramesha *et al.*, 2013). The antifungal potential of *Trichoderma viride, Beauveria bassiana* and plant growth promoting rhizobacteria (Sundar *et al.*, 1995; Babu *et al.*, 2008; Ghosh and Chakraborty, 2012) and some other microorganisms along with different phyto-extracts (Mathur *et al.*, 1997; Jeyalakshmi and Seetharaman, 1998) against *C. gloeosporioides* was evaluated to control anthracnose disease. The use of botanicals from a large number of plant species have also been suggested to control anthracnose disease (Rampersad, 2011; Deshmukh *et al.*, 2012; Kuberan *et al.*, 2012; Saju *et al.*, 2012)

2.1.2. *Pseudocercospora angolensis*

2.1.2.1. Geographical distribution and economic importance

Phaeoramularia leaf and fruit spot disease of citrus caused by *Pseudocercospora angolensis* was first reported in Angola and Mozambique in 1952 (Seif and Hillocks, 1993; Kuate, 1998; Timmer *et al.*, 2003a). The disease has spread in 21 countries in the tropical Africa (Mohammed, 2013). It has been reported in the Comoros Island and Yemen (Kuate, 1998;

Bella-Manga *et al*., 1999). This disease is a potential threat to the major citrus producing countries in the subtropics (Seif and Hillocks 1993; Dewdney and Timmer, 2009). The fungus is a quarantine pest for Europe and the Mediterranean region (EPPO, 2013). *Phaeoramularia* leaf and fruit spot disease of citrus is a great concern for citrus producing areas in warm humid regions like Florida (Chung and Timmer, 2009; Chalkley, 2012). Its presence in Yemen and the possible spread to Asia is also another potential threat (Kuate, 1999).

2.1.2.2. Symptoms and damage

Phaeoramularia leaf and fruit spot disease causes numerous spots and lesions of varying sizes on leaves, fruits and young twigs of citrus (Fig 2.3) (Seif and Hillocks, 1993; Kuate, 1998; Pretorius, 2005; Mohammed, 2013). Lesions on leaves are initially circular, mostly solitary and with greenish-yellow patches. The lesions are often coalesced, with a light-brown or grayish centre during the dry season, and become gray to black when the fungus sporulates at the onset of rain. Lesions are usually surrounded by dark margins and yellow chlorotic halos. Sometimes, the center of the lesion may detach and cause a shot-hole (Seif and Hillocks, 1993; Kuate, 1998; Timmer *et al*., 2003a; Chung and Timmer, 2009). Young lesions on leaves appear similar to those of citrus bacterial canker, but they differ in being flat or shrunken (Seif and Hillocks, 1993). Individual leaf lesion has similarity to those induced by *Alternaria*, but lesions are more numerous and tend to coalescence (Chung and Timmer, 2009).

Fig 2.3. *Pseudocercospora angolensis* lesions on leaf and fruit of citrus.
Adopted from Pretorius (2005).

On fruits, lesions are circular to irregular, discrete or coalescent. On young fruits, infection often creates a type of hyperplasia producing raised tumor-like growths surrounded by a yellow halo, and a mass of gray fungal sporulation can be observed (Seif and Hillocks, 1993; Chung and Timmer, 2009; Chalkley, 2012). At the early stage, brown necrotic lesions may be formed which are usually circular, slightly sunken at the center, with a surrounding ring of raised epicarp, giving the fruit a blistered appearance. Lesions on mature fruits are normally flat but sometimes have a slightly sunken brown centre, turn dark brown to blackish brown in color, their texture becomes rough, and the whole fruit hardens (Emechebe, 1981; Kuate, 1998; Chung and Timmer, 2009; Chalkley, 2012).

Lesions at the stems are not frequent, but heavy attacks on leaves mostly spread to small and young branches as an extension of lesions on the petiole and cause general necrosis. Occurrence of several such dark-brown lesions at the stem tip may coalesce, causing dieback or resulting in the formation of corky and cracked intermodal lesions (Emechebe, 1981; Seif and Hillocks, 1993; Kuate, 1998, 1999).

The most devastating effect of *Phaeoramularia* leaf and fruit spot on various citrus species is substantial premature abscission of leaves and fruits. Fruits may become blemish and remain on the tree. Infected fruits produce poor quality juice, or become very hard, juiceless and unattractive which are not suitable for market (Seif and Hillocks, 1993; Kuate, 1998; Timmer *et al*., 2003a; Chung and Timmer, 2009). The loss of leaves and desiccation of shoots can have a significant debilitating effect on the tree, which affects subsequent fruit yield (Kuate, 1999). The yield losses due to *Phaeoramularia* leaf and fruit spot can reach 50 to 100%, especially in higher elevations where the disease intensity are very high (Seif and Hillocks, 1993; Kuate, 1999; Kuate *et al.*, 2003). In Ethiopia, complete crop losses were observed and many farmers abandoned their citrus trees (Eshetu, 1999; Mohammed, 2007). The disease also affects the yield and quality of essential oils extracted from the fruit (Kuate *et al.*, 2003).

2.1.2.3. Taxonomy and biology

The causal organism of *Phaeoramularia* leaf and fruit spot disease was first identified in 1953 by De Carvalho and Mendes as *Cercospora angolensis* (Kuate, 1998; Chalkley, 2012). Subsequently, it was reported as *Phaeoisariopsis* species in Nigeria (Emechebe, 1981). The fungus was later renamed as *Phaeoramularia angolensis* (Kirk, 1986) based on its pale-brown solitary or catenulate conidia (Kuate, 1998) and its conspicuous and slightly pigmented scars (Pretorius *et al*., 2003). However, Braun (1999) assigned the fungus to *Pseudophaeoramularia*, due to its unthickened or only slightly thickened but somewhat darkened-refractive scars, and the conidiogenous loci do not fit with the former genus. Crous and Braun (in Pretorius *et al*., 2003) carried out morphological and molecular analyses. The conidiophore morphology and the molecular sequence analysis showed that the fungus was not distinct from that of the genus *Pseudocercospora*. Therefore, the fungus has been renamed as *Pseudocercospora angolensis*. It belongs to the family Mycosphaerellaceae, of the class Dothideomycetes, of the phylum Ascomycota in the Fungi kingdom under the domain Eukaryota (Chalkley, 2012).

Pathotype specification of *P. angolensis* has not been well understood. It is only known from its anamorph, and no teleomorph (sexual stage) has been reported (Kuate, 1998; Chung and Timmer, 2009). The fungus can be isolated and cultured from lesions of infected leaves or fruits. It can grow well and sporulate properly on various artificial culture media (Seif and Hillocks, 1993; Pretorius *et al*., 2003; Chung and Timmer, 2009). The colony surface is grayish in appearance, often velvety and raised at the center, forming a gnarled mat. The color of the underside of the colony is dark green. The colony diameter of 14-day-old cultures on different culture media ranged from 3.5-5.0 mm (Seif and Hillocks, 1993).

The fungus can be recognized by fasciculate or tufts of light chestnut-colored or pale-brown to brown, solitary or unbranched, smooth, multiseptate conidiophores, 27 to 240 μm high by 3 to 7 μm wide, that usually arise from a large dark stroma, 30 to 60 μm diameter. Conidiophores that bear conidia emerge through stomata on the lower leaf surface.

Conidiogenous cells are integrated, terminal, sympodial, slightly geniculate and cicatrized; scars are conspicuous but unthickened or minutely thickened and only slightly pigmented. Conidia are acrogenous, solitary or catenulate, borne in simple or branched chain of 2 to 4, cylindrical to narrowly obclavate, rounded at the apex, truncate at the base, straight or slightly flexuous to more or less curved, smooth, hyaline to very pale-brown, 1 to 6 (usually 3 to 4) septet, 23 to 87 µm long by 3 to 7 µm wide (Fig 2.4). The basal and apical scars are slightly thickened and pigmented (Seif and Hillocks, 1993; Pretorius *et al*., 2003; Timmer *et al.*, 2003a; Chalkley, 2012).

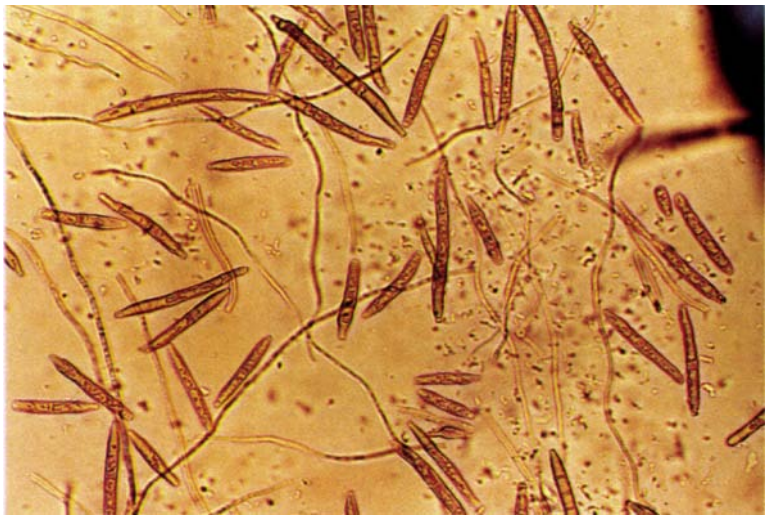

Fig 2.4. Conidia of *Pseudocercospora angolensis*. Adopted from Kuate (1998).

2.1.2.4. Epidemiology and disease cycle

Very limited studies on field epidemiology of *Phaeoramularia* leaf and fruit spot disease have been reported, but detailed information on the inoculum sources and disease cycle are lacking (Emechebe, 1981; Seif and Hillocks, 1993; Kuate, 1998). The disease is mainly restricted to the warm and humid tropics in Africa (Seif and Hillocks, 1993). Elevation appears to play an important role in the epidemiology of citrus leaf and fruit spot disease (Chalkley, 2012). The disease causes extensive damage and the fungus prefers regions with higher elevations

(Chung and Timmer, 2009). In Cameroon, the fungus was most common and the disease was severe at elevations above 200 meters above sea level (m.a.s.l) (Kuate, 1998; Ndo *et al*., 2010). In Guinea, the disease was serious on citrus groves located at above 400 m.a.s.l altitude while the lowland areas appeared to be disease free (Diallo, 2001). In Kenya, the disease was serious at altitudes above 600 m.a.s.l (Seif and Hillocks, 1993). Citrus species, soil and vegetation types were also found important factors influencing disease development and severity (Bella-Manga *et al.*, 1999; Mohammed, 2007; Ndo *et al*., 2010). Disease severity was higher on trees of grapefruit situated on sandy soils in semi-forested land in Cameroon (Ndo *et al*., 2010). In Ethiopia, the severity of *P. angolensis* varied among citrus species; and citrus groves in areas with high moisture and humidity were severely affected (Mohammed, 2007). Fruits can be attacked throughout their development stage, whereas leaves are less affected as they get mature (Seif and Hillocks, 1999; Chalkley, 2012) and no longer receptive to the fungus after 5 to 8 weeks (Kuate, 1998).

Although the survival mechanisms of *P. angolensis* are not well known (Kuate, 1998), the fungus is able to survive over seasons (Eshetu, 1999; Timmer *et al*., 2003a; Mohammed, 2007), probably in dormant lesions on infected material until the onset of conditions conducive to sporulation (Dempsey *et al*., 2002; Chalkley, 2012). However, under natural conditions the spread and infection processes of the fungus are similar to other citrus fungal pathogens whose asexual spores are the primary sources of inoculum for new infection (Kuate, 1998; Seif and Hillocks, 1997; Chung and Timmer, 2009). The old lesions on infected plant tissues appear to be the source of inoculum when conditions favor infection, and start a new disease cycle. Since leaf lesions produce more conidia than those on the fruit, infected leaves may be the main source of infections in infected areas; while fruit lesions serve as subsidiary inoculum source (Seif and Hillocks, 1993; Kuate, 1998; Chalkley, 2012). Neglected groves can also be a large source of spores (Dewdney and Timmer, 2009).

The fungus requires moisture for infection and the production of air-borne conidia (Chalkley, 2012). Disease infection is favored by wind-blown rain or prolonged wet weather conditions followed by dry spells and with moderately cool temperatures, 22 to 26°C, that stimulate the

production of new susceptible flush (Seif and Hillocks, 1993; Timmer *et al*., 2003a; Chung and Timmer, 2009; Dewdney and Timmer, 2009). The rainy seasons are more conducive to disease development, but disease incidence varies with the amount of rainfall (Kuate, 1998; Mohammed, 2007; Chalkley, 2012). At the onset of a rainy season, new disease-free leaves are emerged, but various non-sporulating lesions may be present on older leaves (Seif and Hillocks, 1993). Lesions produced in the previous season begin to sporulate within 3 to 5 weeks after the beginning of the rainy season. These spores infect the new leaves and symptoms appear 2 to 3 weeks later (Seif and Hillocks, 1993; Dewdney and Timmer, 2009; Chalkley, 2012). In Ethiopia, more lesions were counted during the wet and humid seasons; and the number of lesions on both leaves and fruits was higher around the lower tree canopy than in the middle and upper canopies of the trees which could be due to high moisture and humidity around the lower canopy (Mohammed, 2007).

Wind-borne conidia are the apparent means of natural long-distance dispersal of the fungus. The local dispersal of the fungus such as spread within a grove, and vertical spread on new leaves or fruits within a tree is primarily by raindrops laden with spores and/or rain-splash (Seif and Hillocks, 1993; Kuate, 1998; Timmer *et al.*, 2003a; Chung and Timmer 2009; Dewdney and Timmer, 2009). Inadvertent transport of infected fruits and propagating materials from infected areas can play some role in the spread of the fungus (Seif and Hillocks, 1993; Kuate, 1998; Ndo *et al.*, 2010). Insect transmission has not been reported, but the possibility of insect vectors cannot be discounted. The possibility of wild hosts need also be examined (Kuate, 1998; Chalkley, 2012).

2.1.2.5. Host range and susceptibility

P. angolensis was first reported on sweet orange (*Citrus sinensis* [L.] Osbeck). However, the fungus has been observed attacking all the major *Citrus* species including grapefruit (*C. paradisi* Macf.), mandarin (*C. reticulate* Blanco), lemon (*C. limon* [L.] Burm. f.), small-fruit acid lime (*C. aurantifolia* [Christm.] Swing.), pummelo (*C. grandis* L.) and sour orange (*C. aurantium* L.) (Seif and Hillocks, 1993; Kuate, 1998; Bella-Manga *et al*., 1999; Chung and

Timmer, 2009). Virtually all species of cultivated *Citrus* appear to be susceptible, though small-fruited acid lime, lemon, Tahiti lime (*C. latifolia* Tan.) and Satsuma (*C. unshiu* Marc.) are often reported to be relatively resistant (Kuate, 1998; Chalkley, 2012). Of the other members of the Rutaceae, *Citropsis tanakae* (Kuate, 1998) and *Fortunella japonica* (round kumquat) (Chalkley, 2012) are known to be infected. The susceptibility of the many wild *Citrus* species in Asia remains unknown (USDA-ARS, 2009). Any alternate host has not yet been reported (Chung and Timmer, 2009; Mohammed, 2013).

The reaction of citrus to *P. angolensis* infection has been shown to vary among and within the species (Chung and Timmer, 2009; Ndo *et al*., 2010). Grapefruit, many mandarins and oranges were highly susceptible, while some mandarins, lemon, Tahiti lime and the Satsuma group were less susceptible (Kuate, 1998). In Cameroon, grapefruits and some tangerines were observed very susceptible while lemons and pummelo were less susceptible to the fungus (Bella-Manga *et al*., 1999). Another study in Cameroon also indicated that disease severity increased in ascending order for pummelo, tangerine, lemon, orange and grapefruit (Ndo *et al*., 2010). In Kenya, susceptibility varied among the commonly grown *Citrus* spp. where grapefruit manifested the most disease followed by sweet oranges, tangelo, tangerines, lemons and limes in descending order (Seif and Hillocks, 1999). A study conducted in Ethiopia demonstrated that grapefruit was the most susceptible followed by sweet oranges, mandarins, lemon and lime (Mohammed, 2007).

Marked differences can be observed between cultivars within a species in their susceptibility to *P. angolensis* (Bella-Manga *et al*., 1999; Seif and Hillocks, 1999). Among lemon cultivars tested, Meyer was found the most susceptible to the fungus (Kuate, 1998). In Ethiopia, Jaffa and Campbell Valencia cultivars were found moderately resistant while Washington Naval, Pineapple and Hamlin were moderately susceptible to *P. angolensis* (Kassahun *et al*., 2006). Susceptibility may also vary among plant parts (Kuate, 1998). Fruits and leaves are much more susceptible than stems (Kuate, 1999). In Cameroon, citrus fruits often suffered more damage than leaves (Bella-Manga *et al*., 1999). In Kenya, disease incidence was found generally higher on fruits than foliage; but young leaves and fruits appeared to be more

susceptible than older leaves (Seif and Hillocks, 1999). In Ethiopia, fruits were more damaged than leaves, though the severity varied from area to area (Eshetu, 1999). In another study in Ethiopia, severity was more intense on leaves than on young fruits at the early fruiting stage; but severity of the disease increased on fruits at the later crop stages (Mohammed, 2007).

2.1.2.6. Disease management

Efforts have been made to manage the *Phaeoramularia* leaf and fruit spot disease in different tropical African countries. Yet, complete disease control was not made possible in any of the countries affected by *P. angolensis* (Mohammed, 2013). Among the recommended disease management methods, quarantine measures, cultural and sanitary practices, plant extracts, host resistance, and chemical control have been reported from different countries.

Quarantine measures such as prevention of the transport of infected citrus planting materials and fruits from contaminated areas are crucial to inhibit the spread of the fungus in and from citrus-growing countries (Seif and Hillocks, 1993; Kuate, 1999; Ndo *et al.*, 2010; Chalkley, 2012). Although the major citrus production areas in Africa occur outside the range of *P. angolensis* distribution (Seif and Hillocks, 1993) and the relative aridity of the Mediterranean climate may make it unsuitable for wind-disseminated pathogens like *P. angolensis*, the periods of interrupted leaf wetness provided by dew may be sufficient for infection (Vicent and Garcia-Jimenez, 2008). In the United States, it is illegal to bring citrus propagation materials into areas like Florida without the approval of the State Department of Agriculture and Consumer Services, the Division of Plant Industry (Chung and Timmer, 2009).

Some cultural and sanitary practices have been recommended to manage *Phaeoramularia* leaf and fruit spot disease (Chalkley, 2012; Mohammed, 2013). In Kenya, the major cultural practices suggested include collection and destruction of affected and fallen leaves and fruits to reduce the inoculum pressure in the field; planting of windbreaks around orchards to minimize the impact of wind which is the primary dispersal agent for spores; judicious pruning of affected shoots to allow light penetration into and free aeration within the tree

canopy to make the environment less conducive for disease development; and discouraging inter-planting in affected orchards with mature trees, fostering a microclimate of relatively cool temperatures and high relative humidity to prevent disease development (Seif and Kungu, 1989). In Ethiopia, sanitation measures such as removal of infected fruits and leaves, and pruning operations to eliminate dead branches and twigs have been used to reduce inoculum density. Improvement of the poor nutritional status of citrus groves of most smallholders was also suggested (Eshetu, 1999; Mohammed, 2007). In Zimbabwe, eradication of infected trees and plant parts, and abandoning of neglected orchards have been recommended to reduce the inoculum source (Pretorius, 2005).

The anti-pathogenic properties of plant extracts have been known for a long time. Recent attention has focused on the possibility of using the natural oils from plants in place of synthetic chemicals, which should be relatively cheap, available and safer in the environment. Oils extracted from fruits of more resistant species of *Citrus*, like Tahiti lime and lemon, strongly reduced the growth of an isolate of *P. angolensis in vitro* better than the extracts of susceptible species such as grapefruit and sweet orange. A total inhibition occurred at 2500 parts per million (ppm) and 4000 ppm for lime and lemon respectively (Jazet Dongmo *et al*., 2002). Among the 22 varieties of cultivated *Citrus* in Cameroon, oils extracted from tolerant varieties were the most effective in reducing radial growth of *P. angolensis* irrespective of the dose. Oils extracted from highly susceptible varieties significantly reduced sporulation of the fungus at 1000 ppm while at the higher doses of about 2000 to 2500 ppm, oils from tolerant varieties could completely inhibit sporulation. The marked dose effect in reducing sporulation suggests that there may be different compounds acting with changing dose (Kuate *et al.*, 2006). In Cameron, oil extracted from Tahiti lime leaf with Minimal Inhibitory Concentration (MIC), the lowest concentration of essential oil in which no growth occurred, of 1600 ppm showed both fungistatic and fungitoxic effects. At this dose, the growth of *P. angolensis* was totally inhibited (Jazet Dongmo *et al*., 2008a). Oils extracted from three varieties of lime in Cameroon with MIC of 1400 to 1500 ppm inhibited the radial growth of *P. angolensis*. The antifungal activity of oils extracted from lime was suggested to be due to their high content of neral and geranial (Jazet Dongmo *et al*., 2009a). Other than the *Citrus* group, oil extracts from

the leaves of two *Eucalyptus* species had MIC of 6000 and 6500 ppm against *P. angolensis* on agar medium, and completely inhibited mycelium growth (Jazet Dongmo *et al*., 2008b). Essential oils of the bottlebrush plants, *Callistemon citrinus* and *Callistemon rigidus*, showed similar activity (Jazet Dongmo *et al*., 2009b). Essential oil extracts from the grass *Cymbopogon citratus* with MIC of 500 ppm were the most effective in inhibiting fungal growth in the laboratory, and presented comparable efficacy to that of a reference fungicide with MIC 600 ppm (Tchinda *et al*., 2009).

A series of fungicides have also been screened and recommended by several researchers to control the fungus *P. angolensis*. Preventive application of several fungicides could provide effective control against *P. angolensis* (Seif and Hillocks, 1997; Chung and Timmer, 2009; Dewdney and Timmer, 2009). Application of Benzimidazoles was effective against *P. angolensis* in Cameroon (Kuate, 1998). Among a number of contact and systemic fungicides evaluated in Kenya, Triazoles were most inhibitory to *P. angolensis in vitro*, but Fluzilazole provided the best control in the field. Application of Benomyl by alternating with copper-fungicides was also recommended to control the fungus (Seif and Hillocks, 1997). Field application of Chlorothalonil, Copper Hydroxide, Flusilazole and Propinebe provided good control of *P. angolensis* (Seif, 2000). In Ethiopia, application of Chlorothalonil was relatively effective in controlling the fungus (Eshetu, 1999). A mixture of Benomyl and Chlorothalonil applied before fruit set at 15-day intervals was also effective in controlling leaf and fruit spot disease on sweet orange, compared to a mixture of Benomyl and Copper Hydroxide or any of the fungicides alone (Kassahun *et al.*, 2006). Another study in Ethiopia demonstrated that application of Benlate, followed by Score and Cuproxat significantly reduced citrus leaf and fruit spot disease incidence and severity (Mohammed, 2007). Pretorius and Holtz (2008) reported that a combination of Trifloxystrobin, Mancozeb and Mineral Spray Oil, applied in November, January and March, provided the best control of the disease on foliage in Zimbabwe; but applications of Mancozeb in the rainy season were not effective. Seif and Hillocks (1997) recommended spraying after rainfall, rather than on a fixed schedule, because rain stimulates spore production and favors infection.

Repeated applications of recommended fungicides, nearly every two weeks, are required to protect fruit during the period of susceptibility that lasts several months after bloom. Most producers in many citrus growing countries of tropical Africa are not able to afford repeated application of expensive fungicides such as Benzimidazoles and Chlorothalonil, especially smallholder farmers that are typical of the region (Kuate, 1998; Seif and Hillocks, 1997; Mohammed, 2007; Chung and Timmer, 2009; Dewdney and Timmer, 2009; Ndo *et al*., 2010). Extensive and repeated use of fungicides like Benomyl alone may lead to the development of resistant strains and risks of negative effects on the environment (Kuate, 1998; Ndo *et al*., 2010). Therefore, it is necessary to apply expensive systemic fungicides alternated with copper-based fungicides to avoid pathogen resistance and to reduce fungicide application costs (Seif and Hillocks, 1993; Kassahun *et al*., 2006; Mohammed, 2007).

The development and use of resistant varieties would benefit more to growers with small orchards or a few trees, who cannot afford fungicide treatments (Eshetu, 1999; Kassahun *et al*., 2006; Mohammed, 2007). Although all citrus species are affected by *P. angolensis*, cultivated varieties vary in susceptibility to the fungus (Kuate, 1998; Bella-Manga *et al*., 1999; Seif and Hillocks, 1999). Nevertheless, progress in this effort is impeded by absence of strong resistance, loss of apparent resistance in different ecological zones or seasons, and the need to evaluate the susceptibility of leaves and fruits which may be costly and time-consuming. Yet, new hybridization techniques such as using somatic hybrids have been indicated to be promising (Kuate, 1998). However, host resistance alone does not offer a desirable solution to the disease problem. It is necessary to implement an integrated management approach to sustainably control *P. angolensis* (Seif and Hillocks, 1999; Kassahun *et al.*, 2006; Ndo *et al*., 2010).

2.1.3. *Alternaria alternata*

2.1.3.1. Geographical distribution and economic importance

Alternaria is a cosmopolitan fungal genus that occurs worldwide (Laemmlen, 2001; Thomma, 2003; Peever *et al.*, 2004). Plant pathogenic species of *Alternaria* infect a broad range of economically important crops such as citrus, apple, pear, tomato and potato at preharvest stages (Peever *et al.*, 2004). *Alternaria* spp. are also well known as postharvest pathogens (Pegg *et al.*, 2014). In citrus, *Alternaria* spp. cause four different diseases: Alternaria brown spot, Alternaria leaf spot, black rot, and *Mancha foliar de los citricos* of Mexican lime (Akimitsu *et al.*, 2003; Timmer *et al.*, 2003b; Peever *et al.*, 2004).

Alternaria brown spot is one of the most important Alternaria diseases of citrus in humid and semi-arid regions of the world. It causes substantial economic losses in all citrus growing areas (Solel, 1991; Canihos *et al.*, 1997; Timmer *et al.*, 2003b; Kakvan *et al.*, 2012). Alternaria brown spot was first described on Emperor mandarin in Australia in 1903 (Pegg, 1966). It is caused by the fungus *A. alternata* (Fr.) Keissler, formerly *A. citri* Ellis and Pierce (Kiely, 1964; Solel, 1991; Akimitsu *et al.*, 2003). The disease has been subsequently reported in the United States (Whiteside, 1976), Israel (Solel, 1991), Colombia (Castro *et al.*, 1995), Turkey (Canibos *et al.*, 1997), South Africa (Swart *et al.*, 1998), Spain (Vicent *et al.*, 2000), Italy (Bella *et al.*, 2001), Brazil and Argentina (Peres *et al.*, 2003), Greece (Elena, 2006), Iran (Golmohammadi *et al.*, 2006), and China (Wang *et al.*, 2010).

Alternaria diseases are generally characterized by great environmental flexibility and tolerance of a broad range of climatic conditions (Bassimba *et al.*, 2014). Under favorable environmental conditions, significant losses occur both in terms of yield and marketability of citrus fruit (Timmer *et al.*, 2003b). *Alternaria* spp. attack young leaves, twigs and fruits, typically causing brown to black necrotic lesions surrounded by a yellow halo. Lesions may expand rapidly. Severely affected leaves and fruits may drop, and entire shoots may wilt and die (Peever *et al.*, 2002; Akimitsu *et al.*, 2003). The high severity and the difficulty to control

the disease, especially in high humidity regions, may lead to abandon orchards of susceptible citrus varieties (Timmer *et al.*, 2000).

2.1.3.2. Symptoms and damage

Alternaria brown spot attacks the aerial parts of the hosts. It causes leaf spots or blotches, blighting and blackening of young shoots, and sunken dark necrotic lesions on fruits and stems (Fig 2.5). Characteristic symptoms of *Alternaria* infection start as small, circular, brown to black necrotic spots which can vary considerably in size, color and shape. As the disease progresses, the spots enlarge and usually become gray, gray-tan, or near black in color. Spots are often surrounded by yellow halos, induced by a toxin produced by the pathogen. Chlorosis and necrosis may also continue to expand along the veins due to the spread of the host-selective ACT-toxin produced by *A. alternata*. The fungus sporulates in these cankers, causing a fine, black, velvety growth of mycelia and spores. On fruits, lesions vary from minute necrotic spots to large sunken pockmarks. Mature lesions on fruits have a corky appearance. In older lesions the center may dislodge leaving tan colored pockmarks. Alternaria fruit lesions can crack around the outer edge, giving a moat-like appearance. Severe infection can lead to premature defoliation, twig wilting and dieback, and fruitlet drop (Kohmoto *et al.*, 1993; Laemmlen, 2001; Schultz *et al.*, 2013; Pegg *et al.*, 2014), resulting in substantial yield losses, and blemishes on the fruit reduce its value for the fresh market (Akimitsu *et al.*, 2003; Timmer *et al.*, 2003b; Peres and Timmer, 2006; Tsuge *et al.*, 2012). Symptoms of Alternaria brown spot disease are often very similar to those caused by other fungal pathogens such as anthracnose (Pegg *et al.*, 2014).

Fig 2.5. Alternaria necrotic brown spots with yellow halos on citrus leaves, young fruits and shoots. Adopted from Mahdavian (2013) and Pegg *et al*. (2014).

2.1.3.3. Taxonomy and biology

The genus *Alternaria* occurs worldwide as saprophytes, endophytes, plant pathogens, and as human pathogens especially in immuno-compromised patients (Laemmlen, 2001; Thomma, 2003; Peever *et al.*, 2004). *Alternaria* was first described in 1816 by Nees (Stuart *et al.*, 2009). The taxonomic classification of citrus-associated *Alternaria* species is unclear (Peever *et al.*, 2004; Stuart *et al.*, 2009). However, *Alternaria* spp. were usually classified into the division of the mitosporic fungi or the phylum Fungi Imperfecti (Thomma, 2003). Most *Alternaria* spp. do not have known teleomorphic phase (Stuart *et al.*, 2009).

The fungi causing Alternaria brown spot and Alternaria leaf spot diseases were originally identified as *A. citri* based on their morphological similarities to isolates causing black rot (Kiely, 1964; Pegg, 1966). Later, they were considered to represent a distinct strain based on their ability to infect leaves and young fruit and produce host-specific toxins (Kiely, 1964; Whiteside, 1976). These pathogens were treated as *A. alternata* based on a published description of morphology and size of conidia (Kohmoto *et al.*, 1979; Nishimura and Kohmoto, 1983). The fungi were referred to as *A. alternata* f.sp. *citri* (Fr.) Keissel (Solel, 1991) to differentiate them from saprophytic isolates of *A. alternata*, and as *A. alternata* f.sp. *citri* tangerine (Thomma, 2003) to differentiate them from isolates that infect rough lemon.

A. alternata include seven pathotypes, of which the two distinct pathotypes associated with *Citrus* have been described based on differences in host-specificity and toxin production. The tangerine pathotype is specific to tangerines, tangelos and tangors, and produces a host selective ACT-toxin causing Alternaria brown spot; whereas, the rough lemon pathotype is specific to rough lemon, and produces a host-specific ACRL-toxin causing Alternaria leaf spot (Kohmoto *et al.*, 1991; Akimitsu *et al.*, 2003; Yago *et al*., 2011). Both host-specific toxins have been demonstrated to be required for pathogenicity to each host (Ajiro *et al.*, 2010; Miyamoto *et al.*, 2010; Tsuge *et al*., 2012), and these two pathotypes retain clearly distinct host ranges due to the production of different host selective toxins (Kohmoto *et al.*, 1991; Akimitsu *et al.*, 2003; Mahmoudi, 2010).

Alternaria is easy to isolate and grow in culture. Fungal colonies are usually black, gray or green (Pegg *et al.*, 2014). *Alternaria* spores have a distinctive appearance that makes them easy to recognize. Spores are ovoid to obclavate, darkly pigmented, club-shaped, and multi-celled with longitudinal and transverse septations (Fig 2.6). Spores are broader near the base, but become taper to the apex. They are produced singly, or branched chains at the apex of conidiophores (Laemmlen, 2001; Pegg *et al.*, 2014).

Fig 2.6. Asexual spores of *A. alternata*. Adopted from Timmer *et al*. (2003b)

2.1.3.4. Epidemiology and disease cycle

Plant pathogenic *Alternaria* spp. can be host specific or can attack a wide range of hosts. The disease cycle is relatively simple as there is no teleomorph known for *A. alternata*. The fungus survives between crops as spores and mycelium in infected plant tissues, on or within the seeds from infected plants, and in dead organic residues in the soil (Timmer *et al.*, 2003b; Pegg *et al.*, 2014). The fungus can also survive in susceptible weeds or perennials between cropping cycles or seasons. The major sources of transport of these pathogens are by infected plant materials (Rotem, 1994; Pegg *et al.*, 2014).

The most favorable conditions for *Alternaria* infection is rain or heavy dew occurring when temperature is 25 to 28°C (Pegg *et al.*, 2014). The optimum temperature for infection is 27°C. As temperature declines, longer wetting periods are needed for infection to occur (Canihos *et al.*, 1999). Ultraviolet light has been shown to be essential for spore formation (Laemmlen, 2001). Most often, the fungus grows and sporulates on plant tissues or residues during periods of rain, heavy dew, or under conditions of good soil moisture. The fungus is reproduced by means of spores formed on lesions. Spores are dispersed by wind, rain splash, tools and animals. The spores must have free moisture to germinate and infect susceptible plant tissues. Tissues that are stressed, weak, old or wounded are more susceptible to invasion than vigorous tissues. Penetration of the host can be direct with the formation of appressoria, through wounds, or via stomata (Akimitsu *et al.*, 2003; Schultz *et al.*, 2013; Bassimba *et al.*, 2014; Pegg *et al.*, 2014). Studies in Florida reported the penetration only through stomata without formation of appressoria (Akimitsu *et al.*, 2003). However, a study performed in Israel revealed that penetration was consistently associated with the formation of appressoria (Solel and Kimchi, 1997). Penetration was through appressoria formation on leaves of Murcott tangor in Brazil (Stuart *et al.*, 2009). After penetration, necrotic halos are observed surrounding appressoria due to the rapid spread of ACT-toxin in tissues. The effect of host-specific ACT-toxin in host cells is extremely rapid, and the time required for symptom expression is very short (Timmer *et al.*, 2003b; Schultz *et al.*, 2013; Tsuge *et al.*, 2012). The fungus grows on lesions and conidiophores produce spores that are released to start a new

disease cycle on plant tissues (Stuart *et al.*, 2009). The disease cycle continues throughout the season as long as favorable conditions persist and a suitable host is present (Pegg *et al.*, 2014).

2.1.3.5. Host range and susceptibility

The *Alternaria* fungus has a very wide host range of fruits, vegetables and ornamentals. Many other hosts can also be affected (Pegg *et al.*, 2014). Important host plants of *Alternaria* species include citrus, apples, broccoli, cauliflower, carrots, potatoes, Chinese cabbage, tomatoes, and many plants used as ornamentals and a number of weeds (Laemmlen, 2001). However, *A. alternata* causing Alternaria brown spot affects mostly tangerines and their hybrids. Hybrids of Dancy tangerine, some tangors (Murcott and Ortanique), and in some cases Red Blush and Sunrise grapefruit are susceptible (Timmer *et al.*, 2003b; Souza *et al.*, 2009). The disease was reported affecting Africa do Sul and Daisy tangerines, Nova tangelo and Ortanique tangor, as well as Temple x Dancy tangerine hybrids and Satsuma x Murcott 4 hybrids (Souza *et al.*, 2009; Stuart *et al.*, 2009). Clementine and Cleopatra tangerine, limes, lemons and sweet oranges are generally resistant to Alternaria brown spot in the field. However, under laboratory conditions, symptoms were induced in Valencia, Shamouti and Washington Navel oranges as well as in Volkameriana lemon after inoculating *A. alternata* "tangerine pathotype" obtained from Minneola tangelo (Solel and Kimchi, 1997).

The host specificity of the tangerine and rough lemon pathotypes of *A. alternata* depends upon the production of distinct host selective toxins that possess the same selectivity as the fungi (Kohmoto *et al.*, 1979). Dancy tangerine is the parent of most of the hybrids and tangelos that are susceptible to the disease. It is speculated that the susceptibility is inherited from Dancy as a dominant characteristic, while resistance is thought to be recessive (Kohmoto *et al.*, 1991). Reciprocal crosses between resistant Clementine and its susceptible Clementine x Minneola tangelo supported the hypothesis that resistance to *A. alternata* is controlled by a single recessive allele inherited from Clementine (Dalkilic *et al.*, 2005). However, the different degrees of resistance or susceptibility between closely related hybrids suggest that other genes may be involved in the interaction between *Citrus* and *A. alternata*

(Peever *et al.*, 2000). A quantitative resistance loci explaining 30% of the phenotypic characteristics was identified in a citrus linkage map after analyzing 143 hybrids of susceptible Murcott tangor and resistant Pera sweet orange showing a phenotypical 3:1 (susceptible : resistance) segregation ratio, considering the percentage of affected twigs. This quantitative resistance loci is in accordance with the idea that another gene should be involved in the resistance of citrus against *A. alternata* (Stuart *et al.*, 2009).

2.1.3.6. Disease management

Disease control is a key in an effective crop management program (Agrios, 2005). The leaf symptoms of *Alternaria* diseases are often very similar to those caused by other fungal pathogens. Therefore, accurate diagnosis and identification of the fungal pathogen is critical for disease control (Pegg *et al.*, 2014).

Alternaria brown spot is very difficult to control (Schultz *et al.*, 2013). However, various management practices are helpful in reducing disease severity (Bassimba *et al.*, 2014). The disease is controlled primarily by the use of cultural practices and foliar fungicides. Adoption of appropriate hygiene practices is the key to effective management of *Alternaria* (Pegg *et al.*, 2014). Destruction of infected crop debris and eradication of weed hosts reduce inoculum (Laemmlen, 2001; Agrios, 2005). Crop rotation is useful in reducing disease incidence (Laemmlen, 2001). Other practices such as the use of disease-free nursery stock, pruning of affected twigs to remove spores source and reduce fruit infection, promoting air circulation by using wider spacing, pruning tree skirts, avoiding overhead irrigation, the use of under-tree irrigation systems, and avoiding excessive watering and nitrogen fertilization can help to reduce severity of Alternaria brown spot disease in the field (Timmer *et al.*, 2003b; Stuart *et al.*, 2009; Schultz *et al.*, 2013; Bassimba *et al.*, 2014; Pegg *et al.*, 2014).

Despite the good cultural practices used, several fungicide sprays per year are necessary for the economic control of Alternaria brown spot. The most widely used fungicides are Maneb, Mancozeb, Chlorothalonil, Copper-based fungicides and Iprodione. These products are

applied to prevent infection from occurring or to protect uninfected stock after having removed infected plant material. Systemic fungicides with post-infection activity like Benomyl can be used as a protectant but always rotate between products from multiple chemical groups (Solel *et al.*, 1997; Timmer, 2003; Schultz *et al.*, 2013; Bassimba *et al.*, 2014; Pegg *et al.*, 2014). Fungicides like Dithiocarbamates, Dicarboximides, Strobilurins and Conazoles are also effective against *A. alternata* (Timmer, 2003; Stuart *et al.*, 2009). Where the disease is severe and environmental conditions such as humidity, heat and wetness are favorable for the pathogen, several applications (up to 15 times) of fungicides are usually required to acceptable control (Timmer *et al.*, 2003b; Bassimba *et al.*, 2014). Due to the short incubation period of the pathogen and the lack of curative activity of the fungicides, the efficacy of control programs depends on the proper synchronization between spray timing and infection periods. Therefore, site-specific epidemiological knowledge of the disease is critical to develop efficient fungicide schedules (Alva and Graham, 1991; Bassimba *et al.*, 2014)

Application of chemical fungicides may produce serious problems, with residues on the fruit, appearance of fungicide resistant strains of *A. alternata* and their possible accumulation in human adipose tissue constituting an additional health threat (Vicent *et al.*, 2009). An alternative way to fight against these infections might be to modulate the natural defense mechanisms of the plant (Laemmlen, 2001; Kakvan *et al.*, 2012). Some studies have described the mechanisms involved in the defense response of citrus fruit against fungal infection, among which are the induction of the expression of defense-related genes, phytoalexins and other antifungal secondary metabolites that induce protection (Afek *et al.*, 1999; Feng and Zheng, 2007). Similarly, Arcas *et al.* (2000), Ortuno *et al.* (2002) and Del Rio *et al.* (2004) have showed a possible role that phenolic compounds might play as phytoalexins in some *Citrus* species. The susceptibility to *A. alternata* depends on the citrus species. It has been demonstrated that cultivars of mandarins and their hybrids, including Minneola and Orlando tangelo, the Tangor Murcott, and the hybrids Fortune, Nova and Lee are susceptible to the tangerine pathotype of *A. alternata* (Kohmoto *et al*., 1991; Vicent *et al.*, 2004). In contrast, the Satsumas (*C. unshiu*) and the Clementines (*C. clementina*) show a certain degree of resistance while other species, such as sweet orange, lemon and *C.*

margarita are resistant to the pathogen (Gardner *et al.*, 1986; Dalkilic *et al.*, 2005). A study conducted in Iran also indicated that some citrus species cultivated in the country have shown certain degrees of resistance to *A. alternata* (Golmohammadi *et al.*, 2006).

2.2. GENETIC DIVERSITY OF PATHOGEN POPULATION

Plant pathogenic fungi include a very large and heterogeneous group of organisms that have great importance in agriculture and natural plant communities. Many pathogenic fungi can survive for long periods of time on dead host tissue or saprophytically in soil; others rely entirely on living host cells for sustenance (Burdon and Silk, 1997). They show an enormous diversity in life-history strategies and the ways in which they interact with their hosts. These interactions range from species that establish perennial, systemic infections that castrate their hosts to pathogens that kill their hosts with considerable rapidity to pathogens that form discrete lesions whose individual effects are very limited (Burdon, 1993).

It is important to understand the sources of variation that contribute to the diversity of pathogen populations and some of their mechanisms (Burdon and Silk, 1997). Plant pathogenic fungi rely on the processes of mutation and recombination as the ultimate source of genetically based variation (Fig 2.7). Within a species, gene flow between populations supplements these processes as pathogens spread from one epidemiological area to another and from one deme to the next (Burdon and Silk, 1997).

Migration and subsequent gene flow has been suggested to play an important role in contributing to genotype diversity in pathogen populations. The process of migration and gene flow leading to the founding of new pathogen populations in areas in which they previously were absent has been recorded on many occasions (Brasier, 1990; Boland, 1992; Fry *et al*., 1992; Burdon *et al*., 1995). However, where a pathogen population already exists, the recognition of such events tends to be restricted to instances that lead to obvious shifts in the genetic structure of the recipient deme (Burdon and Silk, 1997).

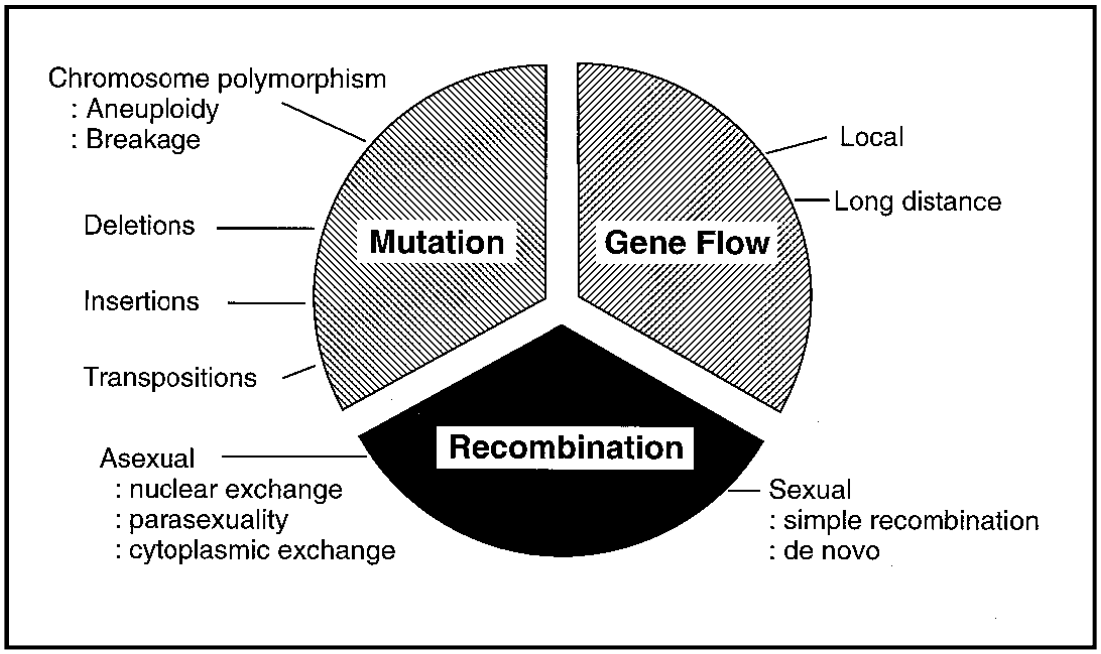

Fig 2.7. Sources of gene and genotype diversity in pathogen population. Adopted from Burdon and Silk (1997).

Recombination in plant pathogens occurs either through sexual reproduction (Roelfs and Groth, 1980; Burdon and Roelfs, 1985) or through a process of somatic hybridization (Burdon *et al*., 1982; Menzies and MacNeill, 1986; Spiers and Hopcroft, 1994), in which nuclear and cytoplasmic material may be exchanged (Boland, 1992; Van Horn and Clay, 1995). In turn, nuclear exchange may be followed by nuclear fusion and recombination; also known as parasexual cycle (Newman and Owen, 1985). All of these mechanisms may generate increased genotypic diversity in a pathogen population, but their importance varies both within and among species (Burdon and Silk, 1997).

The extent to which mutation contributes to the effective diversity of populations is affected by the inherent rate of mutation, the ploidy level of the pathogen (haploid versus diploid or dikaryotic), the size of the pathogen population, and the selective advantage conferred by the mutant phenotype (Burdon, 1992). Despite this complexity, spontaneous mutation is a powerful source of novel variation in many pathogen populations (Wellings and McIntosh, 1990; Goodwin et al., 1995; Burdon and Silk, 1997). The interplay of selection, genetic drift,

migration, and mutation has a major effect on the genetic structure and diversity of all pathogen populations. The relative roles of these factors may change markedly between different pathogen-host associations, between stages in the epidemiological cycle, and between associations in agricultural and natural ecosystems. In doing so, they may result in marked differences in the genotypic diversity of comparable fungal pathogens and, certainly, in the distribution of genotypic diversity within and among different populations (Burdon and Silk, 1997).

Detection and analysis of the genetic variation in living organisms helps to understand the molecular basis of various biological phenomena (Agarwal *et al*., 2008). Studying the polymorphic diversity of plant pathogen population provides significant information relating to the pathosystem (Benali *et al*., 2011). The genetic variability of both the host and the pathogen should also be analyzed using different markers to develop effective control practices (Werlemark *et al*., 2006; Silva *et al*., 2008). These markers are broadly classified into morphological and molecular markers (Bagali *et al.*, 2010; Bhat *et al.*, 2010).

2.2.1. Morphological markers

Morphological markers (also called phenotypic markers) are distinguishable traits with naked eyes. It is easy and quick to detect and identify a morphological marker for a trait because these markers differ among genus, species and varieties (Bagali *et al.*, 2010; Datta *et al.*, 2011). Traditional plant pathology studies have gone through many phases where numerous conventional markers were used for detection, identification, quantification of pathogen species and for evaluation of the genetic variation at individual, population, or species level (McLaughlin *et al*., 1981; Vunsh *et al*., 1990; Bridge *et al*., 2003). These techniques include morphology which is dependent primarily on fruiting body characteristics, and physiological tests. Most of the methods of species characterization based on phenotypic and physiological traits have been used to differentiate *formae speciales* or races of a pathogen. These methods take into account the capacity of the strains to utilize specific nutrients, produce different types of secondary metabolites such as enzymes or toxins, resist antibiotics or other toxic

compounds (Leung *et al*., 1993; Atkins and Clark, 2004; Cooke and Lees, 2004; Coetzee *et al*., 2009).

Pathotypic analyses such as mating tests, virulence, aggressiveness and fungicide resistance have been informative phenotypic traits of morphological characters most often used for genetic studies of plant pathogens and have provided a clear picture of race diversity and distribution (Lopez *et al*., 2003; Schaad *et al*., 2003; Cooke and Lees, 2004). Differences in ploidy levels, antibiotic resistance and temperature response are also additional phenotypic characters that can be considered (Cooke and Lees, 2004). Furthermore, vegetative compatibility which refers to the ability of individual fungal isolates to undergo hyphal fusion and form a stable heterokaryon (Caten and Jinks, 1966) can also help to identify fungal isolates (Coetzee *et al*., 2009; Edel-Hermann *et al*., 2012).

Despite their great importance in life sciences, morphological characters and physiological tests are costly and time consuming (Atkins and Clark, 2004; Coetzee *et al*., 2009). It has been reported that vegetative compatibility requires long time to obtain mutants and pair them with reference strains until a heterokaryon is detected (Correll *et al*., 1987). Moreover, these markers are not considered reliable due to their instability and variability with the environment (Annesi *et al*., 2003; Atkins and Clark, 2004; Coetzee *et al*., 2009; Weir *et al*., 2012). Over the years, solutions were found to avoid some of these problems by using DNA-based markers (Atkins and Clark, 2004).

2.2.2. DNA based molecular markers

Genetic markers and their correlation to phenotypes provide essential landmarks for elucidation of genetic variation (Agarwal *et al*., 2008). The construction of human genetic maps using restriction fragment length polymorphisms was the first reported DNA based molecular marker technique in the detection of DNA polymorphism (Botstein *et al*., 1980). Since then several DNA based marker techniques have been developed and widely used in the various fields of biological sciences (Ippolito *et al*., 2004; Singh and Hughes, 2006). The use of DNA polymorphisms as a trait has added a new dimension to understand the genetic

variability in fungi (Bridge *et al*., 2003; Schaad *et al*., 2003). DNA based markers offer advantages over conventional phenotypic alternatives. They are rapid, highly specific, stable and detectable in all tissues regardless of their growth and development stage, phenotypically neutral, and are not confounded by the environment, pleiotropic (influence of a single gene on multiple traits), and epistatic (suppression of a gene by the effect of an unrelated gene) effects (Agarwal *et al*., 2008; Bagali *et al*., 2010; Benali *et al*., 2011).

Several DNA markers systems have been commune in the study of plant pathology (Schaad *et al*., 2003; Ippolito *et al*., 2004; Singh and Hughes, 2006). The basic DNA based markers are well established (Agarwal *et al*., 2008). Six DNA based molecular techniques have been widely used in plant pathology because of their economic importance and value as biological research tools (Milgroom, 1997; McCartney *et al*., 2003; Cooke and Lees, 2004; Benali *et al*., 2011). These DNA markers are generally classified into two categories based on the techniques employed for their detection and amplification. These are the markers associated with a known gene or known sequence DNA which are visualized by hybridization of the restriction enzyme-digested DNA to a known labeled probes, like restriction fragment length polymorphism (RFLP); and the markers that are associated with anonymous genomic sequence and involve amplification of a particular sequence or loci with a specific or arbitrary primers sequence, such as random amplified polymorphic DNA (RAPD), internal transcribed spacer (ITS), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR) and single nucleotide polymorphism (SNP) (McCartney *et al*., 2003; Cooke and Lees, 2004; Benali *et al*., 2011; Datta *et al*., 2011).

The hybridization (non-PCR) based markers such as RFLP were initially developed for human genomes (Botstein *et al*., 1980). In RFLP, DNA polymorphism is detected by hybridizing chemically labeled DNA probe or alternatively by a Southern blot of DNA digested by sequence-specific restriction endonucleases, resulting in differential DNA fragment profiles (Southern, 1975; Jacobson and Gordon, 1991; Kim *et al*., 1992). This differential genetic profile or polymorphism is generated by a gain or loss in restriction sites resulting from nucleotide substitutions or DNA sequence rearrangements like insertion or

deletion or single nucleotide polymorphisms which could be detected by the variation in the length of restriction fragments (Agarwal *et al*., 2008; Benali *et al*., 2011). The RFLP presents more advantages compared to the non-DNA based molecular markers. It is ideally suited to genetic diversity studies as RFLP is relatively highly polymorphic, co-dominantly inherited and unaffected by the environment, high abundance throughout the genome, high locus specificity and highly reproducible. The method provides opportunity to simultaneously screen numerous samples from DNA of any source. They are robust and readily transferable between laboratories (Agarwal *et al*., 2008; Bagali *et al*., 2010; Benali *et al*., 2011). However, RFLP technique is not very widely used because it is time consuming and labor intensive to apply to numerous individuals (only one or few loci are detected per assay), involves expensive and radioactive or toxic reagents, and requires large amount of high quality DNA (50-200µg). The requirement of prior sequence information for probe generation increases the complexity of the methodology (Agarwal *et al*., 2008; Benali *et al*., 2011). These limitations led to the development of less technically complex PCR-based techniques.

The RAPD technique, which is based on differential PCR amplification of genomic DNA, deduces DNA polymorphisms produced by rearrangements or deletions at or between oligonucleotide primer binding sites in the genome using short random oligonucleotide sequences, mostly 10 bases long (Williams *et al*., 1990). Arbitrarily primed PCR (Welsh and McClelland, 1990) and DNA amplification fingerprinting (Williams *et al*., 1990) are the first independently developed PCR-based marker techniques, which are variants of RAPD. The first variant of RAPD was not very popular because it involved autoradiography; but it has been simplified as fragments can be fractionated using agarose gel electrophoresis (Williams *et al*., 1990). The second technique involves the use of single arbitrary primers shorter than 10 nucleotides for amplification (Caetano-Anolles and Bassam, 1993) and the amplicons are analyzed using polyacrylamide gel along with silver staining (Agarwal *et al*., 2008). The RAPD can be employed across species using universal primers because the approach requires no prior knowledge of the genome (Williams *et al*., 1993). The technique is easy and quick to assay. It is applicable to large number of isolates and enables analysis of variation at more than one locus. The technique is very sensitive and needs very small quantity of DNA (about

5-20ng per reaction). It is low cost and no radioactivity (Williams *et al*., 1993; Bentley *et al*., 1995). The main shortcoming of RAPD is that the profiling is dependent on the reaction conditions and DNA quality, which may vary between two different laboratories and sometimes within laboratory for similar species (Jones *et al*., 1997; Bardakci, 2001). The RAPD is dominant and several discrete loci in the genome are amplified by each primer. As a result, profiles are not able to distinguish heterozygous from homozygous individuals (Bardakci, 2001; Bagali *et al*., 2010; Benali *et al*., 2011).

The AFLP technology (Vos *et al*., 1995) was developed to overcome the limitation of reproducibility associated with RAPD. It combines the power of RFLP with the flexibility of PCR based technology by ligating primer-recognition sequences (oligonucleotide adaptors) to the restricted DNA and selective PCR amplification of restriction fragments using a limited set of primers (Vos *et al*., 1995; Abdel-Satar *et al*., 2003). The AFLP technique generates multiple fingerprints of any DNA regardless of its source, and without any prior knowledge of DNA sequence information for primer construction (Vos *et al*., 1995; Rafalsiki *et al*., 1996). It provides neutral markers and allows analysis of variation at more than one locus (Baayen *et al*., 2000). It has high reproducibility (Kiprop *et al*., 2002). Most AFLP fragments correspond to unique positions on genome, which can be exploited as landmarks in genetic and physical mapping (Vos *et al*., 1995). However, incomplete restriction of DNA samples can result in detection of differences in banding patterns that do not reflect true polymorphisms. The type of frequent cutter used can also reduce the power of this technique (Vos *et al*., 1995). It has the difficulty to recognize alleles which leads to an overestimation of variation because allelic fragments are scored as independent, loss of restriction sites due to point mutations, and changes of fragment sizes due to indels that result in an overestimation of variation (Majer *et al*., 1998). The AFLP is scored as dominant marker, which reduces its information content. It is technically demanding, time consuming and expensive (Benali *et al*., 2011).

Different sequence-specific molecular markers have been designed to correlate the DNA sequence information with particular phenotypes. The ITS is one of the important sequence-specific molecular markers that are widely utilized for genomic analysis (Agarwal *et al*.,

2008). Ribosomal DNA (rDNA) in eukaryotes typically present in several hundred tandemly repeated multiple copies. The region is part of the rDNA cistron which consists of 18S, first internal transcribed spacer (ITS1), 5.8S, second transcribed spacer (ITS2), and 28S sequences. Both the ITS1 and ITS2 are non-coding regions that lie between the 18S and 5.8S, and between the 5.8S and 28S coding regions respectively (Atkins and Clark, 2004; Brunner *et al*., 2007). Multiple copies make it easy to amplify rDNA even from small amounts of samples. Since each of the ITS1 and ITS2 are flanked by highly conserved coding regions of rDNA, primers can be designed for the amplification of both (Benali *et al*., 2011). Amplified products are resolved by electrophoresis on stained agarose gel. The samples are directly sequenced on automated sequencer. Data analyses of complementary strands for the ITS region are assembled using software and checked for homology using Basic Local Alignment Search Tool program (Selig *et al*., 2008; Schultz and Wolf, 2009; NCBI, 2009).

Simple sequence repeats (microsatellites) are repetitions of very short nucleotide motifs that range from one to six base pairs in length (Matsuoka *et al*., 2002; Cai *et al.*, 2013). They are available in both the coding and non-coding regions of genomic DNA (Matsuoka *et al*., 2002; Sharopova, 2008). Variation in the number of tandemly repeated units is mainly due to strand slippage during DNA replication where the repeats allow matching via excision or addition of repeats (Schlotterer and Tautz, 1992). Microsatellite assays show extensive inter-individual length polymorphisms during PCR analysis of unique loci using discriminatory primers sets (Agarwal *et al.*, 2008). Microsatellites can be easily amplified using PCR from DNA extracted from a variety of sources. Polymorphisms can be visualized on a sequencing gel, and the availability of automatic DNA sequencers allows high-throughput analysis of a large number of samples (Jarne and Lagoda, 1996; Goldstein and Schlotterer, 1999). Microsatellites are highly popular genetic markers due to their multi-allelic loci, co-dominant inheritance, high abundance, even distribution throughout the genome, and ease of assessing SSR size variation by PCR with pairs of flanking primers (Morgante *et al*., 2002; Karaoglu *et al.*, 2005; Benali *et al.*, 2011). Microsatellites are highly reproducible and can be used efficiently by different research laboratories to produce consistent data (Saghai Maroof *et al*., 1994). They have been widely used in the taxonomic and population genetic studies of various fungi

(Dusabenyagasani *et al*., 1998; Burgess *et al*., 2001) based on their allelic frequencies (Bruford and Wyne 1993; Villarea *et al*., 2002).

The traditional methods of developing microsatellites, which are still used by many laboratories today, generally involve several steps (Cai *et al*., 2013). The whole process may require several months of work and considerable resources due to lengthy cloning and plasmid screening procedures (Morgante *et al*., 2002; Zane *et al*., 2002). In fungi, the traditional approach is more time- and resource-consuming due to their lower densities of microsatellite loci and shorter alleles with fewer polymorphisms as compared to many other organisms (Dutech *et al.*, 2007). The amplification of accessory bands commonly known as stutter bands which are scored when resolved on gel electrophoresis needs great care in the employment of SSR at large scale (Morgante *et al*., 2002; Benali *et al*., 2011). Microsatellites also differ from genotype to genotype due to high mutation rate in the motifs associated with unequal crossing during meiosis and replication slippage or misreading (Richards and Sutherland, 1992; Matsuoka *et al*., 2002). However, advances in sequencing technology are changing many aspects of the methods to develop microsatellite markers. The high-throughput and low cost of next-generation sequencing technologies enable the efficient generation of large amounts of genome sequence data to identify microsatellite markers (Capote *et al.*, 2012; Cai *et al.*, 2013). These approaches can be applied either for the development of microsatellite markers alone or in combination with genome sequencing projects that produce much deeper genome coverage (Cai *et al.*, 2013).

The SNP represents one of the new classes of DNA markers that have become popular and highly proffered in genomic studies (Chen *et al*., 2002). The SNPs describe polymorphism between individuals caused by single nucleotides (point mutation) which gives rise to different alleles with a usual alternative of two possible bases at a given position (Agarwal *et al.*, 2008). The SNPs are highly abundant, spread both in the coding and non-coding regions of the genome (Wang *et al*., 1998; Vignal *et al*., 2002; Teneva, 2009). A variety of analytical procedures have been developed for detection and genotyping SNPs rapidly and accurately (Benali *et al*., 2011). However, SNPs are chosen if there is sufficient amount of sequence

information and if analysis tool is available to assign gene function (Bagali *et al*., 2010; Datta *et al*., 2011).

Despite the presence of a range of molecular markers, the choice of an appropriate genetic marker depends on the experience and competence of researchers, on laboratory facilities, and mostly on the question on which are being sought (McCartney *et al*, 2003). There is no unique and ideal marker applicable for purposes facing the plant pathologists, rather these markers could often complement each other (Benali *et al*., 2011).

So far, the causal pathogen of leaf and fruit spot disease of citrus in Ethiopia is not well-studied, and its populations have not yet characterized. There are no markers developed for the genetic characterization of the pathogen. These shortcomings have prompted the need for reliable techniques to examine and understand the pathogen in Ethiopia.

REFERENCES

Abang, M.M., S. Winter, H.D. Mignouna, K.R. Green and R. Asiedu. 2003. Molecular taxonomic, epidemiological and population genetic approaches to understanding yam anthracnose disease. *African Journal of Biotechnology*, 2:486-496.

Abdel-Satar, M.A., M.S. Khalil, I.N. Mohmed, K.A. Abd-Elsalam and J.A. Verreet. 2003. Molecular phylogeny of *Fusarium* species by AFLP fingerprint. *African Journal of Biotechnology*, 2:51-55.

Adhikary, N.K., S. Dey and J. Tarafdar. 2013. Studies on morphology of mango anthracnose disease causing fungus *Colletotrichum gloeosporioides* and efficacy of azoxystrobin against the fungus under *in vitro* and *in vivo* condition. *The Bioscan*, 8:493-497.

Afanador-Kafuri, L., D. Minz, M. Maymon and S. Freeman. 2003. Characterization of *Colletotrichum* isolates from tamarillo, *Passiflora*, and mango in Colombia and identification of a unique species from the genus. *Phytopathology*, 93:579-587.

Afek U., J. Orenstein, S. Carmeli, V. Rodov and M.B. Joseph. 1999. Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*. *Phytochemistry*, 50:1129-1132.

Agarwal, M., N. Shrivastava and H. Padh. 2008. Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Report*, 27:617-631.

Agostini, J.P, L.W Timmer and D.J. Mitchell. 1992. Morphological and pathological characteristics of strains of *Colletotrichum gloeosporioides* from citrus. *Phytopathology*, 82:1377-1382.

Agrios, G.N. 2005. Plant Pathology. 5th ed. Elsevier Academic Press, London, UK.

Aiello, D., R. Carrieri, V. Guarnaccia, A. Vitale, E. Lahoz and G. Polizzi. 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology*, 163:168-177.

Ajiro, N., Y. Miyamoto, A. Masunaka, T. Tsuge, M. Yamamoto, K. Ohtani, T. Fukumoto, K. Gomi, T.L. Peever, Y. Izumi, Y. Tada and K. Akimitsu. 2010. Role of the host-selective ACT-toxin synthesis gene ACTTS2 encoding an enoyl-reductase in pathogenicity of the tangerine pathotype of *Alternaria alternata*. *Phytopathology*, 100:120-126.

Akimitsu, K., T.L. Peever and L.W. Timmer. 2003. Molecular, ecological and evolutionary approaches to understanding *Alternaria* diseases of citrus. *Molecular Plant Pathology*, 4:435-446.

Alva, A.K. and J.H. Graham. 1991. The role of copper fungicides in citriculture. *Advances in Agronomy*, 1:145-170.

Alvarez, E., L. Ganan, A. Rojas-Trivino, J.F. Mejia, G.A. Llano and A. Gonzalez. 2014. Diversity and pathogenicity of *Colletotrichum* species isolated from soursop in Colombia. *European Journal of Plant Pathology*, 139:325-338.

Annesi, T., R. Coppola and E. Motta. 2003. Isozyme analysis on some wood decay fungi. *Journal of Plant Pathology*, 85:87-90.

Arcas, M.C., J.M. Botia, A. Ortuno and J.A. Del Rio. 2000. UV irradiation alters the levels of flavonoids involved in the defense mechanisms of *Citrus aurantium* fruits against *Penicillium digitatum*. *European Journal of Plant Pathology*, 106:617-622.

Atkins, S.D. and I.M. Clark. 2004. Fungal molecular diagnostics: a mini review. *Journal of Applied Genetics*, 45:3-15.

Baayen, R.P., K. O'Donnell, P.J.M. Bonants, E. Cigelnik, L.P.N.M. Kroon, E.J.A. Roebroeck and C. Waalwijk. 2000. Gene genealogies and AFLP analyses in the *Fusarium oxysporium* complex identify monophyletic and non-monophyletic *formae speciales* causing wilt and rot disease. *Phytopathology*, 90:891-900.

Babu, L.J., H.N. Gour and S. Pankaj. 2008. Efficacy of phyto-extracts and bioagents against *Colletotrichum gloeosporioides* causing banana fruit rot. *Journal of Mycology and Plant Pathology*, 38:635-637.

Bagali, P.G., P.D.A.H. Prabhu, K. Raghavendra, P.G. Bagali, S. Hittalmani and J.S.Vadivelu. 2010. Application of molecular markers in plant tissue culture. *Asia-Pacific Journal of Molecular Biology and Biotechnology*, 18(1):85-87.

Bailey, J.A., R.J. O'Connell, R.J. Pring and C. Nash. 1992. Infection strategies of *Colletotrichum* species. pp. 88-120. In: J.A. Bailey and M.J. Jeger (eds), *Colletotrichum*: Biology, Pathology and Control. CABI, Wallingford, UK.

Bardakci, F. 2001. Random amplified polymorphic DNA (RAPD) markers. *Turkish Journal of Biology*, 25:185-196.

Bassimba, D.D.M, J.L. Mira and A. Vicent. 2014. Inoculum sources, infection periods, and effects of environmental factors on Alternaria brown spot of mandarin in Mediterranean climate conditions. *Plant Disease*, 98:409-417.

Bella, P., C. Guarino, R. LaRosa and A. Catara. 2001. Severe infections of *Alternaria* spp. on a mandarin hybrid. *Journal of Plant Pathology*, 83:231.

Bella-Manga, B., C. Dubois, J. Kuate, M.M. Ngbwa and J.Y. Rey. 1999. Susceptibility to *Phaeoramularia angolensis* of some citrus varieties grown in the Cameroon rain forests. *Fruits*, 54:167-176.

Benali, S., B. Mohamed, H.J. Eddine and C. Neema. 2011. Advances of molecular markers application in plant pathology research. *European Journal of Scientific Research*, 50:110-123.

Bentley, S., K.G. Pegg and J.L. Dale. 1995. Genetic variation among a world-wide collection of isolates of *Fusarium oxysporium* f.sp. *cubense* analyzed by RAPD-PCR fingerprinting. *Mycological Research*, 99:1378-1384.

Bhat, P.R., V. Krishnakumar, P.S. Hendre, P. Rajendrakumar, R.K. Varshney and R.K. Aggarwal. 2005. Identification and characterization of expressed sequence tags-derived simple sequence repeats markers from robusta coffee variety 'CxR' (an interspecific hybrid of *Coffea canephora* x *Coffea congensis*). *Molecular Ecology Notes*, 5:80-83.

Boland, G. 1992. Hypovirulence and double-stranded RNA in *Sclerotinia sclerotiorum*. *Canadian Journal of Plant Pathology*, 14:10-17.

Botstein, D., R.L. White, M. Skolnick and R.W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32:314-331.

Brasier, C.M. 1990. The unexpected element: Mycovirus involvement in the outcome of two recent pandemics, Dutch elm disease and chestnut blight. pp. 289-307. In: J.J. Burdon and S.R. Leather (eds.), Pests, Pathogens and Plant Communities. Blackwell Scientific Publications, Oxford.

Braun, U. 1999. Taxonomic notes on some species of the *Cercospora* complex (VI). *Cryptogamie Mycologie*, 20:155-177.

Bridge, P.D., T. Singh and D.K. Arora. 2003. The application of molecular markers in the epidemiology of plant pathogenic fungi. pp. 93-113. In: D.K. Arora, P.D. Bridge and D. Bhatnagar (eds.), Fungal Biotechnology in Agricultural, Food, and Environmental Applications. Mycology Series, Vol. 21, Marcel Dekker Inc.

Browning, H.W., R.J. McGrovern, L.K. Jackson, D.V. Calvert and W.F. Wardowski. 1995. Florida citrus diagnostic guide. Florida Science Source Inc., Florida, USA.

Bruford, M.W. and R.K. Wyne. 1993. Microsatellites and their application to population genetic studies. *Current Opinion in Genetics and Development*, 3:939-943.

Brunner, K., S. Zeilinger and R.L. Mach. 2007. Environmental and microbial relationships, 2nd ed. The Mycota IV C.P. Kubicek and I.S. Druzhinina (eds.) Springer-Verlag Berlin Heidelberg.

Burdon, J.J. 1992. Genetic variation in pathogen populations and its implication for adaptation to host resistance. pp. 41-56. In: Th. Jacobs and J.E. Parlevliet (eds.), Durability of Disease Resistance. Kluwer Academic Publishers, Amsterdam.

Burdon, J.J. 1993. The structure of pathogen populations in natural plant communities. Annual Review of Phytopathology, 31:305-323.

Burdon, J.J., L. Ericson and W.J. Muller. 1995. Temporal and spatial relationships in a metapopulation of the rust pathogen *Triphragmium ulmariae* and its host, *Filipendula ulmaria*. *Journal of Ecology*, 82:979-989.

Burdon, J.J. and J. Silk. 1997. Sources and patterns of diversity in plant-pathogenic fungi. *Phytopathology*, 87(7):664-669.

Burdon, J.J. and A.P. Roelfs. 1985. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. *Phytopathology*, 75:1068-1073.

Burdon, J.J., D.R. Marshall, N.H. Luig and D.J.S. Gow. 1982. Isozyme studies on the origin and evolution of *Puccinia graminis* f. sp. *tritici* in Australia. *Australian Journal of Biological Science*,. 35:231-238.

Burgess, T., M.J. Wingfield and B.D. Wingfield. 2001. Simple sequence repeat markers distinguish among morphotypes of *Sphaeropsis sapinea*. *Applied and Environmental Microbiology*, 67:354-362.

Caetano-Anolles, G. and B.J.D.N.A. Bassam. 1993. Amplification fingerprinting using arbitrary oligonucleotide primers. *Applied Biochemistry and Biotechnology*, 42:189-200.

Cai, L., K.D. Hyde, P.W.J. Taylor, B.S. Weir, J. Waller, M.M. Abang, J.Z. Zhang, Y.L. Yang, S. Phoulivong, Z.Y. Liu, H. Prihastuti, R.G. Shivas, E.H.C. McKenzie and P.R. Johnston. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Diversity*, 39:183-204.

Cai, Z., G. Li, C. Lin, T. Shi, L. Zhai, Y. Chen and G. Huang. 2013. Identifying pathogenicity genes in the rubber tree anthracnose fungus *Colletotrichum gloeosporioides* through random insertional mutagenesis. *Microbiological Research*, 68:340-350.

Canihos Y., A. Erkilic and L.W. Timmer. 1997. First report of Alternaria brown spot of Minneola tangelo in Turkey. *Plant Disease*, 81:1214.

Canihos, Y., T.L. Peever and L.W. Timmer. 1999. Temperature, leaf wetness, and isolate effects on infection of Minneola tangelo leaves by *Alternaria* species. *Plant Disease*, 83:429-433.

Cannon, P.F., U. Damm, P.R. Johnston and B.S. Weir. 2012. *Colletotrichum* – current status and future directions. *Studies in Mycology*, 73:181-213.

Capote, N., A.M. Pastrana, A. Aguado and P. Sanchez-Torres. 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. pp. 151-202. In: C.J. Cumagun (ed.), Plant Pathology. InTech, Rijeka, Croatia.

Castro, B.L., J. Leguizamon and A. Lopez. 1995. Determination of the causal agent of leaf spot in citrus plants in Colombian coffee growing region. *Fitopatologia Colombiana*, 19:31-35.

Caten, C.E. and J.L. Jinks. 1966. Heterokaryosis: its significance in wild homothallic Ascomycetes and fungi imperfecti. *Transactions of the British Mycological Society*, 49:81-93.

Chalkley, D. 2012. Invasive fungi: angular leaf spot of citrus - *Pseudocercospora angolensis*. Systematic Mycology and Microbiology Laboratory, ARS, USDA. Online: /sbmlweb/fungi/index.cfm. Retrieved July 12, 2012.

Chen, F., P.H Goodwin, A. Khan and T. Hsiang. 2002. Population structure and mating-type genes of *Colletotrichum graminicola* from *Agrostis palustris*. *Canadian Journal of Microbiology*, 48:427-436.

Chung, K.R. and L.W. Timmer. 2009. Citrus diseases exotic to Florida: *Phaeoramularia* fruit and leaf spot (PFLS). Fact Sheet PP-234. University of Florida, Gainesville, USA. Online: http://edis.ifas.ufl.edu/pp155. Retrieved October 28, 2011

Coetzee, M.P.A., L. Maphosa, B.D. Wingfield, E. Mwenje and M.J. Wingfield. 2009. Characterization of *Armillaria* species based on pectic isozyme analyses. *Fungal Diversity*, 36:9-16.

Cooke, D.E.L. and A.K. Lees. 2004. Markers, old and new, for examining *Phytophthora infestans* diversity. *Plant Pathology*, 53:692-704.

Correll, J.C., C.J.R. Klittich and J.F. Leslie. 1987. Nitrate nonutilizing mutants of *Fusarium oxysporum* and their use in vegetative compatibility tests. *Phytopathology*, 77:1640-1646.

Da Silva, C.F.B. and S.J. Michereff. 2013. Biology of *Colletotrichum* spp. and epidemiology of the anthracnose in tropical fruit trees. *Revista Caatinga*, 26:130-138.

Dalkilic Z., L.W. Timmer and F.G. Gmitter. 2005. Linkage of an *Alternaria* disease resistance gene in mandarin hybrids with RAPD fragments. *Journal of American Society of Horticultural Science*, 130:191-195.

Damm, U., P.F. Cannon, J.H.C. Woudenberg, P.R. Johnston, B.S. Weir, Y.P. Tan, R.G. Shivas and P.W. Crous. 2012. The *Colletotrichum boninense* species complex. *Studies in Mycology*, 73:1-36.

Datta, D., S. Gupta, S.K. Chaturvedi and N. Nadarajan. 2011. Molecular markers in crop improvement. Indian Institute of Pulses Research, Kanpur - 208 024.

Dean, R., J.A.L. Van Kan, Z.A. Pretorius, K.E. Hammond-Kosack, A. Di Pietro, P.D. Spanu, J.J. Rudd, M. Dickman, R. Kahmann, J. Ellis and G.D. Foster. 2012. The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13:414-430.

Deising, H.B., S. Werner and M. Wernitz. 2000. The role of fungal appressoria in plant infection. *Microbes and Infection*, 2:1631-1641.

Del Rio, J.A., P. Gomez, G.A. Baidez, M.C. Arcas, J.M. Botia and A. Ortuno. 2004. Changes in the level of polymethoxyflavones and flavonones as part of the defense mechanism of *Citrus sinensis* (cv. Valencia late) fruits against *Phytophthora citrophthora*. *Journal of Agriculture and Food Chemistry*, 52: 1913-1917.

Dempsey S., G. Evans and E. Szandala. 2002. A target list of high risk pathogens of citrus. Agriculture, Fisheries and Forestry, Canberra, Australia.

Dewdney, M. and L.W. Timmer. 2009. Citrus production and diseases in Ghana. Online: http://www.crec.ifas.ufl.edu/academics/faculty/dewdney/PDF/Ghanatrip story.pdf. Retrieved February 19, 2014.

Deshmukh, A.J., B.P. Mehta and V.A. Patil. 2010. *In vitro* evaluation of some known bioagents to control *Colletotrichum gloeosporioides* Penz. and Sacc., causing anthracnose of Indian bean. *International Journal of Pharma and Bio Sciences*, 1:1-6.

Deshmukh, A.J., B.P. Mehta, A.N. Sabalpara and V.A. Patil. 2012. Screening of Indian bean (*Lablab purpureus* L.) varieties/germplasm against anthracnose (*Colletotrichum gloeosporioides* Penz. and Sacc.) under field conditions. *Journal of Biopesticides*, 5:50-52.

Diallo, M.T.S. 2001. Progression of the citrus leaf and fruit spot disease (*Phaeoramularia angolensis*) in Guinea. *Fruits*, 56:37-43.

Doyle, V.P., P.V. Oudemans, S.A. Rehner and A. Litt. 2013. Habitat and host indicate lineage identity in *Colletotrichum gloeosporioides* s.l. from wild and agricultural landscapes in North America. *PLOS ONE*, 8(5):e62394.

Dusabenyagasani, M., N. Lecours and R.C. Hamelin. 1998. Sequence-tagged sites (STS) for studies of molecular epidemiology of scleroderris canker of conifers. *Theoretical and Applied Genetics*, 97:789-796.

Dutech, C., J. Enjalbert, E. Fournier, F. Delmotte, B. Barres, J. Carlier, D. Tharreau and T. Giraud. 2007. Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology*, 44:933-949.

Edel-Hermann, V., N. Gautheron and C. Steinberg. 2012. Genetic diversity of *Fusarium oxysporum* and related species pathogenic on tomato in Algeria and other Mediterranean countries. *Plant Pathology*, 61(4):787-800.

Elena, K. 2006. Alternaria brown spot of Minneola in Greece; evaluation of citrus species susceptibility. *European Journal of Plant Pathology*, 115:259-262.

El-Ghaouth, A., C.L. Wilson, M. Wisniewski, S. Droby, J.L. Smilanick and L. Korsten. 2002. Biological control of postharvest disease of citrus fruits. pp. 289-312. In: S.S. Gnanamanickam (ed.), Biological Control of Crop Diseases. Mared Dekker Inc., New York.

Emechebe, A.M. 1981. Brown spot disease of *Citrus* caused by *Phaeoisariopsis* species. *Annals of Applied Biology*, 97:257-262.

EPPO (European and Mediterranean Plant Protection Organization). 2013. EPPO standards PM 1/2(20): EPPO A1 and A2 lists of pests recommended for regulation as quarantine pests. Paris, France. Online: http://archives.eppo.int/EPPOStandards/PM1-GENERAL/pm102(22)_A1A2_2013.pdf. Retrieved February 19, 2014.

Eshetu Derso. 1999. Occurrence, prevalence and control methods of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 54:225-232.

Farr, D.F., M.C. Aime, A.Y. Rossman and M.E. Palm. 2006. Species of *Colletotrichum* on Agavaceae. *Mycological Research*, 110:1395-1408.

Feng, W. and X. Zheng. 2007. Essential oils to control *Alternaria alternata in vitro* and *in vivo*. *Food Control*, 18:1126-1130.

Freeman, S. and E. Shabi. 1996. Cross-infection of subtropical and temperate fruits by *Colletotrichum* species from various hosts. *Physiology and Molecular Plant Pathology*, 49:395-404.

Freeman, S., T. Katan and E. Shabi. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease*, 82:596-605.

Freeman, S., S. Horowitz-Brown, L. Afanador-Kafuri, M. Maymon and D. Minz. 2013. *Colletotrichum*: host specificity and pathogenicity on selected tropical and subtropical crops. *Acta Horticulturae*, 975:209-216.

Fry, W.E., S.B. Goodwin, J.M. Matuszak, L.J. Spielman and M.G. Milgroom. 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annual Review of Phytopathology*, 30:107-129.

Gardner, J.M., Y. Kono and J.L. Chandler. 1986. Bioassay and host-selectivity of *Alternaria citri* toxins affecting Rouge lemon and mandarins. *Physiological and Molecular Plant Pathology*, 29:293-304.

Gautam, A.K., M. Kant and Y. Thakur. 2013. Isolation of endophytic fungi from *Cannabis sativa* and study their antifungal potential. *Archives of Phytopathology and Plant Protection*, 46:627-635.

Gautam, A.K. 2014. *Colletotrichum gloeosporioides*: biology, pathogenicity and management in India. *Journal of Plant Physiology and Pathology*, 2:2

Ghosh, S.K. and N. Chakraborty. 2012. *In vitro* biological control of *Colletotrichum gloeosporioides*, causal organism of anthracnose of sarpagandha (*Roulvolfia serpentina*). *Agriculture and Biology Journal of North America*, 3:306-310.

Goldstein, D.B. and C. Schlotterer. 1999. Microsatellites: evolution and applications. Oxford University Press. 352p.

Golmohammadi, M., M. Andrew, T.L. Peever, N.A. Peres and L.W. Timmer. 2006. Brown spot of tangerine hybrid cultivars Minneola, Page and Fortune caused by *Alternaria alternata* in Iran. *Plant Pathology*, 55:578.

Goodwin, S.B., L.S. Sujkowski, and W.E. Fry. 1995. Rapid evolution of pathogenicity within clonal lineages of the potato late blight disease fungus. *Phytopathology*, 85:669-676.

Hiremath, S.V., P.C. Hiremath and R.K. Hegde. 1993. Studies on cultural characters of *Colletotrichum gloeosporioides*: a causal agent of Shisham blight. *Karnataka Journal of Agricultural Science*, 6:30-32.

Huang, F., G.Q. Chen, X. Hou, Y.S. Fu, L. Cai, K.D. Hyde and H.Y. Li. 2013. *Colletotrichum* species associated with cultivated citrus in China. *Fungal Diversity*, 61:61-74.

Hubballi, M., S. Nakkeeran, T. Raguchander, T. Anand and P. Renukadevi. 2011. Physiological characterization of *Colletotrichum gloeosporioides*, the incitant of anthracnose disease of noni in India. *Archives of Phytopathology and Plant Protection*, 44:1105-1114.

Hyde, K.D., L. Cai, P.F. Cannon, J.A. Crouch, P.W. Crous, U. Damm, P.H. Goodwin, H. Chen, P.R. Johnston, E.B.G. Jones, Z.Y. Liu, E.H.C. McKenzie, J. Moriwaki, P. Noireung, S.R. Penny cook, L.H. Pfenning, H. Prihastuti, T. Sato, R.G. Shivas, Y.P. Tan, P.W.J. Taylor, B.S. Weir, Y.L. Yang and J.Z. Zhang. 2009. *Colletotrichum* – names in current use. *Fungal Diversity*, 39:147-182.

Ippolito, A., L. Schena, F. Nigro, V.S. Ligorio and T. Yaseen. 2004. Real time detection of *Phytophthora nicotinanae* and *P. citrophtora* in citrus roots and soil. *European Journal of plant pathology*, 110:833-843.

Jacobson, D.J. and T.R. Gordon. 1991. *Fusarium oxysporium* f.sp. *melonis*: a case study of diversity within a *forma specialis*. *Phytopathology*, 81:1064-1067.

Jarne, P. and P.J.L. Lagoda. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11:424-429.

Jazet Dongmo, P.M., J. Kuate, F.F. Boyom, D. Ducelier, F. Damesse, P.H.A. Zollo, C. Menut, J.M. Bessiere. 2002. Chemical composition and *in vitro* antifungal activity against *Phaeoramularia angolensis* of *Citrus* essential oils. *Fruits*, 57:95-104.

Jazet Dongmo, P.M., N.L. Tatsadjieu, E. Tchinda Sonwa, J. Kuate, P.H. Amvam Zollo and C. Menut. 2008a. Antiradical potential and antifungal activities of essential oils of the leaves of *Citrus latifolia* against *Phaeoramularia angolensis. African Journal of Biotechnology*, 7:4045-4050.

Jazet Dongmo, P.M., L.T. Ngoune, B.N. Dongmo, J. Kuate, P.H. Amvam Zollo and C. Menut. 2008b. Antifungal potential of *Eucalyptus Saligna* and *Eucalyptus Camaldulensis* essential oils from Cameroon against *Phaeoramularia Angolensis*. *European Journal of Scientific Research*, 24:348-357.

Jazet Dongmo, P.M., L.N. Tatsadjieu, E. Tchinda Sonwa, J. Kuate, P.H. Amvam Zollo and C. Menut. 2009a. Essential oils of *Citrus aurantifolia* from Cameroon and their antifungal activity against *Phaeoramularia angolensis*. *African Journal of Agricultural Research*, 4:354-358.

Jazet, P.M.D., L.N. Tatsadjieu, B.D. Ndongson, J. Kuate, P.H. Amvam Zollo and C. Menut. 2009b. Correlation between chemical composition and antifungal properties of essential oils of *Callistemon rigidus* and *Callistemon citrinus* of Cameroon against *Phaeoramularia angolensis*. *Journal of Medicinal Plants Research*, 3:009-015.

Jeyalakshmi, C. and K. Seetharaman. 1998. Biological control of fruit rot and dieback of chili with plant products and antagonistic microorganisms. *Plant Disease Research*, 13:46-48.

Johnston, P.R., S.R. Penny cook and M.A. Manning. 2005. Taxonomy of fruit-rotting fungal pathogens: what's really out there? *New Zealand Plant Protection*, 58:42-46.

Jones, C.J., K.J. Edwards, S. Castaglione, M.O. Winfield, F. Sala, C. Wiel, G. van de Bredemeijer, B. Vosman, M. Matthes, A. Daly, R. Brettschneider, P. Bettini, M. Buiatti, E. Maestri, A. Malcevschi, N. Marmiroli, R. Aert, G. Volckaert, J. Rudea, R. Linacero, A. Vazquez and A. Karp. 1997. Reproducibility testing of RAPD, AFLP and SSR markers in plants by a network of European laboratories. *Molecular Breeding*, 3:381-390.

Joshee, S., B.C. Paulus, D. Park and P.R. Johnston. 2009. Diversity and distribution of fungal foliar endophytes in New Zealand Podocarpaceae. *Mycological Research*, 113:1003-1015.

Kakvan, N., Z. Hamidreza, M. Bahar, H. Shahab and T. Hossein. 2012. Evaluation of citrus cultivars resistance to *Alternaria alternata*, the causal agent of brown spot disease, using RAPD-PCR. *Journal of Research in Agricultural Science*, 8:69-76.

Karaoglu, H., C.M.Y. Lee and W. Meyer. 2005. Survey of simple sequence repeats in completed fungal genomes. *Molecular Biology and Evolution*, 22:639-649.

Kassahun Tessega, Temam Hussien and P.K. Sakhuja. 2006. Management of *Phaeoramularia* fruit and leaf spot disease of citrus in Ethiopia. *Agricultura Tropica et Subtropica*, 39:242-248.

Kiely, T.B. 1964. Brown spot of Emperor mandarin. *Agricultural Gazette N.S.W.*, 75:854-856.

Kim, D.H., R.D. Martyn and C.W. Magill. 1992. Restriction fragment length polymorphism groups and physical map of mitochondrial DNA from *Fusarium oxysporium* f.sp. *niveum*. *Phytopathology*, 82:346-353.

Kiprop, E.K., J.P. Baudoin, A.W. Mwangombe, P.M. Kimani, G. Mergeai and A. Maquet. 2002. Characterization of Kenyan isolates of *Fusarium udum* from pigeon pea (*Cajanus cajan* [L.] Millsp.) by cultural characteristics, aggressiveness and AFLP analysis. *Journal of Phytopathology*, 150:517-525.

Kirk, P.M. 1986. *Phaeoramularia angolensis*. CMI Descriptions of Pathogenic Fungi and Bacteria, No. 863. *Mycopathologia*, 94:177-178.

Kohmoto, K., R.P. Scheffer and J.O. Whiteside. 1979. Host-selective toxins from *Alternaria citri*. *Phytopathology*, 69:667-671.

Kohmoto, K., Y. Itoh, N. Shimomura, Y. Kondoh, H. Otani, M. Kodama, S. Nishimura and S. Nakatsuka. 1993. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. *Phytopathology*, 83:495-502.

Kuate, J. 1998. Citrus leaf and fruit spot disease caused by *Phaeoramularia angolensis* (Cercospariose des agrumes causee par *Phaeoramularia angolensis*). *Cahiers Agricultures*, 7:121-129.

Kuate, J. 1999. Fruit and leaf spot disease caused by *Phaeoramularia angolensis*: a threat to citrus production. Research and Methods, FRuiTROP, No. 61.

Kuate, J., P.M. Jazet Dongmo, D. Ducelier, F, Damesse, C. Menut, J.M. Bessiere. 2003. Effect of citrus leaf spot disease (*Phaeoramularia angolensis)* on the content and chemical composition of essential oils from orange peel. *Fruits,* 58:143-149.

Kuate, J., J. Foko, S.A. Ndindeng, P.M. Jazet Dongmo, E. Foure, F. Damesse, Bella-Manga and D. Ducelier. 2006. Effect of essential oils from citrus varieties on in vitro growth and sporulation of *Phaeoramularia angolensis* causing citrus leaf and fruit spot disease. *European Journal of Plant Pathology*, 114:151-161.

Kuberan, T., A. Balamurugan, R. Vidhyapallavi, P. Nepolean, R. Jayanthi, T. Beulah and R. Premkumar. 2012. *In vitro* evaluation certain plant extracts against *Glomerella cingulata* causing brown blight disease of tea. *World Journal of Agricultural Sciences*, 8:464-467.

Laemmlen, F. 2001. *Alternaria* diseases. University of California, Agricultural and Natural Resources. Publication 8040. Online: http://anrcatalog.ucdavis.edu/. Retrieved June 1, 2015.

Leung, H., R.J. Nelson and J.E. Leach. 1993. Population structure of plant pathogenic fungi and bacteria. *Advances in Plant Pathology*, 10:157-205.

Lima, W.G., M.B. Sposito, L. Amorim, F.P. Golcalves and P.A.M. de Filho. 2011. *Colletotrichum gloeosporioides*, a new causal agent of citrus post-bloom fruit drop. *European Journal of Plant Pathology*, 131:157-165.

Lima, N.B., W.G. Lima, J.M. Tovar-Pedraza, S.J. Michereff and M.P.S. Camara. 2015. Comparative epidemiology of *Colletotrichum* species from mango in northeastern Brazil. *European Journal of Plant Pathology*, 141:679-688.

Lopez, C.E., I.F. Acosta, C. Jara, P. Pedraza, E. Gaitan-Solis, G. Gallego, S. Beebe and J. Thome. 2003. Identifying resistance gene analogs associated with resistance to different pathogens in common bean. *Phytopathology*, 93:88-95.

MacKenzie, S.J., T.E. Seijo, D.E. Legard, L.W. Timmer and N.A. Peres. 2007. Selection for pathogenicity to strawberry in populations of *Colletotrichum gloeosporioides* from native plants. *Phytopathology*, 97:1130-1140.

Mahdavian, S.E. 2013. Occurrence and infection rate of the Alternaria brown spot disease of the Minneola tangelo in the west part of Mazandaran province. *Scholarly Journal of Agricultural Science*, 3:167-171.

Mahmoudi, E. 2010. Evaluation of citrus cultivars susceptibility to leaf spot disease caused by *Alternaria alternata* under *in vitro* conditions. *Journal of Research in Agricultural Science*, 6:75-81.

Majer, D., B.G. Lewis and R. Mithen. 1998. Genetic variation among field isolates of *Pyrenopeziza brassicae*. *Plant Pathology*, 47:22-28.

Manner, H.I., R.S. Buker, V.E. Smith, D. Ward and C.R. Elevitch. 2006. *Citrus* (citrus) and *Fortunella* (kumquat). Species Profiles for Pacific Island Agroforestry. Online: www.traditionaltree.org. Retrieved May 10, 2011.

Mathur, K., R.P. Thakur and V.P. Rao. 1997. A strain of *Colletotrichum gloeosporioides* causing anthracnose on yellow sorghum. *International Sorghum and Millets Newsletter*, 38:69-70.

Matsuoka, Y., S.E. Mitchell, S. Kresovich, M. Goodman and J. Doebley. 2002. Microsatellites in *Zea* variability, patterns of mutations, and use for evolutionary studies. *Theoretical and Applied Genetics*, 104:436-450.

McCartney, H.A., S.J. Foster, B.A. Fraaije and E. Ward. 2003. Molecular diagnostics for fungal plant pathogens. *Pest Management Science*, 59:129-142.

McLaughlin, M.R., O.W. Barnett, P.M. Burrows and R.H. Baum. 1981. Improved ELISA conditions for detection of plant viruses. *Journal of Virological Methods*, 3:13-25.

Menzies, J.G., and B.H. MacNeill. 1986. Asexual recombination in *Erysiphe graminis* f. sp. *tritici*. *Canadian Journal of Plant Pathology*, 8:400-404.

Milgroom, M.G. 1997. Genetic variation and the application of genetic markers for studying plant pathogen populations. *Journal of Plant Pathology*, 78:1-13.

Miyamoto, Y., A. Masunaka, T. Tsuge M. Yamamoto, K. Ohtani, T. Fukumoto, K. Gomi, T.L. Peever, Y. Tada, K. Ichimura and K. Akimitsu. 2010. ACTTS3 encoding a polyketide synthase is essential for the biosynthesis of ACT-toxin and pathogenicity in the tangerine pathotype of *Alternaria alternata*. *Molecular Plant-Microbe Interactions*, 23:406-414.

Mohammed Yesuf. 2007. Distribution and management of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 62:99-106.

Mohammed Yesuf, Wondirad Mandefro, Eshetu Ahmed, Girma Adugna, Dereje Tadesse, Temam Hussen and Meki Shehabu. 2009. Review of pathological research on fruit crops in Ethiopia. pp. 231-252. In: Abraham Tadesse (ed.), Increasing Crop Production through Improved Plant Protection, Vol. II. Plant Protection Society of Ethiopia (PPSE). EIAR, Addis Ababa, Ethiopia.

Mohammed Yesuf. 2013. Pseudocercospora leaf and fruit spot disease of citrus: achievements and challenges in the citrus industry: a review. *Agricultural Sciences*, 4:324-328.

Morgante, M., H. Hanafey and W. Powell. 2002. Microsatellites are preferentially associated with non-repetitive DNA in plant genome. *Nature Genetics*, 30:194-200.

Moriwaki, J., T. Sato and T. Tsukiboshi. 2003. Morphological and molecular characterization of *Colletotrichum boninense* species nov. from Japan. *Mycoscience*, 44:47-53.

Naqvi, S.A.M.H. 2004. Diseases of fruits and vegetables: diagnosis and management. Vol. I. Kluwer Academic Publishers, the Netherlands.

Ndo, E.G.D., F. Bella-Manga, S.A. Ndindeng, M. Ndoumbe-Nkeng, A.D. Fontem and C. Cilas. 2010. Altitude, tree species and soil type are the main factors influencing the severity of *Phaeoramularia* leaf and fruit spot disease of citrus in the humid zones of Cameroon. *European Journal of Plant Pathology*, 128:385-397.

Newman, P.L. and H. Owen. 1985. Evidence of asexual recombination in *Rhynchosporium secalis*. *Plant Pathology*, 34:338-340.

Nicholson, R.L. and W.B.C. Moraes. 1980. Survival of *Colletotrichum graminicola*: importance of the spore matrix. *Phytopathology*, 70:255-261.

NCBI (National Center for Biotechnology Information). 2009. Entrez cross-database search engine. Maryland, USA. Online: http://www.ncbi.nlm.nih.gov/sites/gquery.

Ortuno A., M.C. Arcas, J.M. Botia, M.D. Fuster and J.A. Del Rio. 2002. Increasing resistance against *Phytophthora citrophthora* in tangelo Nova fruits by modulating polymethoxyflavones levels. *Journal of Agriculture and Food Chemistry*, 50: 2836-2839

Padman, M. and G.R. Janardhana. 2012. Screening for inhibitory activities of essential oils on the growth of *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc., the causal agent of leaf spot disease of *Murraya koenigii* L. *Archives of Phytopathology and Plant Protection*, 45:1575-1581.

Nishimura, S. and K. Kohmoto. 1983. Host-specific toxins and chemical structures from *Alternaria* species. *Annual Review of Phytopathology*, 21:87-116.

Patel, D.S. 2009. Chemical management of fruit spot of pomegranate caused by *Colletotrichum gloeosporioides* Penz. and Sacc. *Indian Phytopathology*, 62:252-253.

Peever, T.L., L. Olsen, A. Ibanez and L.W. Timmer. 2000. Genetic differentiation and host specificity among populations of *Alternaria* spp. causing brown spot of grapefruit and tangerine × grapefruit hybrids in Florida. *Phytopathology*, 90:407-414.

Peever, T.L., A. Ibanez, K. Akimitsu and L.W. Timmer. 2002. Worldwide phylogeography of the citrus brown spot pathogen, *Alternaria alternata*. *Phytopathology*, 92:794-802.

Peever, T.L., G. Su, L. Carpenter-Boggs and L.W. Timmer. 2004. Molecular systematics of citrus-associated *Alternaria* species. *Mycologia*, 96:119-134.

Pegg, K.G. 1966. Studies of a strain of *Alternaria citri* Pierce, the causal organism of brown spot of Emperor mandarin. *Queensland Journal of Agricultural Animal Science,* 23:14-18.

Pegg, K., J. Duff and A. Manners. 2014. *Alternaria* diseases in production nurseries. Agri-science Queensland, Department of Agriculture, Fisheries and Forestry, Australia.

Peres, N.A.R., J.P. Agostini and L.W. Timmer. 2003. Outbreaks of Alternaria brown spot of citrus in Brazil and Argentina. *Plant Disease*, 87:750.

Peres, N.A., L.W. Timmer, J.E. Adaskaveg and J.C. Correll. 2005. Lifestyles of *Colletotrichum acutatum*. *Plant Disease,* 89:784-796.

Peres, N.A. and L.W. Timmer. 2006. Evaluation of the Alter-Rater model for spray timing for control of Alternaria brown spot on Murcott tangor in Brazil. *Crop Protection*, 25:454-460.

Pretorius, M.C., P.W. Crous, J.Z. Groenewald and U. Braun. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. *Sydowia*, 55:286-305.

Pretorius, M.C. 2005. Epidemiology and control of *Pseudocercospora angolensis* fruit and leaf spot disease on citrus in Zimbabwe**.** M.Sc. Thesis, University of Stellenbosch, South Africa. 70p.

Pretorius, M.C. and G. Holtz. 2008. Distribution and control of *Pseudocercospora angolensis* on citrus in Zimbabwe and Mozambique. *Phytopathology*, 98:S127.

Prusky, D. 1996. Pathogen quiescence in postharvest diseases. *Annual Review of Phytopathology*, 34:413-434.

Rafalski, J.A., J.M. Vogel, M. Morgante, W. Powell, C. Andre and S.V. Tingey. 1996. Generating and using DNA markers in plants. pp. 75-134. In: B. Birren and E. Lai (eds.), Non-Mammalian Genomic Analysis: A Practical Guide. Academic Press, New York.

Ramesha, A., V.H. Sunitha and C. Srinivas. 2013. Antimicrobial activity of secondary metabolites from endophytic fungi isolated from *Nerium oleander*. *International Journal of Pharma and Bio Sciences*, 4: 683-693.

Rampersad, S.N. 2011. Molecular and phenotypic characterization of *Colletotrichum* species associated with anthracnose disease of papaya in Trinidad. *Plant Disease*, 95:1244-1254.

Rawal, R.D. 1998. Management of fungal diseases in tropical fruits. In: R.K. Arora and V. Ramanatha Rao (eds.), Tropical Fruits in Asia: Diversity, Maintenance, Conservation and Use. Proceedings of the IPGRI-ICAR-UTFANET Regional Training Course on the Conservation and Use of Germplasm of Tropical Fruits in Asia, 18-31 May 1997, Indian Institute of Horticultural Research, Bangalore, India.

Richards, R.I., and G.R. Sutherland. 1992. Heritable unstable DNA sequences. *Nature Genetics*, 1:7-9.

Roelfs, A.P. and J.V. Groth. 1980. A comparison of virulence phenotypes in wheat stem rust populations reproducing sexually and asexually. *Phytopathology*, 70:855-862.

Rojas, E.I., S.A. Rehner, G.J. Samuels, S.A. Van Bael, E.A. Herre, P. Cannon, R. Chen, J. Panq, R. Wang, Y. Zhang, Y.Q. Peng and T. Sha. 2010. *Colletotrichum gloeosporioides* s.l. associated with *Theobroma cacao* and other plants in Panama: multilocus phylogenies distinguish pathogen and endophyte clades. *Mycologia*, 102:1318-1338.

Rotem, J. 1994. The genus *Alternaria*: biology, epidemiology and pathogenicity. American Phytopathological Society, St. Paul, MN.

Saghai Maroof, M.A., R.M. Biyashev, G.P. Yang, Q. Zhang and R.W. Allard. 1994. Extraordinarily polymorphic microsatellite DNA in barley: species diversity, chromosomal locations, and population dynamics. *Proceedings of the National Academy of Science USA*, 91:5466-5470.

Saju, K.A., T.N. Deka, U. Gupta, A.K. Biswas and M.R. Sudharshan. 2012. *In vitro* evaluation of biocontrol agents, botanicals and fungicides against *Colletotrichum gloeosporioides* infecting large cardamom. *Plant Disease Research,* 27:49-53.

Schaad, N.W., R.D. Frederick, J. Shaw, W.L. Schneider, R. Hickson, M.D. Petrillo and D.G. Luster. 2003. Advances in molecular based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annual Review of Phytopathology*, 41:305-324.

Schena, L., S. Mosca, S.O. Cacciola, R. Faedda, S.M. Sanzani, G.E. Agosteo, V. Sergeeva and G. Magnano di San Lio. 2014. Species of the *Colletotrichum gloeosporioides* and *C. boninense* complexes associated with olive anthracnose. *Plant Pathology*, 63:437-446.

Schlotterer, C. and D. Tautz. 1992. Slippage synthesis of simple sequence DNA. *Nucleic Acids Research*, 20:2231-2235.

Schultz, D., M. Rybak, R. Rybak and R.D. French. 2013. Alternaria brown spot. Texas A&M AgriLife Extension Service, PLPA-Cit013-04. Online: http://sickcitrus.tamu.edu. Retrieved June 1, 2015.

Schultz, J. and M. Wolf. 2009. ITS2 sequence-structure analysis in phylogenetics: a how-to manual for molecular systematic. *Molecular Phylogenetics and Evolution*, 52:520-523.

Seif, A.A. and J.N. Kungu. 1989. The current status of cercosporiose of citrus in Kenya. In: Proceedings of the Second Conference of the Kenya Agricultural Research Institute, 4-7 September 1989, Pan Africa Hotel, Nairobi, Kenya.

Seif, A.A. 2000. *Phaeoramularia* fruit and leaf spot. pp. 29-30. In: L.W. Timmer, S.M. Garnsey and J.H. Graham (eds.), Compendium of Citrus Diseases. American Phytopathological Society, APS Press Inc., USA.

Seif, A.A. and J.N. Kungu. 1990. Association of *Colletotrichum* species with the fruit and leaf spot of citrus caused by *Phaeoramularia angolensis*. National Agricultural Research Laboratories, Annual Report of Plant Pathology Department for 1990.

Seif, A.A and R.J. Hillocks. 1993. *Phaeoramularia* fruit and leaf spot of citrus with special reference to Kenya. *International Journal of Pest Management,* 39:44-50.

Seif, A.A and R.J. Hillocks. 1997. Chemical control of *Phaeoramularia* fruit and leaf spot of citrus in Kenya. *Crop Protection*, 16:141-145.

Seif, A.A. and R.J. Hillocks. 1999. Reaction of some citrus cultivars to *Phaeoramularia* fruit and leaf spot in Kenya. *Fruits*, 54:323-329.

Seifu Gebre-Mariam. 2003. Status of commercial fruit production in Ethiopia. Ethiopian Agricultural Research Organization, Addis Ababa, Ethiopia. 91p.

Selig, C., M. Wolf, T. Muller, T. Dandekar and J. Schultz. 2008. The ITS2 database II: homology modeling RNA structure for molecular systematic. *Nucleic Acids Research*, 36D:377-380.

Sharma, P.N., M. Kaur, O.P. Sharma, P. Sharma and A. Pathania. 2005. Morphological, pathological and molecular variability in *Colletotrichum capsici*, the cause of fruit rot of chilies in the subtropical region of northwestern India. *Journal of Phytopathology*, 153:232-237.

Sharopova, N. 2008. Plant simple sequence repeats: distribution, variation, and effects on gene expression. *Genome*, 51:79-90.

Silva, K.J.D., E.A. Souza, A. Sartorato and C.N. Souza-Freire. 2008. Pathogenic variability of isolates of *Pseudocercospora griseola*, the cause of common bean angular leaf spot, and its implications for resistance breeding. *Journal of Phytopathology*, 156:602-606.

Singh, P.K. and G.R. Hughes. 2006. Genetic similarity among isolates of *Pyrenophora triticirepentis*, causal agent of tan spot of wheat. *Journal of Phytopathology*, 154:178-184.

Singh, A., K.S. Verma and C. Mohan. 2009. Comparative performance of guava cultivars against *Colletotrichum gloeosporioides* causing anthracnose. *Journal of Mycology and Plant Pathology*, 39:436-438.

Sisay Bekele Mekbib. 2007. Identification of citrus (*Citrus sinensis*) postharvest pathogens from Ethiopia and their control. Ph.D. Dissertation, University of Pretoria, Pretoria, South Africa. 225p.

Smith, B.J., and L. Black. 1990. Morphological, cultural, and pathogenic variation among *Colletotrichum* species isolated from strawberry. *Plant Disease*, 74:69-76.

Solel, Z. 1991. Alternaria brown spot on Minneola tangelos in Israel. *Plant Pathology*, 40:145-147.

Solel, Z. and M. Kimchi. 1997. Susceptibility and resistance of citrus genotypes to *Alternaria alternata* pv. *citri*. *Journal of Phytopathology*, 145:389-391.

Solel Z., Y. Oren and M. Kimchi. 1997. Control of Alternaria brown spot of Minneola tangelo with fungicides. *Crop Protection*, 16:659-664.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *Journal of Molecular Biology*, 98:503-517.

Souza, M.C. E.S. Stuchi and A. de Goes. 2009. Evaluation of tangerine hybrid resistance to *Alternaria alternata*. *Scientia Horticulturae*, 123:1-138.

Spiers, A.G. and D.H. Hopcroft. 1994. Comparative studies of the poplar rusts *Melampsora medusae*, *M. larici-populina* and their interspecific hybrid *M. medusae-populina*. *Mycological Research*, 98:889-903.

Stuart, R.W., M. Bastianel, F.A. De Azevedo and M.A. Machado. 2009. Alternaria brown spot. *LARANJA, Cordeiropolis*, 30:29-44.

Sundar, A.R., N.D. Das and D. Krishnaveni. 1995. In-vitro antagonism of *Trichoderma* spp. against two fungal pathogens of castor. *Indian Journal Plant Protection*, 23:152-155.

Sutton, B.C. 1980. The Coelomycetes: fungi imperfecti with pycnidia, acervulli and stromata. Commonwealth Mycological Institute, Kew, UK.

Sutton, B.C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. pp. 1-26. In: J.A. Bailey and M.J. Jeger (eds), *Colletotrichum*: Biology, Pathology and Control. CAB International, Wallingford, UK.

Swart, S.H., M.J. Wingfield, W.J Swart and G.C. Schutte. 1998. Chemical control of Alternaria brown spot on Minneola tangelo in South Africa. *Annals of Applied Biology*, 133:17-30.

Tchinda, E.S., P.M.D. Jazet, L.N. Tatsadjieu, B.D. Ndongson, P.H.Z. Amvam and C. Menut. 2009. Antifungal activity of the essential oil of *Cymbopogon citratu*s (Poaceae) against *Phaeoramularia angolensis*. *Journal of Essential Oil-Bearing Plants*, 12:218-224.

Teneva, A. 2009. Molecular markers in animal genome analysis. *Biotechnology in Animal Husbandry*, 25:1267-1284.

Tesfaye Tedilla and Habtu Assefa. 1985. A review of research activities on fruit crop diseases in Ethiopia. pp. 263-276. In: Tsedeke Abate (ed.), Proceedings of the First Ethiopian Crop Protection Symposium, 4-7 February 1985, Addis Ababa, Ethiopia.

Thomma, B.P.H.J. 2003. *Alternaria* spp.: from general saprophyte to specific parasite. *Molecular Plant Pathology*, 4:225-236.

Timmer, L.W., H.M. Darhower, S.E. Zitko, T.L. Peever, A.M. Ibanez and P.M. Bushong. 2000. Environmental factors affecting the severity of Alternaria brown spot of citrus and their potential use in timing fungicide applications. *Plant Disease*, 84:638-643.

Timmer, L.W. 2003. Florida citrus pest management guide. University of Florida, IFAS, Publication number SP-43, USA.

Timmer, L.W., S.M. Garnsey and P. Broadbent. 2003a. Diseases of citrus. pp. 163-195. In: R.C. Ploetz (ed.), Diseases of Tropical Fruit Crops. CAB International, Wallingford, UK.

Timmer, L.W., T.L. Peever, Z. Solel and K, Akimitsu. 2003b. Alternaria diseases of citrus - novel pathosystems. *Phytopathologia Mediterranea*, 42:3-16.

Tsuge, T., Y. Harimoto, K. Akimitsu, K. Ohtani, M. Kodama, Y. Akagi, M. Egusa, M. Yamamoto and H. Otani. 2012. Host-selective toxins produced by the plant pathogenic fungus *Alternaria alternata*. *FEMS Microbiology Review*, 37:44-66.

Udayanga, D., D.S. Manamgoda, X. Liu, E. Chukeatirote and K.D. Hyde. 2013. What are the common anthracnose pathogens of tropical fruits? *Fungal Diversity*, 61:165-179.

USDA-ARS. 2009. Germplasm resources information network (GRIN), online database. National Germplasm Resources Laboratory, Beltsville, USA. Online: http://www.ars-grin.gov/cgi-bin/npgs/html/tax_search.pl. Retrieved December 26, 2012.

Van Horn, R. and K. Clay. 1995. Mitochondria DNA variation in the fungus *Atkinsonella hypoxylon* infecting sympatric *Danthonia* grasses. *Evolution*, 49:360-371.

Vicent A., J. Armengol, R. Sales and J. Garcia-Jimenez. 2000. First report of Alternaria brown spot of citrus in Spain. *Plant Disease*, 84:1044.

Vicent, A., J. Badal, M.J. Asensi, N. Sanz, J. Armengol and J. Garcia-Jimenez. 2004. Laboratory evaluation of citrus cultivars susceptibility and influence of fruit size on Fortune mandarin to infection by *Alternaria alternata* pv. *citri*. *European Journal of Plant Pathology*, 110:245-251.

Vicent, A. and J. Garcia-Jimenez. 2008. Risk of establishment of non-indigenous diseases of citrus fruit and foliage in Spain: an approach using meteorological databases and tree canopy climate data. *Phytoparasitica*, 36:7-19.

Vicent, A., J. Armengol and J. Garcia-Jimenez. 2009. Protectant activity of reduced concentration copper sprays against Alternaria brown spot on 'Fortune' mandarin fruit in Spain. *Crop Protection*, 28:1-6.

Vignal, A., D. Milian, M. SanCristobal and A. Eggen. 2002. A review on SNP and other types of molecular markers and their use in animal genetics. *Genetic Selection and Evolution*, 34:275-305.

Villareal, L.M.M.A., C. Lannou, C. de Vallavieille-Pope and C. Neema. 2002. Genetic variability in *Puccinia striiformis* f.sp. *tritici* populations sampled on a local scale during natural epidemics. *Applied and Environmental Microbiology*, 68:6138-6145.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research*, 23:4407-4414.

Vunsh, R.A., A. Rosner and A. Stein. 1990. The use of the polymerase chain reaction (PCR) for detection of bean yellow mosaic virus in gladiolus. *Annals of Applied Biology*, 117:561-569.

Wang, X.F., Z.A. Li, K.Z. Tang, C.Y. Zhou and L. Yi. 2010. First report of Alternaria brown spot of citrus caused by *Alternaria alternata* in Yunnan Province, China. *Plant Disease*, 94:375-375.

Wang, D.G., J.B. Fan, C.J. Siao, A. Berno, P. Young, R. Sapolsky, G. Ghandour, N. Perkins, E. Winchester, J. Spencer, L. Kruglyak, L. Stein, L. Hsie, T. Topaloglou, E. Hubbell, E. Robinson, M. Mittmann, M.S. Morris, N. Shen, D. Kilburn, J. Rioux, C. Nusbaum, S. Rozen, T.J. Hudson, R. Lipshutz, M. Chee and E.S. Lander. 1998. Large-scale identification, mapping, and genotyping of single-nucleotide polymorphisms in the human genome. *Science*, 280:1077-1082.

Weir, B.S., P.R. Johnston and U. Damm. 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*, 73:115-180.

Welsh, J. and M. McClelland. 1990. Fingerprint genomes using PCR with arbitrary primers. *Nucleic Acids Research*, 18:7213-7218.

Werlemark, G., B.U. Carlson-Nilsson and C.G. Davidson. 2006. Genetic variation in the rose pathogen *Marssonina rosae* estimated by RAPD. *International Journal of Horticultural Science*, 12:63-67.

Whiteside, J.O. 1976. A newly recorded Alternaria-induced brown spot disease on Dancy tangerines in Florida. *Plant Disease Reporter*, 60:326-329.

Williams, J.G., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*, 18:6531-6535.

Williams, J.G.K., M.K. Hanafey, J.A. Rafalski and S.V. Tingey. 1993. Genetic analysis using random amplified polymorphic DNA markers. Methods *in Enzymology*, 218:704-740.

Wellings, C.R. and R.A. McIntosh. 1990. Puccinia striiformis f. sp. *tritici* in Australasia: Pathogenic changes during the first ten years. *Plant Pathology*, 39:316-325.

Yago, J.I., C.H. Lin and K.R. Chung. 2011. The SLT2 mitogen-activated protein kinase-mediated signaling pathway governs conidiation, morphogenesis, fungal virulence and production of toxin and melanin in the tangerine pathotype of *Alternaria alternata*. *Molecular Plant Pathology*, 12:653-665.

Yuan, Z.L., Z.Z. Su, L.J. Mao, Y.Q. Peng, G.M. Yang, F.C. Lin and C.L. Zhang. 2011. Distinctive endophytic fungal assemblage in stems of wild rice (*Oryza granulata*) in China with special reference to two species of Muscodor (Xylariaceae). *The Journal of Microbiology*, 49:15-23.

Zane, L., L. Bargelloni and T. Patarnello. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11:1-16.

Zivkovic, S., N. Trkulja, T. Popovic, V. Oro and Z. Ivanovic. 2012. Morphological and molecular identification of *Colletotrichum gloeosporioides* from *Citrus reticulata*. pp. 292-298. In: D. Marisavljevic (ed.), Proceedings of International Symposium on Current Trends in Plant Protection, 25-28 September 2012, Institute for Plant Protection and Environment, Belgrade, Serbia.

3. DISTRIBUTION, INCIDENCE AND SEVERITY OF LEAF AND FRUIT SPOT DISEASE OF CITRUS IN ETHIOPIA

3.1. ABSTRACT

Citrus leaf and fruit spot disease has been reported to cause high yield and quality losses on citrus in Ethiopia. A total of forty-nine citrus orchards in twenty-eight districts were surveyed between June 2012 and May 2013 in the major citrus growing areas of the country to assess the distribution, incidence and severity of citrus leaf and fruit spot disease. Disease incidence and severity on citrus leaves and intact fruits were determined using random sampling techniques. In the present surveys, the status of citrus production, major production constraints and management practices were assessed using questionnaire and field observations. The survey results showed that about 59.2% of the orchards (29 out of 49) and 53.6% (15 out of 28) of the districts surveyed were affected by the leaf and fruit spot disease. The disease prevalence in the districts surveyed ranged from zero to 100%. The damage of leaf and fruit spot disease varied with citrus species. The overall mean incidences of the disease were on leaves of sweet orange (38.2%), mandarin (21.5%), lemon (17.1%) and lime (16.3%) while disease severities were 39.1%, 33.3%, 25% and 16.7%, respectively. Similarly, the average incidences and severities on fruits were 63.9%, 29.4%, 18.0% and 16.7%, and 71.6%, 50%, 25% and 25%, in the same order. Disease incidences in the different orchards ranged from zero to 81.7% on leaves and from zero to 100% on fruits. Disease severity also varied from zero to 75% on leaves and from zero to 100% on fruits. Disease incidences and severities were high (ranged from 28% to 60.4% and 25% to 75% on leaves while 40.7% to 100% and 49% to 100% on fruits) in Jimma town, Abeshege, Aleta Wendo, Kebena, Mana, Gomma, Ginbo and Debre Werk districts. The questionnaire assessment and field observations indicated that various citrus species (sweet orange being the major species) and varieties with two to seventy years of age were produced by smallholder and commercial farmers. The assessment also showed that diseases and insect pests, poor agronomic and irrigation practices, and shortage of adapted high yielding varieties were the major citrus

production constraints in the country. In the present surveys, it has been observed that commercial citrus orchards practice field sanitation, pruning, irrigation, and fertilizer and pesticide applications. However, most of the orchards of the smallholders were not well managed. The present findings showed that leaf and fruit spot disease of citrus has widely distributed in the wet humid districts in the south, southwest, central and northwest parts of Ethiopia. The results also clearly indicated that the citrus orchards in low rainfall areas in the northeast, central rift valley and eastern parts of the country were not affected by the disease. To manage the disease, integrated disease management approaches need to be in place. Frequent disease monitoring and precautions are essential. Care should be taken during the movement of planting materials and fruits from affected areas to locations where the disease is not recorded. Citrus growers should improve the fertility status of their orchards, and practice general hygiene and sanitary measures. Application of relatively safe fungicides could reduce the damage due to the disease. It is also necessary to investigate the reactions of the available citrus cultivars to the pathogen and select disease tolerant scions.

3.2. INTRODUCTION

A variety of fruit crops are grown in Ethiopia, and citrus has been cultivated for several decades throughout the country (Seifu, 2003; Kassahun *et al*., 2006; Mohammed, 2007). However, production and productivity of citrus is very low as compared to the potential that could be attained due to various production constraints. One of the major limitations to citrus production in the country is lack of good quality and high yielding varieties. Most fruit crops are relatively new to Ethiopian agriculture, and the culture of production and utilization is not well developed. Smallholder farmers predominantly use traditional technologies and field operation practices that contributed to the low fruit yield and quality (Seifu, 2003; Joosten *et al.*, 2011). Most farmers do not use agricultural inputs such as fertilizers and chemicals for fruit crops including citrus (Asmare and Derbew, 2013).

Citrus production and productivity is seriously threatened by various pathogenic diseases (Mohammed *et al.*, 2009) and numerous insect pests (Ferdu *et al*., 2009) resulting in declining

of citrus in the country. In the recent past decades, a number of surveys have been conducted in different parts of Ethiopia, and a large number of pathogens and insect pests have been identified and documented (Eshetu, 2006; Ferdu *et al.*, 2009; Mohammed *et al.*, 2009). Viruses and virus-like diseases (Tesfaye and Habtu, 1985; Lemma, 1994), citrus canker (*Xanthomonas axonopodis pv. citri*) (Eshetu and Sijam, 2007), and different fungal diseases (Tesfaye and Habtu, 1985; Eshetu, 1999; Mohammed, 2007; Sisay, 2007; Mohammed *et al.*, 2009) have been reported in various citrus orchards in the country. Some of these diseases are responsible for significant yield and quality losses in citrus resulting in decreased income for producers (Seifu, 2003; Mohammed, 2007; Sisay, 2007).

Citrus leaf and fruit spot is one of the major diseases causing serious damage in many citrus producing areas of Ethiopia (Eshetu, 1999; Yigzaw and Gelelbelu, 2002; Mohammed, 2007). Eshetu (1999) carried out a survey in southwest Ethiopia on sweet oranges and reported 100% incidence, and 43% severity on leaves and 65% on fruits. Mohammed (2007) conducted another survey in the south, southwest and northwest Ethiopia and reported incidences that ranged from 64.5 to 98.6% on leaves and 78.6 to 94.2% on fruits, and severity which varied from 52.4 to 84.0% on leaves and 65.6 to 90.4% on fruits.

Pseudocercospora angolensis was reported to cause leaf and fruit spot disease on citrus in tropical African countries and Yemen since its first record in Angola and Mozambique (Seif and Hillocks, 1993; Kuate, 1999; Pretorius *et al*., 2003; Mohammed, 2013). Due to the traditional trade of infected fruit and planting material, and the wind-borne dispersal nature of the fungal spores, the disease is a potential threat to the Mediterranean basin and South Africa (Seif and Hillocks 1993; Kuate, 1999), and to the major producing American and Asian countries that supply more than 70% of the world's citrus production (Ndo *et al*., 2010).

Citrus leaf and fruit spot disease affects virtually all citrus species (Kirk, 1986; Kuate, 1999; Dewdney and Timmer, 2009). It has become a serious citrus production constraint due to its impact on yield, quality and international trade (Mohammed, 2013). The disease occurs at all development stages and indiscriminately attacks leaves, fruits and young twigs. It causes

devastating lesions that result in considerable premature leaf and fruit drop, and blemish fruits that remain on the tree. It can weaken trees and jeopardize production in severe conditions (Eshetu, 1999; Timmer *et al*., 2003; Mohammed, 2007). The disease can cause 50 to 100% yield loss; and a single lesion usually renders the fruit unsalable (Seif and Hillocks, 1993, 1999; Kuate *et al.*, 2002; Chung and Timmer, 2009). It also affects the yield and quality of essential oils extracted from citrus fruit peel (Kuate *et al.*, 2003).

Assessment of geographical distribution of a disease and its economic importance is crucial for appropriate monitoring and to devise effective disease management strategy (McDonald and Linde, 2002; Mohammed, 2007; Sisay, 2007). However, comprehensive information on the current distribution of citrus leaf and fruit spot disease, and the extent of damage due to this disease is lacking. Therefore, this study was initiated to assess the distribution, incidence and severity of leaf and fruit spot disease of citrus in the major citrus growing areas of Ethiopia. In the surveys, general information on the present status of citrus production and management practices were also generated.

3.3. MATERIALS AND METHODS

3.3.1. Description of study areas

The geographic distribution and the climatic conditions of the citrus orchards surveyed are described in Fig 3.1 and Table 3.1. The geographic coordinates of the locations were taken using Global Positioning System (GPS 72H, Garmin Ltd., Taiwan) receiver. The areas covered in the surveys lie between 6.417° North latitude at Guangua town (Dilla area) in the south and 11.831° North latitude at Woldiya town in the northeast Ethiopia, while 35.593° East longitude at Bebeka in the southwest and 41.862° East longitude at Dire Dawa in the eastern Ethiopia. The locations surveyed have altitudes ranging from 900 m.a.s.l at Bebeka to 2000 m.a.s.l at Agaro, with a wide range of soil types.

According to the climatic information collected during the surveys (Table 3.1), the mean minimum temperature ranged from 10°C at Nura Era in the central rift valley to 21°C at Lado around Lake Abaya in the south. Similarly, the mean maximum temperature varied from 21°C at Bikolo in the northwest to 37.5°C at Nura Era. The mean annual rainfall for the surveyed areas ranged from 316 millimeter (mm) at Nura Era to 1750 mm at Bebeka. The major commercial citrus producing areas have long growing seasons with low precipitation, but most of them have a river, lake or ground water supply. However, the period of the rainy season and the precipitation intensity and distribution vary from place to place. The mean annual rainfall of the major commercial citrus producing areas was between 316 and 500 mm.

Land holding of citrus orchards in surveyed areas varied from 0.15 ha by smallholder farmers to 10,030 ha by Bebeka Coffee Estate farm. The area coverage of citrus plantation for these orchards ranged from a few citrus trees in the backyards of smallholders to 1,000 ha at Nura Era citrus farm. About 83.7% of the orchards surveyed had citrus trees of 10 ha or less, and 12.2% of the orchards were with citrus trees covering between 10 and 100 ha. Only a few citrus orchards had area coverage greater than 100 ha.

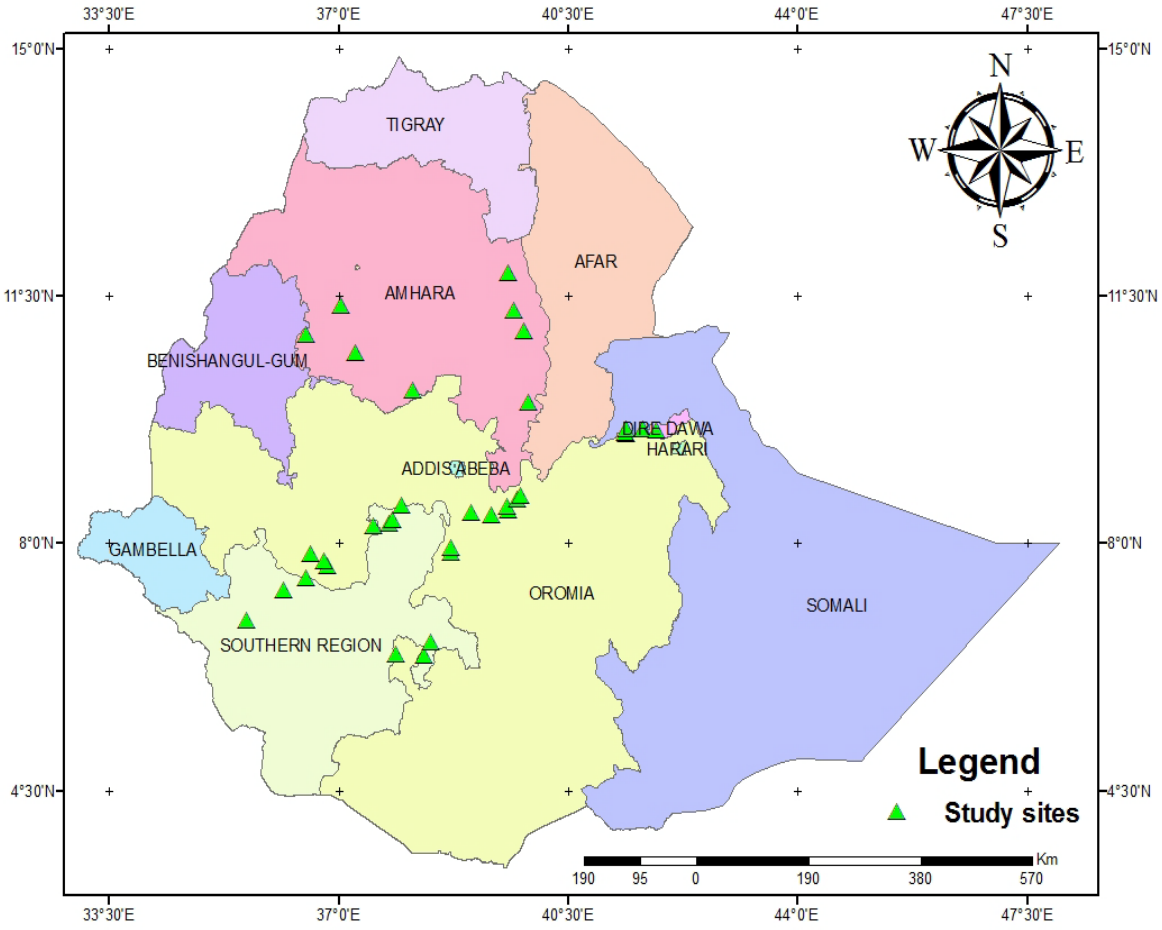

Fig 3.1. Map of Ethiopia showing the areas covered in citrus leaf and fruit spot disease surveys during 2012 and 2013.

Table 3.1. Geographic locations and climatic conditions of citrus orchards surveyed in 2012 and 2013 in Ethiopia

Citrus orchards	Regional State	Zone	District	Coordinates (Degree decimals)	Distance from AA (km)[a]	Altitude (m.a.s.l)	Mean temperature range (°C)	Mean annual rainfall (mm)	Relative humidity (%)	Soil type
Horizon Plantation, MIDROC										
Bebeka	SNNP	Bench-Maji	Debre Werk	6.904 N, 35.593 E	590 SW	900-1200	15-30	1750	No data	Vertisol
Nura Era	Oromia	East Shewa	Boset	8.670 N, 39.779 E	188 E	1100-1205	10-37.5	316	44.3	Clay to sandy loam
Merti	Oromia	Arsi	Merti	8.623 N, 39.722 E	177 E	1100	11-34	383	No data	Black soil
Abadeshka-Jeju	Oromia	Arsi	Jeju	8.514 N, 39.569 E	172 E	1100	11-34	383	No data	Black soil
Erer Gota Citrus Farms										
Erer	Somali	Shinele	Erer	9.567 N, 41.383 E	563 E	1120	18-27	425	No data	Sandy loam
Fetuli	Somali	Shinele	Erer	9.616 N, 41.395 E	567 E	1180	18-27	500	No data	Sandy loam
Gota	Somali	Shinele	Erer	9.550 N, 41.389 E	571 E	1120	18-26	425	No data	Sandy loam
Federal Prison Administration Citrus Farms										
Ziway	Oromia	East Shewa	Adami Tulu	7.945 N, 38.712 E	164 S	1680	13-28	600	52	Sandy loam
Shewarobit	Amhara	North Shewa	Kewet	10.002 N, 39.899 E	225 N	1320	16-30	425	44.5	Black heavy
Tifhste Genet, AfricaJuice Tibila Share Company	Oromia	Arsi	Jeju	8.470 N, 39.589 E	157 E	1240	16-36	700	No data	Clay loam
Gibe, Ethiopian Seed Enterprise	Oromia	Jimma	Sekoru	8.248 N, 37.540 E	185 SW	1100	15-35	800	No data	Vertisol
Government Fruit Nurseries										
Guangua	Oromia	Borena	Abaya	6.417 N, 38.308 E	365 S	1620-1680	18-24	1000	No data	Sandy soil
Bikolo	Amhara	West Gojjam	Mecha	11.367 N, 37.033 E	512 NW	1900	12-21	828	No data	Brown soil
Chagni	Amhara	Awi	Guangua	10.950 N 36.500 E	504 NW	1750	15-28	1665	No data	Red to brown
Finote Selam	Amhara	West Gojjam	Jabitehnan	10.700 N, 37.267 E	387 NW	1800	14-26	1270	No data	Red soil
Kurar	Amhara	East Gojjam	Dejen	10.167 N, 38.133 E	220 NW	1600	15-29	750	No data	No data
Harbu	Amhara	South Wello	Kalu	11.000 N, 39.833 E	355 N	1560	13-25	800	No data	Black soil
Citrus Foundation Blocks of the Research System										
Melkassa Research Center	Oromia	East Shewa	Adama	8.400 N, 39.333 E	115 SE	1550	13-28	750	77	Sandy loam
Jarre/Hayk Research Center	Amhara	South Wello	Tehuledere	11.300 N, 39.683 E	440 N	1700	14-28	500	No data	Clay loam
Jimma University	Oromia	Jimma Special	Jimma City	7.685 N, 36.832 E	350 SW	1780	14-30	1150	No data	Red soil
Tony Farm, Haramaya University	Dire Dawa	Dire Dawa	Dire Dawa	9.592 N, 41.862 E	515 E	1280	18-28	595	42.2	Sandy loam

Table 3.1. Continued.

Citrus orchards	Regional State	Zone	District	Coordinates (Degree decimals)	Distance from AA (km)	Altitude (m.a.s.l)	Mean temperature range (°C)	Mean annual rainfall (mm)	Relative humidity (%)	Soil type
Private Medium-Scale Commercial Citrus Farms										
Ethioflora PLC	Oromia	East Shewa	Adami Tulu	7.868 N, 38.726 E	171 S	1600	13-28	543	52	Sandy soil
Woldiya	Amhara	North Wello	Guba Lafto	11.831 N, 39.600 E	528 N	1760	14-27	800	No data	Brown soil
Koka	Oromia	East Shewa	Lume	8.434 N, 39.031 E	93 S	1595	13-24	880	No data	Sandy loam
Hurso, Military Training Center	Somali	Shinele	Erer	9.614 N, 41.643 E	550 E	1130	18-28	500	No data	Sandy loam
Orchards of Smallholders										
Omacho Chawa	SNNP	Sidama	Aleta Wendo	6.600 N, 38.417 E	337 S	1900	12-26	1400	No data	Red soil
Laygnaw Tatessa and Jejeba ena Gasorie	SNNP	Gurage	Abeshege	8.283 N, 37.783 E	160 SW	1830-1860	10.3-25.9	1244	No data	Red to brown
Aregita	SNNP	Gurage	Kebena	8.333 N, 37.833 E	148 SW	1780	10-26	1240	No data	Vertisol
Balewold and Megenagna	SNNP	Kaffa	Ginbo	7.333 N, 36.167 E	430 SW	1440-1500	11.9-26.4	1760	No data	Red brown sandy loam
Lado, Lake Abaya	Oromia	Borena	Abaya	6.433 N, 37.883 E	480 S	1280	21-32	900	No data	Clay black
Kishe-Kosta	Oromia	Jimma	Shebe Senbo	7.506 N, 36.514 E	394 SW	1440	13-27	1760	No data	Black soil
Agaro, Elbu, Genji Elbu and Koye Seja	Oromia	Jimma	Gomma	7.850 N, 36.583 E	390-405 SW	1680-2000	12-27.5	1760	No data	Red to Brown
Gube Bosoka (Yebu)	Oromia	Jimma	Mana	7.756 N, 36.776 E	370 NW	1620-1640	11.6-27.1	1640	No data	Red soil
Adami Wedessa	Oromia	Southwest Shewa	Goro	8.533 N, 37.967 E	134 SW	1860	8.8-23.3	1260	No data	Black soil
Harbu Tropical Fruits Propagator Farmers Association	Amhara	South Wello	Kalu	11.000 N, 39.833 E	355 N	1600	13-25	800	No data	Black soil

[a] AA = Addis Ababa

3.3.2. Sampling design, method of data collection and analysis

3.3.2.1. Prevalence, incidence and severity of leaf and fruit spot of citrus

A multistage sampling technique was used to select sample units (orchards, trees, and leaves and fruits). Five main citrus production regions were selected from the country, namely the South Nations, Nationalities and People (SNNP), Oromia, Amhara and Somali Regional States, and Dire Dawa City Administration. Then, four zones from SNNP, five zones from Oromia, six zones from Amhara, one zone from Somali, and Dire Dawa were selected based on citrus area coverage and annual production. A total of twenty-eight major citrus producing districts were selected from these seventeen citrus production zones of the country. All orchards in these districts that have five or more citrus trees were considered in the study. To this effect, a total of 49 citrus orchards were surveyed to assess the distribution, incidence and severity of leaf and fruit spot disease. The survey included citrus trees in the backyards of smallholder farmers, groves of Farmers' Association and Federal Prison Administration of Ethiopia, private and public medium and large scale commercial farms, fruit nurseries of the Ministry of Agriculture, and citrus foundation blocks in research centers. The field surveys were conducted between June 2012 and May 2013.

The status of citrus leaf and fruit spot disease was assessed by taking unsprayed citrus trees at each orchard by means of disease prevalence, incidence and severity. Samples were collected using destructive sampling method on leaves while intact fruits were used to avoid fruit loss due to picking (Eshetu, 1999). Geographic coordinates were used to map the distribution of leaf and fruit spot disease of citrus in the country.

Disease prevalence was assessed as the percentage of orchards in which leaf and fruit spot disease symptoms were observed to the total number of orchards surveyed in each district (Amata *et al*., 2009).

$$\text{Prevalence} = \frac{\text{Number of orchards with the disease symptoms}}{\text{Total number of orchards surveyed in each district}} \times 100$$

Five to ten representative citrus trees were selected randomly diagonally across the field in each orchard to determine disease incidence and severity of leaves and fruits (Seif and Hillocks, 1999; Mohammed, 2007). To estimate disease incidence on the foliage, leaves on eight randomly selected terminal shoots from the upper and lower halves of the canopy in the four directions of each tree were selected. The incidence was calculated by counting visibly infected leaves expressed as a percentage of the total number of leaves considered. Incidence on fruits was similarly assessed using five to forty randomly selected intact fruits per tree depending on availability. Incidence was calculated based on the presence or absence of visible disease symptoms on each fruit.

Disease incidence was calculated using the following formula:

$$\text{Disease Incidence} = \frac{\text{Number of infected leaves or fruits}}{\text{Total number of leaves or fruits assessed}} \times 100$$

Assessment of severity on the foliage and fruits was done on the same samples used for disease incidence. Severity on leaves was estimated based on a zero-to-four scoring scale, where 0 = no symptoms, 1 = 1 to 25%, 2 = 26 to 50%, 3 = 51 to 75% and 4 = above 75% of leaf area infected (Amadi, 2008; Ezeibekwe, 2011). The scoring system for fruit severity was: 0 = healthy, 1 = less than 5%, 2 = 5 to 20%, 3 = 21-50% and 4 = above 50% of fruit surface affected (Seif and Hillocks, 1999).

Disease severity was estimated using the formula suggested by Chaube and Singh (1991):

$$\text{Disease Severity} = \frac{\text{Sum of numerical ratings}}{\text{Total number of plants assessed} \times \text{maximum disease score}} \times 100$$

Disease incidence and severity data were angularly transformed to stabilize homogeneity of variance. Transformed data were subjected to analysis using Statistical Analysis System v9.2 (SAS Institute, Carey, North Carolina, USA). Pearson's correlation analysis was run to estimate the relationship between disease incidence or severity, and agro-climatic variables.

3.3.2.2. Status of citrus orchards in Ethiopia

During the surveys, questionnaire (Appendix Table 3.1) was used to collect general information about each citrus orchard from farmers, development workers, horticulture experts, and researchers. The questionnaire was compiled in two sections. The first section covered the history of each citrus orchard, such as farm size, rootstock and scion source, cultivars planted, orchard and/or tree age, ownership, flora composition around the orchard, soil type and application of fertilizer. The second section comprised the diseases and insect pests, and the management practices used in each orchard. Information collected from all citrus orchards was combined for each question and was summarized to give an overview of citrus production and field management practices in the country.

3.4. RESULTS

3.4.1. Leaf and fruit spot disease of citrus

3.4.1.1. Prevalence and distribution

The results of the field surveys indicated the current occurrence and distribution of leaf and fruit spot disease of citrus in Ethiopia (Fig 3.2 and Table 3.2). The disease was widely distributed in the south, southwest, central, and northwest parts of the country. The disease was found prevalent in 53.6% of the districts surveyed (15 of the 28 districts). Based on visual observations, no symptoms of citrus leaf and fruit spot disease were recorded in citrus orchards in thirteen districts of East Gojjam, North Shewa, North Wello, East Shewa, Shinele and Dire Dawa zones (Table 3.2). In South Wello zone, field symptoms of leaf and fruit spot-like disease were observed at Jarre.

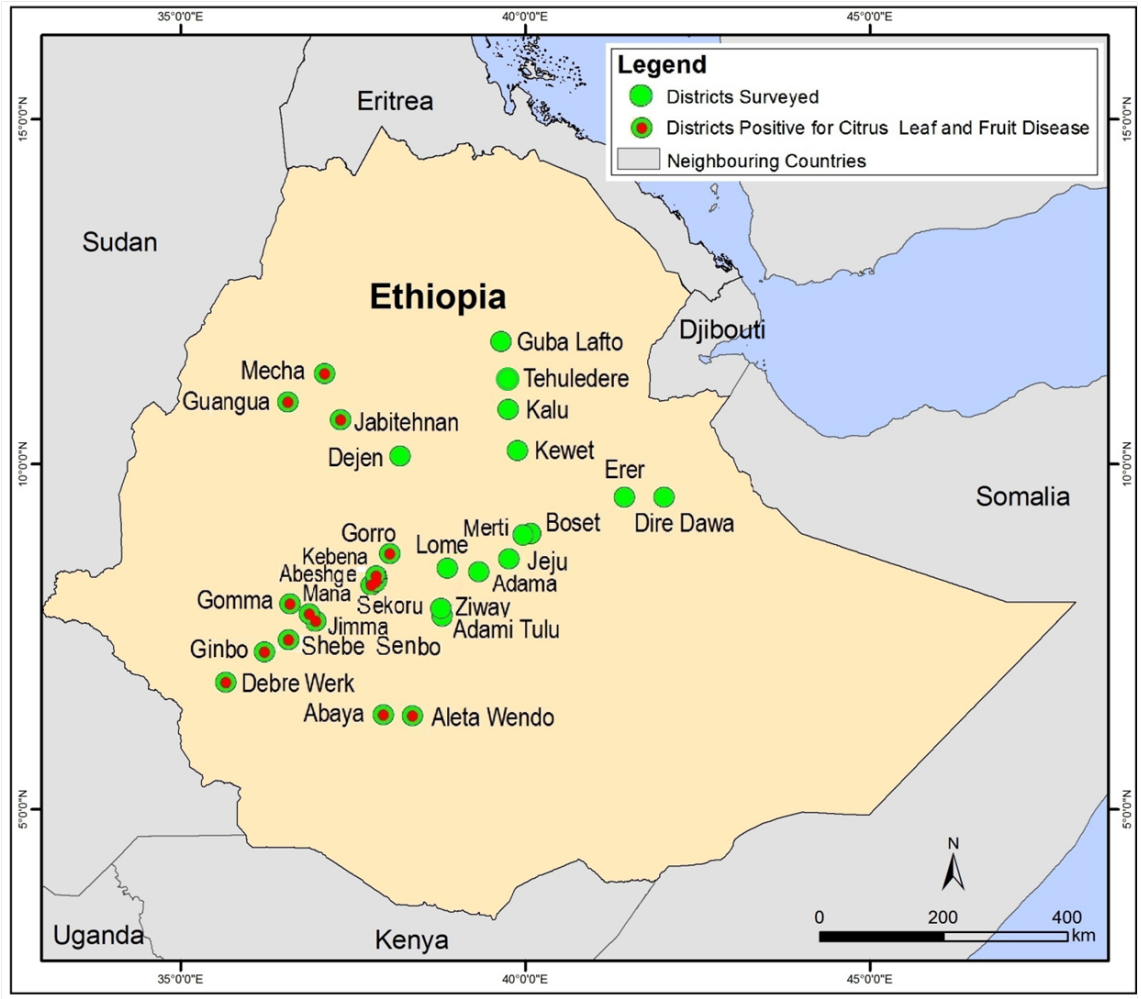

Fig 3.2. Map of Ethiopia showing the districts surveyed, and where the citrus leaf and fruit spot disease were recorded.

3.4.1.2. Disease incidence and severity

Citrus leaf and fruit spot disease affects the different parts of the plant. In the orchards surveyed, the disease commonly affected leaves and fruits. Symptoms were also observed on twigs of sweet oranges and mandarins. During the surveys, citrus growers indicated that the disease begins infection on leaves and then progresses to fruits and twigs. The disease caused leaf spots, blemish fruits that remain on the tree, premature leaf and fruit drop, and drying of tips of twigs. The characteristic field symptoms of citrus leaf and fruit spot disease are indicated in Fig 3.3A to F.

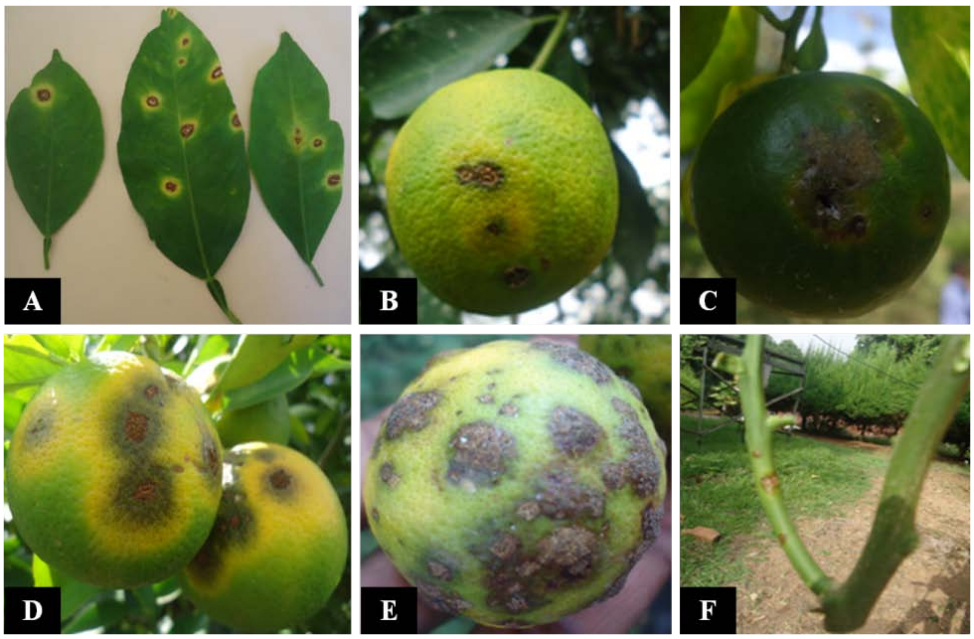

Fig 3.3. Symptoms of citrus leaf and fruit spot disease in Ethiopia. (A) Leaves with spots, (B to E) symptomatic fruits, and (F) young twig with lesions.

Field symptoms of the spot-like disease was observed on leaves and some fruits of sweet orange at Jarre orchard in south Wello zone (Fig 3.4A). Jarre is the only orchard in the northeast part of the country where spot-like disease symptoms were observed. Similarly, at Bebeka, spot-like symptoms that are similar to the citrus leaf and fruit spot disease were observed on a coffee shade tree (Fig 3.4B and C).

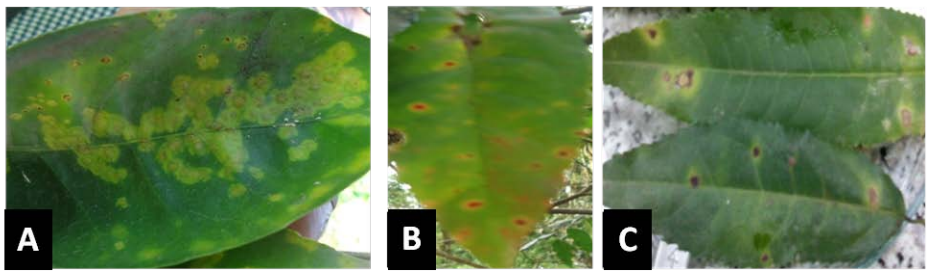

Fig 3.4. Leaf and fruit spot-like symptoms. (A) On sweet orange leaf at Jarre orchard, south Wello, and (B, C) on leaves of a coffee shade tree at Bebeka orchard, Bench Maji zone.

In the surveyed districts, the percentage of citrus leaf and fruit spot disease incidences and severities in the various citrus orchards ranged from zero to 100% (Table 3.2). The highest mean incidence on leaves was 60.4% in Jimma town followed by 58.6%, 56.1% and 50.2% in Abeshege, Aleta Wendo, and Kebena, respectively. The maximum incidence (100%) on fruits was recorded in Aleta Wendo. From 50 to 92% average disease incidences on fruits were recorded in Abeshege, Debre Werk, Mana, Kebena, Ginbo, Jimma town and Shebe Senbo districts. Similarly, the highest severity on leaves (75%) and fruits (100%) were recorded in Jimma town and Aleta Wendo, respectively (Table 3.2). Above 46% disease severity on leaves were recorded in Aleta Wendo, Abeshege and Debre Werk districts. Higher disease severity on fruits that ranged from 75% to 91.7% were recorded in six districts. In Aleta Wendo and several citrus orchards of small-scale farmers in Abeshege and Mana districts, complete damage of fruits was observed. Disease incidence and severity were nil on citrus leaves and fruits in 13 and 14 districts, respectively (Table 3.2).

In the current surveys, leaves were generally attacked less severely than fruits. Disease severity in Jimma town and Aleta Wendo was high on both citrus leaves and fruits. In Goro, it was observed that only citrus leaves were affected. However, severity on citrus leaves and fruits varied from district to district. Accordingly, mean incidence values on leaves and fruits at district level were 29.5% and 45.2%, while severity values were 31.9% and 52.6%, respectively. Jimma town, Abeshege, Aleta Wendo, Mana, Ginbo, Debre Werk and Kebena were the most seriously affected districts.

Table 3.2. Prevalence, incidence and severity of leaf and fruit spot disease of citrus in the major citrus growing districts of Ethiopia surveyed in 2012 and 2013.

	Prevalence	Incidence (%)[a]		Severity (%)[a]	
District	**(%)**	**Foliage**	**Fruit**	**Foliage**	**Fruit**
Jimma Town	100	60.4 (7.7)	75.0 (0.0)	52.0 (5.6)	90.0 (13.7)
Abeshege	100	58.6 (22.8)	50.0 (8.1)	92.3 (9.0)	90.0 (12.6)
Aleta Wendo	100	56.1 (20.8)	70.0 (11.2)	100 (0.0)	100 (0.0)
Kebena	100	50.2 (8.9)	25.0 (0.0)	72.0 (8.4)	75.0 (0.0)
Mana	100	48.1 (5.9)	40.0 (12.7)	75.3 (9.9)	91.7 (12.2)
Ginbo	100	34.1 (5.5)	25.0 (0.0)	64.0 (34.8)	75.0 (36.6)
Gomma	100	30.5 (13.6)	37.0 (14.8)	40.7 (25.8)	48.9 (31.5)
Debre Werk	100	28.0 (9.9)	46.9 (16.0)	81.5 (7.3)	75.0 (23.1)
Abaya	75.0	23.5 (18.4)	23.8 (19.0)	19.0 (34.0)	6.3 (13.8)
Shebe Senbo	66.67	22.7 (17.5)	18.3 (14.8)	62.5 (46.3)	65.0 (48.0)
Jabitehnan	100	21.4 (17)	41.7 (32.3)	29.7 (22.9)	58.3 (44.0)
Mecha	100	19.6 (3.3)	25.0 (0.0)	28.0 (5.6)	50.0 (0.0)
Sekoru	100	17.5 (8.2)	18.8 (11.1)	6.8 (12.3)	6.3 (11.1)
Guangua	100	14.9 (6.3)	23.0 (6.9)	29.1 (16.3)	50.0 (28.0)
Goro	100	2.1 (2.6)	10.4 (12.9)	0.0 (0.0)	0.0 (0.0)
Tehuledere	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Adama	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Adami Tulu	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Boset	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Dejen	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Dire Dawa	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Erer	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Guba Lafto	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Jeju	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Kalu	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Kewet	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Lume	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Merti	0.0	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Mean		29.5 (20.6)	31.9 (20.2)	45.2 (36.9)	52.6 (40.5)

[a] Figures in parenthesis are standard deviation values.

The summary of disease incidences and severity on citrus species is presented in Table 3.3. The mean disease incidence was higher on sweet oranges (38.2% on leaves and 63.9% on fruits) followed by mandarins (21.5% on leaves and 29.4% on fruits). Disease incidence was

relatively less on lemons (17.1% on leaves and 18.0% on fruits) and limes (16.3% on leaves and 16.7% on fruits). Disease symptoms were not observed on grapefruit and citron leaves and fruits, and on tangor/tangelo fruits. Disease severity followed the same trend as in disease incidence (Table 3.3). Higher disease severity were recorded on sweet orange (39.1% on leaves and 71.6% on fruits) and mandarin (33.3% on leaves and 50% on fruits); but it was less on lemon and lime trees.

Table 3.3. Average incidence and severity of leaf and fruit spot disease on different citrus species in 2012 and 2013 in Ethiopia

	Incidence (%)		**Severity (%)**	
Citrus species	**Foliage**	**Fruit**	**Foliage**	**Fruit**
Sweet orange	38.2	39.1	63.9	71.6
Mandarin	21.5	33.3	29.4	50.0
Lemon	17.1	25.0	18.0	25.0
Lime	16.4	16.7	16.7	25.0
Sour orange	23.3	26.9	3.1	3.8
Tangor/Tangelo	4.0	15.0	0.0	0.0
Grapefruit	0.0	0.0	0.0	0.0
Citron	0.0	0.0	0.0	0.0

Disease incidence on sweet orange ranged from zero to 81.7% on leaves and from zero to 100% on fruits, while disease severity varied from zero to 75% on leaves and from zero to 100% on fruits (Table 3.4). The disease prevailed on all sweet orange fruits sampled from citrus orchards in Aleta Wendo, Ginbo, Shebe Senbo and Welkite areas. In these areas, the disease severity was 100% and leaf and fruit spot disease caused nearly complete fruit drop. However, many citrus orchards surveyed did not show any incidence of the disease on both leaves and fruits of sweet orange. Sweet orange fruits were more severely affected by the disease than the leaves. Based on the field observations, among sweet orange varieties Washington Naval was highly attacked by citrus leaf and fruit spot while Jaffa and Hamlin were less affected.

Table 3.4. Incidence and severity of leaf and fruit spot disease on sweet orange at the different locations in 2012 and 2013 in Ethiopia

	Incidence (%)[a]		Severity (%)[a]	
Orchard	**Foliage**	**Fruits**	**Foliage**	**Fruits**
Welkite (Layignaw Tatessa) 2	81.7 (3.0)	100 (0)	55.0 (11.0)	100 (0)
Welkite (Layignaw Tatessa) 1	76.7 (1.8)	100 (0)	50.0 (0)	100 (0)
Jimma	60.4 (7.7)	52.0 (6.0)	75.0 (0)	90.0 (14.0)
Omacho Chawa	56.1 (20.8)	100 (0)	70.0 (11)	100 (0)
Yebu (Gube Bosoka 3)	52.4 (2.7)	72.0 (11.0)	50.0 (0)	90.0 (14.0)
Welkite (Aregita)	50.2 (8.9)	72.0 (8.0)	25.0 (0)	75.0 (0)
Yebu (Gube Bosoka 2)	48.4 (3.3)	82.0 (8.0)	40.0 (14)	95.0 (11.0)
Welkite (Jejeba ena Gasorie) 2	44.8 (5.4)	85.3 (6.0)	50.0 (0)	80.0 (11.0)
Agaro town	43.2 (10.7)	66.7 (12.0)	45.0 (11.0)	60.0 (29.0)
Guangua (Government nursery)	43.0 (10.9)	76.0 (9.0)	45.0 (21.0)	25.0 (18.0)
Finote Selam	39.5 (2.7)	40.0 (8.0)	75.0 (0)	100 (0)
Ginbo 2 (Megenagna)	38.0 (3.9)	100 (0)	25.0 (0)	100 (0)
Shebe (Kishe Kosta 2)	37.2 (4.3)	87.5 (9.0)	30.0 (11.0)	95.0 (11.0)
Agaro (Elbu 2)	36.7 (5.5)	42.0 (19.0)	30.0 (11.0)	55.0 (21.0)
Ginbo 1 (Balewold)	35.9 (4.5)	72.0 (11.0)	25.0 (0)	100 (0)
Agaro (Genji Elbu)	31.7 (1.9)	48.0 (4.0)	50.0 (0)	70.0 (11.0)
Welkite (Jejeba ena Gasorie) 1	31.1 (13.1)	84.0 (8.0)	45.0 (11.0)	80.0 (11.0)
Shebe (Kishe Kosta 1)	31.0 (8.0)	100 (0)	25.0 (0)	100 (0)
Bebeka	28.0 (9.9)	81.5 (7.0)	46.9 (16.0)	75.0 (23)
Gibe	25.5 (4.7)	27.0 (6.0)	25.0 (0)	25.0 (0)
Chagni	20.0 (1.1)	40.0 (4.0)	25.0 (0)	75.0 (0)
Bikolo	19.6 (3.3)	28.0 (6.0)	25.0 (0)	50.0 (0)
Agaro (Elbu 1)	18.9 (14.2)	22.5 (36.0)	25.0 (0)	30.0 (41.0)
Welliso (Adami Wedessa)	5.0 (0.8)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Guangua (Farmer)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Ethioflora	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Ziway Prison Farm	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Tibila (Tifhste Genet)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Abadeshka	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Merti	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Nura Era	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Woldiya	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Hayk/Jarre	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Harbu (Government nursery)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Harbu (Tropical Fruits Propagator Farmers Association)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Shewarobit	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Table 3.4. Continued.

	Incidence (%)[a]		Severity (%)[a]	
Orchard	**Foliage**	**Fruits**	**Foliage**	**Fruits**
Melkassa	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Tony Farm	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Erer	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Gota	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Fetuli	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Kurar	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Koka	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

[a] Figures in parenthesis are standard deviation values.

Among the locations surveyed, only three of them had leaf and fruit spot disease incidences on mandarin trees (Table 3.5). Higher disease incidence and severity were recorded at Finote Selam and Chagni in the northwest Ethiopia. No incidence of leaf and fruit spot disease was observed on fruits at Gibe.

Disease incidences on lemons and limes occurred at Chagni, Ginbo and Gibe orchards (Table 3.5). Similar trend was observed for disease severity for lemon and lime. Based on the results of the survey, lemons seemed relatively more susceptible to citrus leaf and fruit spot disease than limes. Lemons and limes at many places were not infected by the disease.

Symptoms of leaf and fruit spot disease occurred on leaves and fruits of sour orange in Yebu and Agaro areas; but it was not observed only on leaves at Lado, near Lake Abaya (Table 3.5). Incidence on leaves of tangor/tangelo trees was recorded only at Chagni (Table 3.5). During the present surveys, no incidence of citrus leaf and fruit spot disease was observed on grapefruit at Gota and Melkassa, and on citron at Goro and Melkassa orchards.

Table 3.5. Incidence and severity of leaf and fruit spot disease on mandarin, lime, lemon, citrus hybrids and sour orange at different orchards in 2012 and 2013 in Ethiopia

Citrus species	Orchard	Incidence (%)[a]		Severity (%)[a]	
		Foliage	Fruits	Foliage	Fruits
Mandarin	Finote Selam	24.6 (3.5)	49.0 (8.2)	50.0 (0.0)	75.0 (0.0)
	Gibe	20.7 (4.0)	0.0 (0.0)	25.0 (0.0)	0.0 (0.0)
	Chagni	19.2 (1.2)	39.3 (4.3)	25.0 (0.0)	75.0 (0.0)
	Ziway Prison Farm	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Tibila (Tifhste Genet)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Abadeshka	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Merti	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Nura Era	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Woldiya	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Hayk/Jarre	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Harbu (Government nursery)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Melkassa	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Tony Farm	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Gota	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Lime	Ginbo 3 (Megenagna)	28.4 (2.6)	20.0 (0.0)	25.0 (0.0)	25.0 (0.0)
	Chagni	14.2 (2.4)	30.0 (9.5)	35.0 (0.0)	50.0 (0.0)
	Gibe	6.5 (2.5)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Shebe (Kishe Kosta 3)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Welliso (Adami Wedessa)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Nura Era	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Harbu (Government nursery)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Melkassa	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Lemon	Gibe	17.2 (5.7)	0.0 (0.0)	25.0 (0.0)	0.0 (0.0)
	Chagni	16.9 (2.5)	36.0 (8.9)	25.0 (0.0)	50.0 (0.0)
	Ziway Prison Farm	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Hayk/Jarre	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Melkassa	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Tony Farm	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Finote Selam	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Woldiya	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

Table 3.5. Continued.

Citrus species	Orchard	Incidence (%)[a]		Severity (%)[a]	
		Foliage	Fruits	Foliage	Fruits
Sour Orange	Agaro (Koye Seja)	25.1 (20.9)	28.0 (25.9)	31.3 (23.9)	40.0 (37.9)
	Yebu (Gube Bosoka 1)	43.5 (7.5)	72.0 (8.4)	30.0 (11.2)	90.0 (13.7)
	Lado 1 (Farmer)	29.3 (15.6)	0.0 (0.0)	25.0 (0.0)	0.0 (0.0)
	Lado 2 (Farmer)	21.5 (5.4)	0.0 (0.0)	25.0 (0.0)	0.0 (0.0)
Tangor/ Tangelo	Chagni	4.0 (3.7)	0.0 (0.0)	15.0 (13.7)	0.0 (0.0)
	Abadeshka	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Merti	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Nura Era	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
	Melkassa	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)

[a] Figures in parenthesis are standard deviation values.

Disease incidence and severity in relation to agro-climatic conditions

Temperatures of the orchards had weak and negative correlation with the incidence and severity of leaf and fruit spot disease of citrus (Table 3.6). Citrus orchards affected by leaf and fruit spot disease had average temperatures that ranged from 12 to 35°C. However, the amount of rainfall had highly significant positive correlation with leaf and fruit spot disease incidence and severity (Table 3.6). Orchards with leaf and fruit spot incidences had mean annual rainfall of 500 to 1750 mm. Citrus orchards that received high rainfall exhibited high severity of leaf and fruit spot disease, while those areas that did not exhibit the disease had low annual rainfall. More severe leaf and fruit spot attacks were recorded in districts with high annual rainfall like Jimma town, Aleta Wendo, Welkite, Yebu, Ginbo and Bebeka. The disease was reported to be more severe during and right after the end of the rainy season, particularly in September and October. Altitude of the areas surveyed had also a positive relationship with the incidence and severity of the disease (Table 3.6). Districts with leaf and fruit spot incidences had altitudes varying from 900 to 2000 m.a.s.l. Those districts that were free from leaf and fruit spot incidences had temperatures ranging from 10 to 37.5°C, mean annual rainfall of 316 to 750 mm, and altitudes from 1100 to 1760 m.a.s.l. However, further studies in controlled environment are needed to validate these results.

Table 3.6. The relationship of disease incidence and severity of citrus leaf and fruit spot and the agro-climatic conditions of citrus orchards in Ethiopia

Incidence and severity	Mean temperature (°C)[a]	Mean annual rainfall (mm)[a]	Altitude (m)[a]
Disease incidence on foliage	-0.135 (0.492)	0.703 (0.000)	0.418 (0.027)
Disease severity on foliage	-0.170 (0.388)	0.749 (0.000)	0.401 (0.035)
Disease incidence on fruit	-0.277 (0.153)	0.750 (0.000)	0.339 (0.078)
Disease severity on fruit	-0.242 (0.216)	0.788 (0.000)	0.419 (0.0267)

[a] Figures in parenthesis are P values.

3.4.2. Assessment of citrus orchards in Ethiopia

3.4.2.1. Citrus production in Ethiopia

The orchards considered in the surveys represented plantings with ages ranging from two to 70 years. About 60% of the citrus orchards surveyed constituted younger tree age, 20 and less years while the rest (40%) of the orchards had citrus trees of over 20 years of age. Various citrus species and varieties are cultivated in the country. In many of the orchards, more than one citrus species were produced. The information obtained from the respondents showed that sweet orange, mandarin, lime, lemon, grapefruit, citrus hybrids, sour orange and citron were produced in 48, 18, 15, 13, 5, 3, 3 and 2 of the 49 orchards surveyed, respectively (Appendix Table 3.2). It was also indicated that the most dominant sweet orange variety produced in the country was Valencia (in 61.1% of the orchards surveyed) followed by unknown variety (58.3%), Washington Naval (47.2%), Hamlin (44.4%), Pineapple (38.9%), and Jaffa (16.7%). Algerian Tangerine (in 36.1% of the locations), Fairchild (30.6%) and Dancy (27.8%) were the most widely produced mandarin varieties. Among limes and lemons, UCR Meyer (in 25% of the locations), Bears (22.2%), Allen Eureka (13.9%) and Mexican Lime (11.1%) varieties were produced (Appendix Table 3.2). Citrus hybrids, grapefruit and citron varieties were reported as the least produced.

According to the information obtained from the respondents, citrus trees that were grown by smallholder farmers were directly from seeds and ungrafted. On the other hand, citrus trees in state and private owned orchards, government nurseries and research foundation blocks were grafted. The original sources of scions and rootstocks of most old orchards were unknown, but 33.3% of the respondents did not have available information. However, the major sources of the recent citrus plantations included University of California at Riverside, Melka Sedi Farm in the Middle Awash, Upper Awash Agro Industry Enterprise, Research Centers, ICRISAT, and local growers. The most commonly used rootstocks were Sour orange, Volkameriana and Troyer Citrange (Appendix Table 3.2).

Ownership of citrus orchards considered in the survey was also assessed. Nearly half of the orchards (24 out of 49) were owned by smallholder farmers. Eighteen orchards were state-owned (some of them were in the process of privatization), while six orchards were owned by private companies. Only one orchard was owned by Farmers' Association. However, in terms of area coverage, farms of the private companies, the government and Farmers' Association represented 1115.1 (68.5%), 503.52 (30.9%) and 8.5 (0.5%) ha, respectively. Smallholder farmers in total contributed only 1.0 ha (0.1%), because they grow a few or several citrus trees in their backyards.

According to the information obtained from the survey, several fruit crops (mainly mango, banana, avocado and papaya), vegetable crops (primarily tomato and onion), coffee, and maize were the major crops intercropped with most of citrus plantings and/or grown around citrus orchards.

3.4.2.2. Production constraints and management practices

Irrigation and fertilization

The mean annual rainfall reported for most commercial citrus orchards was below 500 mm (Table 3.1). These citrus orchards (24 out of 49) used nearby rivers or lakes for irrigation. The

frequency of irrigation water application in most of these orchards averaged once or twice per month using furrow irrigation. However, serious moisture stress which has caused leaf wilting, fruit cracking and premature fruit drop was observed at Gota, Nura Era, Abadeshka-Jeju, Jarre, Kurar and Goro citrus orchards.

Nutrient imbalance symptoms such as light color between veins and mottled leaves, green veins and light green to yellow background leaves, mid-vein blotches and leaf chlorosis, narrow and pointed leaves, and excessive defoliation and lateral growth, supposed to be caused due to deficiency of trace elements were observed in many of the orchards (65.3% of the orchards surveyed), especially in farms owned by smallholder farmers and by the state (Appendix Table 3.3). At Chagni, nutrient imbalance symptoms were observed on all citrus trees regardless of the species. Although there were no soil and plant tissue analyses, salinity problem was suspected at Ziway orchard. According to the information from the respondents, fertilizer application was not practiced in most of the citrus orchards, particularly those orchards owned by smallholder farmers (71.4%, or 35 out of 49). The commercial citrus producing orchards (18.4%, or 9) reported applying diammonium phosphate (DAP) and Urea fertilizers to their citrus trees (Appendix Table 3.3). The use of both chemical fertilizers and animal manure was reported by Ethioflora, Melkassa and Hurso citrus orchards. Application of animal manure and compost to citrus trees was reported by Woldiya and Bikolo citrus orchards, respectively. However, fertilizer application in all citrus orchards in the country was not based on soil and plant tissue nutrient analyses.

Preharvest diseases and disorders of citrus

Many of the respondents differentiated between damages caused by insect pests and diseases in their orchards. However, they have difficulties to differentiate diseases caused by pathogens from abiotic disorders. According to the information from the respondents and field observations during surveys, most citrus orchards had suffered from complex of diseases (Appendix Table 3.3). Virus and virus-like diseases including tristeza, greening and exocortis, and unknown diseases were reported to cause tree decline and dieback at Erer Gota, Hurso,

Tony, Nura Era, Merti, Melkassa, Koka, Ziway, Ethioflora, Shewarobit, Harbu, Gibe, Ginbo, and Bebeka citrus orchards. In these orchards, symptoms of leaf yellowing, mottling and cupping, dying of twigs, stunting and deterioration of trees were clearly observed.

Citrus trees at Fetuli, Hurso, Tony, Nura Era, Merti, Abadeshka-Jeju, Melkassa, Ethioflora, Shewarobit and Gibe orchards were affected by several diseases caused by plant pathogens including *Phytophthora* (primarily on sweet oranges and mandarins), citrus anthracnose (on mandarins) and citrus bacterial canker (on acid limes). Field symptoms of citrus leaf and fruit spot disease were observed in twenty-seven citrus orchards (55.1%) in the south, southwest, central and northwest parts of the country (Appendix Table 3.3). Symptoms of lichens (fungus and algae that grow together) on leaves and stems of most citrus trees at Bebeka and Ginbo, melanose at Gibe and Lado, sooty mold at Melkassa and Lado, and fruit rots at Melkassa and Tony citrus orchards were recorded during the survey. Nematode related problem was seen only at Tony farm. Deformation and scion-rootstock incompatibility (overgrowth of rootstocks) were observed at Ethioflora and Kurar citrus orchards.

Insect pests of citrus

Insect pests were found to be equally important to diseases. Survey results and field observation showed that leaf miners (in 77.6% of the orchards surveyed), red scales (in 57.1% of the orchards), citrus thrips (in 24.5% of the orchards), cottony cushion scales (in 16.3% of the orchards), Mediterranean fruit fly (in 14.3% of the orchards), and woolly whitefly (in 10.2% of the orchards) were reported as the major insect pests on citrus (Appendix Table 3.3). Attacks by false codling moth, mealy bug, bud mite, orange dog and termites were reported from several citrus orchards. Red scale infestation at Tibila citrus farm was extremely severe; it attacked leaves and fruits, made the twigs dry and led the trees deteriorate. Both red scale and leaf miner were the most serious pests attacking citrus trees at Melkassa orchard. At Nura Era, woolly white fly was found the most important pest of citrus.

According to the information obtained from the respondents, the start of insect attack and extent of damage in citrus orchards was found to vary depending on tree age and fruit maturity. Many respondents indicated very high leaf miner infestation on leaves of young plants and on newly emerging leaves of older citrus trees. Respondents also reported initial infestation of scale insects and Mediterranean fruit fly attacks during fruit development and ripening stages.

Citrus orchard management

Citrus trees in most of the orchards, especially those owned by smallholder farmers, were not well-managed. The citrus orchard at Bebeka had been neglected for the past several years due to citrus leaf and fruit spot disease. The disease severely affected the trees. In some other orchards like Guangua, weed infestation was very serious. In some orchards, only sanitations were practiced against citrus leaf and fruit spot disease. Citrus orchards at Bikolo, Chagni, Finote Selam, Nura Era and Merti did practice back pruning of dried twigs and branches. At Erer Gota, the second largest citrus farm next to Nura Era in area coverage, pruning of dead branches and uprooting of dead trees were practiced. Most medium and large scale citrus plantations were sprayed with pesticides. According to the information obtained from the respondents, government nurseries such as Bikolo, Chagni and Finote Selam stopped to propagate and distribute citrus planting materials due to citrus leaf and fruit spot disease.

About 43% of the citrus orchards surveyed apply pesticides as a major means of disease and/or insect pest control (Appendix Table 3.3). The remaining orchards, especially those owned by the smallholder farmers, do not use pesticides. Kocide 101, Mancozeb, Bayleton, Ridomil MZ, Ridomil 5G, and Daconil fungicides, as well as Diazinon, Karate, Selecron, Suprathion, Endosulfan, Ultracide, Confider, White mineral oil, Diazol, Thiodan, Sumithion, Dimethoate and Methidathion insecticides were applied to control diseases and insect pests in the orchards surveyed. Of these pesticides, Kocide 101, Mancozeb and Bayleton fungicides, and Diazinon and Karate insecticides were the most widely applied pesticides for citrus disease and insect pest control, respectively.

3.5. DISCUSSION

Citrus farming in Ethiopia is a mixed agriculture (Seifu, 2003; Sisay, 2007), in which, many horticultural, field and forage crops, and shade trees are grown within and around citrus plantations. According to the information from the present surveys, sweet orange, mandarin, lime, and lemon were the dominant citrus species produced in the country. Citrus hybrids, grapefruit and citron varieties were the least produced. The original sources of scions and rootstocks of most old orchards were either unknown, or the respondents did not have information (Asmare and Derbew, 2013). The lack of information in this regard could complicate management and breeding programs aimed at improving citrus production (Sisay, 2007). Establishment of new citrus plantations should be with known scions and rootstocks.

Ethiopia is endowed with diverse and extensive climate conditions that are suitable for the production of various crops (Efrem, 2006). Almost all citrus orchards surveyed have temperature ranges which are ideal for citrus production. The optimum temperature to induce flowering in citrus is between 13°C and 24°C with a tolerance range of plus or minus 3°C (Davies and Albrigo, 1994). Many parts of Ethiopia has a bimodal rainfall defining two seasons (Hailu, 2008). However, the period of the rainy season and the precipitation intensity and distribution vary from place to place. The mean annual rainfall of the major commercial citrus producing areas were between 316 mm and 500 mm, which necessitate the application of supplementary irrigation. Ethiopia has enormous water resources with high potential for irrigation (Sileshi *et al*., 2007; Hailu, 2008). Most of the major commercial citrus producing areas are with nearby river, lake or ground water supply used for irrigation purposes. The frequency of irrigation water application in most of these orchards averaged once or twice per month using double ring basin method of furrow irrigation (Seifu, 2003; Sisay, 2007).

Despite the available water potential, serious moisture stress which has caused leaf wilting, fruit cracking and premature fruit drop was observed at several citrus orchards. Application of surface water at wider intervals creates moisture stress during early spring while the tree is at flowering stage. This could result in excessive drop of flowers and immature fruits. Drought

followed by good rains could also lead to out-of season flowering and fruit set (Sisay, 2007). Long and wet rainy seasons favor the development of high disease pressure and increase preharvest fruit loss of citrus (Seifu, 2003; Sisay, 2007). Saturated and poorly drained citrus orchard soils can contribute to root rot and tree dieback, which may ultimately result in total yield loss (Sisay, 2007). Improper use of the irrigation system can create a direct contact between tree bark and surface water, which results in increased soil borne disease infections and eventually tree dieback (Caruso and Wilcox, 1990; Oudemans, 1999).

During the present surveys, disease and insect pest attacks, and weed infestation were observed in many of the citrus orchards. In the past, viruses and virus-like diseases including psorosis, tristeza and greening were reported to be of great economic importance and thought to play a significant role in the decline of citrus plantation in the country (Tesfaye and Habtu, 1985; Lemma, 1994; Mohammed and Getachew, 1995). Citrus canker, caused by a bacterium, is a serious disease of most commercial citrus cultivars and some citrus relatives (Schubert and Miller, 2000). Eshetu and Sijam (2007) reported the occurrence of citrus canker disease on Mexican lime and sour orange in some citrus orchards. Various fungal pathogens including *Phytophthora*, *Penicillium*, *Colletotrichum* and *Pseudocercospora* species were reported to seriously affect citrus trees in Ethiopia (Eshetu, 1999; Seifu, 2003; Mohammed, 2007; Sisay, 2007; Mohammed *et al.*, 2009).

Intrinsic factors such as lack of certified planting materials, inappropriate use of cultural practices, and adverse edaphic conditions may increase the rate of infection and the spread of diseases (Salerno and Cutuli, 1982). It has been observed that *Phytophthora* spp. cause the most serious problem and are economically important soil-borne diseases of citrus worldwide (Graham and Timmer, 1994) and in Ethiopia (Seifu, 2003; Sisay, 2007). The use of inappropriate irrigation system can result in increased *Phytophthora* infection and ultimately tree dieback (Caruso and Wilcox, 1990; Oudemans, 1999). Poor rootstock-scion combinations could also be attributed to high levels of gummosis and *Phytophthora* root rot (Ippolito *et al*., 1996).

Citrus leaf and fruit spot disease has been reported in various areas in the south, southwest, central and northwest Ethiopia (Eshetu, 1999; Yigzaw and Gelelbelu, 2002; Kassahun *et al*., 2006; Mohammed, 2007). The present survey showed that the disease was spread to new areas in the northwest like Bikolo and Bahir Dar. This was a clear indication for the need for continuous monitoring, and putting in place an internal quarantine system to avoid the spread of the disease in to new areas. The disease incidence and severity were higher in areas located in the south and southwest than those in the northwest Ethiopia. This could be due to the favorable weather conditions of the areas and the long time disease buildup since its first introduction in the south Ethiopia. Farmers in Aleta Wendo stated that symptoms of citrus leaf and fruit spot disease were first appeared in 1985. Yimenu (1993) reported the first observation of characteristic field symptoms of leaf and fruit spot disease on citrus trees in Aleta Wendo and Dale districts in 1988. According to farmers, citrus fruit used to be one of their major income sources; three to four trucks of sweet orange fruits from Aleta Wendo district alone had been supplied to the local markets daily before the disease affected their citrus plantations. Farmers indicated that in the past 20 years many citrus trees were abandoned, and replaced by other crops like coffee. The production environment also seemed to influence the prevalence of the disease. More severe citrus leaf and fruit spot disease attacks were recorded in areas with high annual rainfalls. No symptoms of citrus leaf and fruit spot disease were recorded in citrus orchards found in low rainfall areas of the country despite their proximity to areas highly affected by the disease. In the present surveys, the disease was reported to be more severe during and right after the end of the rainy season. These results were consistent with previous reports (Eshetu, 1999; Yigzaw and Gelelbelu, 2002; Mohammed, 2007).

It has been reported that citrus leaf and fruit spot disease affects fruits, leaves and young twigs of citrus (Seif and Hillocks, 1993; Eshetu, 1999; Mohammed, 2007). In the orchards surveyed, the disease commonly affected leaves and fruits, but severity varied from district to district. The variation of disease severity percentage on leaves and fruits from location to location was reported by Eshetu (1999) and Mohammed (2007). In Cameroon, susceptibility was suggested to vary with different periods of the year and with locations (Kuate, 1998).

Symptoms were also observed on twigs of sweet oranges and mandarins. During the surveys, citrus growers indicated that the disease begins infection on leaves and then progresses to fruits and twigs. In the field, the disease caused leaf spots, blemish fruits that remain on the tree, premature leaf and fruit drop, and drying of tips of twigs.

Sweet oranges and mandarins were more severely affected than sour orange, grapefruit, tangor/tangelo, lemon and lime. The disease did not affect citron trees. Moreover, the disease affected fruits more severely than leaves of the major citrus species. The observed differences in the incidence and severity of citrus leaf and fruit spot disease among the citrus species, and varieties within a species might be due to the differences in their resistance capabilities and the antifungal activities of their essential oils which prevent the infection of the pathogen. The results were consistent with the findings of previous studies (Emechebe, 1981; Kuate, 1998; Eshetu, 1999; Seif and Hillocks, 1999; Diallo *et al.*, 2003; Mohammed, 2007). Essential oils extracted from *Citrus latifolia* var Tahiti, and *C. aurantifolia* inhibited the growth of *Pseudocercospora angolensis* on artificial medium (Jazet Dongmo *et al.*, 2008, 2009). The variation in resistance between varieties of a species often resides in a physiological or biochemical differences between them (Wutscher, 1997). Sisay (2007) recommended that apart from efficient management practices, farmers should plant resistant varieties budded on resistant stocks, and adopt a comprehensive integrated approach to disease control involving biological, cultural and chemical methods. Trees planted around the periphery of the orchards are thought to be the potential sources of wind-borne conidia of the pathogen (Whiteside *et al*., 1988). Although the use of windbreak trees seems important from agro-ecological point of view and pest trap, field disease control by sanitation and clearing of inoculum source is important (Sierra *et al*., 1993; Pretorius, 2005).

Field symptoms of spots observed on leaves and some fruits of sweet orange at Jarre orchard in South Wello zone were different from the symptoms in other orchards. Pretorius (2005) reported similar symptoms in Zimbabwe as concentric ring blotch on citrus leaves caused by grey mite. However, grey mites were not observed at Jarre during the surveys. At Bebeka, spot-like symptoms that are similar to the citrus leaf and fruit spot disease were observed on a

coffee shade tree. It needs to be confirmed by characterization of the pathogen, and clarified whether the coffee shade tree is an alternate host.

Insect pests on citrus were found to be equally important to diseases. Sisay (2007) reported more than 50% preharvest fruit damage due to citrus insect pests. In the past few decades, large number of insect pests have been identified and documented. Accordingly, the major insect pests of citrus in the country included scales, woolly white fly, Mediterranean fruit fly, false codling moth, citrus thrips, leaf miner and other fruit flies (Emana *et al.*, 2003; Ferdu *et al.*, 2009). In the present surveys, all these insect pests were recorded at various orchards. . The start of insect attack and extent of damage in citrus orchards was found to vary depending on tree age and fruit maturity. In the current study, many respondents indicated very high leaf miner infestation on leaves of young plants and on newly emerging leaves of older citrus trees. Respondents also reported initial infestation of scale insects and Mediterranean fruit fly attacks during fruit development and ripening stages. In citrus cultivation, regular monitoring of orchard practices from land preparation to fruit maturity and harvesting can provide sufficient information to control infestations of citrus trees (Taylor, 1997).

As part of disease and insect pest management measures, government nurseries in Amhara region stopped to propagate and distribute citrus planting materials to limit the spread of citrus leaf and fruit spot disease. Some orchards practice back pruning of dried twigs and branches to rejuvenate new branches, and uproot dead trees. The use of proper irrigation methods and selection of disease resistant/tolerant rootstocks may reduce the risk of infection by soil borne pathogens (Salerno and Cutuli, 1982). Most medium and large scale citrus plantations apply pesticides as a major means of disease and insect pest control. Application of insecticides should not be only during the first observation of the pest because it may lead to ineffective control and can result in buildup of the inoculum over time and eventually disease outbreak in an area (Fry, 1977). It is required to investigate into alternative natural plant products (Tripathi and Dubey, 2004), microbial antagonists (Droby *et al.*, 1991) and the application of improved sanitary practices (Sierra *et al*., 1993; Wilson *et al*., 1995) in order to reduce the risks associated with ineffective application of pesticides and its environmental and

health considerations. In Ethiopia, cultural practices, a number of fungicides, and host plant resistance have been suggested for the management of citrus diseases (Mohammed *et al.*, 2009). Similarly, cultural practices, bait sprays and attractants, a number of insecticides, parasitoids and predators, and botanicals have been recommended for the management and control of major citrus insect pests (Tsedeke 1983, 1991; Ferdu *et al*. 2009).

In summary, disease and insect pests, insufficient improved varieties, and poor agronomic and other crop management practices were the major citrus production constraints. Proper orchard management practices such as irrigation, fertilization, spacing, and disease and insect pest control measures should be applied to improve citrus production and productivity in Ethiopia. Leaf and fruit spot disease is a serious threat to citrus production in the country. The disease has widely distributed in most citrus producing areas with high rainfalls. Citrus orchards in the south and southwest were more severely affected by the disease than those in the central and northwest parts of the country. The disease caused severe leaf and fruit damages which rendered significant defoliation and fruit drop. In some areas, complete loss of fruit yield was recorded. In the present study, the disease severity varied among different citrus species and locations. Disease severity was higher on sweet orange and mandarin. To manage the disease spread, citrus producers should maintain the nutritional status of their orchards, and practice general hygiene and sanitary measures such as removal of infected leaves and fruits, pruning of dead branches and twigs, and clearing of neglected orchards to reduce inoculum source. Domestic quarantine system should be in place to limit the disease spread to new areas through planting materials and/or fruits. Field application of relatively safe fungicides could reduce the damage of the disease. Differences in susceptibility were observed among and within species which necessitate studying the reactions of the available citrus varieties to the pathogen. Thus, selection and use of disease resistant/tolerant scion cultivars may be possible. Reliable and quick disease identification and monitoring techniques, and integrated disease management strategies need to be in place in the country.

REFERENCES

Amadi, J.E. 2008. Studies of the etiology of leaf spot of disease of rough lemon (*Citrus jambhiri*-B. Jamir; H. Jhambhiri). *Bio-Research*, 6:343-345.

Amata, R.L., M.J. Otipa, M. Waiganjo, M. Wabule, E.G. Thuranira, M. Erbaugh and S. Miller. 2009. Incidence, prevalence and severity of passion fruit fungal diseases in major production regions of Kenya. *Journal of Applied Biosciences*, 20:1146-1152.

Asmare Dagnew and Derbew Belew. 2013. Production, research and development status of fruits in Ethiopia. pp. 97-135. In: Brhane Gebrekidan, Seme Debela, Solomon Bekure, Taye Bezuneh, Solomon Hailemariam and Gete Zeleke (eds.), Proceedings of the State of Agricultural Science and Technology in Ethiopia, 28-30 November 2011, the Ethiopian Academy of Sciences (EAS), ILRI Campus, Addis Ababa, Ethiopia.

Cai, L., K.D. Hyde, P.W.J. Taylor, B.S. Weir, J. Waller, M.M. Abang, J.Z. Zhang, Y.L. Yang, S. Phoulivong, Z.Y. Liu, H. Prihastuti, R.G. Shivas, E.H.C. McKenzie and P.R. Johnston. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Diversity*, 39:183-204.

Caruso, F.L. and W.F. Wilcox. 1990. *Phytophthora cinnamomi* as a cause of root rot and dieback of cranberry in Massachusetts. *Plant Disease*, 74:664-667.

Chaube, H.S. and U.S. Singh. 1991. Plant disease management: principles and practices. CRS Press, Boca Raton, USA. 319 pp.

Chung, K.R. and L.W. Timmer. 2009. Citrus diseases exotic to Florida: *Phaeoramularia* fruit and leaf spot (PFLS). Fact Sheet PP-234. University of Florida, Gainesville, USA. Online: http://edis.ifas.ufl.edu/pp155. Retrieved October 28, 2011.

Crouch, J.A., B.B. Clarke and B.I. Hillman. 2009. What is the value of ITS sequence data in *Colletotrichum* systematics and species diagnosis? a case study using the falcate-spored graminicolous *Colletotrichum* group. *Mycologia*, 101:648-656.

Davies, F.S. and L.G. Albrigo. 1994. Citrus. CAB International, Wallingford, UK.

Dewdney, M. and L.W. Timmer. 2009. Citrus production and diseases in Ghana. In: Citrus Industry. Online: http://www.crec.ifas.ufl.edu/academics/faculty/dewdney/PDF/Ghanatripstory.pdf. Retrieved February 19, 2014.

Diallo, M.T.S., M. Camara, M.Y. Diane, A.S. Bah, A.M. Pivi and L. Traore. 2003. Towards control of citrus cercosporiose in Guinea. *Fruits*, 58:329-344.

Droby, S., E. Chalutz and C.L. Wilson. 1991. Antagonistic microorganisms as biological control agents of postharvest diseases of fruits and vegetables. *Postharvest News and Information*, 2:169-173.

Efrem Bechere. 2006. Agricultural research and development in Ethiopia. International Conference on a Development Strategy of Horn of Africa, Rural and Agricultural Development Panel, 20-22 October 2006, University of Texas, Arlington Engineering Hall.

Emana Getu, Eshetu Ahmed and Mohammed Yesuf. 2003. Woolly white fly: a new pest of citrus orchards in Ethiopia. The Eleventh Annual Conference of the Crop Protection Society of Ethiopia (CPSE), 5-6 June 2003, Addis Ababa, Ethiopia (Abstract).

Emechebe, A.M. 1981. Brown spot disease of *Citrus* caused by *Phaeoisariopsis* species. *Annals of Applied Biology*, 97:257-262.

Eshetu Bekele. 2006. Experience with management of major plant diseases in Ethiopia. pp. 25-34. In: Eshetu Bekele, Ferdu Azerefegn and Tsedeke Abate (eds.), Facilitating the Implementation and Adoption of Integrated Pest Management (IPM) in Ethiopia. Drylands Coordination Group Proceedings No. 17. Planning Workshop, 13-15 October 2003, Melkassa, Ethiopia.

Eshetu Derso. 1999. Occurrence, prevalence and control methods of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 54:225-232.

Eshetu Derso and K. Sijam. 2007. Citrus canker: a new disease of Mexican lime (*Citrus aurantifolia*) and sour orange (*C. aurantium*) in Ethiopia. *Fruits*, 62:89-98.

Ezeibekwe, I.O. 2011. Study of citrus disease prevalence on four citrus varieties at the National Institute of Horticultural Research (NIHORT) Mbato, Okigwe, Imo State, Nigeria. *African Journal of Plant Science*, 5:360-364.

Ferdu Azerefegne, Mohammed Dawd, Difabachew Belay and Bezawork Mekonnen. 2009. Review of entomological research on fruit crops in Ethiopia. pp. 69-92. In: Abraham Tadesse (ed.), Increasing Crop Production through Improved Plant Protection, Vol. 2. Plant Protection Society of Ethiopia (PPSE), EIAR, Addis Ababa.

Fry, W.E. 1977. Management with chemicals. pp. 213-332. In: J.G. Horsfall and E.B. Cowling (eds.), Plant Disease: An Advanced Treatise. Vol. I. How Disease Is Managed. Academic Press Inc., New York.

Graham, J.H. and L.W. Timmer. 1994. *Phytophthora* diseases of citrus. SL127, Reviewed June 2003. Institute of Food and Agricultural Sciences (IFAS), University of Florida, Gainesville, Florida. Online: http://orange.ifas.ufl.edu/mg/mg_compendium/pdffiles/ch/CH08700.pdf. Retrieved April 8, 2014.

Hailu Beyene Abera. 2008. Adoption of improved tef and wheat production technologies in crop-livestock mixed systems in northern and western Shewa Zones of Ethiopia. PhD Dissertation, University of Pretoria, Pretoria, South Africa.

Ippolito, A., F. Nigro and G. Lima. 1996. Influence of the scion on the response of sour orange rootstock to experimentally induced *Phytophthora* gummosis and root rot. *Proceedings of the International Society of Citriculture*, 1:385-388.

Jazet Dongmo, P.M., N.L. Tatsadjieu, E. Tchinda Sonwa, J. Kuate, P.H. Amvam Zollo and C. Menut. 2008. Antiradical potential and antifungal activities of essential oils of the leaves of *Citrus latifolia* against *Phaeoramularia angolensis. African Journal of Biotechnology*, 7:4045-4050.

Jazet Dongmo, P.M., N.L. Tatsadjieu, E. Tchinda Sonwa, J. Kuate, P.H. Amvam Zollo and C. Menut. 2009. Essential oils of *Citrus aurantifolia* from Cameroon and their antifungal activity against *Phaeoramularia angolensis*. *African Journal of Agricultural Research*, 4:354-358.

Joosten, F., D. Boselie, Bekele Wolde and Lemma Dessalegn. 2011. Exporting fruit and vegetables from Ethiopia: assessment of development potentials and investment options in the export-oriented fruit and vegetable sector. Ethiopia-Netherlands Horticulture Partnership Programme.

Kassahun Tessega, Temam Hussien and P.K. Sakhuja. 2006. Management of *Phaeoramularia* fruit and leaf spot disease of citrus in Ethiopia. *Agricultura Tropica et Subtropica*, 39:242-248.

Kirk, P.M. 1986. *Phaeoramularia angolensis*. CMI Descriptions of Pathogenic Fungi and Bacteria, No. 863. *Mycopathologia*, 94:177-178.

Kuate, J. 1999. Fruit and leaf spot disease caused by *Phaeoramularia angolensis*: a threat to citrus production. Research and Methods, FRuiTROP, No. 61.

Kuate, J. 1998. Citrus leaf and fruit spot disease caused by *Phaeoramularia angolensis*. *Cahiers Agricultures*, 7:121-129.

Kuate, J., P.M. Jazet Dongmo, D. Ducelier, F. Damesse, C. Menut and J.M. Bessiere. 2003. Effect of citrus leaf spot disease (*Phaeoramularia angolensis)* on the content and chemical composition of essential oils from orange peel. *Fruits*, 58:143-149.

Kuate, J., E. Foure, J. Foko, D. Ducelier and F. Tchio. 2002. Citrus phaeoramulariosis due to *Phaeoramularia angolensis* in Cameroon: parasitic expression at different altitudes. *Fruits*, 57:207-218.

Lemma Gebeyehu. 1994. Research on fruit crops diseases. pp. 232-242. In; E. Herath and Lemma Dessalegn (eds.), Horticulture Research and Development in Ethiopia. Proceedings of the Second National Horticulture Workshop, 1-3 December 1992, Addis Ababa, Ethiopia.

McDonald, B. and C. Linde. 2002. Pathogen population genetics, evolutionary potential, and durable resistance. *Annual Review of Phytopathology*, 40:349-379.

Mohammed Yesuf. 2013. *Pseudocercospora* leaf and fruit spot disease of citrus: achievements and challenges in the citrus industry: a review. *Agricultural Sciences*, 4:324-328.

Mohammed Yesuf. 2007. Distribution and management of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 62:99-106.

Mohammed Yesuf, Wondirad Mandefro, Eshetu Ahmed, Girma Adugna, Dereje Tadesse, Temam Hussen and Meki Shehabu. 2009. Review of pathological research on fruit crops in Ethiopia. pp. 231-252. In: Abraham Tadesse (ed.), Increasing Crop Production through Improved Plant Protection, Vol. II. Plant Protection Society of Ethiopia (PPSE). EIAR, Addis Ababa, Ethiopia.

Mohammed Yesuf and Getachew Ayana. 1995. A review of vegetables and fruit crops diseases research: achievements and prospects. pp. 129-140. In: Habtu Assefa (ed.), Proceedings of the 25th Anniversary of Nazareth Agricultural Research Center: 25 Years

of Experience in Lowland Crops Research, 20-23 September 1995. Nazareth Agricultural Research Center, Nazareth, Ethiopia.

Ndo, E.G.D., F. Bella-Manga, S.A. Ndindeng, M. Ndoumbe-Nkeng, A.D. Fontem and C. Cilas. 2010. Altitude, tree species and soil type are the main factors influencing the severity of *Phaeoramularia* leaf and fruit spot disease of citrus in the humid zones of Cameroon. *European Journal of Plant Pathology*, 128:385-397.

Oudemans, P.V. 1999. *Phytophthora* species associated with cranberry root rot and surface irrigation water in New Jersey. *Plant Disease*, 83:251-258.

Pretorius, M.C., P.W. Crous, J.Z. Groenewald and U. Braun. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. *Sydowia*, 55:286-305.

Pretorius, M.C. 2005. Epidemiology and control of *Pseudocercospora angolensis* fruit and leaf spot disease on citrus in Zimbabwe. MSc Thesis, Stellenbosch University, South Africa.

Salerno, M. and G. Cutuli. 1982. The management of fungal and bacterial disease of citrus in Italy. *Proceedings of the International Society of Citriculture*, 1981, 1:360-362.

Schubert, T.S. and J.W. Miller. 2000. Bacterial citrus canker. Florida Department of Agricultural Conservation Service (FDACS), Division of Plant Industry, Plant Pathology Circular 377 revised. Gainesville, Florida.

Seif, A.A. and R.J. Hillocks. 1999. Reaction of some citrus cultivars to *Phaeoramularia* fruit and leaf spot in Kenya. *Fruits*, 54:323-329.

Seif, A.A. and R.J. Hillocks. 1993. *Phaeoramularia* fruit and leaf spot of citrus with special reference to Kenya. *International Journal of Pest Management*, 39:44-50.

Seifu Gebre-Mariam. 2003. Status of commercial fruit production in Ethiopia. Ethiopian Agricultural Research Organization (EARO), Addis Ababa, Ethiopia.

Sierra, C.C., E.B. Molina, C.P. Zaldivar, L.P. Flores and L.P. Garcia. 1993. Effect of harvesting season and postharvest treatments on storage life of Mexican limes (*Citrus aurantifolia* Swingle). *Journal of Food Quality*, 16:339-354.

Sileshi Bekele Awulachew, Aster Denekew Yilma, Mekonnen Loulseged, W. Loiskandl, Mekonnen Ayana and Tena Alamirew. 2007. Water resources and irrigation development

in Ethiopia. Working Paper 123. International Water Management Institute, Colombo, Sri Lanka.

Sisay Bekele Mekbib. 2007. Identification of citrus (*Citrus sinensis*) postharvest pathogens from Ethiopia and their control. PhD Dissertation, University of Pretoria, South Africa. 225p.

Taylor, M.S. 1997. The future of citrus fruits in the fresh produce world: a discerning customer's view. *Proceedings of the International Society of Citriculture, The 8th International Citrus Congress*, 1:15-18.

Tesfaye Tedilla and Habtu Assefa. 1985. A review of research activities on fruit crop diseases in Ethiopia. pp. 263-276. In: Tsedeke Abate (ed.), Proceedings of the First Ethiopian Crop Protection Symposium, 4-7 February 1985, Addis Ababa, Ethiopia.

Timmer, L.W., S.M. Garnsey and P. Broadbent. 2003. Diseases of citrus. pp. 163-195. In: R.C. Ploetz (ed.), Diseases of Tropical Fruit Crops. CAB International, Wallingford.

Tripathi, P. and N.K. Dubey. 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biology and Technology*, 32:235-245.

Tsedeke Abate. 1983. Insecticides for the control of red scale *Aonidiella aurantii* (Maskell) (Homoptera: Diaspididae) in the Awash Valley, Ethiopia. *Ethiopian Journal of Agricultural Science*, 5:32-42.

Tsedeke Abate. 1991. Entomophagous arthropods on Ethiopia: a catalogue. Technical Manual No. 4. Institute of Agricultural Research (IAR), Addis Ababa.

Whiteside, J.O., S.M. Garnsey and L.W. Timmer. 1988. Compendium of citrus diseases. American Phytopathological Society Press, MN, USA.

Wilson, G.L., M.D. Boyette and E.A. Estes. 1995. Postharvest handling and cooling of fresh fruits, vegetables, and flowers for small farms. Horticulture Information Leaflet. North Carolina State University College of Agriculture, Cooperative Extension Service, USA. Online: http://www.ces.ncsu.edu/hil/hil-800.html. Retrieved April 7, 2014.

Wutscher, H.K. 1997. Soil acidity and citrus blight. *Communications in Soil Science and Plant Analysis*, 28:603-612.

Yigzaw Dessalegn and Gelelbelu Girma. 2002. *Phaeoramularia angolensis*: a citrus disease in Northwest Ethiopia. *AgriTopia*, 17(1):12-13.

Yimenu J. 1993. Preliminary survey report on leaf spot disease of citrus in Sidamo. Coffee and Tea Authority, Addis Ababa, Ethiopia.

4. IDENTIFICATION OF CAUSAL PATHOGEN OF LEAF AND FRUIT SPOT DISEASE OF CITRUS IN ETHIOPIA

4.1. ABSTRACT

The study was conducted to identify the causal pathogens of citrus leaf and fruit spot disease in Ethiopia, and analyze the species complexes. Fungal isolates were isolated from infected citrus leaves and fruits. Isolates were characterized based on their cultural, morphological and pathogenic characteristics. The fungal isolates produced colonies with compact, medium or sparse density. The average daily colony growth rate ranged from 0.04 to 2.3 cm. Some isolates were very slow-growing, whereas most cultures had characteristic fast-growing compact aerial mycelia. The colonies varied from white to dark gray in color. The majority of the isolates produced circular, wooly or cottony colonies with pale brown or grayish white color. Most isolates produces hyaline, ovoid to oblong, slightly curved or dumbbell shaped conidia. Pathogenicity tests were employed on detached leaves of different citrus cultivars, and necrotic lesions were developed. Based on cultural, morphological and pathogenic characters, several fungal pathogens were identified that could be associated with leaf and fruit spot disease of citrus. The identities and phylogenetic relationships of the isolates were further analyzed using three sets of universal primers that span internal transcribed spacers, portion of long subunit regions of the nuclear ribosomal DNA and partial actin gene sequences. The isolates were identified as *Alternaria*, *Cladosporium*, *Cercospora*, *Colletotrichum*, *Mycosphaerella*, *Penicillium*, *Podospora*, *Phoma*, *Pseudocercospora angolensis*, and unidentified fungal species. The majority of the fungal isolates (81%) belonged to *Colletotrichum gloeosporioides* species complex. These findings provide information on the causal pathogen of citrus leaf and fruit spot disease in Ethiopia and suggest the need for in-depth studies to determine the role of *C. gloesporioides* species complex in citrus leaf and fruit spot disease epidemiology. The information will also be useful in developing disease management measures against *C. gloesporioides*.

4.2. INTRODUCTION

Manner *et al.* (2006) have observed that numerous diseases are known to affect citrus. *Phytophthora* spp., *C. gloeosporioides*, *C. acutatum*, *Alternaria citri*, *Mycosphaerella citri*, *Elsinoe fawcetti*, *Botrytis cinerea*, *Diplodia natalensis*, and *Phomopsis citri* are the major fungal pathogens causing preharvest diseases on citrus worldwide (Browning *et al.*, 1995; El-Ghaouth *et al.*, 2002; Naqvi, 2004; Manner *et al.*, 2006; Lima *et al.*, 2011). *C. boninense* is also well established as preharvest anthracnose pathogen of a diverse range of host plants including citrus (Moriwaki *et al.*, 2003; Johnston *et al.*, 2005; Damm *et al.*, 2012a).

In Ethiopia, the major fungal diseases that have been reported to affect citrus at preharvest stage are foot rot and *Phytophthora* gummosis (*Phytophthora* spp.), anthracnose (*Colletotrichum* spp.), citrus leaf and fruit spot (*Pseudocercospora angolensis*), Alternaria leaf spot (*Alternaria citri*), and melanose (*Diaporthe* spp.) (Tesfaye and Habtu, 1985; Mohammed *et al.*, 2009). Among these diseases, citrus leaf and fruit spot has been reported to cause significant losses in many citrus producing areas of Ethiopia (Eshetu, 1999; Yigzaw and Gelelbelu, 2002; Seifu, 2003; Mohammed, 2007). Eshetu (1999) identified the causal pathogen of citrus leaf and fruit spot disease as *Phaeoramularia angolensis* based on its cultural and morphological characteristics. However, spot diseases on citrus could also be due to other fungal pathogens such as *Colletotrichum* spp. (Peres *et al.*, 2005; Da Silva and Michereff, 2013; Damm *et al.*, 2012a), or *Alternaria alternata* (Kohmoto *et al.*, 1993; Schultz *et al.*, 2013; Pegg *et al.*, 2014). The association of *Colletotrichum* species with the leaf and fruit spot of citrus was also reported in Kenya (Seif and Kungu, 1990). Therefore, it is crucial to accurately identify species and pathotypes that cause or contribute to initiation and development of a disease in order to understand the disease epidemiology and to devise and implement effective disease control measures (Freeman *et al.*, 1998; Sisay, 2007; Amata *et al*., 2009; Cai *et al.*, 2009; Crouch *et al*., 2009).

Morphological, cultural and host-preference criteria have been used for species identification and delimitation (Silva *et al.*, 2012). The shapes and the surface textures of fungal colonies provide useful information to determine the species. However, highly variable morphology and cultural characteristics depending upon experimental conditions, host, and variability in pathogenicity make reliable identification of pathogens very difficult (Atkins and Clark, 2004; Cai *et al.*, 2009; Coetzee *et al*., 2009; Phoulivong *et al.*, 2010). Moreover, a single species of a pathogen can cause diseases on various crops (Peres *et al.*, 2005; Da Silva and Michereff, 2013). However, it has been observed that different pathogen species or biotypes of a pathogen can be associated with a single host (Freeman *et al.*, 1998; Peres *et al.*, 2002). To overcome some of these limitations, DNA-based technologies have been extensively employed, and have become preferred methods in plant pathology (Bridge *et al*., 2003; Schaad *et al*., 2003).

Molecular markers have improved precision of identification and classification of pathogenic fungi (Weir *et al.*, 2012). The majority of studies employed for species identification have relied on the ITS region of the nuclear ribosomal DNA (Peres *et al.*, 2002; Cai *et al.*, 2009). However, the resolution provided by the ITS barcode may not be adequate to discriminate among individuals of a species complex, and to effectively differentiate physiological races (Weir *et al.*, 2012). The ITS sequence data available in public domains can also cause considerable confusion as sequence data are often entered and named incorrectly, and identical sequences have been recorded under different names (Crouch *et al*., 2009). Therefore, the use of multilocus nucleotide sequences has been recommended to diagnose and understand pathogen species (Farr *et al*., 2006; Cai *et al.*, 2009; Crouch *et al*., 2009).

At present, the available information on the causal pathogen of citrus leaf and fruit spot disease in Ethiopia is very limited (Mohammed, 2013). This shortcoming has prompted the need for analyzing the fungal pathogen species associated with citrus leaf and fruit spot disease. Therefore, the objective of the study was to identify the causal pathogen of citrus leaf and fruit spot disease in Ethiopia using conventional and molecular methods.

4.3. MATERIALS AND METHODS

4.3.1. Isolation of fungal isolates

A total of 223 fungal isolates (Appendix Table 4.1) were isolated on water agar from symptomatic citrus leaf and fruit samples collected from major citrus production areas of Ethiopia (Fig 3.2) during 2012 to 2014 using the procedures described by Eshetu (1999) with minor modifications. Infected leaf and fruit tissues were surface-sterilized in 70% alcohol for 1 min followed by 1% sodium hypochlorite for 10 min and rinsed three times with sterile distilled water. Sterilized leaf discs or fruit peel pieces were placed on PDA (Oxoid, UK) plates containing 50 ppm streptomycin sulphate and incubated at 25±1°C. Single spore or hyphal tip cultures were developed in water agar and subsequently transferred to PDA. Cultures were incubated at 25±1°C. Stock cultures were maintained on PDA slants in 10% glycerol at 4°C.

4.3.2. Cultural and morphological characteristics

Cultural and morphological characterization of pathogen isolates associated with citrus leaf and fruit spot disease was conducted at the National Biotechnology Center, Holetta, Ethiopia, and at BecA-ILRI hub, Nairobi, Kenya. Fungal growth on PDA was determined after seven days of incubation. Colony development was recorded as presence or absence of fungal growth from each isolated leaf or fruit tissue in each plate. Colony growth was measured as daily increase in diameter for each isolate. The colony growth rate was assessed as the seven day average of mean daily growth. Colony color and density (scored as compact, medium or sparse) were assessed by visual observation after seven days of growth. Isolates were examined for their mycelial and conidial morphological characters by mounting on slides with clear lactophenol with the aid of a stereomicroscope (Eshetu, 1999; Seif and Hillocks, 1999).

4.3.3. Pathogenicity tests

Fungal isolates were tested for pathogenicity on different citrus cultivars (each sweet orange, mandarin, lime, lemon and grapefruit varieties). Pathogenicity tests were carried out following the standard techniques (Kiraly *et al.*, 1974; Agrios, 2005) on apparently healthy detached leaves. Young leaves from two-year old plants were washed in distilled water and surface sterilized in 1% sodium hypochlorite solution for two min. Sterilized leaves were rinsed three times with sterile water. Two sterilized leaves were placed, by keeping the abaxial side up, in each plate containing water agar. Leaves were artificially inoculated with each isolate by placing drops of aqueous suspension of 1×10^6 conidia per milliliter (ml), or mycelia suspension (Eshetu, 1999; Seif and Hillocks, 1999). Conidia were obtained from a week old culture grown on PDA, suspended in sterile water, and filtered through two layers of sterile cheesecloth. Conidia concentration was adjusted using a hemacytometer (Reichert-Jung, Cambridge Instruments, Inc.). Each test isolate was inoculated on leaves in four replications. In all tests, inoculation with sterile water was used as control. Inoculated leaves were incubated for up to three weeks at 26°C, and were assessed daily for the development of disease symptoms (Eshetu, 1999; Amadi, 2008). At the end of each test, symptomatic tissue was surface disinfested and placed on water agar to confirm recovery of the inoculated isolates. Reisolated cultures were examined for growth and morphological characters with the parent cultures. The experiment was conducted twice.

4.3.4. Molecular identification and phylogenetic analysis of isolates

4.3.4.1. DNA extraction

Total genomic DNA was extracted using the procedures described earlier (Murray and Thompson, 1980; Cenis, 1992) with some modifications. Mycelium was scraped from the surface of week-old cultures, added with fine sand and crushed in a mortar and pestle. The finely ground mycelium were transferred into 1.5 ml eppendorf tubes with sterile

glass beads, and were pulverized with GenoGrinder at 25 rotation per minute (rpm) for 3 min in 0.5 ml extraction buffer [3% CTAB, 200 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 10 mM EDTA (pH 8.0), and 2% SDS (preheated at 65°C)]. To each sample, 150 µl of sodium acetate (3 M, pH 5.2) was added and mixed by inverting the tubes. The mixtures were incubated at -20°C for 10 min and centrifuged at 14800 rpm for 10 min at room temperature. The supernatant was transferred into a new eppendorf tube and 300 µl of ice-cold isopropanol was added and mixed by gentle inverting. The mixture was kept at room temperature for 5 min. Precipitated DNA was pelleted by centrifugation. The supernatant was poured off and the DNA pellet was washed with 0.5 ml of 70% ethanol and centrifuged for 5 min. The supernatant was decanted and the pellet was dried at room temperature by placing the tubes face down on paper towels for 20 min. The pellet was re-suspended in 50 µl low-salt TE buffer. The concentration and quality of DNA was determined using NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Walthum, MA), and visualized on a 1% agarose gel stained with SYBR Safe DNA gel stain under ultra-violet light (UVP BioImaging Systems, Upland, CA). The DNA was stored at -20°C.

4.3.4.2. PCR amplification

Three loci including the 5.8S nuclear ribosomal gene with the two flanking ITS regions, the portion of LSU, and partial sequences of the ACT gene were amplified and sequenced using universal primer pairs ITS-1F/ITS-4 (White *et al.*, 1990; Gardes and Bruns, 1993); LROR/LR5 (Vilgalys and Hester, 1990; Moncalvo *et al.*, 1995); and ACT-512F/ACT-783R (Carbone and Kohn, 1999), respectively. Genomic DNA from *Acremonium* species isolate 133 (obtained from Plant Pathology Lab of BecA-ILRI hub, Nairobi) was used as positive control whereas reaction with no DNA template was used as a negative control.

All PCRs were performed in 20 µl reactions containing AccuPower PCR PreMix (Bioneer, Daejeon, Republic of Korea), 0.8 µl of 10 µM of each forward and reverse primers, and 2.0 µl template DNA (20 ng/µl). PCR reactions were performed in a

GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA). The PCR cycling conditions for the ITS regions constituted an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 sec, primer annealing at 48°C for 45 sec, and primer extension at 72°C for 1 min; and a final extension step of 72°C for 10 min. The PCR programs for LSU were an initial denaturation step at 95°C for 5 min, followed by 35 cycles at 94°C for 30 sec, at 43°C for 30 sec, and at 72°C for 1 min; and a final extension step of 72°C for 10 min. PCR reaction profiles for partial ACT gene comprised an initial denaturation at 96°C for 2 min, followed by 35 cycles at 94°C for 30 sec, at 61°C for 45 sec, and at 72°C for 45 sec; and a final extension step of 72°C for 10 min.

PCR products were subjected to electrophoresis in 1.5% agarose gels stained with GelRed at 70 V for 45 min and visualized under UV light. The sizes of amplicons were determined against a 100 bp molecular weight marker (Invitrogen, Carlsbad, CA). The PCR products with the expected sizes were cleaned using the GeneJET (Thermo Fisher Scientific) for ITS and QIAquick (Qiagen, Venlo, The Netherlands) PCR purification kit for LSU and ACT as instructed by the manufacturers. The concentration and quality of the purified PCR products were determined by NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific) and visualized on 1.5% agarose gel electrophoresis.

4.3.4.3. DNA sequencing and alignment

Purified PCR products were sequenced using the same forward and reverse primers used for PCR amplifications with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and were run on an ABI 3130xl DNA analyzer (Applied Biosystems). The nucleotide sequence datasets were inspected, edited and assembled into consensus contigs using CLC Main Workbench v7.5.1 (CLC bio, Prismet, Denmark). The sequences were analyzed using BLASTN v2.2.30 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) program (Altschul *et al.*, 1990) against the GenBank database based on the best hits of the query sequences that were used to assign identities to the test isolates. Multiple

sequence alignments were performed with MAFFT v7.223 (Katoh and Standley, 2013) using the auto alignment strategy with the 200PAM/ K=2 scoring matrix and a gap opening penalty of 1.53 with an offset value of 0.0. The ambiguous regions of each gene sequences were removed with Gblocks v0.91b (Talavera and Castresana, 2007). Resulting sequence alignments were evaluated and manually edited where necessary using MEGA v6.06 (Tamura *et al.*, 2013) software.

4.3.4.4. Phylogenetic analyses

Phylogenetic analyses were performed on each multiple sequence alignment of the ITS, LSU and ACT as well as on the combined dataset of the three loci using statistical methods to differentiate the isolates by species complexes. The Modeltest (Posada and Crandall, 1998) implemented in the MEGA was used to estimate the best-fit models of nucleotide substitution and the corresponding general time-reversible substitution rate parameters, shape of the four-category gamma distribution and fraction of invariable sites for each gene using corrected Akaike Information Criterion and the Bayesian Information Criterion scores. For each locus, 201 sequence datasets were used to reconstruct phylogenetic trees.

To determine whether the three sequence datasets were congruent and combinable, tree topologies of 70% reciprocal Neighbor-joining (NJ) bootstrap with Maximum Likelihood (ML) distances (1000 replicates) with substitution models determined separately for each partition using Modeltest were compared visually (Mason-Gamer and Kellogg, 1996). Individual genes were broadly congruent, and nucleotide alignments of the three genes were concatenated using scripts in Microsoft Office Excel 2007 program.

Phylogenetic analysis using ML method was performed on the combined multilocus alignment. The evolutionary likelihood was computed using the Kimura 2- parameter substitution model (Kimura, 1980) and Subtree-Pruning-Regrafting branch-swapping algorithm with search level 3. All alignment gaps and missing data were excluded from

the analysis and the rate variation among sites was modeled with a gamma distribution. Phylogeny reconstruction was also performed by NJ method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together was evaluated with a bootstrap analysis with 1000 replicates (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2- parameter substitution model (Kimura, 1980). All alignment positions containing gaps and missing data were completely deleted and the rate variation among sites was modeled with a gamma distribution (shape parameter = 5). The resulting phylogenetic trees were drawn and edited using TreeGraph v2.4.0-456 beta (Stover and Muller, 2010). All branches with bootstrap values of less than 50% were collapsed.

4.4. RESULTS

4.4.1. Cultural and morphological characters

Fungi isolates from infected citrus leaf and fruit samples collected from major citrus growing areas of Ethiopia were identified based on cultural and morphological characteristics. The colonies of fungi isolates varied from white to dark gray (Fig 4.1). The majority of the isolates (77% of the 223) produced circular, wooly or cottony colonies with pale brown or grayish white color. The colonies for many cultures were light orange or creamy white. These colony appearances are typical for *Colletotrichum* spp. complex (Fig 4.2A to C). Some isolates (20% of the 223) formed light gray to dark gray colonies which might be *Colletotrichum* or *Alternaria* spp. (Fig 4.2D to F). The colony surface for four cultures was grayish in appearance, velvety and raised at the center, and the underside of the colony was dark green. These colony characteristics are typical for *P. angolensis* (Fig 4.2G).

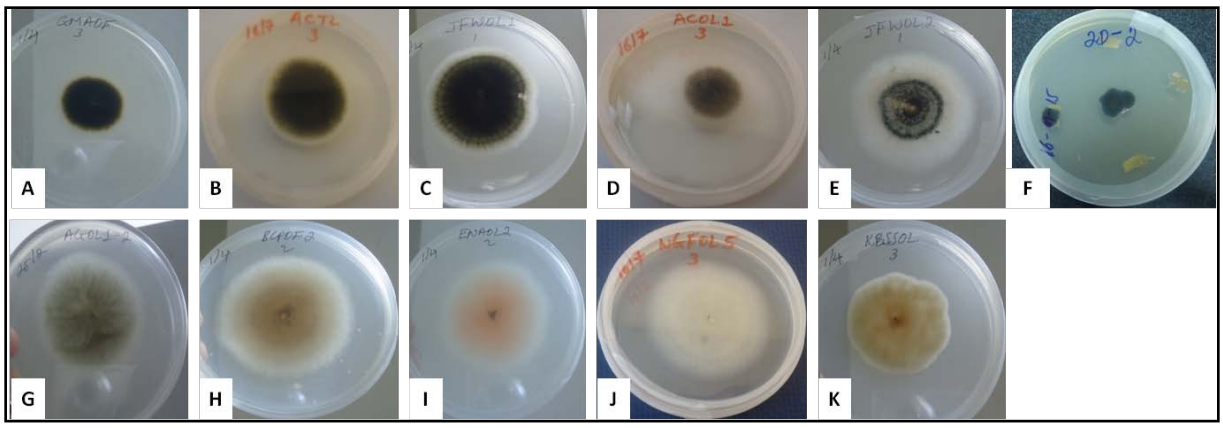

Fig 4.1. Colony characters of fungal isolates obtained from infected citrus leaves and fruits expressing symptoms of spot disease and grown on potato dextrose agar. (A, C) *Alternaria* spp., (B) *Phoma* sp., (D, E, H, I, J, K) *Colletotrichum* spp., and (F) *Pseudocercospora angolensis*.

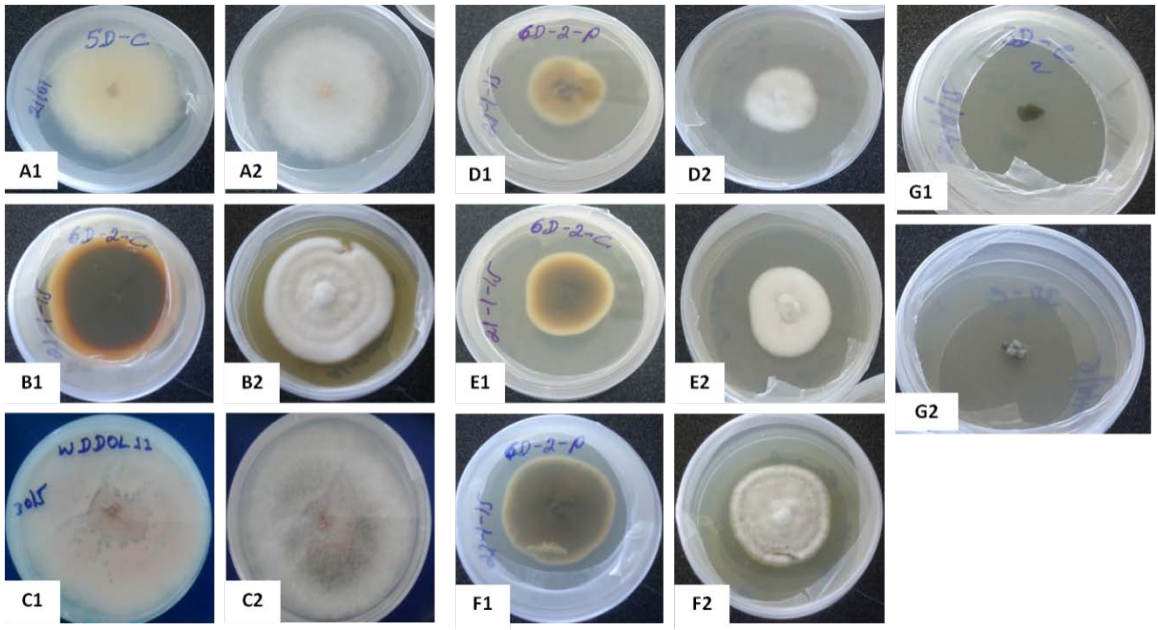

Fig 4.2. Colony appearances of fungal isolates grown on potato dextrose agar. A1 to G1 are the backside of the plates, and A2 to G2 are the front side of the cultures. (A, B, C, D, E) *Colletotrichum* spp., (F) *Phoma* spp., and (G) *Pseudocercospora angolensis*.

The colony density of the isolates varied from compact to sparse (Table 4.1). Among the 223 isolates, 128 produced compact colonies while 84 isolates were with medium density colonies. The remaining 11 isolates had sparse colonies. The daily colony growth rate

ranged from 0.04 to 2.3 cm (Table 4.2). Isolates of *Phoma* spp. and *Pseudocercospora angolensis* were compact and very slow-growing, whereas most isolates of *Colletotrichum* spp. exhibited fast growing. About 80.7% of the isolates had a daily growth rate ranging from 1.1 to 1.5 cm.

Table 4.1. Colony density of fungal isolates from infected citrus leaves and fruits

Colony density	**Number of isolates**	**Percent**
Compact	128	57.4
Medium	84	37.6
Sparse	11	5.0
Total	223	

Table 4.2. Colony growth of fungal isolates from infected citrus leaves and fruits

Number of isolates	**Average daily growth rate (cm)**
6	< 0.5
22	0.5 - 1.0
180	1.1 - 1.5
6	1.6 - 2.0
9	> 2.0
Total = 223	

Conidia were mostly born in single and sometimes in branched chains of two to four (Fig 4.3A and B). Most isolates produce hyaline, ovoid to oblong, slightly curved or dumbbell shaped conidia. However, there was variation in size and shape of conidia. Several isolates had spores that are ovoid to obclavate, club-shaped, broader near the base, but become taper to the apex, and with longitudinal and transverse septations. Many isolates totally failed to sporulate. The mycelium of most of the cultures was hyaline, septet and branched (Fig 4.3C).

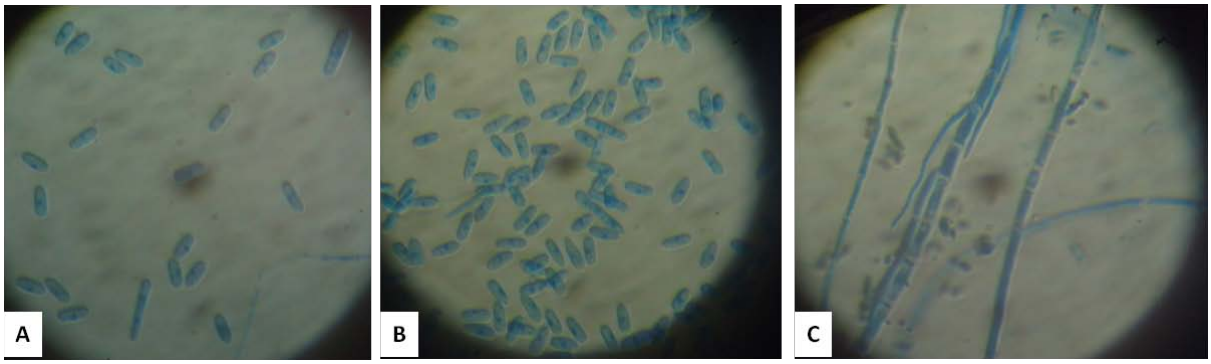

Fig 4.3. Microscopic morphological characters of a *Colletotrichum* isolate representing the majority of the isolates recovered from infected citrus leaves and fruits. (A, B) Conidia or spores, and (C) septated and branched mycelia.

Based on cultural and morphological characters, the isolates were belonged to several fungi species. Although it was difficult to accurately identify each fungal isolate, most of the isolates were not found identical to *P. angolensis* described by Kirk (1986) and Kuate (1998).

4.4.2. Pathogenicity test of fungal isolates

Pathogenicity tests were conducted on apparently healthy detached citrus leaves using Koch's method (Kiraly *et al.*, 1974; Agrios, 2005). Leaf spots on citrus leaves under natural conditions in the field are indicated in Fig 4.4A. Most of the fungal isolates caused foliar disease symptoms on artificially inoculated citrus cultivars (Olinda Valencia, Washington Naval, Clementine, Fairchild, Bears, UCR Mayer, Shamber) after incubation at 25°C for five days to two weeks (Fig 4.4B and C). The fungal isolates were consistently recovered from symptomatic leaf tissues. Many fungal isolates caused death of the entire leaf area, but some isolates did not cause any symptoms on all the inoculated citrus cultivars. Water inoculated controls remained healthy.

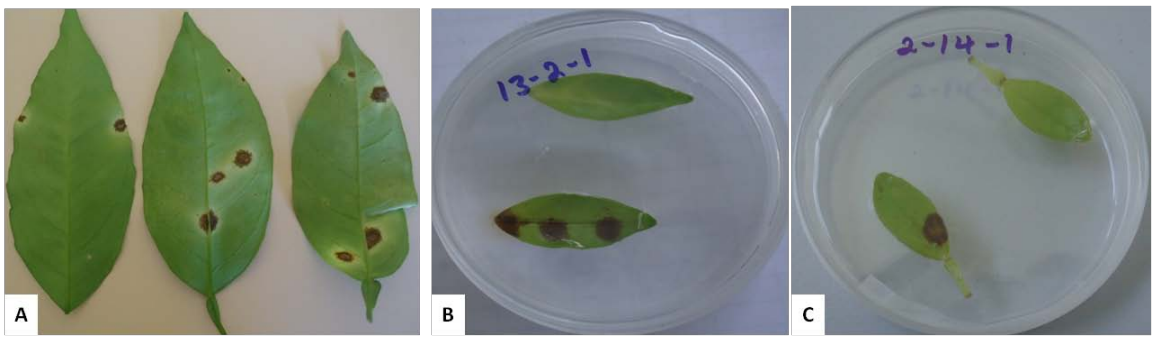

Fig 4.4. Pathogenicity test of two *Colletotrichum* isolates (ETHCTR047 and ETHCTR092 isolated from infected sweet orange leaf and fruit, respectively) on sweet orange leaves. (A) Naturally observed spots, and (B, C) symptoms developed on artificially inoculated leaves.

After molecular identification of the fungi isolates, detached citrus leaves were inoculated with each *C. gloeosporioides* or *P. angolensis* isolates alone, and the two together. Those leaves inoculated with only *C. gloeosporioides* (Fig 4.5A) or with the two together (Fig 4.5B) developed disease symptoms after incubation at room temperature for a week. Whereas, leaves inoculated with *P. angolensis* isolates did not develop any disease symptom after incubation for three weeks (Fig 4.5C).

Fig 4.5. Disease symptoms developed on inoculated detached citrus leaves. (A) *C. gloeosporioides* isolate (ETHCTR200), (B) both *C. gloeosporioides* and *P. angolensis* isolates, and (C) *P. angolensis* isolate (ETHCTR204).

4.4.3. Molecular identification and phylogenetic analyses

Three universal primer pairs (ITS-1F/ITS4, LROR/LR5 and ACT-512F/ACT-783R) were used to amplify the target ITS, LSU and ACT loci. Of the 223 isolates, 201 fungal DNA samples were successfully amplified and sequenced. Twenty-two isolates (ETHCTR005, ETHCTR010, ETHCTR011, ETHCTR030, ETHCTR079, ETHCTR083, ETHCTR087, ETHCTR099, ETHCTR102, ETHCTR135, ETHCTR145, ETHCTR147, ETHCTR149, ETHCTR150, ETHCTR155, ETHCTR171, ETHCTR177, ETHCTR191, ETHCTR195, ETHCTR196, ETHCTR199 and ETHCTR216) failed to amplify. The amplified ITS, LSU and ACT loci were approximately 600 bp, 900 bp and 300 bp in size, respectively (Fig 4.6A to C). The average sizes of assembled sequences of the test isolates used in the present study were 570 bp for ITS, 850 bp for LSU, and 250 bp for ACT gene. Among isolates, there were only slight variations in amplicon size with few inconsistencies due to variable length nucleotide repeats.

The sequences of the ITS, LSU and ACT barcode markers identified all the isolates, except one, to genus levels. Isolates were identified as several fungal species complexes using BLASTN search tool (Table 4.3). Of the 201 isolates, 81% were recognized as *C. gloeosporioides* sensu strico. The results indicated *C. gloeosporioides* was the main pathogen associated with citrus leaf and fruit spot disease in Ethiopia.

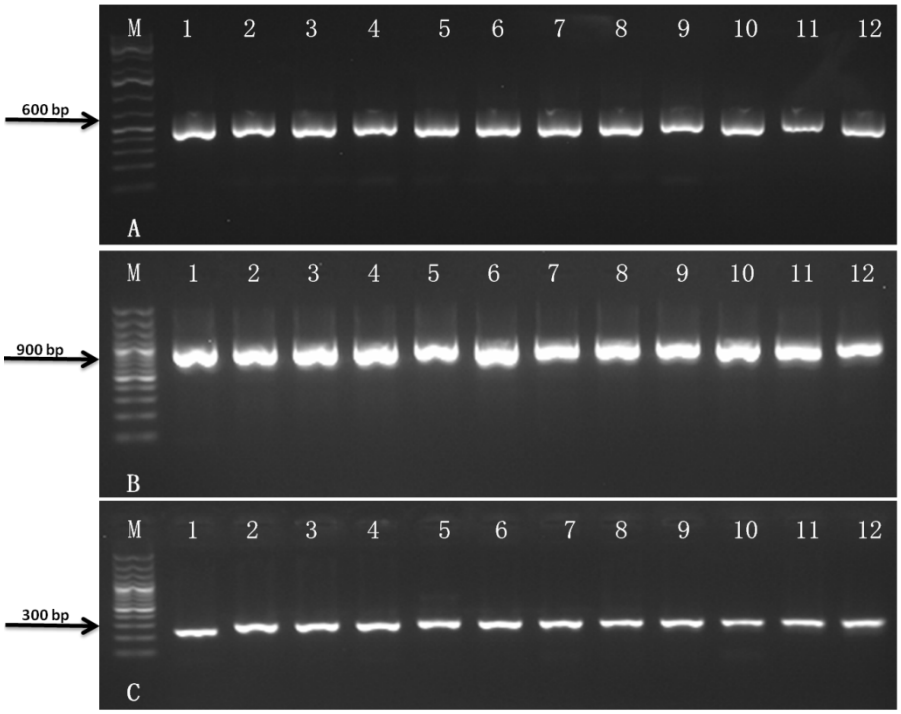

Fig 4.6. Sample gels for the detection of 201 fungal isolates with universal primer sets using polymerase chain reaction. (A) ITS-1F/ITS4, (B) LROR/LR5, and (C) ACT-512F/ACT-783R. Lanes M are GeneRuler 100-bp DNA Ladder, and lanes 1 to 12 are the test isolates (ETHCTR001, ETHCTR002, ETHCTR003, ETHCTR004, ETHCTR006, ETHCTR007, ETHCTR008, ETHCTR009, ETHCTR012, ETHCTR013, ETHCTR014 and ETHCTR015).

The trees drawn from each individual dataset using NJ and ML had similar topology (data not shown) for the 70% reciprocal NJ bootstrap trees, which allowed us to combine them. The phylogenetic analysis of the combined sequences from the three loci using NJ and ML methods resulted in similar grouping of isolates into various species complexes except the position (Figs 4.7 and 4.8). The analysis resulted in delineation of four main clades for the isolates studied. Most of the isolates clustered in the first main clade that consisted of several species complexes including *C. gloeosporioides* sensu lato, *C. boninense* sensu lato, *Podospora* species and undescribed fungus species. The second clade contained only one isolate, representing *Penicillium* species. *P. angolensis*, *Cercospora* and *Mycosphaerella* spp. belonged to the third main clade. The fourth main clade consisted of the *Cladosporium*, *Alternaria* and *Phoma* spp.

Table 4.3. Molecular identification of fungi species isolated from leaf and fruit spot infected citrus tissues in Ethiopia

Fungi species	**Number of isolates**	**Percent**
Colletotrichum gloeosporioides	163	81.0
Colletotrichum fructicola	4	2.0
Cercospora spp.	4	2.0
Mycosphaerella spp.	4	2.0
Phoma spp.	4	2.0
Pseudocercospora angolensis	4	2.0
Alternaria spp.	3	1.5
Colletotrichum boninense	3	1.5
Cladosporium spp.	2	1.0
Colletotrichum aenigma	2	1.0
Colletotrichum aotearoa	2	1.0
Colletotrichum asianum	1	0.5
Colletotrichum karstii	1	0.5
Colletotrichum spp.	1	0.5
Penicillium spp.	1	0.5
Podospora spp.	1	0.5
Unidentified fungus	1	0.5
Total	201	100

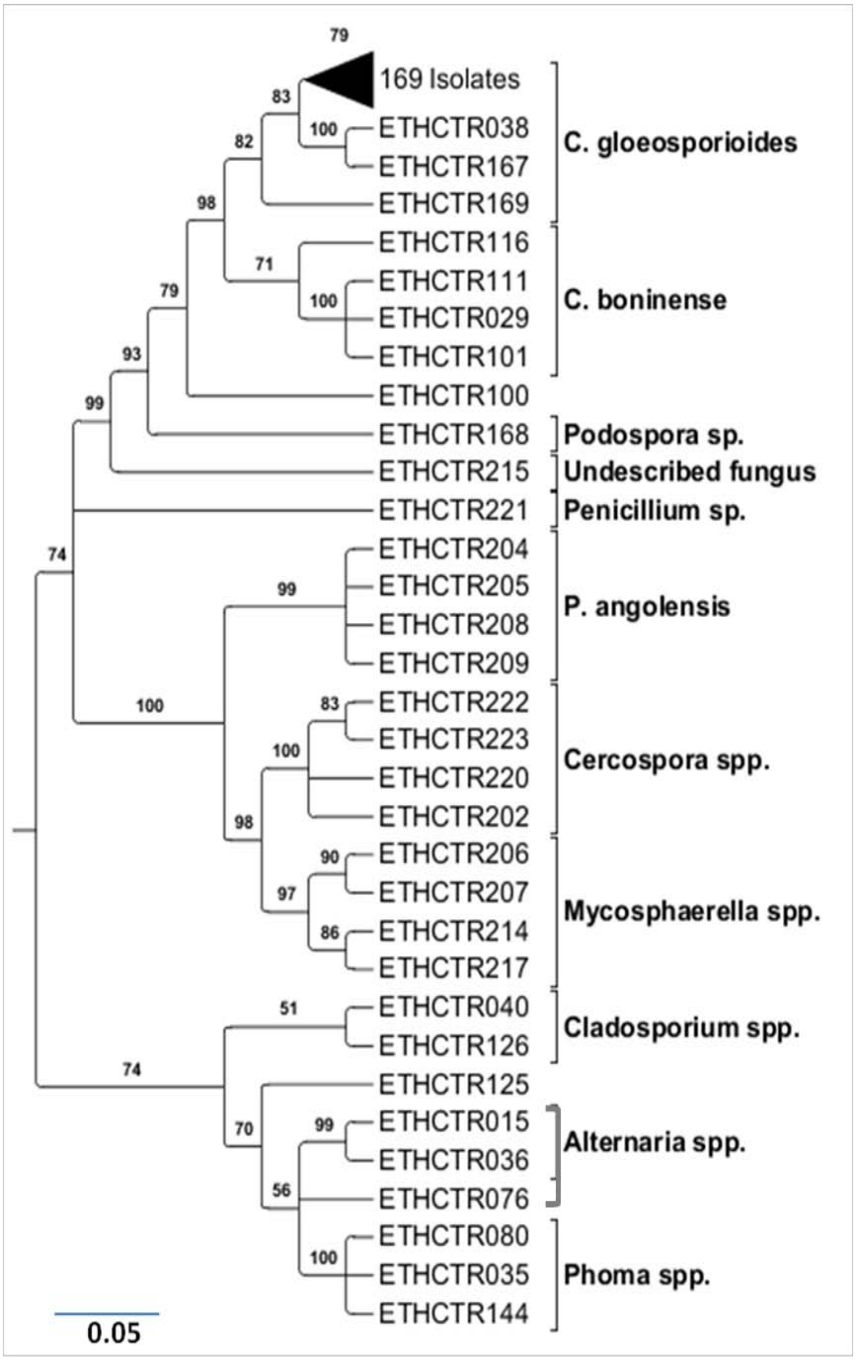

Fig 4.7. Maximum likelihood phylogenetic tree of fungi isolates based on concatenated ITS, LSU and ACT sequences of 201 isolates obtained from citrus in Ethiopia. Numbers above the nodes are bootstrap values (>50%). The scale bar indicates the number of expected nucleotide changes per site.

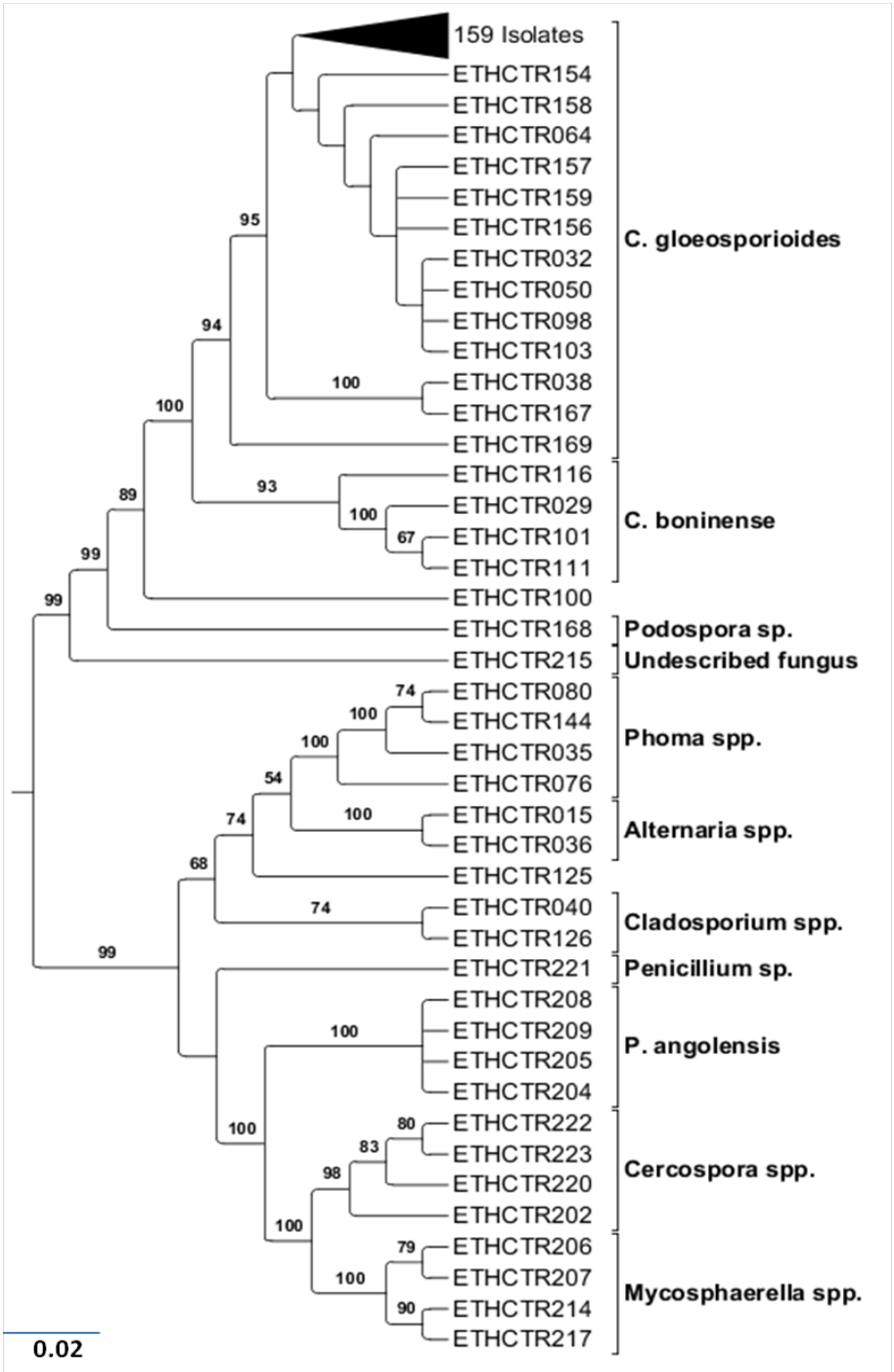

Fig 4.8. Neighbor-joining phylogenetic tree based on combined ITS, LSU and ACT sequences of 201 fungi isolates sampled from citrus in Ethiopia. Numbers above the nodes are bootstrap values (>50%). The scale bar indicates the number of expected nucleotide changes per site.

4.5. DISCUSSSION

The present study reports the cultural, morphological and molecular identification, and pathogenicity tests of fungal isolates associated with citrus leaf and fruit spot disease in Ethiopia. The cultural and morphological characters of the fungal isolates were identified by comparing with those descriptions reported by Kirk (1986) and Kuate (1998) for the *P. angolensis*, Damm *et al.* (2012a, b) and Weir *et al.* (2012) for *Colletotrichum* spp. complexes, and Kohmoto *et al.* (1979) and Pegg *et al.* (2014) for *Alternaria* spp. Based on colony and microscopic conidial characters of the fungal isolates and by comparing with other previous studies, the isolates were identified as different fungal species. Most of the pathogenic isolates showed cultural characters such as colony color, shape and growth on PDA that were similar to that previously described for *Colletotrichum* spp. complex (Damm *et al.*, 2012a; Weir *et al.*, 2012; Zivkovic *et al.*, 2012; Aiello *et al.*, 2015). Conidial morphological characters of these isolates were similar with those described for *C. gloeosporioides* (Weir *et al.*, 2012). Only four isolates showed colonial and morphological features that are similar with those reported in Kenya (Seif and Hillocks, 1993), Cameroon (Kuate, 1998) and Ethiopia (Eshetu, 1999).

Based on the pathogenicity observed after inoculation of apparently healthy citrus leaves with conidial or mycelial suspension, and comparing the results obtained with the symptoms of leaf and fruit spot, it was clear that the *Colletotrichum* isolates were able to cause the disease, while *P. angolensis* did not cause infection. This might be due to the use of inadequate pathogen inoculum (mycelial suspension) to cause infection. All *P. angolensis* isolates did not sporulate on PDA to use conidial suspension for inoculation. *Colletotrichum* spp. cause anthracnose spots and blights of aerial plant parts on various hosts (Dean *et al.*, 2012a). Typical symptoms of anthracnose disease appear as sunken, dark brown spots or lesions, that are semi-circular or angular shaped, surrounded by yellow halo on leaves, and often coalesce to form larger lesions (Agrios, 2005; Peres *et al.*, 2005; Cai *et al.*, 2009; Dean *et al.*, 2012a). These spots enlarge, and lead to wilting,

withering and dying of infected plant tissues (Hiremath *et al.*, 1993). Yet, the symptoms greatly vary from host to host (Gautam, 2014).

The *Colletotrichum* spp. isolated in this study has been reported to show highly variable cultural and morphological characteristics (Johnston et al. 2005; Weir et al. 2012) making these attributes less reliable to determine species complex (Phoulivong et al. 2010). Therefore, multiple gene sequences based molecular identification and phylogeny has been preferred over other methods (Crouch et al. 2009). However, the importance of linking morphological descriptions to multilocus molecular data in pathogen identification was described by Cai *et al.* (2009). The multigene phylogeny approach adopted in the present study identified all the isolates at genera level except one.

In the present study, the multilocus molecular approach reliably differentiated the fungi spp. The isolates obtained from citrus trees with symptoms of leaf and fruit spots were identified using the three DNA barcode markers and were belonged to eleven fungi species complexes (Table 4.3). Based on the analysis, more than 85% of the isolates were recognized as *Colletotrichum* spp. complexes. The *Colletotrichum* isolates were distributed across different taxa within *C. gloeosporioides* and *C. boninense* sensu lato complexes. Multilocus analyses have been demonstrated in various studies for identification and genetic delimitation of *Colletotrichum* spp. attacking various hosts (Farr *et al.*, 2006; Cannon *et al.*, 2012; Damm *et al.*, 2012a, b; Weir *et al.*, 2012; Huang *et al.*, 2013; Udayanga *et al.*, 2013).

Multilocus sequences were used to construct phylogenetic trees for species identification. All the ITS, LSU and ACT sequences were robust to differentiate species complexes. The trees constructed in the present study confirmed the delineation of four main clades and were detailed enough to enable the identification of all isolates (Figures 4.7 and 4.8). The different taxa identified in the present study have been reported as having worldwide geographic distribution and associated with plant diseases in many agriculturally important crops (Kohmoto *et al.*, 1993; Seif and Hillocks, 1993; Pretorius *et al.*, 2003;

Lima *et al.*, 2011; Damm *et al.*, 2012a; Weir *et al.*, 2012; Schultz *et al.*, 2013; Udayanga *et al.*, 2013; Pegg *et al.*, 2014). Different pathogen species or biotypes of a pathogen have been reported to be associated with a single host. It is also common to find a single species infecting multiple hosts (Freeman *et al.*, 1998; Peres *et al.*, 2002).

The results of the present study demonstrated that cultural, morphological and pathogenic characteristics supported by molecular analyses are reliable and useful approaches for species identification. Isolates of the *Colletotrichum* spp. identified in this study were pathogenic and caused leaf and fruit spot disease on citrus. These findings suggest that leaf and fruit spot disease control measures on citrus in Ethiopia should focus on *C. gloeosporioides*. Further in-depth studies are needed to examine roles of other associated pathogens in leaf and fruit spot disease initiation and development.

REFERENCES

Agrios, G.N. 2005. Plant pathology. 5th ed. Elsevier Academic Press, London, UK.

Aiello, D., R. Carrieri, V. Guarnaccia, A. Vitale, E. Lahoz and G. Polizzi. 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology*, 163:168-177.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215:403-410. http://blast.ncbi.nlm.nih.gov/Blast.cgi.

Amadi, J.E. 2008. Studies of the etiology of leaf spot of disease of rough lemon (*Citrus jambhiri*-B. Jamir; H. Jhambhiri). *Bio-Research*, 6:343-345.

Amata, R.L., M.J. Otipa, M. Waiganjo, M. Wabule, E.G. Thuranira, M. Erbaugh and S. Miller. 2009. Incidence, prevalence and severity of passion fruit fungal diseases in major production regions of Kenya. *Journal of Applied Biosciences*, 20:1146-1152.

Atkins, S.D. and I.M. Clark. 2004. Fungal molecular diagnostics: a mini review. *Journal of Applied Genetics*, 45:3-15.

Bridge, P.D., T. Singh and D.K. Arora. 2003. The application of molecular markers in the epidemiology of plant pathogenic fungi. pp. 93-113. In: D.K. Arora, P.D. Bridge and D. Bhatnagar (eds.), Fungal Biotechnology in Agricultural, Food, and Environmental Applications. Mycology Series, Vol. 21, Marcel Dekker Inc.

Browning, H.W., R.J. McGrovern, L.K. Jackson, D.V. Calvert and W.F. Wardowski. 1995. Florida citrus diagnostic guide. Florida Science Source Inc., Florida, USA.

Cai, L., K.D. Hyde, P.W.J. Taylor, B.S. Weir, J. Waller, M.M. Abang, J.Z. Zhang, Y.L. Yang, S. Phoulivong, Z.Y. Liu, H. Prihastuti, R.G. Shivas, E.H.C. McKenzie and P.R. Johnston. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Diversity*, 39:183-204.

Cannon, P.F., U. Damm, P.R. Johnston and B.S. Weir. 2012. *Colletotrichum* – current status and future directions. *Studies in Mycology*, 73:181-213.

Carbone, I. and L.M. Kohn. 1999. A method for designing primer sets for speciation studies in filamentous ascomycetes. *Mycologia*, 91:553-556.

Cenis, J.L. 1992. Rapid extraction of fungal DNA for PCR amplification. *Nucleic Acids Research*, 20:2380.

Coetzee, M.P.A., L. Maphosa, B.D. Wingfield, E. Mwenje and M.J. Wingfield. 2009. Characterization of *Armillaria* species based on pectic isozyme analyses. *Fungal Diversity*, 36:9-16.

Crouch, J.A., B.B. Clarke and B.I. Hillman. 2009. What is the value of ITS sequence data in *Colletotrichum* systematics and species diagnosis? A case study using the falcate-spored graminicolous *Colletotrichum* group. *Mycologia*, 101:648-656.

Da Silva, C.F.B. and S.J. Michereff. 2013. Biology of *Colletotrichum* spp. and epidemiology of the anthracnose in tropical fruit trees. *Revista Caatinga*, 26:130-138.

Damm, U., P.F. Cannon, J.H.C. Woudenberg, P.R. Johnston, B.S. Weir, Y.P. Tan, R.G. Shivas and P.W. Crous. 2012a. The *Colletotrichum boninense* species complex. *Studies in Mycology*, 73:1-36.

Damm, U., P.F. Cannon, J.H.C. Woudenberg and P.W. Crous. 2012b. The *Colletotrichum acutatum* species complex. *Studies in Mycology*, 73:37-114.

Dean, R., J.A.L. Van Kan, Z.A. Pretorius, K.E. Hammond-Kosack, A. Di Pietro, P.D. Spanu, J.J. Rudd, M. Dickman, R. Kahmann, J. Ellisand, G.D. Foster. 2012. The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13:414-430.

El-Ghaouth, A., C.L. Wilson, M. Wisniewski, S. Droby, J.L. Smilanick and L. Korsten. 2002. Biological control of postharvest disease of citrus fruits. pp. 289-312. In: S.S. Gnanamanickam (ed.), Biological Control of Crop Diseases. Marcel Dekker Inc., NY.

Eshetu Derso. 1999. Occurrence, prevalence and control methods of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 54:225-232.

Farr, D.F., M.C. Aime, A.Y. Rossman and M.E. Palm. 2006. Species of *Colletotrichum* on *Agavaceae. Mycological Research*, 110:1395-1408.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39:783-791.

Freeman, S., T. Katan and E. Shabi. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease*, 82:596-605.

Gardes, M. and T.D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. *Molecular Ecology*, 2:113-118.

Gautam, A.K. 2014. *Colletotrichum gloeosporioides*: biology, pathogenicity and management in India. *Journal of Plant Physiology and Pathology*, 2:2

Hiremath, S.V., P.C. Hiremath and R.K. Hegde. 1993. Studies on cultural characters of *Colletotrichum gloeosporioides*: a causal agent of Shisham blight. *Karnataka Journal of Agricultural Science*, 6:30-32.

Huang, F., G.Q. Chen, X. Hou, Y.S. Fu, L. Cai, K.D. Hyde and H.Y. Li. 2013. *Colletotrichum* species associated with cultivated citrus in China. *Fungal Diversity*, 61:61-74.

Johnston, P.R., S.R. Penny cook and M.A. Manning. 2005. Taxonomy of fruit-rotting fungal pathogens: what's really out there? *New Zealand Plant Protection*, 58:42-46.

Katoh, K. and D.M. Standley. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, 30:772-780. http://mafft.cbrc.jp/alignment/software/

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16:111-120.

Kiraly, Z., Z. Klement, F. Solymosy and J. Voros. 1974. Methods in plant pathology. pp. 135-136. Rev. Ed., Elsevier Scientific Publishing Company, New York, USA.

Kirk, P.M. 1986. *Phaeoramularia angolensis*. CMI Descriptions of Pathogenic Fungi and Bacteria, No. 863. *Mycopathologia*, 94:177-178.

Kohmoto, K., Y. Itoh, N. Shimomura, Y. Kondoh, H. Otani, M. Kodama, S. Nishimura and S. Nakatsuka. 1993. Isolation and biological activities of two host-specific toxins from the tangerine pathotype of *Alternaria alternata*. *Phytopathology*, 83:495-502.

Kuate, J. 1998. Citrus leaf and fruit spot disease caused by *Phaeoramularia angolensis*. *Cahiers Agricultures*, 7:121-129.

Lima, W.G., M.B. Sposito, L. Amorim, F.P. Golcalves and P.A.M. de Filho. 2011. *Colletotrichum gloeosporioides*, a new causal agent of citrus post-bloom fruit drop. *European Journal of Plant Pathology*, 131:157-165.

Manner, H.I., R.S. Buker, V.E. Smith, D. Ward and C.R. Elevitch. 2006. *Citrus* (citrus) and *Fortunella* (kumquat). Species Profiles for Pacific Island Agroforestry. 35p. Online: www.traditionaltree.org. Retrieved May 10, 2011.

Mason-Gamer, R.J. and E.A. Kellogg. 1996. Testing for phylogenetic conflict among molecular data sets in the tribe *Triticeae* (Gramineae). *Systematic Biology*, 45:524-545.

Mohammed Yesuf. 2013. *Pseudocercospora* leaf and fruit spot disease of citrus: achievements and challenges in the citrus industry: a review. *Agricultural Sciences*, 4(7):324-328.

Mohammed Yesuf. 2007. Distribution and management of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 62:99-106.

Mohammed Yesuf, Wondirad Mandefro, Eshetu Ahmed, Girma Adugna, Dereje Tadesse, Temam Hussen and Meki Shehabu. 2009. Review of pathological research on fruit crops in Ethiopia. pp. 231-252. In: Abraham Tadesse (ed.), Increasing Crop Production through Improved Plant Protection, Vol. II. Plant Protection Society of Ethiopia. EIAR, Addis Ababa, Ethiopia.

Moncalvo, J.M., H.H. Wang and R.S. Hseu. 1995. Phylogenetic relationships in *Ganoderma* inferred from the internal transcribed spacers and 25S ribosomal DNA sequences. *Mycologia*, 87:223-238.

Moriwaki, J., T. Sato and T. Tsukiboshi. 2003. Morphological and molecular characterization of *Colletotrichum boninense* species nov. from Japan. *Mycoscience*, 44:47-53.

Murray, M.G. and W.F. Thompson. 1980. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Research*, 8:4321-4325.

Naqvi, S.A.M.H. 2004. Diagnosis and management of certain important fungal diseases of citrus. pp. 247-290. In: S.A.M.H. Naqvi (ed.), Diseases of Fruits and Vegetables: Diagnosis and Management, Vol. I. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Pegg, K., J. Duff and A. Manners. 2014. *Alternaria* diseases in production nurseries. Agri-science Queensland, Department of Agriculture, Fisheries and Forestry, Australia.

Peres, N.A., L.W. Timmer, J.E. Adaskaveg and J.C. Correll. 2005. Lifestyles of *Colletotrichum acutatum*. *Plant Disease*, 89:784-796.

Peres, N.A.R.. E.E. Kuramae, M.S.C. Dias and N.L. de Sousa. 2002. Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil. *Journal of Phytopathology*, 150:128-134.

Phoulivong, S., L. Cai, H. Chen, E.H.C. McKenzie, K. Abdelsalam, E. Chukeatirote and K.D. Hyde. 2010. *Colletotrichum gloeosporioides* is not a common pathogen on tropical fruits. *Fungal Diversity*, 44:33-43.

Posada, D. and K.A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14:817-818

Pretorius, M.C., P.W. Crous, J.Z. Groenewald and U. Braun. 2003. Phylogeny of some cercosporoid fungi from *Citrus*. *Sydowia*, 55:286-305.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4:406-425.

Schaad, N.W., R.D. Frederick, J. Shaw, W.L. Schneider, R. Hickson, M.D. Petrillo and D.G. Luster. 2003. Advances in molecular based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annual Review of Phytopathology*, 41:305-324.

Schultz, D., M. Rybak, R. Rybak and R.D. French. 2013. Alternaria brown spot. Texas A&M AgriLife Extension Service, PLPA-Cit013-04. Online: http://sickcitrus.tamu.edu. Retrieved June 1, 2015.

Seif, A.A. and R.J. Hillocks. 1999. Reaction of some citrus cultivars to *Phaeoramularia* fruit and leaf spot in Kenya. *Fruits*, 54:323-329.

Seif, A.A and R.J. Hillocks. 1993. *Phaeoramularia* fruit and leaf spot of citrus with special reference to Kenya. *International Journal of Pest Management*, 39:44-50.

Seif, A.A. and J.N. Kungu. 1990. Association of *Colletotrichum* species with the fruit and leaf spot of citrus caused by *Phaeoramularia angolensis*. National Agricultural Research Laboratories, Annual Report of Plant Pathology Department for 1990.

Seifu Gebre-Mariam. 2003. Status of commercial fruit production in Ethiopia. Ethiopian Agricultural Research Organization, Addis Ababa, Ethiopia. 91p.

Silva, D.N., P. Talhinhas, V. Varzea, L. Cai, O.S. Paulo and D. Batista. 2012. Application of the Apn2/MAT locus to improve the systematics of the *Colletotrichum gloeosporioides* complex: an example from coffee (*Coffea* spp.) hosts. *Mycologia*, 104:396-409.

Sisay Bekele Mekbib. 2007. Identification of citrus (*Citrus sinensis*) postharvest pathogens from Ethiopia and their control. Ph.D. Dissertation, University of Pretoria, Pretoria, South Africa.

Stover, B.C. and K.F. Muller. 2010. TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, 11:7.

Talavera, G. and J. Castresana. 2007. Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. *Systematics Biology*, 56:564-577.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30:2725-2729.

Tesfaye Tedilla and Habtu Assefa. 1985. A review of research activities on fruit crop diseases in Ethiopia. pp. 263-276. In: Tsedeke Abate (ed.), Proceedings of the 1st Ethiopian Crop Protection Symposium, 4-7 February 1985, Addis Ababa, Ethiopia.

Udayanga, D., D.S. Manamgoda, X. Liu, E. Chukeatirote and K.D. Hyde. 2013. What are the common anthracnose pathogens of tropical fruits? *Fungal Diversity*, 61:165-179.

Vilgalys, R. and M. Hester. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology*, 172:4238-4246.

Weir, B.S., P.R. Johnston and U. Damm. 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*, 73:115-180.

White, T.J., T. Bruns, S. Lee and J.W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. pp. 315-322. In: M.A. Innis, D.H.J.J. Gelfand, Sninsky and T.J. White (eds), PCR Protocols: A Guide to Methods and Applications. Academic Press, San Diego, CA.

Yigzaw Dessalegn and Gelelbelu Girma. 2002. *Phaeoramularia angolensis*: a citrus disease in Northwest Ethiopia. *AgriTopia*, 17(1):12-13.

Zivkovic, S., N. Trkulja, T. Popovic, V. Oro and Z. Ivanovic. 2012. Morphological and molecular identification of *Colletotrichum gloeosporioides* from *Citrus reticulata*. Proceedings of International Symposium on Current Trends in Plant Protection, 292-298.

5. MULTILOCUS PHYLOGENY OF *COLLETOTRICHUM* SPECIES ASSOCIATED WITH LEAF AND FRUIT SPOT DISEASE OF CITRUS IN ETHIOPIA

5.1. ABSTRACT

This study was conducted to analyze the phylogenetic relationships of *Colletotrichum* species complex associated with leaf and fruit disease of citrus. A total of 167 isolates were isolated from symptomatic citrus leaves and fruits. Monosporic or single hyphal tip isolates were sequenced for ITS, LSU and ACT genes. Generated sequences were compared with GenBank database using the BLASTN search tool. The phylogenetic analysis of the isolates based on multilocus sequences delineated them as *C. gloeosporioides* sensu lato (broad sense) and *C. boninense* spp. complex. Each single locus sequence analysis identified 163 isolates as *C. gloeosporioides* or its teleomorph *G. cingulata*. The results demonstrated that multilocus sequence analysis is a reliable method for phylogenetic analysis of species within the genus *Colletotrichum*. These findings provide a baseline for further studies on the genetic diversity and population structure of *Colletotrichum* spp. associated with leaf and fruit disease of citrus, and can contribute to devise effective management practice against the disease.

5.2. INTRODUCTION

The genus *Colletotrichum* comprises important plant pathogens, causing significant crop losses in a wide range of plant species worldwide (Sutton, 1980; Freeman, 2000; Cannon *et al.*, 2012; Da Silva and Michereff, 2013), and was ranked as the eighth economically important plant pathogenic fungi (Dean *et al*., 2012). *Colletotrichum* spp. cause devastating preharvest and postharvest diseases in many fruit crops. The most typical disease is anthracnose that infects fruits, leaves and branches of young and mature tissues of numerous economically important crops. Anthracnose symptoms appear as dark spots

or sunken lesions, that are semi-circular or angular shaped, often coalesce to form larger lesions (Freeman, 2000; Agrios, 2005; Cai *et al.*, 2009). Other diseases associated with *Colletotrichum* spp. include fruit and leaf spots, twig dieback, flower abortion, immature fruit drop, rotting of fruits, seedling blight, and root rots (Agrios, 2005; Peres *et al.*, 2005; Da Silva and Michereff, 2013).

Several species of *Colletotrichum* can cause anthracnose diseases on tropical fruit trees including *Citrus* spp. (Peres *et al.*, 2005; Da Silva and Michereff, 2013). Different species or biotypes of *Colletotrichum* can also be associated with a single host (Freeman *et al.*, 1998; Peres *et al.*, 2002). It is important to accurately identify and delineate species and pathotypes, and understand the disease epidemiology to devise disease control measures (Freeman *et al.*, 1998; Cai *et al.*, 2009; Crouch *et al*., 2009). Pathogen species identification and delimitation have been done using morphological and host-preference criteria (Silva *et al.*, 2012). However, morphological characters vary with experimental conditions and the host. Isolates of a pathogen may also have different level of pathogenicity. Therefore, the use of such criteria has been reported as unreliable in the identification and phylogenetic analysis of *Colletotrichum* spp. (Freeman, 2000; Cai *et al.*, 2009; Phoulivong *et al.*, 2010).

Molecular markers have improved precision of identification and classification of pathogenic fungi (Weir *et al.*, 2012). Nucleotide sequence information for different loci has been used as reliable technique to identify *Colletotrichum* spp. (Cai *et al.*, 2009). The majority of the studies which employed phylogenetic analysis for species identification have relied on the ITS region of the nuclear rDNA (Peres *et al.*, 2002; Cai *et al.*, 2009). However, the resolution provided by the ITS barcode may not adequately discriminate among individuals of a species complex (Weir *et al.*, 2012). Moreover, ITS sequence data available in public domains can cause considerable confusion as sequence data are often entered and named incorrectly, or identical sequences may be recorded under different names (Crouch *et al*., 2009). The use of multilocus phylogeny has been recommended to diagnose and understand the relationships among *Colletotrichum* spp. (Farr *et al*., 2006;

Crouch *et al*., 2009). Therefore, the objective of this study was to analyze species complex of the genus *Colletotrichum* associated with leaf and fruit spot disease of citrus using multilocus sequences.

5.3. MATERIALS AND METHODS

5.3.1. Fungal isolates

Symptomatic leaves and fruits samples were collected from citrus production areas of Ethiopia (Fig 3.2). A total of 167 *Colletotrichum* isolates (Appendix Table 5.1) were isolated from infected citrus leaf and fruit samples. Surface-sterilization and culture conditions were as reported in section 4.3.1.

5.3.2. DNA extraction

Fungal genomic DNA was extracted as described in section 4.3.4.1.

5.3.3. PCR amplification

Three loci including the 5.8S nuclear ribosomal gene with the two flanking ITS regions, the portion of LSU, and partial sequences of the ACT gene were amplified and sequenced using universal primer pairs as stated in section 4.3.4.2. Genomic DNA from *Acremonium* species isolate 133 was used as positive control whereas reaction with no DNA template was used as negative control. All PCR reactions, their cycling conditions and electrophoresis were as described in section 4.3.4.2.

5.3.4. DNA sequencing and alignment

Sequencing of purified PCR products, and inspecting, editing and assembling the sequences into consensus contigs were using the same procedures and programs

described in section 4.3.4.3. The sequences were analyzed against the GenBank database using BLASTN v2.2.30 program (Altschul *et al.*, 1990) and multiple sequence alignments were performed with MAFFT v7.223 (Katoh and Standley, 2013) as detailed in section 4.3.4.3. Resulting sequence alignments were evaluated and manually edited where necessary using MEGA v6.06 software (Tamura *et al.*, 2013).

5.3.5. Phylogenetic analyses

Phylogenetic analyses were performed on each multiple sequence alignment of the ITS, LSU and ACT as well as on the combined dataset of the three loci using different statistical methods to differentiate the isolates by species complex. jModelTest v2.1.7 (Darriba *et al.*, 2012) as well as the Modeltest (Posada and Crandall, 1998) implemented in the MEGA6 were used to estimate the best-fit models of nucleotide substitution and the corresponding general time-reversible substitution rate parameters, shape of the four-category gamma distribution and fraction of invariable sites for each gene using corrected Akaike Information Criterion and the Bayesian Information Criterion scores. For each locus, 167 sequence datasets were used to reconstruct phylogenetic trees. Published ITS, LSU and ACT nucleotide sequences of thirteen *Colletotrichum* spp. isolates were retrieved from the GenBank database and included as reference species (Appendix Table 5.2). *Colletotrichum acutatum* J.H. Simmonds (GenBank accession numbers: DQ286124, DQ286125, and JQ949687) (Farr *et al.*, 2006; Damm *et al.*, 2012b) was designated as outgroup in all analyses for the reconstruction of the phylogenetic trees.

To determine whether the three sequence datasets were congruent and combinable, tree topologies of 70% reciprocal NJ bootstrap with ML distances (1000 replicates) with substitution models determined separately for each partition using Modeltest were compared visually (Mason-Gamer and Kellogg, 1996). The analyses showed that individual genes were broadly congruent, thus nucleotide alignments of the three genes were concatenated using scripts in Microsoft Office Excel 2007 program.

Phylogeny reconstruction was performed by NJ method (Saitou and Nei, 1987) using the MEGA6 software. The percentage of replicate trees in which the associated taxa clustered together was evaluated with a bootstrap analysis with 1000 replicates (Felsenstein, 1985). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2- parameter substitution model (Kimura, 1980). All alignment positions containing gaps and missing data were completely deleted and the rate variation among sites was modeled with a gamma distribution (shape parameter = 5). All branches with bootstrap values of less than 50 were collapsed.

The evolutionary history was also inferred using the Maximum Parsimony (MP) method on the combined multilocus alignments using Tree-Bisection-Reconstruction algorithm with search level 3 in which the initial trees were obtained by 10 random sequence additions. Alignment gaps and missing data were eliminated and the rate of variation among sites was modeled with gamma distribution. The confidence values for clades within the resulting tree were determined using a bootstrap analysis with 1000 replicates (Felsenstein, 1985). Tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC) and homoplasy index (HI) were calculated for one of the most parsimony trees.

A Markov Chain Monte Carlo (MCMC) algorithm was used to generate phylogenetic trees with Bayesian probabilities using MrBayes v3.2.1 (Ronquist *et al*., 2012) for the combined multilocus sequence datasets. Based on the results of the jModelTest, the Bayesian analysis for all loci was performed using the Dirichlet (1,1,1,1) nucleotide frequency distribution, and general time-reversible model with gamma-distributed rate variation across sites and a proportion of invariable sites. The analyses of two MCMC chains on the full data set were run from random trees for 4×10^6 generations and sampled from the posterior every 1000 generations until the split frequency reached below 0.02. The first 25% of the trees were discarded as burn-in phase of the analysis and posterior probabilities determined from the remaining trees. The effective sample size

and traces of all parameters and convergence of the two runs were checked using the internal diagnostics of the standard deviation of split frequencies and performance scale reduction factors, and then externally with Tracer v1.6 (Rambaut *et al.*, 2014). A summary maximum clade credibility species tree was built with TreeAnnotator v1.7.1 (Drummond *et al*., 2012) using a 25% burn-in and a posterior probability limit of 0.5. The resulting phylogenetic trees were drawn and edited using TreeGraph v2.4.0-456 beta (Stover and Muller, 2010). All tree branches with values of less than 0.50 were collapsed.

Sequences derived in the present study were deposited in the GenBank with accession numbers from KT282463 to KT282629 (ACT), from KT282630 to KT282796 (ITS), and from KT282797 to KT282963 (LSU), whereas alignments and trees were deposited into TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17920).

5.4. RESULTS

5.4.1. PCR amplification

Three universal primer pairs (ITS-1F/ITS4, LROR/LR5 and ACT-512F/ACT-783R) were used to amplify the target ITS, LSU and ACT loci. All 167 fungal DNA samples were successfully amplified and sequenced. The amplified ITS, LSU and ACT loci were of approximately 600 bp, 900 bp and 300 bp in size, respectively. The average sizes of assembled sequences of the test isolates were 570 bp for ITS, 855 bp for LSU, and 250 bp for ACT gene. Among isolates, there were only slight variations in amplicon size with few inconsistencies due to variable length nucleotide repeats.

5.4.2. Identification of the fungal test isolates

Among the 167 fungal isolates, 163 were identified as *C. gloeosporioides* (or its teleomorph *G. cingulata*) species complex using BLASTN search tool. Four isolates were recognized as *C. boninense* species complex.

The ITS sequence analysis delineated 163 isolates as *C. gloeosporioides* species complex (*C. aenigma* = 2, *C. aotearoa* = 2, and *C. gloeosporioides* sensu stricto [in strict sense] = 159). One isolate was resolved as *C. karstii*. Three isolates were identified as *C. truncatum*. The ITS sequence data resolved all the isolates at species level.

The analysis of portion of LSU revealed that 93 isolates were recognized as *C. gloeosporioides* (*C. asianum* = 3, *C. fructicola* = 4, and *C. gloeosporioides* sensu stricto = 86), while four isolates were identified as *C. boninense* species complex. The rest 70 isolates were belonged to *G. cingulata*. The partial LSU data identified all the isolates at species level.

The partial ACT nucleotide sequence data resolved 163 isolates as *C. gloeosporioides* (*C. aenigma* = 2, *C. asianum* = 1, *C. crassipes* = 1, *C. fructicola* = 4, *C. siamense* = 1, and *C. gloeosporioides* sensu stricto = 154), one isolate as *C. karstii*, and three isolates as *C. magnisporum*. The partial ACT sequences discriminated all the isolates at species level.

The sequences of the ITS, LSU and ACT barcode markers discriminated all the *Colletotrichum* isolates to the species level. The results further indicated that citrus leaf and fruit disease in Ethiopia is associated mainly with *C. gloeosporioides* species complex.

5.4.3. Phylogenetic analyses

The trees drawn from each individual dataset (ITS, LSU and ACT loci) using NJ and MP had similar topology (data not shown) for the 70% reciprocal NJ bootstrap trees, which allowed us to combine them. The phylogenetic analysis of the combined sequences from the three loci using NJ, MP and Bayesian methods resulted similar grouping of isolates (Figs 5.1 to 5.3) into *C. gloeosporioides* (Weir *et al.*, 2012) and *C. boninense* (Damm *et al.*, 2012a) spp. complex.

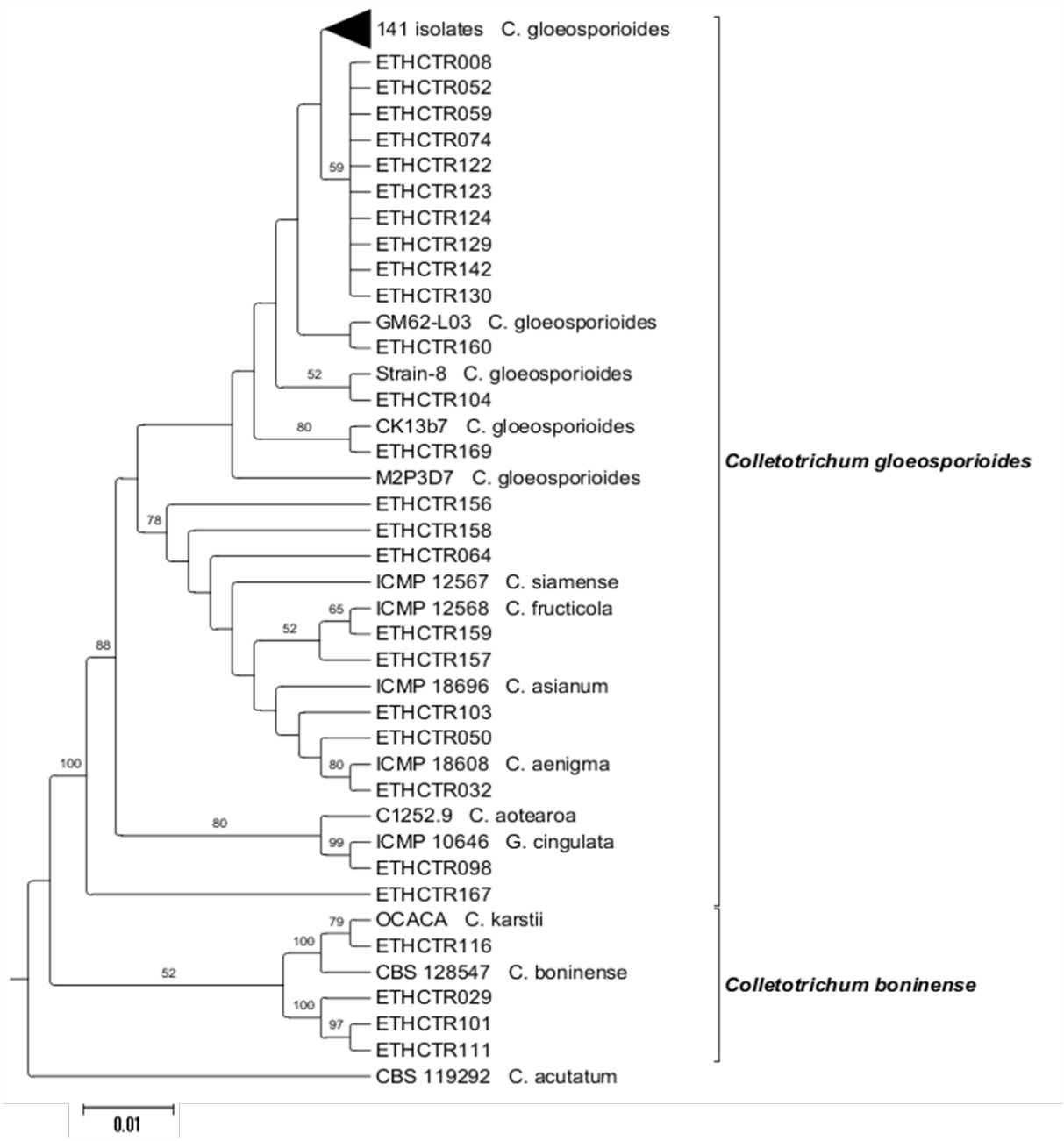

Fig 5.1. Neighbor-joining phylogenetic tree of concatenated ITS, LSU and ACT sequences of 167 *Colletotrichum* isolates generated in this study and 13 isolates from other studies, retrieved from the GenBank. Bootstrap support values ≥50 are shown above the nodes. The tree was rooted with *C. acutatum* (CBS 119292) as outgroup. The scale bar indicates the number of expected nucleotide changes per site.

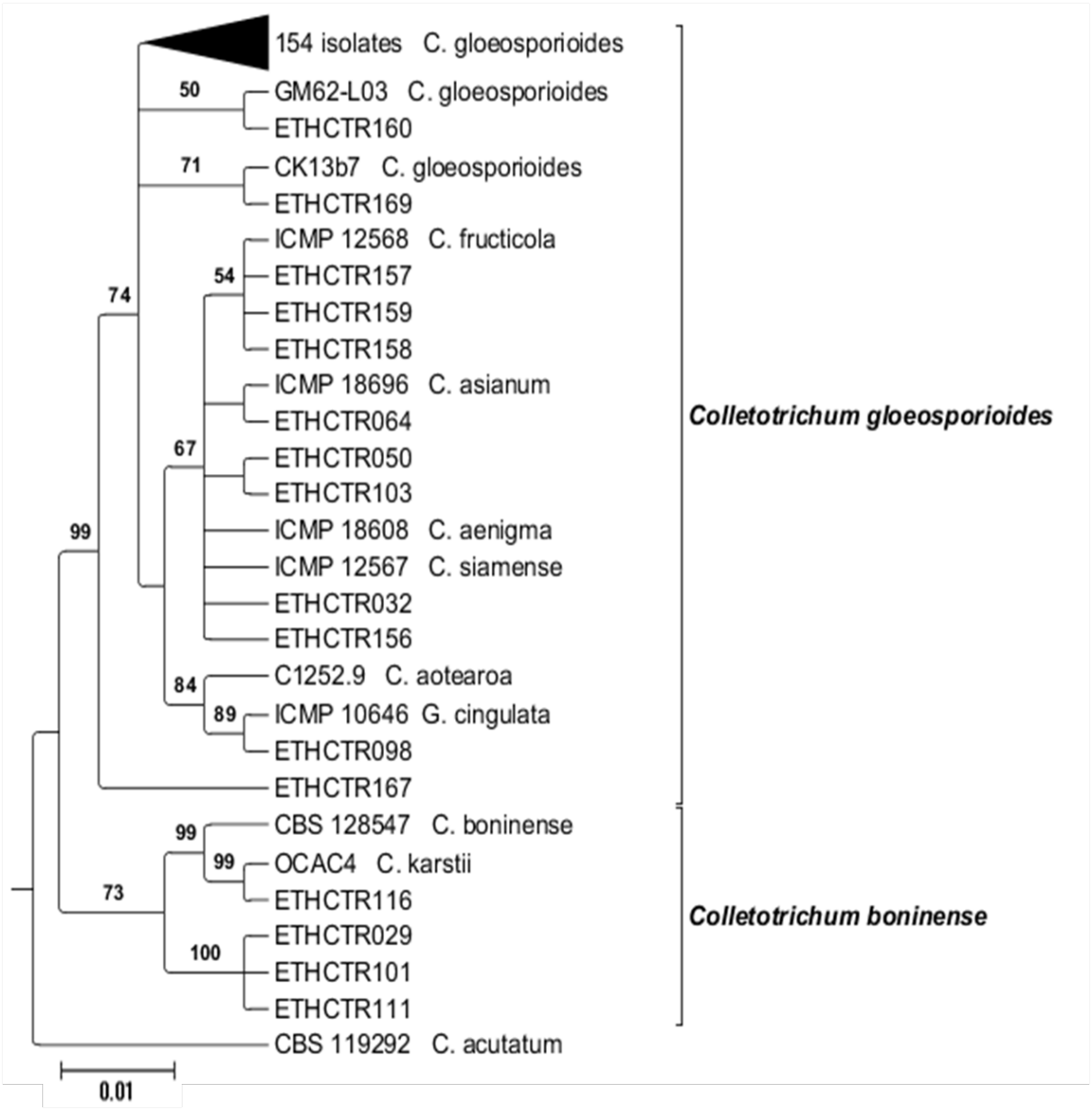

Fig 5.2. One of the most parsimonious trees obtained from a heuristic search of combined ITS, LSU and ACT sequences of 167 isolates generated in this study and 13 published reference isolates from the GenBank in the *C. gloeosporioides* and *C. boninense* species complexes. Bootstrap support values of≥50 are shown at the nodes. *C. acutatum* (CBS 119292) is used as outgroup. The scale bar indicates the number of expected changes per site.

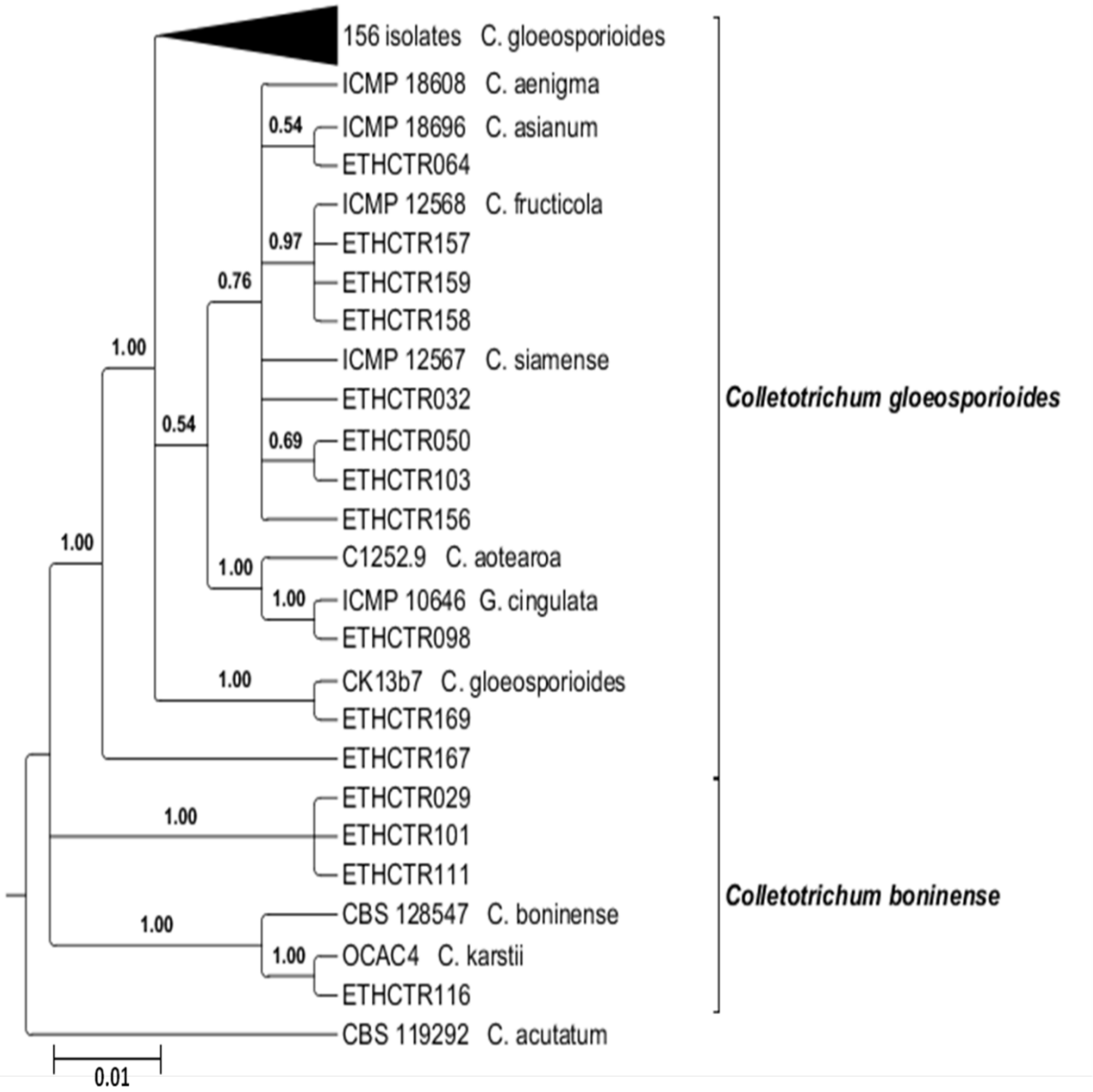

Fig 5.3. A Bayesian inference phylogenetic tree which illustrates the relationships of 167 isolates generated in this study and 13 published reference isolates from the GenBank in the *C. gloeosporioides* and *C. boninense* species complexes. The tree was built using concatenated sequences of the ITS, LSU and LSU genes, each with a separate models of DNA evolution. Bayesian posterior probability values of 0.5 are shown above the nodes. *C. acutatum* (CBS 119292) is used as outgroup. The scale bar indicates the number of expected changes per site.

In the combined multilocus analyses (gene boundaries of ITS: 1–527, LSU: 528-1385, ACT: 1386-1623) of 181 isolates (including 13 references) of *C. gloeosporioides* and related *Colletotrichum* spp. complex, as well as the outgroup (*C. acutatum* CBS 119292), 1913 characters including the alignment gaps were processed, of which 143 characters were parsimony informative, 236 parsimony uninformative and 1364 constant. Parsimony analysis resulted in three most parsimonious trees. One of the most parsimonious trees (tree length = 341, CI = 0.584615, RI = 0.829921, RC = 0.485185, and HI = 0.415385) obtained with the combined multiple sequence alignment of the three loci using MP method is presented in Fig 5.2.

The overall topology of all of the equally most parsimonious trees was similar; they differed in the position of isolates within the clades. Out of the 8002 trees, 3001 trees were used to calculate the consensus tree and posterior probabilities. The analysis resulted in the delineation of four main clades within the isolates studied in this study (Fig 5.3). Most of the isolates clustered in the first clade (*C. gloeosporioides* sensu lato) with a bootstrap support and Bayesian posterior probability values of 74/1.0. The first main clade consists of several closely related species including *C. aenigma*, *C. asianum*, *C. fructicola* and *C. siamense* (67/0.76), *C. aotearoa* and *G. cingulata* (84/1.0), and *C. gloeosporioides* sensu stricto. The second clade contained only one isolate, representing C. *gloeosporioides* sensu lato (99/1.0). *C. karstii* (99/1.0), and *C. boninense* (99/1.0) belonged to the third main clade. The fourth main clade consisted of the *C. acutatum* isolate used as outgroup for the phylogenetic analysis.

5.5. DISCUSSSION

The present study reports the molecular phylogenetic analyses of *Colletotrichum* spp. associated with leaf and fruit disease of citrus in main citrus production areas of Ethiopia. The *Colletotrichum* spp. identified in this study have been reported to show highly variable cultural and morphological characteristics (Johnston *et al.*, 2005; Weir *et al.*,

2012). These characteristics cannot be used as reliable method to determine species complex (Phoulivong *et al.*, 2010). Molecular phylogenetic analysis based on multiple gene sequences is preferred to employ either morphology or single gene sequence alone (Crouch *et al.*, 2009). It is also important to link morphological descriptions to multilocus molecular data (Cai *et al.*, 2009). Multilocus analyses have been demonstrated in various studies for identification and genetic delimitation of *Colletotrichum* spp. attacking different hosts (Farr *et al.*, 2006; Cannon *et al.*, 2012; Damm *et al.*, 2012a, b; Weir *et al.*, 2012; Huang *et al.*, 2013; Udayanga *et al.*, 2013).

In the present study, the multilocus molecular approach reliably differentiated the *Colletotrichum* spp. The isolates obtained from citrus trees with symptoms of leaf and fruit lesions were identified using the three DNA barcode markers and were belonged to *C. gloeosporioides* and *C. boninense* spp. complex. Based on the analyses, the genetic diversity of *Colletotrichum* in Ethiopia was high as the isolates associated with leaf and fruit disease of citrus were distributed across different taxa: *C. aenigma*, *aotearoa, C. asianum, C. boninense* sensu stricto, *C. crassipes*, C. *fructicola*, *C. gloeosporioides* sensu stricto, *C. karstii, C. magnisporum, C. siamense*, *C. truncatum* and *Glomerella cingulata*. Although *C. acutatum* was reported worldwide as the main causal agent of citrus anthracnose disease (Lima *et al.*, 2011), no isolate generated from the present study belonged to this species.

Multilocus sequences were used to construct a phylogenetic tree for species identification in order to exploit the potential accuracy of ITS, LSU and ACT loci. The ITS regions have been proposed as universal DNA barcode marker for fungal species identification (Schoch *et al.*, 2012). However, the ITS is not universally accepted as DNA barcode marker, because they are not always discriminant (Kiss, 2012). Many phylogenetically closely related species may have sequences identical or differing only by a few nucleotide positions at the ITS level (Nilsson *et al.*, 2008; Kovacs *et al.*, 2011). In this study, all the ITS, LSU and ACT sequences were robust to differentiate species complex. The tree constructed in the present study largely confirmed those of the defined *C.*

gloeosporioides (Weir *et al*., 2012) and *C. boninense* (Damm *et al*., 2012a) spp. complex, and was detailed enough to enable the identification of all isolates.

However, GenBank BLASTN analysis with ACT sequence of one isolate revealed a high similarity with sequences from an isolate sourced from *Pleione bulbocodioides* in China and reported as *C. crassipes* (CORCS3) (Yang *et al.*, 2011), but it was clustered in *C. gloeosporioides* complex. Three isolates with ACT sequences also showed high similarity with sequences from unknown sourced isolate from China and reported as *C. magnisporum* (CBS 398.84) (Liu *et al.*, 2014), but they were grouped in *C. boninense* complex. Three isolates with ITS sequences showed high similarity with sequences from an isolate (GenBank code as AY548234.1) sourced from *Asclepias* in England and reported as C. *truncatum* (Hobden and Walker, 2004), but clustered in *C. boninense* species complex.

The various taxa identified in the present study have been reported as having worldwide geographic distribution, and many isolates have been associated with plant diseases in many agriculturally important crops (Damm *et al.*, 2012b; Weir *et al.*, 2012; Udayanga *et al.*, 2013; Lima *et al.*, 2015). Many cases have been reported on the association of several *Colletotrichum* spp. or biotypes with a single host. It is also common to find a single *Colletotrichum* species infecting multiple hosts (Freeman *et al.*, 1998).

Colletotrichum gloeosporioides complex has been reported to cause both postharvest anthracnose (Freeman and Shabi, 1996) and preharvest diseases such as wither tip on twigs, tear stain (Benyahia *et al.*, 2003) and fruit stem-end rot disease (Kaur *et al.*, 2007) in various plant species. *C. gloeosporioides* has been commonly associated with Key Lime Anthracnose (KLA) and postharvest diseases on citrus species (Weir *et al.*, 2012). It has also been reported as a causal agent of post-bloom fruit drop (PFD) on sweet orange in Brazil (Lima *et al.*, 2011). The ability of *C. fructicola* and *C. gloeosporioides* strains to cause anthracnose on citrus fruits has been demonstrated in China (Huang *et al.*, 2013). In the tropical Asia, *C. siamense* has also been identified as a pathogenic species

causing anthracnose disease on a wide range of tropical fruits, such as *Ficus racemosa*, *Azadirachta indica* and *Mangifera indica* (Udayanga *et al.*, 2013). Recently, *C. gloeosporioides* and *C. karstii* have been reported to cause severe lesions on fruits of sweet orange at preharvest stage in Italy, where *C. gloesporioides* was more aggressive to cause lesions on fruits than *C. karstii* (Aiello *et al.*, 2015). The virulence and pathogenicity of *C. asianum*, *C. fructicola*, and *C. karstii* have been demonstrated on various plant species including mango, papaya, banana, guava and bell pepper cultivars in Brazil (Lima *et al.*, 2015).

Colletotrichum boninense, once considered to belong to the *C. gloeosporioides* complex, was first described from *Crinum asiaticum* var. *sinicum* and *Cucumis melo* collected from Bonin Islands in Japan, where the species was associated with a variety of host plants (Moriwaki *et al.*, 2003). Since then, this species has been reported as a pathogen causing fruit and leaf anthracnose on different host plants (Johnston *et al.*, 2005; Damm *et al.*, 2012a). It has been observed that *C. boninense* found to be associated with diseases of Proteaceae in Australia and Zimbabwe, and with *Eucalyptus* in South Africa (Lubbe *et al.*, 2004), *Dracaena* and *Pachira* in China, *Passiflora* in New Zealand and *Hippeastrum* in Brazil and the Netherlands (Farr *et al.*, 2006), berries and twigs of *Coffea* in Vietnam (Nguyen *et al.*, 2009), and avocado in Mexico (Silva-Rojas and Ávila-Quezada, 2011). *C. boninense* and *C. gloesporioides* have been shown to infect *Protea* leaves and stems in South Africa (Lubbe *et al.*, 2006).

In conclusion, the results of the present study demonstrated that multilocus sequence analysis is a reliable and useful method for species phylogenetic analyses. It was observed that the phylogenetic analysis of the isolates based on both each single locus sequence and multilocus sequences grouped them as *C. gloeosporioides* and *C. boninense* spp. Most of the known taxa identified in this study represent the first report of these species as pathogens associated with leaf and fruit disease of citrus in Ethiopia. Further in-depth studies on the etiology of the pathogens are needed to define effective disease management strategies.

REFERENCES

Agrios, G.N. 2005. Plant Pathology. 5th ed. Elsevier Academic Press, London, UK.

Aiello, D., R. Carrieri, V. Guarnaccia, A. Vitale, E. Lahoz and G. Polizzi. 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology*, 163:168-177.

Altschul, S.F., W. Gish, W. Miller, E.W. Myers and D.J. Lipman. 1990. Basic local alignment search tool. *Journal of Molecular Biology*, 215:4034-10.

Benyahia, H., A. Jrifi, C. Smaili, M. Afellah and L.W. Timmer. 2003. First report of *Colletotrichum gloeosporioides* causing wither tip on twigs and tear stain on fruit of citrus in Morocco. *Plant Pathology*, 52:798.

Cai, L., K.D. Hyde, P.W.J. Taylor, B.S. Weir, J. Waller, M.M. Abang, J.Z. Zhang, Y.L. Yang, S. Phoulivong, Z.Y. Liu, H. Prihastuti, R.G. Shivas, E.H.C. McKenzie and P.R. Johnston. 2009. A polyphasic approach for studying *Colletotrichum*. *Fungal Diversity*, 39:183-204.

Cannon, P.F., U. Damm, P.R. Johnston and B.S. Weir. 2012. *Colletotrichum* – current status and future directions. *Studies in Mycology*, 73:181-213.

Crouch, J.A., B.B. Clarke and B.I. Hillman. 2009. What is the value of ITS sequence data in *Colletotrichum* systematics and species diagnosis? A case study using the falcate-spored graminicolous *Colletotrichum* group. *Mycologia*, 101:648-656.

Da Silva, C.F.B. and S.J. Michereff. 2013. Biology of *Colletotrichum* spp. and epidemiology of the anthracnose in tropical fruit trees. *Revista Caatinga*, 26:130-138.

Damm, U., P.F. Cannon, J.H.C. Woudenberg, P.R. Johnston, B.S. Weir, Y.P. Tan, R.G. Shivas and P.W. Crous. 2012a. The *Colletotrichum boninense* species complex. *Studies in Mycology*, 73:1-36.

Damm, U., P.F. Cannon, J.H.C. Woudenberg and P.W. Crous. 2012b. The *Colletotrichum acutatum* species complex. *Studies in Mycology*, 73:37-114.

Darriba, D., G.L. Taboada, R. Doallo and D. Posada. 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nature Methods*, 9:772.

Dean, R., J.A.L. Van Kan, Z.A. Pretorius, K.E. Hammond-Kosack, A. Di Pietro, P.D. Spanu, J.J. Rudd, M. Dickman, R. Kahmann, J. Ellis and G.D. Foster. 2012. The top 10 fungal pathogens in molecular plant pathology. *Molecular Plant Pathology*, 13:414-430.

Drummond, A.J., M.A. Suchard, D. Xie and A. Rambaut. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. *Molecular Biology and Evolution*; 29:1969-1973.

Farr, D.F., M.C. Aime, A.Y. Rossman and M.E. Palm. 2006. Species of *Colletotrichum* on Agavaceae. *Mycological Research*, 110:1395-1408.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39:783-791.

Freeman, S. 2000. Genetic diversity and host specificity of *Colletotrichum* species on various hosts. pp. 131-144. In: D. Prusky, S. Freeman and M.B. Dickman (eds.), *Colletotrichum*: Host Specificity, Pathology, and Host-Pathogen Interaction. APS Press, St. Paul, Minnesota.

Freeman, S., T. Katan and E. Shabi. 1998. Characterization of *Colletotrichum* species responsible for anthracnose diseases of various fruits. *Plant Disease*, 82:596-605.

Freeman, S. and E. Shabi. 1996. Cross-infection of subtropical and temperate fruits by *Colletotrichum* species from various hosts. *Physiology and Molecular Plant Pathology*, 49:395-404.

Hobden, E.M. and L.A. Walker. 2004. Sequence of unknown organism from host *Asclepias* (CSL number 20317144). Mycology, Plant Health Group, Central Science Laboratory, Sand Hutton, York, North Yorkshire YO41 1LZ, England (Unpublished).

Huang, F., G.Q. Chen, X. Hou, Y.S. Fu, L. Cai, K.D. Hyde and H.Y. Li. 2013. *Colletotrichum* species associated with cultivated citrus in China. *Fungal Diversity*, 61:61-74.

Johnston, P.R., S.R. Penny cook and M.A. Manning. 2005. Taxonomy of fruit-rotting fungal pathogens: what's really out there? *New Zealand Plant Protection*, 58:42-46.

Katoh, K. and D.M. Standley. 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution*, 30:772-780. Online: http://mafft.cbrc.jp/alignment/software/.

Kaur, R., H.S. Rewal and A. Sethi. 2007. Pre-harvest stem-end rot in citrus cultivars due to *Colletotrichum gloeosporioides*. *European Journal of Horticultural Science*, 72:20-25.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16:111-20.

Kiss, L. 2012. Limits of nuclear ribosomal DNA internal transcribed spacer (ITS) sequences as species barcodes for Fungi. *Proceedings of the National Academy of Sciences, USA*, 109:E1811.

Kovacs, G.M., T. Jankovics and L. Kiss. 2011. Variation in the nrDNA ITS sequences of some powdery mildew species: Do routine molecular identification procedures hide valuable information? *European Journal of Plant Pathology,* 131:135-141.

Lima, N.B., W.G. Lima, J.M. Tovar-Pedraza, S.J. Michereff and M.P.S. Camara. 2015. Comparative epidemiology of *Colletotrichum* species from mango in northeastern Brazil. *European Journal of Plant Pathology,* 141:679-688.

Lima, W.G., M.B. Sposito, L. Amorim, F.P. Golcalves and P.A.M. de Filho. 2011. *Colletotrichum gloeosporioides*, a new causal agent of citrus post-bloom fruit drop. *European Journal of Plant Pathology*, 131:157-165.

Liu, F., L. Cai, P.W. Crous and U. Damm. 2014. The *Colletotrichum gigasporum* species complex. *Persoonia*, 33:83-97.

Lubbe, C.M., S. Denman, S.C. Lamprecht and P.W. Crous. 2006. Pathogenicity of *Colletotrichum* species to *Protea* cultivars. *Australasian Plant Pathology*, 35:37-41.

Lubbe, C.M., S. Denman, P.F. Cannon, J.Z. Groenewald, S.C. Lamprecht and P.W. Crous. 2004. Characterization of *Colletotrichum* species associated with diseases of Proteaceae. *Mycologia*, 96:1268-1279.

Mason-Gamer, R.J. and E.A. Kellogg. 1996. Testing for phylogenetic conflict among molecular data sets in the tribe *Triticeae* (Gramineae). *Systematic Biology*, 45:524-545.

Moriwaki, J., T. Sato and T. Tsukiboshi. 2003. Morphological and molecular characterization of *Colletotrichum boninense* species nov. from Japan. *Mycoscience*, 44:47-53.

Nguyen, T.H.P., T. Sall, T. Bryngelsson and E. Liljeroth. 2009. Variation among *Colletotrichum gloeosporioides* isolates from infected coffee berries at different locations in Vietnam. *Plant Pathology*, 58:898-909.

Nilsson, R.H., E. Kristiansson, M. Ryberg, N. Hallenberg and K.H. Larsson. 2008. Intraspecific ITS variability in the Kingdom *Fungi* as expressed in the international sequence databases and its implications for molecular species identification. *Evolutionary Bioinformatics*, 4:193-201.

Peres, N.A., L.W. Timmer, J.E. Adaskaveg and J.C. Correll. 2005. Lifestyles of *Colletotrichum acutatum*. *Plant Disease*, 89:784-796.

Peres, N.A.R.. E.E. Kuramae, M.S.C. Dias and N.L. de Sousa. 2002. Identification and characterization of *Colletotrichum* spp. affecting fruit after harvest in Brazil. *Journal of Phytopathology*, 150:128-134.

Phoulivong, S., L. Cai, H. Chen, E.H.C. McKenzie, K. Abdelsalam, E. Chukeatirote and K.D. Hyde. 2010. *Colletotrichum gloeosporioides* is not a common pathogen on tropical fruits. *Fungal Diversity*, 44:33-43.

Posada, D. and K.A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14:817-818.

Rambaut, A., M.A. Suchard, D. Xie and A.J. Drummond. 2014. Tracer v1.6. Online: http://beast.bio.ed.ac.uk/Tracer.

Ronquist, F., M. Teslenko, P. van der Mark, D.L. Ayres, A. Darling, S. Hohna, B. Largeto, L. Liu, M.A. Suchard and J.P. Huelsenbeck. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61:539-542.

Saitou, N. and M. Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*, 4:406-425.

Schoch, C.L., K.A. Seifert, S. Huhndorf, V. Robert, J.L. Spouge, C.A. Levesque, W. Chen and Fungal Barcoding Consortium. 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for *Fungi*. *Proceedings of the National Academy of Sciences, USA*, 109:6241-6246.

Silva, D.N., P. Talhinhas, V. Varzea, L. Cai, O.S. Paulo and D. Batista. 2012. Application of the Apn2/MAT locus to improve the systematics of the *Colletotrichum gloeosporioides* complex: an example from coffee (*Coffea* spp.) hosts. *Mycologia*, 104:396-409.

Silva-Rojas, H.V. and G.D. Avila-Quezada. 2011. Phylogenetic and morphological identification of *Colletotrichum boninense*: a novel causal agent of anthracnose in avocado. *Plant Pathology*, 60:899-908.

Stover, B.C. and K.F. Muller. 2010. TreeGraph 2: Combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics*, 11:7.

Sutton, B.C. 1980. The Coelomycetes: fungi imperfecti with pycnidia, acervulli and stromata. Commonwealth Mycological Institute, Kew, UK.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution*, 30:2725-2729.

Udayanga, D., D.S. Manamgoda, X. Liu, E. Chukeatirote and K.D. Hyde. 2013. What are the common anthracnose pathogens of tropical fruits? *Fungal Diversity*, 61:165-179.

Weir, B.S., P.R. Johnston and U. Damm. 2012. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*, 73:115-180.

Yang, Y.L., L. Cai, Z.N. Yu, Z.Y. Liu and K.D. Hyde. 2011. *Colletotrichum* species on Orchidaceae in southwest China. *Cryptogamie*, 32:229-253.

6. GENOME-WIDE SURVEY OF MICROSATELLITE LOCI AND DEVELOPMENT OF SIMPLE SEQUENCE REPEAT (SSR) MARKERS FOR *COLLETOTRICHUM GLOEOSPORIOIDES*

6.1. ABSTRACT

Colletotrichum gloeosporioides causes anthracnose and other fruit and leaf diseases of citrus. This study was conducted to analyze the distribution and frequency of microsatellite loci in *C. gloeosporioides* genome, and to develop polymorphic SSR markers. Whole genome sequences were generated from *C. gloeosporioides* isolate using Illumina sequencing platform, and illumina reads were *de novo* assembled into a draft genome of approximately 56.77 Mbp. Fifty SSR loci across the genome were selected and first-pass screening was done using thirteen geographically representative isolates. The results showed that a genome-wide microsatellite database of 5030 microsatellite motifs were identified at 4506 loci, including 274 compound loci. Of these motifs, 4756 were perfect motifs. Trinucleotide repeats were the most frequent; whereas penta- and hexanucleotide motifs were the least abundant. The number of motifs decreased as the number of the repeats increased. Among the mononucleotide microsatellites, A/T repeats were more abundant than G/C repeats in the *C. gloeosporioides* genome. The most frequent di-, tri- and tetranucleotide repeats were AG/CT, AGC/GCT, and AT-GC balance motifs, respectively. In penta- and hexanucleotide repeats, GC-rich motifs were predominant. Forty-six loci (92%) were successfully amplified and yielded scorable single amplicons. Four loci were either failed to generate specific amplicons or generated no amplicons. Twenty-one markers showed polymorphism and demonstrated allele diversity among the tested isolates. The present approach has been successful for microsatellite discovery and development of SSR markers for the genetic analysis of *C. gloeosporioides* populations from citrus in Ethiopia.

6.2. INTRODUCTION

Microsatellites are stretches of DNA consisting of tandemly repeated short units, usually one to six base pairs (Jarne and Lagoda, 1996; Matsuoka *et al.*, 2002; Cai *et al.*, 2013). They are valuable tools in many research areas, such as population biology, genome mapping, genealogy, genetic diversity and population genetic studies. They are multi-allelic, inherited co-dominantly, usually abundant, and cover most or all parts of the genome (Morgante *et al.*, 2002; Karaoglu *et al.*, 2005; Benali *et al.*, 2011; Peixoto-Junior *et al.*, 2014). The traditional method to develop microsatellite markers, which is still used by many laboratories today, generally involves several steps: enrich microsatellite-containing sequences from genomic DNA; clone the microsatellite-enriched DNA; extract plasmids; sequence the inserts through Sanger sequencing; design primers; and screen individual loci (Cai *et al.*, 2013). The whole process may require several months of work and considerable resources (Morgante *et al*., 2002; Zane *et al*., 2002). In fungi, the traditional approach is even more time- and resource-consuming, because fungal species usually have lower densities of microsatellite loci and the alleles are often shorter with fewer polymorphisms, compared to many other organisms (Dutech *et al.*, 2007).

Advances in sequencing technology are changing many aspects of the biological sciences, including methods to develop microsatellite markers. The high-throughput and low cost of next-generation sequencing technology enables the efficient generation of large amounts of genome sequence data from which microsatellite markers can be identified (Capote *et al.*, 2012; Cai *et al.*, 2013). The 454 sequencing platform (454 Life Sciences, Roche) has been used most frequently for this purpose due to its production of longer reads of DNA. Many reads containing microsatellites have sufficiently long flanking sequences to allow the design of primers to amplify the target microsatellite loci (Abdelkrim *et al.*, 2009; Allentoft *et al.*, 2009; Castoe *et al.*, 2010; Yu *et al.*, 2011; Ji *et al.*, 2012).

In contrast, the Illumina sequencing platform generates shorter reads, but recent progress extends read length up to 250 bp on the Illumina MiSeq platform and up to 150 bp on other platforms (www.illumina.com). Furthermore, the read length can be extended with paired-end sequencing. The Illumina platform generally operates with much higher throughput and lower cost than the 454 platform; thus, it has become an attractive sequencing platform for use to identify large numbers of microsatellite loci (Cai *et al.*, 2013). Castoe *et al*. (2012) used paired-end sequencing to generate 114 bp x 2 reads for a python and 116 bp x 2 reads for two bird genomes. They searched for microsatellites on these reads and designed primers using their flanking sequences. Their approach proved to be valuable, but with two limitations. The first limitation was that many reads did not have long enough flanking sequences to allow primer design as microsatellite loci were located toward one end. In the three species they examined, primers were successfully designed for only 32.7% to 40.1% of the loci. In comparison, a 454-generated library allowed successful primer design for 49.6% of the loci. The second limitation was the need to identify and filter out primers that could amplify multiple PCR products. This challenge was alleviated bioinformatically by counting the occurrence of the primers in the dataset (Castoe *et al*., 2012).

Cai *et al.* (2013) used an alternative approach to Castoe *et al*. (2012). Instead of trying to design primers directly from the Illumina reads, they first performed a genome assembly of the organism to achieve longer contiguous sequences, and then performed the microsatellite search and primer design. Although genome assembly requires access to more sequence data than that needed in the approach used by Castoe *et al*. (2012), advances in Illumina technology in terms of higher throughput and lower cost make this approach readily applicable. Furthermore, multiplexing allows multiple small genomes to be sequenced in a single run (Cai *et al.*, 2013). The Illumina Genome Analyzer IIX and HiSeq platforms have even lower per-base sequencing cost than MiSeq (www.illumina.com). The approach can be applied either for the development of microsatellite markers alone or in combination with genome sequencing projects that produce much deeper genome coverage (Cai *et al.*, 2013).

In this study, the approach used by Cai *et al.* (2013) was applied to the fungus *C. gloeosporioides*, which has been found associated with citrus leaf and fruit spot disease in Ethiopia. The genus *Colletotrichum* represents a group of plant pathogenic fungi that can infect a wide range of plant species (Sreenivasaprasad and Talhinhas, 2005; Gan *et al.*, 2013). *Colletotrichum gloeosporioides* is one of the most common and widely distributed plant pathogen worldwide (Sutton, 1992; Cannon *et al.*, 2000). It is economically important pathogen of a wide range of fruit crops including citrus (Adaskaveg and Forster, 2000; Timmer and Brown, 2000; Ramos *et al*., 2006; Aiello *et al.*, 2015). However, little is known about the population biology and genetic diversity of *C. gloeosporioides* from citrus. Such information is vital to define the populations of the pathogen and to support disease resistance breeding program (Belayneh *et al.*, 2010). Therefore, the objectives of this work were to construct the whole genome sequence, identify microsatellite loci, and develop polymorphic SSR markers for *C. gloeosporioides*, the major pathogen associated with leaf and fruit spot disease of citrus using illumina sequencing and *de novo* assembly.

6.3. MATERIALS AND METHODS

6.3.1. Fungal isolates

Five *C. gloeosporioides* isolates (ETHCTR016, ETHCTR064, ETHCTR092, ETHCTR193 and ETHCTR194), obtained from different geographic origin of Ethiopia (Appendix Table 5.1), were used to construct whole genome libraries for microsatellite loci search and SSR markers development. Thirteen geographically representative *C. gloeosporioides* isolates (ETHCTR032, ETHCTR050, ETHCTR092, ETHCTR111, ETHCTR114, ETHCTR116, ETHCTR129, ETHCTR153, ETHCTR189, ETHCTR193, ETHCTR194, ETHCTR197 and ETHCTR198) (Appendix Table 5.1) were used in the screening of the SSR markers.

6.3.2. DNA extraction

The total genomic DNA was extracted using the procedures described previously in section 4.3.4.1 of chapter 4. The concentration and purity of extracted DNA samples were checked by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific). The integrity of the DNA was visualized on 1% agarose gel stained with GelRed under ultra violet-light (UVP BioImaging systems).

6.3.3. Genome sequencing and assembly

Whole genome DNA libraries were constructed using next-generation Illumina MiSeq sequencing platform according to the manufacturer's instructions. The libraries were sequenced to obtain paired-end reads. Quality and nucleotide distribution of the sequences were explored using FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) and FastQC version 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) installed on a high performance computing (hpc) Linux server. The adapters indexed at the beginning and end of each sequence were removed using Scythe version 0.994-beta (https://github.com/vsbuffalo/scythe). Poor quality sequences at both ends were trimmed and the reads with 'N's were filtered out using SolexaQA++ version 3.1.2 (http://solexaqa.sourceforge.net) (Cox *et al.*, 2010). The genome was assembled with SPAdes version 3.1.1 (Bankevich *et al.*, 2012) genome assembler program with K-mer size 55 using the trimmed reads to achieve longer contiguous sequences for microsatellite search and SSR markers development.

6.3.4. Microsatellite mining and primer design

Genome-wide microsatellite database was developed for *C. gloeosporioides*. The genome assembly was fed to the PERL script MIcroSAtellite identification tool (MISA) (Thiel *et al.*, 2003), simple sequence repeat locator (SSRLocator) version 1 (Da Maia *et al.*, 2008), and a web-based simple sequence repeat identification tool (SSRIT) (Temnykh *et al.*,

2001) to search for microsatellite motifs (unit size 1 to 6 bp). For a unit size of 1 bp, the minimal repeat number required was 10; for a unit size of 2 bp, the minimal repeat number required was 6 bp, and for a unit size of 3 to 6 bp, the minimal repeat number required was 5 bp. The results obtained using these three search tools were compared and validated using tandem repeats finder (TRF) version 4.07b (http://tandem.bu.edu/trf/trf.advanced.submit.html) (Benson, 1999). The outputs were processed using a custom PERL script modified from "P3in.pl" (http://pgrc.ipkgatersleben.de/misa/primer3.html) and primers were designed for selected SSR loci using Primer3 (Rozen and Skaletsky, 2000) in a batch mode on hpc Linux server and validated using Primer Premier version 6.22 (PREMIER Biosoft package, Palo Alto, CA) software. The parameters used for primer design were: product size of 70 to 300 bp; primer size of 18 to 22 bp with optimal length 20 bp; primer melting temperature (Tm) of 50°C to 60°C with an optimum at 55°C; and primers were at least 5 bp away from the SSR locus. PrimerDigital (http://primerdigital.com/tools/) (Kalendar *et al.*, 2011), a Java based tool with multiple options was used to predict the annealing temperature and the amplified products for each primer pair.

6.3.5. PCR amplification and genotyping

SSR markers were developed and tested on *C. gloeosporioides* isolates for polymorphism. The 5' end of the forward primers of all SSR loci were labeled with fluorescent dyes (6-FAM = blue, PET = red, VIC = green, and NED = yellow). Amplification for each SSR loci was performed in standard PCR to determine the appropriate annealing temperature. Each PCR was done in 10 µl reactions containing AccuPower PCR PreMix without dye (Bioneer), additional 0.5 mM $MgCl_2$ (Promega), 0.05 to 0.15 µM each forward and reverse primers, and 2.0 µl template DNA (20 ng/µl). PCR reaction without template DNA was used as control. When clear bands were obtained, multiplex PCRs were performed using four to five SSR primer pairs in each PCR based on annealing temperature. Amplifications were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) using the following PCR cycling

conditions: initial denaturation step at 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C, 51°C or 54°C for 1 min, and extension at 72°C for 2 min; and a final extension step at 72°C for 10 min. To reveal polymorphism and allele identification, PCR products were separated in 2% agarose gel stained with GelRed and visualized under UV light. The sizes of PCR amplicons were estimated using a 100-bp ladder (Invitrogen).

Those loci which produced bands of expected size were selected, and fragment sizes and allele variations in the repeats were further analyzed on an ABI 3730 xl automated DNA sequencer (Applied Biosystems). The multiplex PCR products were mixed with 8.87 µl Hi-Di formamide and 0.135 µl fluorescent-labeled GeneScan™-500 LIZ size standard (Applied Biosystems) in a 96-well microtiter plate. The mixed products were denatured at 95°C for 3 min and snap-chilled on ice for 5 min. The products were then electrophoresed using an ABI 3730xl DNA sequencer. Allele sizes of the fragments were determined using GeneMapper software version 4.1 (Applied Biosystems).

6.3.6. SSR polymorphism

The SSR markers were evaluated for their polymorphism using thirteen *C. gloeosporioides* isolates. The major allele frequency, number of alleles per locus, gene diversity and polymorphic information content (PIC) were determined using PowerMarker version 3.25 (Liu and Muse, 2005). For each SSR marker, the degree of polymorphism estimated by gene diversity or expected heterozygosity (Weir, 1996) was calculated for the test isolates. To estimate the discriminatory power of the microsatellite loci, the PIC was calculated for each locus (Botstein *et al.*, 1980; Anderson *et al.*, 1993).

6.4. RESULTS

6.4.1. Genome sequencing and assembly

The *C. gloeosporioides* libraries were sequenced on the Illumina MiSeq sequencing platform and produced 301 bp × 2 paired-end reads. The average read length after quality control was 267 bp. Each isolate with a total reads (Table 6.1), including 99.7 to 99.87% in pairs were generated for the *C. gloeosporioides* libraries. The reads were shown to be of good quality (Fig 6.1).

Table 6.1. Summary of Illumina genome sequencing data for *C. gloeosporioides* isolates.

	Isolate				
	016	**064**	**092**	**193**	**194**
Read length (bp)	2x 301	2x 301	2x 301	2x 301	2x 301
Trimmed read length (bp)	15-281	15-281	15-281	15-281	15-281
Raw data (bp)	6400078	6827614	3824958	9287698	6055858
Trimmed data (bp)	6392928	6810366	3817332	9269764	6050346
Retained (%)	99.89	99.74	99.8	99.81	99.91
GC (%)	52	51	52	50	52

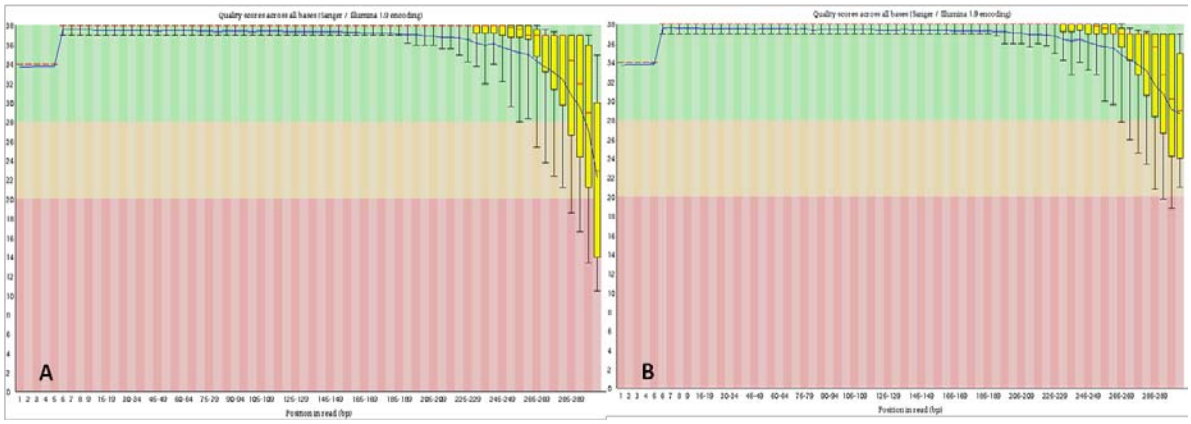

Fig 6.1. Quality of reads for an isolate *of C. gloeosporioides*. (A) Raw reads generated from Illumina MiSec sequencing, and (B) reads after trimming.

The final assembled parameters are listed in Table 6.2. Scaffolds N50 ranging from 15,767 to 86,296 bp were achieved. The assembled draft genome was ranged from 55.8 to 59.3 Mb including gaps in the scaffold. In reality, there is no one true genome sequence for a species because of individual genomic variation (Ekblom and Wolf, 2014). Therefore, usually only a single individual is sequenced (Wheeler *et al.*, 2008).

Table 6.2. Summary of the genome assembly of *C. gloeosporioides*.

	Isolate				
Scaffolds	**016**	**064**	**092**	**193**	**194**
Total length (>=0 bp)	56.99	59.58	57.40	57.96	57.32
Total length (>=0.5 kbp)	56.77	59.30	57.15	57.75	57.00
Total length (>=1 kbp)	56.29	58.36	56.54	57.53	56.39
Contigs count (>=0 bp)	2940	7942	6002	2169	3201
Contigs count (>=0.5 kbp)	2445	7333	5445	1691	2479
Contigs count (>=1 kbp)	1708	5973	4539	1332	1527
Largest contig (bp)	324,997	86,739	146,390	351,061	427,002
N50	74,988	15,767	21,981	86,296	75,263
N75	39,509	8,593	11,725	43,545	38,995
N90	18,459	4,278	5,595	22,156	19,549
Number of N's	1,282	0	0	717	100
No. of N's per 100 kbp	2.26	0	0	1.24	0.18

6.4.2. Construction of genome-wide microsatellite markers database

The flow chart presented in Fig 6.2 illustrates the steps used to construct the genome-wide database of microsatellite markers in *C. gloeosporioides*. A summary of microsatellite mining database is presented in Table 6.3. The total of 4,676 to 5,218 SSR motifs were identified from *C. gloeosporioides* genome at 4,266 to 4,629 loci, including 209 to 256 compound loci in which two motifs were located within 100 bp from each other. Of these SSR motifs, 230 to 284 motifs were in compound forms. More than one SSR motifs were located at 278 to 506 loci. Overall, the tested isolates constituted from 161 to 171 SSR motifs per Mbp genome.

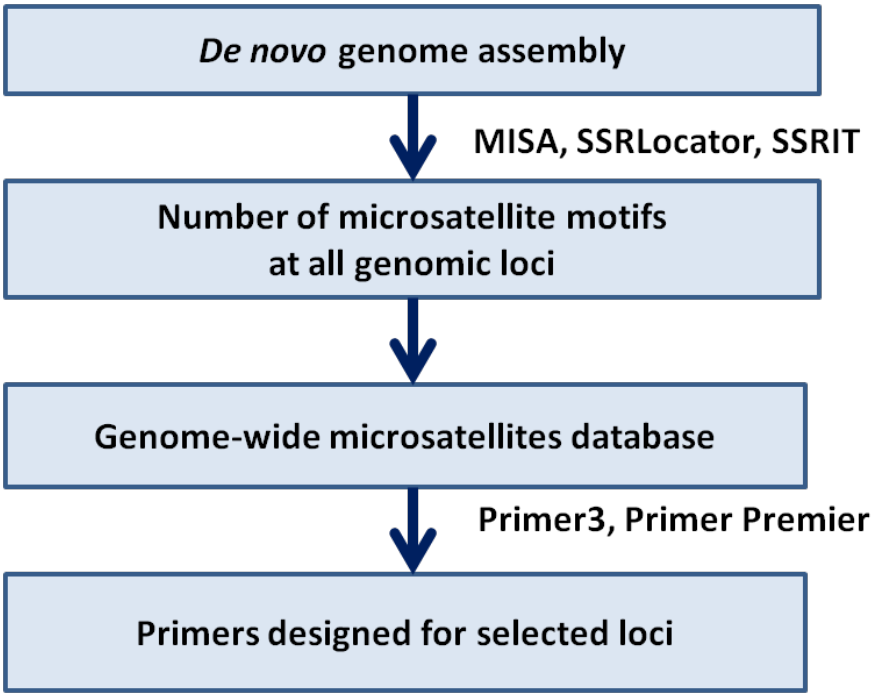

Fig 6.2. Flow chart describing the bioinformatics pipeline used to construct a genome-wide microsatellite markers database for *C. gloeosporioides*.

Table 6.3. Summary of microsatellite mining using MISA tool in the assembled genome of *C. gloeosporioides*.

	Isolate				
SSR mining	**016**	**064**	**092**	**193**	**194**
Total length of sequences analyzed (Mbp)	28.78	25.74	27.61	27.71	28.94
Total number of sequences examined	40,487	45,222	40,573	42,240	39,121
No. of sequences containing SSRs	4,506	4,315	4,266	4,543	4,629
No. of sequences with more than one SSRs	455	379	378	462	506
SSRs in compound forms	274	241	230	284	255
Perfect SSRs identified	4,756	4,497	4,446	4,796	4,963
Total SSRs	5,030	4,738	4,676	5,080	5,218

6.4.3. Frequency and distribution of microsatellite loci in *Colletotrichum gloeosporioides* genome

The distribution and frequency of perfect microsatellites with a minimum length of 5-bp and a unit size of 1 to 6-bp were analyzed. The results of the microsatellite mining database are summarized in Table 6.4. Among the different unit sizes, trinucleotide repeats were the most common. They constituted 38.1 to 41.4% of all SSRs, followed by mono- (28.3 to 34.9%) and dinucleotide repeats (22.1 to 24.0%) (Table 6.4 and Fig 6.3). The number of di-nucleotide motifs was 56.7 to 59.7% of that of the tri-nucleotide motifs. Penta- and hexa-nucleotide motifs were the least abundant.

Table 6.4. Summary of the occurrence of different types of microsatellite loci with perfect repeats in the *C. gloeosporioides* genome.

	016		**064**		**092**		**193**		**194**	
SSR Type	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Mono-nucleotide	1612	33.9	1347	28.3	1416	29.8	1597	33.6	1661	34.9
Dinucleotide	1050	22.1	1081	24.0	1012	22.8	1102	23.0	1163	23.4
Tri-nucleotide	1853	39.0	1862	41.4	1785	40.1	1855	38.7	1889	38.1
Tetra-nucleotide	183	3.8	172	3.8	170	3.8	178	3.7	189	3.8
Penta-nucleotide	46	1.0	28	0.6	53	1.2	51	1.1	45	0.9
Hexa-nucleotide	12	0.3	7	0.2	10	0.2	13	0.3	16	0.3
Total	4756	100	4497	100	4446	100	4796	100	4963	100

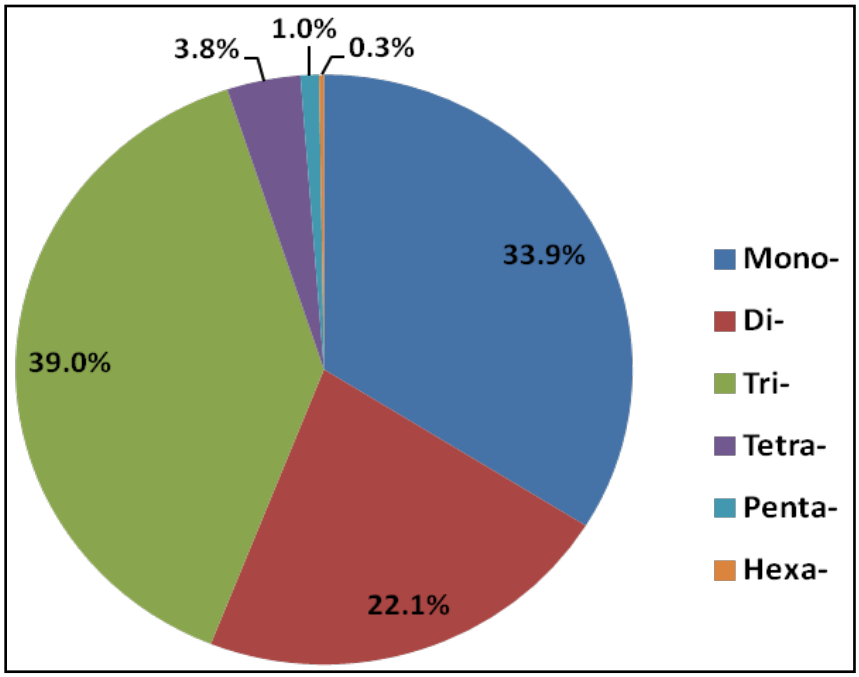

Fig 6.3. Graphical frequency distribution of microsatellite loci in a *C. gloeosporioides* isolate (016) by motif length.

The distribution of microsatellite motifs in *C. gloeosporioides* by the number of repeats are summarized in Fig 6.4. The number of motifs decreased as the number of the repeats increased. Up to 24 repeat units for mono-motifs, 23 for di-motifs, and 12 or fewer for longer motifs were observed. A more detailed investigation of individual repeat types was performed and presented in Fig 6.5. To analyze individual motif types, shifted motifs

and their reverse-complement motifs were grouped together. Results showed that A/T repeats were more abundant than G/C repeats in the *C. gloeosporioides* genome. The most frequent dinucleotide repeat unit was AC/GT, while CG/GC was very rare. In tri-motifs, the AGC/GCT and CAG/CTG repeats were the most frequent (152 and 156 in that order), followed by the ACC/GGT repeats (132). The predominant tetranucleotide repeat was AGAC, whereas repeats like CCAG, TTGA and AGGT were rare. In penta- and hexanucleotide repeats, GC-rich motifs were predominant.

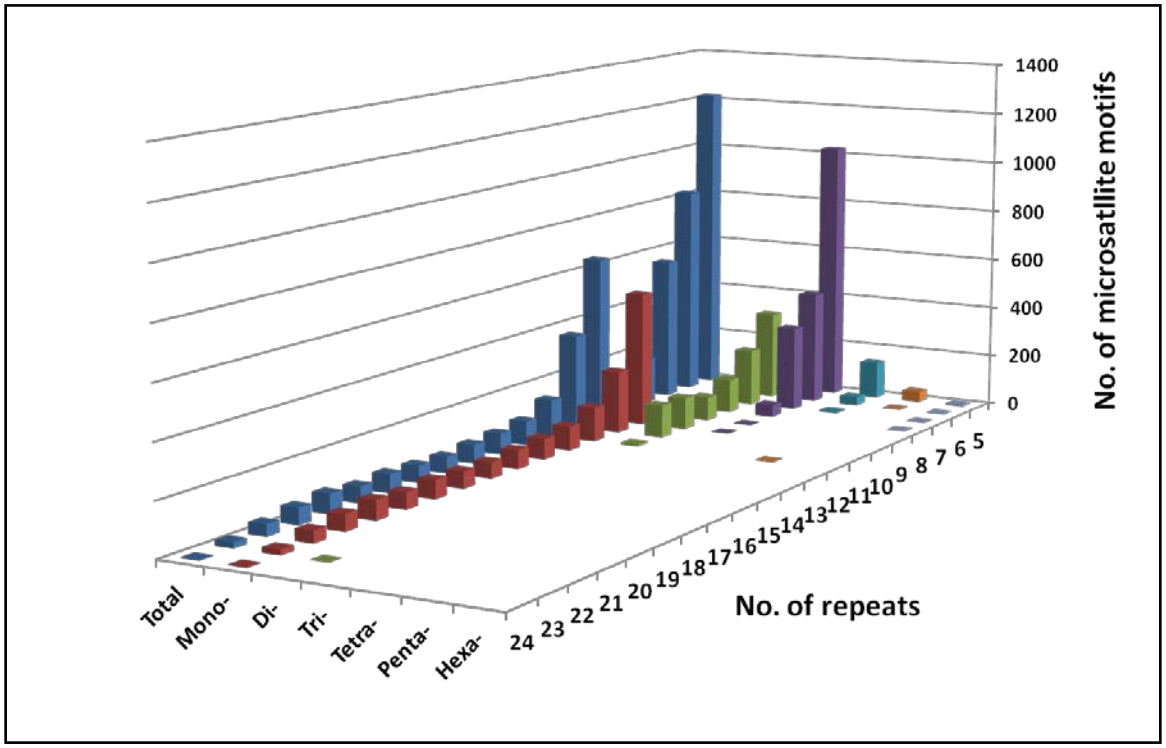

Fig 6.4. Distribution of mono- to hexa-microsatellite motifs in the genome of *C. gloeosporioides* by number of repeats.

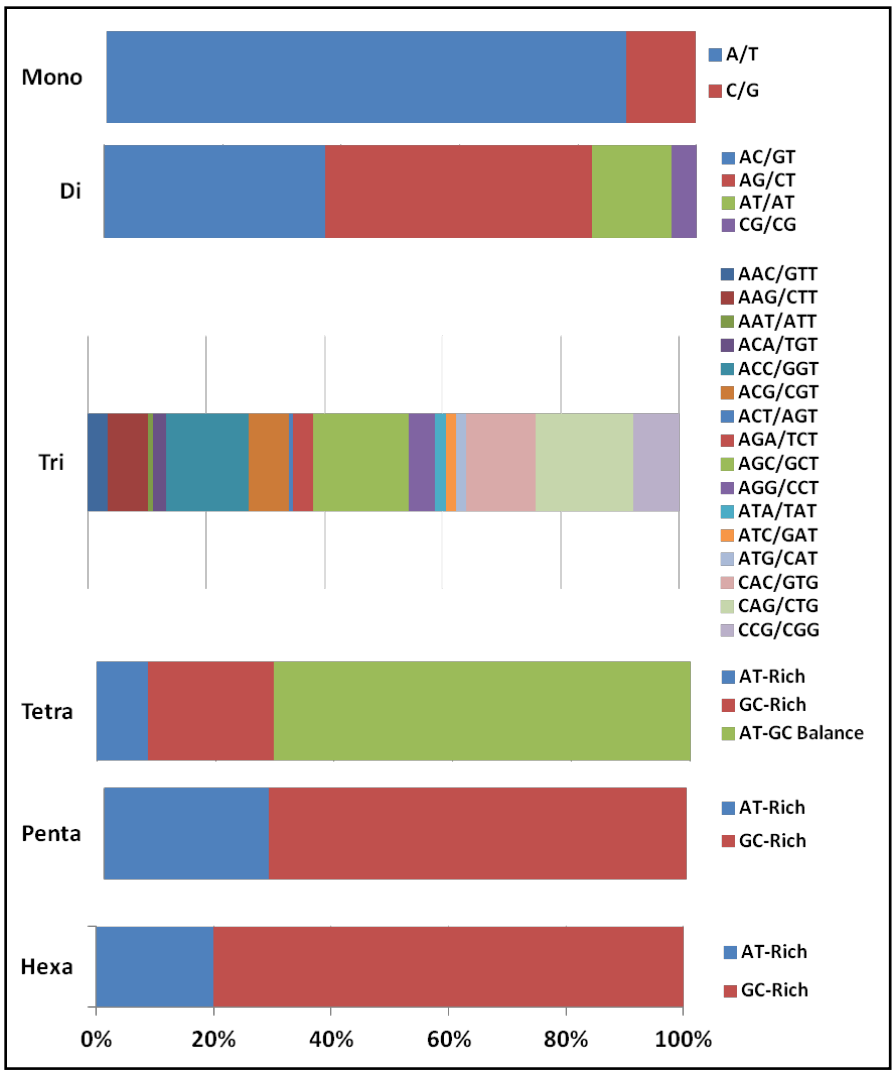

Fig 6.5. Frequency distribution of mono- to hexa- microsatellite motifs in *C. gloeosporioides* by motif sequences.

6.4.4. SSR marker development and primer design

One of the primary objectives of this study was to develop SSR markers for genetic analysis of *C. gloeosporioides*. Primers were designed for selected di- and tri-nucleotide repeats. A total of 284 SSR primer pairs were successfully designed using Primer3 (Rozen and Skaletsky, 2000). Primer pairs produced by Primer3 were validated using Primer Premier (PREMIER Biosoft package) and the best 50 primer pairs were chosen for further analyses. The details of the selected SSR primer pairs, including sequences,

Tm of forward and reverse primers, and the size of expected amplicons predicted by PrimerDigital (Kalendar *et al.*, 2011) is presented in Appendix Table 6.1.

6.4.5. SSR polymorphism

Due to resource limitation, twenty eight loci were selected and evaluated for polymorphism. Some of these loci did not show polymorphisms among the tested isolates. However, most of them demonstrated polymorphism and allelic diversity in the samples tested. Statistics of individual loci are summarized in Table 6.5. The frequency of the major allele in each polymorphic locus varied from 0.4231 to 0.9231. The number of alleles per locus were 2 to 4, the average being 2.8. The 21 SSR markers detected a total of 59 alleles in the thirteen *C. gloeosporioides* isolates studied. Gene diversity or expected heterozygosity ranged from 0.1420 to 0.6627, with an average of 0.38. The PIC values varied from 0.1319 to 0.6039, with a mean of 0.34 per marker.

Table 6.5. Summary statistics of polymorphic microsatellite markers in thirteen isolates of *C. gloeosporioides*

Marker	Major allele frequency	No. of alleles	Gene diversity	PIC[a]
CG1	0.9231	2	0.1420	0.1319
CG2	0.9231	2	0.1420	0.1319
CG3	0.4615	4	0.6627	0.6039
CG4	0.5385	3	0.5562	0.4652
CG6	0.8462	3	0.2722	0.2552
CG7	0.7692	4	0.3905	0.3693
CG9	0.8462	3	0.2722	0.2552
CG11	0.5385	4	0.6036	0.5395
CG14	0.7692	3	0.3787	0.3434
CG16	0.4615	4	0.6272	0.5556
CG18	0.9231	2	0.1420	0.1319
CG20	0.7000	2	0.4200	0.3318
CG21	0.6923	2	0.4260	0.3353
CG22	0.4231	4	0.6302	0.5576
CG27	0.7692	2	0.3550	0.2920
CG28	0.7692	3	0.3787	0.3434
CG29	0.8462	2	0.2604	0.2265
CG30	0.8889	2	0.1975	0.1780
CG32	0.4615	3	0.6154	0.5353
CG36	0.8462	3	0.2722	0.2552
CG37	0.8000	2	0.3200	0.2688

[a] Polymorphic Information Content

6.5. DISCUSSION

Genome sequencing and assembly has been a challenging task that requires time, resources and expertise. However, rapid advances in high-throughput sequencing technology and bioinformatic tools have revolutionized genome sequencing and the development of microsatellite markers (Cai *et al.*, 2013; Ekblom and Wolf, 2014). The 454 platform, with its longer reads, was the first choice in the earlier days. However, the high-throughput and lower per-base cost of the Illumina platform has become preferable, especially when used to generate large numbers of genome-wide microsatellite loci. Its shortcoming, in comparison to the 454 platform, is its shorter reads. To circumvent this limitation, Cai *et al.* (2013) applied an alternative approach to the fungus *Anisogramma anomala*, which first required genome assembly from the raw Illumina sequence reads, and then identification of microsatellites from the draft assembly. This approach overcame the read length problem and resulted in primer pairs successfully designed for the identified loci, and the designed primer pairs amplified their targets.

In the present study, it has been successfully generated pair-end genomic sequences of *C. gloeosporioides* using Illumina MiSec platform, *de novo* assembled the draft genome, and analyzed the distribution, frequency and density of microsatellites with motifs of 1 to 6 bp long and minimum of 5 repeats. A total of 5,030 microsatellite motifs were detected in *C. gloeosporioides* genome sequence at 4,506 loci, accounting for 0.024% of the assembled genome, with an overall density of 165 perfect SSRs/Mbp (Table 6.3). In this study, the distribution and frequency of the SSRs have been analyzed (Table 6.4, and Figs. 6.3 and 6.4). Trinucleotide motifs were the most frequent followed by mono- and dinucleotide motifs. Penta- and hexanucleotide motifs were the least abundant. The number of motifs decreased as the number of the repeats increased. Karaoglu *et al.* (2005) analyzed SSRs in completely sequenced genomes of nine taxonomically different fungal species. In all of the genomes they studied, the occurrence, abundance, and relative density of SSRs varied and was not influenced by the genome sizes. In most genomes they analyzed, mono-, di-, and trinucleotide repeats were more abundant than

the longer repeated SSRs. In each fungal organism they investigated, the occurrence, relative abundance, and relative density of SSRs decreased as the repeat unit increased. Their analysis also showed that the relative abundance of SSRs in fungi is low, and longer SSRs are rare. However, there is no known explanation for the lower occurrence of SSRs in fungi. Zhi-Fang *et al.* (2013) identified a total of 5,418 SSR loci in the genome of *Verticillium dahliae* which causes *Verticillium* wilt on cotton. Zhang *et al.* (2015) also identified 2,462 SSR loci in the genome of *Ustilago maydis*, causative agent of smut disease on maize, with 125 SSRs/Mbp. Roughly, SSRs occupy between 0.08% and 0.67% of the fungal genomes (Karaoglu *et al.*, 2005). Genome-wide analysis of microsatellites and information on their distribution provide insights into possible roles of microsatellites in gene regulation and genome organization, and provide abundant markers for genetic, genomic and evolutionary studies (Cavagnaro *et al.*, 2010).

In this study, A/T repeats were more abundant than C/G repeats in the *C. gloeosporioides* genome. AC/GT, AGC/GCT and CAG/CTG, and AGAC were the most frequent repeat units among di-, tri- and tetra motifs, respectively. In penta- and hexanucleotide repeats, GC-rich motifs were predominant. Toth *et al.* (2000) examined microsatellite frequencies in several eukaryotic organisms including fungi, and reported higher overall microsatellite density in intergenic regions and introns as compared to exons in all the taxa investigated. Variations inherent to the genomes examined in their study may account for large part of the observed differences. Some of these differences may also be due to variations in the search parameters and algorithms used for detection of microsatellites in DNA sequence, and differences in the type and size of the data sets used (Cavagnaro *et al.*, 2010). Toth *et al.* (2000) used custom Perl scripts to detect mono- to hexanucleotide SSR motifs of at least 12 bp and two repeat units, and expressed the results in bp of repeats/Mbp of sequence instead of count/Mbp. These minor differences in procedure can strongly influence microsatellite distributions and impede direct comparison among studies (Cavagnaro *et al.*, 2010). Toth *et al.* (2000) reported that trinucleotide motifs were most abundant in protein-coding sequences. A/T, AT, and AAT repeats were the most frequent mono-, di- and trinucleotide repeats in fungi genomes

studied. Karaoglu *et al.* (2005) also found a strong overrepresentation of A/T compared with C/G sequences in the genomes of nine fungi species investigated. The relative abundance of trinucleotides over other SSR types could be attributed to negative selection against frame-shift mutations in the coding regions and positive selection for specific single amino-acid stretches (Morgante *et al.*, 2002).

In this study, more than twenty SSR markers were developed (Table 6.5) that could be useful to analyze genetic diversity and population structure of *C. gloeosporioides* isolates affecting citrus. The polymorphisms detected by these SSR markers were in the range of slightly informative to highly informative. They displayed allelic diversity among the tested isolates, with 2 to 4 alleles per locus, and the PIC values ranged from 0.1319 to 0.6039. The PIC provides an estimate of the discriminatory power of a locus by taking into account the number and the relative frequencies of the alleles (Marulanda *et al.*, 2014). The use of microsatellites as genetic markers has been very popular due to their abundance and length variation between different individuals (Karaoglu *et al.*, 2005). Ciampi *et al.* (2011) demonstrated the better discriminatory power of SSR markers in comparison to the commonly used universal markers such as the ITS to estimate genetic diversity in *C. acutatum*. Polymorphic SSR markers have been developed from whole genome sequences of different fungi species such as *Aspergillus nidulans*, *Cryptococcus neoformans*, *Encephalitozoon cuniculi*, *Fusarium graminearum*, *Magnaporthe grisea*, *Neurospora crassa*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Ustilago maydis* (Karaoglu *et al.*, 2005), *Anisogramma anomala* (Cai *et al.*, 2013), *Verticillium dahliae* (Zhi-Fang *et al.*, 2013), and *Ustilago maydis* (Zhang *et al.*, 2015).

In conclusion, this study has contributed to a detailed characterization of microsatellite loci in *C. gloeosporioides* genome. The occurrence of SSRs in *C. gloeosporioides* was found to be comparatively less frequent than in other eukaryotic genomes. The data and information presented here increase the genomic resources available in *C. gloeosporioides* by adding a large set of designed microsatellite primers. The small-scale population study could serve as a proof-of-concept showing that the approach was

successfully applied to the *C. gloeosporioides*. The informative markers developed here are useful to characterize and better understand the genetic diversity and population structure of this fungal pathogen.

REFERENCES

Abdelkrim, J., B.C. Robertson, J.A.L. Stanton and N.J. Gemmell. 2009. Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. *BioTechniques*, 46:185-191.

Adaskaveg, J.E. and H. Forster. 2000. Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California. pp. 317-336. In: D. Prusky, S. Freeman and M.B. Dickman (eds.), *Colletotrichum*: Host Specificity, Pathology and Host-Pathogen Interaction. APS Press, St. Paul, MN.

Aiello, D., R. Carrieri, V. Guarnaccia, A. Vitale, E. Lahoz and G. Polizzi. 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology*, 163:168-177.

Allentoft, M.E., S.C. Schuster, R.N. Holdaway, M.L. Hale, E. McLay, C. Oskam, M.T.P. Gilbert, P. Spencer, E. Willerslev and M. Bunce. 2009. Identification of microsatellites from an extinct moa species using high-throughput (454) sequence data. *BioTechniques*, 46:195-200.

Anderson, J.A., G.A. Churchill, J.E. Autrique, S.D. Tanksley and M.E. Sorrels. 1993. Optimizing parental selection for genetic linkage maps. *Genome*, 36:181-186.

Bankevich, A., S. Nurk, D. Antipo, A.A. Gurevich, M. Dvorkin, N. Kulikov, et al. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, 19:455-477.

Belayneh Admassu, W. Friedt and F. Ordon. 2010. Genetic characterization of *Puccinia graminis f.sp. tritici* populations from Ethiopia by SSRs. *Journal of Phytopathology*, 158:806-812.

Benali, S., B. Mohamed, H.J. Eddine and C. Neema. 2011. Advances of molecular markers application in plant pathology research. *European Journal of Scientific Research*, 50:110-123.

Benson, G. 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acid Research*, 27:573-580.

Botstein, D., R.L. White, M. Skolnick and R.W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32:314-331.

Cai, Z., G. Li, C. Lin, T. Shi, L. Zhai, Y. Chen and G. Huang. 2013. Identifying pathogenicity genes in the rubber tree anthracnose fungus *Colletotrichum gloeosporioides* through random insertional mutagenesis. *Microbiological Research*, 68:340-350.

Cannon, P.F., P.D. Bridge and E. Monte. 2000. Linking the past, present, and future of *Colletotrichum* systematics. pp. 1-20. In: D. Prusky, S. Freeman and M. Dickman (eds.), *Colletotrichum*: Host specificity, Pathology and Host-pathogen interaction. APS Press, St. Paul, MN, USA.

Capote, N., A.M. Pastrana, A. Aguado and P. Sanchez-Torres. 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. pp. 151-202. In: C.J. Cumagun (ed.), Plant Pathology. InTech, Rijeka, Croatia.

Castoe, T.A., A.W. Poole, A.P.J. de Koning, K.L. Jones, D.F. Tomback, S.J. Oyler-McCance, J.A. Fike, S.L. Lance, J.W. Streicher, E.N. Smith and D.D. Pollock. 2012. Rapid microsatellite identification from Illumina paired-end genomic sequencing in two birds and a snake. *PLOS ONE*, 7(2): e30953.

Castoe, T.A., A.W. Poole, W.J. Gu, A.P.J. De Koning, J.M. Daza, E.N. Smith And D.D. Pollock. 2010. Rapid identification of thousands of copperhead snake (*Agkistrodon contortrix*) microsatellite loci from modest amounts of 454 shotgun genome sequence. *Molecular Ecology Resources*, 10:341-347.

Cavagnaro, P.F., D.A. Senalik, L. Yang, P.W. Simon, T.T. Harkins, C.D. Kodira, S. Huang and Y. Weng. 2010. Genome-wide characterization of simple sequence repeats in cucumber (*Cucumis sativus* L.). *BMC Genomics*, 11:569-586.

Ciampi, M.B., C. Baldauf, B.B.Z. Vigna, A.P. Souza, M.B. Sposito and L. Amorim. 2011. Isolation and characterization of microsatellite loci in *Colletotrichum acutatum*, the causal agent of post-bloom fruit drop on citrus. *Conservation Genetics Resources*, 3:651-654.

Cox, M.P., D.A. Peterson and P.J. Biggs. 2010. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics*, 11:485.

Da Maia, L.C., D.A. Palmieri, V.Q. de Souza, M.M. Kopp, F.I.F. de Carvalho and A.C. de Oliveira. 2008. SSR Locator: tool for simple sequence repeat discovery integrated with primer design and PCR simulation. *International Journal of Plant Genomics*, 2008:412696. doi:10.1155/2008/412696

Dutech, C., J. Enjalbert, E. Fournier, F. Delmotte, B. Barres, J. Carlier, D. Tharreau and T. Giraud. 2007. Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology*, 44:933-949.

Ekblom, R. and J.B.W. Wolf. 2014. A field guide to whole-genome sequencing, assembly and annotation. *Evolutionary Applications*, 7:1026-1042.

Gan, P., K. Ikeda, H. Irieda, M. Narusaka, R.J. O'Connell, Y. Narusaka, Y. Takano, Y. Kubo and K. Shirasu. 2013. Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. *New Phytologist*, 197:1236-1249.

Jarne, P. and P.J.L. Lagoda. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11:424-429.

Ji, P.F., Y. Zhang, C. Li, Z.X. Zhao, J. Wang, J. Li, P. Xu and X. Sun. 2012. High throughput mining and characterization of microsatellites from common carp genome. *International Journal of Molecular Sciences*, 13:9798-9807.

Kalendar, R., D. Lee and A.H. Schulman. 2011. Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis. *Genomics*, 98:137-144.

Karaoglu, H., C.M.Y. Lee and W. Meyer. 2005. Survey of simple sequence repeats in completed fungal genomes. *Molecular Biology and Evolution*, 22:639-649.

Liu, K. and S.V. Muse. 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics*, 21:2128-2129.

Marulanda, M.L., A.M. Lopez, L. Isaza and P. Lopez. 2014. Microsatellite isolation and characterization for *Colletotrichum* spp., causal agent of anthracnose in Andean blackberry. *Genetics and Molecular Research*, 13:7673-7685.

Matsuoka, Y., S.E. Mitchell, S. Kresovich, M. Goodman and J. Doebley. 2002. Microsatellites in *Zea* variability, patterns of mutations, and use for evolutionary studies. *Theoretical and Applied Genetics*, 104:436-450.

Morgante, M., H. Hanafey and W. Powell. 2002. Microsatellites are preferentially associated with non-repetitive DNA in plant genome. *Nature Genetics*, 30:194-200.

Peixoto-Junior, R.F., S. Creste, M.G.A. Landell, D.S. Nunes, A. Sanguino, M.F. Campos, R. Vencovsky, E.V. Tambarussi and A. Figueira. 2014. Genetic diversity among *Puccinia melanocephala* isolates from Brazil assessed using simple sequence repeat markers. *Genetics and Molecular Research*, 13:7852-7863.

Ramos, A.P., Z. Merali, P. Talhinhas, S. Sreenivasaprasad and H. Oliveira. 2006. Molecular and morphological characterization of *Colletotrichum* species involved in citrus anthracnose in Portugal. *Bulletin OILB/SROP*, 29(3):317-326.

Rozen, S. and H.J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. pp. 365-368. In: S. Krawetz Misener (ed.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ.

Sreenivasaprasad, S. and P. Talhinhas. 2005. Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Molecular Plant Pathology*, 6:361-378.

Sutton, B.C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. pp. 1-26. In: J.A. Bailey and M.J. Jeger (eds.), *Colletotrichum*: Biology, Pathology and Control. CAB international, Wallingford, UK.

Temnykh, S., G. DeClerck, A. Lukashova, L. Lipovich, S. Cartinhour and S. McCouch. 2001. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): frequency, length variation, transposon associations, and genetic marker potential. *Genome Research*, 11:1441-1452.

Thiel, T., W. Michalek, R.K. Varshney and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics*, 106:411-422.

Toth, G., Z. Gaspari and J. Jurka. 2000. Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Research*, 10:967-981.

Timmer, L.W. and G.E. Brown. 2000. Biology and control of anthracnose diseases of citrus. pp. 300-316. In: D. Prusky, S. Freeman and M.B. Dickman (eds.), *Colletotrichum*: Host Specificity, Pathology, and Host-Pathogen Interaction. APS Press, St. Paul, MN.

Weir, B.S. 1996. Genetic data analysis II: methods for discrete population genetic data. Sinauer Associates, Inc. Sunderland, Massachusetts.

Wheeler, D.A., M. Srinivasan, M. Egholm, Y. Shen, L. Chen, A. McGuire, W. He, Y-J. Chen, V. Makhijani, G.T. Roth, X. Gomes, K. Tartaro, F. Niazi, C.L. Turcotte, G.P. Irzyk, J.R. Lupski, C. Chinault, X. Song, Y. Liu, Y. Yuan, L. Nazareth, X. Qin, D.M. Muzny, M. Margulies, G.M. Weinstock, R.A. Gibbs and J.M. Rothberg. 2008. The complete genome of an individual by massively parallel DNA sequencing. *Nature*, 452:872-876.

Yu, J-N., C. Won, J. Jun, Y. Lim and M. Kwak . 2011. Fast and cost-effective mining of microsatellite markers using NGS technology: an example of a Korean water deer *Hydropotes inermis argyropus*. *PLOS ONE*, 6:e26933.

Zhang, M., Y. Chen, J. Yuan and Q. Meng. 2015. Development of genomic SSR markers and analysis of genetic diversity of 40 haploid isolates of *Ustilago maydis* in China. *International Journal of Agriculture and Biology*, 17:369-374.

Zhi-Fang, L., Z. He-Qin, F. Zi-Li, Z. Li-Hong, S. Yong-Qiang and Y. Zhi-Xin. 2013. Development of polymorphic microsatellite markers from *Verticillium dahliae*, a pathogenic fungus of cotton. *African Journal of Microbiology Research*, 7:3556-3558.

Zane, L., L. Bargelloni and T. Patarnello. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11:1-16.

7. GENETIC DIVERSITY AND POPULATION STRUCTURE OF *COLLETOTRICHUM GLOEOSPORIOIDES* INFECTING CITRUS USING SSR MARKERS

7.1. ABSTRACT

Twenty three polymorphic microsatellite markers were used to analyze the genetic diversity and population structure of 163 isolates of *C. gloeosporioides* from four different geographic regions of Ethiopia. These loci produced a total of 118 alleles with an average of 5.13 alleles per microsatellite marker. The polymorphic information content values ranged from 0.104 to 0.597 with an average of 0.371. The average observed heterozygosity across all loci varied from 0.046 to 0.058. The gene diversity among the loci ranged from 0.106 to 0.664. Unweighted Neighbor-joining and model-based population structure analyses grouped these 163 isolates into three major groups. The clusters were not according to the geographic origin of the isolates. Analysis of molecular variance showed 85% of the total variation within populations and only 5% among populations. There was low genetic differentiation in the total populations (F_{ST} = 0.049) as evidenced by high level of gene flow estimate (N_m = 4.8 per generation) among populations. The results showed that Ethiopian *C. gloeosporioides* populations are generally characterized by a low level of genetic diversity. The microsatellite markers developed and used in this study were helpful in analyzing the genetic diversity and population structure of the *C. gloeosporioides* populations. Information obtained from this study could be useful as a base to design strategies such as resistance breeding, biocontrol and integrated disease management practices for the control of this pathogen in Ethiopia.

7.2. INTRODUCTION

Members of the genus *Colletotrichum* represent a group of plant pathogenic fungi that can infect a wide range of plant species including many commercially cultivated crops

(Sreenivasaprasad and Talhinhas, 2005; Gan *et al.*, 2013). *C. gloeosporioides* species complex is one of the most common and widely distributed plant pathogens worldwide (Sutton, 1992; Cannon *et al.*, 2000). It is an important pathogen associated with more than 470 different host species, either as a primary disease causing organism, or isolated from deteriorated plant parts (Hyde *et al.*, 2009). It is economically important pathogen of a wide range of fruit crops, including citrus (Adaskaveg and Forster, 2000; Timmer and Brown, 2000; Ramos *et al*., 2006; Aiello *et al.*, 2015), apple (Carvalho *et al*., 2000), avocado (Freeman *et al*., 2000), mango (Afanador-Kafuri *et al*., 2003), olive (Martin and Garcia-Figueres, 1999), papaya (Rampersad, 2013), passion fruit (Afanador-Kafuri *et al*., 2003) and strawberry (Smith and Black, 1990; Buddie *et al*., 1999; Xie *et al*., 2010).

Colletotrichum species was reported as one of the fungal pathogens affecting citrus in Ethiopia (Seifu, 2003; Mohammed, 2007; Sisay, 2007). In the present study, *C. gloeosporioides* has been found associated with leaf and fruit spot disease of citrus across citrus growing regions in the country. Identification of sources of resistance and epidemiological studies are the priority areas of citrus research in Ethiopia (Mohammed, 2007). Disease resistant varieties could serve as essential part of sustainable and long-term disease management strategies (Abang *et al.*, 2003). However, resistance breeding requires extensive information on the genetics of host-pathogen interactions (Belayneh *et al.*, 2010). Little is known about the population biology and genetic diversity of *C. gloeosporioides* from citrus. Such information is vital to define the regional populations of the pathogen and to support disease resistance breeding efforts (Marulanda *et al.*, 2014).

DNA markers are in wide use for analyzing the dynamics of plant pathogen populations due to their high precision levels (Milgroom and Peever, 2003). They are rapid, highly specific and can be detected using minute quantity of DNA (Schaad *et al.*, 2003; Benali *et al.*, 2011). Microsatellites (Litt and Luty, 1989), also known as SSRs (Tautz, 1989) or short tandem repeats (STR) (Edwards *et al*., 1991), are one of the highly versatile genetic markers that have been widely used for the genetic study of plant pathogens (Benali *et al.*, 2011; Capote *et al.*, 2012). Microsatellites comprise tandemly repeated nucleotide motifs of one to six base pairs

long (Tautz and Renz, 1984; Jarne and Lagoda, 1996; Matsuoka *et al.*, 2002; Zane *et al*., 2002; Capote *et al.*, 2012). They are relatively abundant, co-dominant, ubiquitous, and exhibit extensive levels of polymorphisms in prokaryotic and eukaryotic genomes (Tautz, 1989; Weber, 1990; Weising *et al.*, 1995; Powell *et al.*, 1996; Toth *et al.*, 2000; Katti *et al.*, 2001; Morgante *et al.*, 2002). Microsatellites are found in both coding and non-coding regions (Toth *et al.*, 2000; Katti *et al.*, 2001; Matsuoka *et al*., 2002; Ellegren, 2004; Sharopova, 2008), but they are more abundant in non-coding regions (Hancock, 1995; Zane *et al*., 2002; Capote *et al.*, 2012). Microsatellites enable higher statistical power and discrimination among genotypes (Halkett *et al.*, 2005).

The high levels of polymorphisms of SSR markers and the relative ease of detection of these polymorphisms by PCR amplification has led to the wide applications of SSRs as genetic markers (Karaoglu *et al.*, 2005). Microsatellites have proven to be invaluable in many fields of biology that span from forensic DNA studies to genome mapping, paternity testing, population genetics and biological resources conservation (Jarne and Lagoda, 1996; Schuler *et al.*, 1996; Knapik *et al.*, 1998; Luikart *et al.*, 2003). One of the major limitations of SSR markers is high mutation rates ranging from 10^{-3} to 10^{-6} per generation (Weber and Wong, 1993; Schug *et al.*, 1997; Xu *et al.*, 2000) due to slipped-strand miss-pairing and subsequent resulting errors during DNA replication, repair and recombination (Levinson and Gutman, 1987; Schlotterer and Tautz, 1992; Schlotterer, 2000). The major drawbacks of the traditional methods to develop SSR markers are that they require prior knowledge of the DNA sequences of the flanking regions, they are expensive and time-consuming, and they have low throughput due to difficulties for automation and data management (Morgante and Olivieri, 1993; Brouwer and Osborn, 1999; Morgante *et al*., 2002; Zane *et al*., 2002). A high number of microsatellites loci are also required for a reliable phylogenetic reconstruction (Capote *et al.*, 2012). At present, these problems are partly resolved with the advent of high-throughput next-generation sequencing technologies and multiplexing microsatellites (Capote *et al.*, 2012; Cai *et al.*, 2013).

Fungal genomes appear to contain fewer SSR sequences than other eukaryotes (Dutech *et al.*, 2007). However, when polymorphic loci are available, they can be very useful for genome mapping, and genetic diversity and population genetic studies (Peixoto-Junior *et al.*, 2014). Accordingly, SSRs have been used for the study of the genetic diversity of various plant pathogenic fungi including *Ascochyta rabiei* (Bayraktar *et al.*, 2007), *Ceratocystis fimbriata* (Rizatto *et al.*, 2010), *Macrophomina phaseolina* (Jana *et al.*, 2005), *Puccinia graminis* and *P. triticina* (Szabo, 2007; Szabo and Kolmer, 2007; Belayneh *et al.*, 2010), *Sclerotinia subarctica* and *S. sclerotiorum* (Winton *et al.*, 2007), and *C. gloeosporioides* from Andean blackberry (Marulanda *et al.*, 2014).

The objective of this study was to analyze the genetic diversity and population structure of *C. gloeosporioides* populations from the major citrus production areas of Ethiopia using SSRs.

7.3. MATERIALS AND METHODS

7.3.1. Fungal isolates and culture conditions

A total of 163 *C. gloeosporioides* isolates were isolated on water agar from symptomatic citrus leaf and fruit samples collected from major citrus production areas of Ethiopia during 2012 to 2014 as described in chapter 4. Single spore or hyphal tip cultures were developed in water agar and subsequently transferred to PDA supplemented with antibiotics (100 ppm of ampicillin, 50 ppm of chloramphenicol and 50 ppm of streptomycin sulphate). Isolates were assigned into four populations based on their geographic origin (Table 7.1). The details of isolates used in this study are presented in Appendix Table 7.1.

Table 7.1. Geographic origin of *C. gloeosporioides* populations, the number of isolates represented in each population and their hosts

Region	No. of isolates	Origin (Districts)	Host	Year of collection
Central Ethiopia	43	Abeshege	*Citrus sinensis*	2012-2014
		Cheha	*C. sinensis*	
		Geta	*C. sinensis*	
		Goro	*C. sinensis*	
		Kebena	*C. sinensis*	
		Sekoru	*C. sinensis* and *C. reticulata*	
		Wolliso	*C. sinensis* and *C. aurantium*	
Northwest Ethiopia	43	Guangua	*C. sinensis*	2013
		Jabitehnan	*C. sinensis* and *C. reticulata*	
South Ethiopia	30	Abaya	*C. sinensis* and *C. aurantium*	2013-2014
		Aleta Wendo	*C. sinensis*	
		Boloso Sore	*C. sinensis*	
		Damot Pulasa	*C. sinensis*	
Southwest Ethiopia	47	Debre Werk	*C. sinensis*	2013
		Ginbo	*C. sinensis*	
		Gomma	*C. sinensis*	
		Jimma	*C. sinensis*	
		Mana	*C. sinensis*	
		Shebe Senbo	*C. sinensis*	
Total	163			

7.3.2. DNA extraction

The total genomic DNA was extracted from lyophilized mycelia obtained from 10-day-old cultures grown on PDA as described by Ghimire *et al.* (2011) using Qiagen MagAttract96 DNA Plant Core Kit according to the manufacturer's instructions. The quality and concentration of extracted DNA were estimated using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific) and the integrity of the DNA was visualized in 1% agarose gel stained with SYBR Safe DNA gel stain under ultra violet-light (UVP BioImaging Systems). DNA was stored at -20°C until further use.

7.3.3. PCR amplification and genotyping

A total of 23 polymorphic SSR markers were used for analysis (Table 7.2). The 5' end of the forward primers of all SSR loci were labeled with fluorescent dyes (6-FAM, PET, VIC and NED). Multiplex PCR amplifications were performed in a total volume of 10 µl containing AccuPower PCR PreMix without dye (Bioneer), additional 0.5 mM $MgCl_2$ (Promega), 0.05 to 0.15 µM forward primer, 0.05 to 0.15 µM reverse primer, and 2.0µl template DNA (20 ng/µl). PCR reaction without template DNA was used as control. Multiplex PCR amplifications were performed using four to five SSR primer pairs in each PCR based on their annealing temperatures. Amplifications were performed in a GeneAmp PCR System 9700 thermocycler (Applied Biosystems) using the following PCR cycling conditions: initial denaturation step at 94°C for 3 min; followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 50°C or 51°C (Table 7.2) for 1 min, and extension at 72°C for 2 min; and a final extension step at 72°C for 10 min. To reveal polymorphism and allele identification, the PCR products were separated in 2% agarose gel stained with GelRed and visualized under UV light. The sizes of the PCR amplicons were estimated using a 100-bp ladder (Invitrogen).

In addition to agarose gel electrophoresis, fragment sizes of the PCR products and allele variations in the repeats were assessed by capillary electrophoresis of amplicons and by sequencing the amplified loci. The multiplexed PCR products were mixed with 8.87 µl Hi-Di formamide and 0.135 µl fluorescent-labeled GeneScan™-500 LIZ internal size standard (Applied Biosystems, Warrington, UK) in a 96-well microtiter plate. The mixed products were denatured at 95°C for 3 min and snap-chilled on ice for 5 min. The products were then electrophoresed using an ABI PRISM 3730xl automated genetic analyzer (Applied Biosystems). Scoring of fragment allele size was performed manually using GeneMapper software version 4.1 (Applied Biosystems). The SSR markers were scored for the presence or absence of the corresponding bands for the tested isolates.

Table 7.2. Characteristics of 23 polymorphic SSR markers used for population diversity analysis of *C. gloeosporioides* isolates.

Locus	Forward primer sequences (5'- 3')	Reverse primer sequences (5'- 3')	Ta (ºC)[a]	Allele size range (bp)	Repeat motifs
CG1	CAAGCAGTCTTTCTGGTCTT	AAAACAACTTCTCTCGTCCA	51	125-129	(TG)6
CG2	TCACCTTCACTCACACTTGA	CTACTTCGAGACAAGCACG	51	201-203	(CT)6
CG3	GGTTTTCTCATTCTCAACA	CGACATGATCCATAGCAAG	50	247-253	(AT)6
CG4	AACTCAAGATCAAGAGCAGC	ATGTACAGACGCTCACACAA	51	150-161	(TG)6
CG6	AGAGCAAGACAGGTGGAATA	ATCCCTGACTGCATAAACC	51	201-220	(AC)6
CG7	ATCTCCAGAGAGAACACAGC	GAGACCTCACGGAATTGAC	51	158-187	(TG)7
CG9	GTCTTGATGCTGAAGTCCAC	CACTCCTTCATAGAACACCC	51	218-257	(TG)6
CG11	CAGTGAAGATAGGGAAGCAG	ACCACTCAGCGTATGAGAAA	51	107-152	(GT)8
CG14	ACATGACATCAAACCAGCTT	CTCTTGACCCGATGTTCTAT	51	173-179	(TC)7
CG16	CCATTCTTTGTACTGGTCGT	GACATCAGACATCCATCCTC	51	186-196	(TG)6
CG18	TCCAGACGGATAGCTTACAC	GAGGTATTGCGTCCACTAAG	51	197-204	(CT)7
CG20	CATAGTCCGTCCAGTCTCAT	CTAATGAAAAGTCGTGGAGC	51	218-235	(GA)8
CG21	GTCTCACTCAGTCTCAAGCC	AACACAGTCTGAGAGGCAAT	51	223-229	(AT)9
CG22	CTTCGAGTCACCTCTTCAAC	CAGAGTGGTAAAGGTGGTGT	51	158-241	(AC)7
CG23	TATTAGATCCCGACCTTGTG	ATCCTGGTCACCATAATCC	51	160-182	(GA)6
CG27	CCTGTTGATCCATGATGTAA	GAAAGGCTGACTTGTGAACT	50	126-138	(GT)6
CG28	CATATCTCTTCGTACCTCGC	GGTTTGTTGTCTGCTTCTCT	51	163-170	(AG)8
CG29	TTTCAACTACATCCCACCTC	GTATTTGAGGCTGAAGCAAG	51	55-74	(AC)7
CG30	CGTCATTTTCTGGATTCACT	ATCCATTGGGCTGTCCAT	50	137-163	(GT)9
CG32	TTGTTAGCATCGTGAGTCAG	GCAGTTGATTGAGCAGTACA	51	209-217	(AG)10
CG33	GGCATCTATGGACTAGCAGA	TCATACACCAAAGCTTCCTC	51	150-229	(GC)6
CG36	CCACTCAATTCAATGACAGA	TGAGAGAGTTGTGTCCATCA	50	225-265	(AC)7
CG37	TTATATGCCCCATACTCACC	GGGTCATCTTACACCGTTAC	50	214-231	(CA)8

[a] Ta = Annealing temperature

7.3.4. SSR polymorphism and genetic diversity

Twenty-three polymorphic SSR markers were used to analyze the genetic diversity of 163 *C. gloeosporioides* isolates. The basic statistics, such as the major allele frequency, number of alleles per locus, gene diversity, heterozygosity, and PIC were determined using PowerMarker version 3.25 (Liu and Muse, 2005). For each SSR marker, the degree of polymorphism estimated by gene diversity (Nei, 1987) was calculated for all the 163 isolates. To estimate the discriminatory power of the microsatellite loci, the PIC for each locus was computed using the formula PIC = 1 - ΣPi^2, where Pi^2 is the sum of the *i*th allele frequency of each microsatellite locus for the genotypes (Botstein *et al.*, 1980; Anderson *et al.*, 1993).

The number of different alleles per locus, number of effective alleles per locus, number of private alleles, observed heterozygosity, expected heterozygosity (Weir, 1996), and Shannon's Information Index were computed for each population using GenAlEx version 6.501 (Peakall and Smouse, 2012). Allelic richness and private allelic richness were computed using the rarefaction method (Hulbert, 1971) implemented in HP-Rare version 1.1 software (Kalinowski, 2005). The exact tests to estimate the deviation from Hardy-Weinberg equilibrium (HWE) and genotypic linkage disequilibrium for all pairs of loci were computed using GenePop version 4.3 (Rousset, 2008) and corrected using the sequential Bonferroni procedure (Hochberg, 1988).

7.3.5. Population structure and gene flow

The Sokal and Michener dissimilarity index was used to generate dissimilarity matrices (Sokal and Michener, 1958), based on the set of SSR markers. To assess the distribution of gene diversity and estimate the components of variances of the populations, analysis of molecular variance (AMOVA) based on co-dominant SSR loci was computed using GenAlEx 6.501 (Peakall and Smouse, 2012). To investigate population differentiation, Wright (1951) fixation index (F_{ST}) of the total populations and pairwise F_{ST} among all pairs of populations were computed, and significance was tested based on 1000 bootstraps. Principal Coordinate

Analysis (PCoA) was done using the same software to show the pattern of genetic differentiation of the populations of *C. gloeosporioides* isolates. Gene flow among populations was estimated using indirect method based on the number of migrants per generation (N_m) using the formula, $N_m = 0.25(1 – F_{ST}) /F_{ST}$.

Frequency-based genetic distances were calculated using shared alleles distance matrix (Jin and Chakraborty, 1994), and used to construct Unweighted Neighbor-joining dendrogram for the 163 isolates belonging to the four populations of *C. gloeosporioides* using DARwin version 6.0.010 (http://darwin.cirad.fr) (Perrier *et al.*, 2003). The resulting tree was bootstrapped with 1000 replicates (Felsenstein, 1985) and viewed using TreeView version 1.6.6 (Page, 2001; available at http://taxonomy.zoology.gla.ac.uk/rod/rod.html).

The pattern of population structure and detection of admixture were inferred using a Bayesian model-based clustering algorithm implemented in STRUCTURE version 2.3.4 (Pritchard *et al.*, 2000; Falush *et al.*, 2003) using the SSR loci data. For this, two separate analyses were run with and without prior information about the populations. The first was done by assigning the site of collection as the putative population origin for each individual and the second run was without giving such information and letting the STRUCTURE software assign each individual into a population. The admixture model with correlated allele frequencies was used as suggested in the manual. To determine most appropriate number of populations (*K*), a burn-in period of 25,000 was used in each run, and data were collected over 100,000 MCMC replications from $K = 1$ to $K = 10$. The probability values were averaged across runs for each cluster. This procedure clusters individuals into populations and estimates the proportion of membership in each population for each individual (Falush *et al.*, 2003). The *K* value was determined by the log probability of data (Ln P(D)) based on the rate of change in LnP(D) between successive *K*. The optimum *K* value was predicted following the simulation method of Evanno *et al*. (2005) using the web-based software STRUCTURE HARVESTER version 0.6.92 (Earl and vonHoldt, 2012).

7.4. RESULTS

7.4.1. SSRs polymorphism and gene diversity

The polymorphism and diversity of the different SSR loci are presented in Table 7.3. Availability of alleles in each locus (the proportion of loci without missing alleles) was one for all the loci. The 23 SSR markers detected a total of 118 alleles in the 163 *C. gloeosporioides* isolates studied. Of the 118 alleles detected, 59 (50%) were rare (with frequency ≤ 0.05). The number of alleles per locus ranged from 3 to 8, with an average of 5.13 alleles. The allele size ranged from 55 to 265 bp (Table 7.2). The PIC values varied from 0.104 (CG29) to 0.597 (CG30), with an average of 0.371 per marker. Nine SSR loci were highly informative (PIC$\geq$ 0.5), seven were reasonably informative (0.5 <PIC > 0.25), and seven were slightly informative (PIC < 0.25). The frequency of the major allele in each locus varied from 0.41 to 0.94, with a mean of 0.69. The number of effective alleles was in the range of 1.0 to 2.3. Gene diversity, defined as the probability that two randomly chosen alleles from the population are different (Kiran Babu *et al.*, 2013), varied from 0.106 (CG29) to 0.664 (CG30), with an average of 0.41. Low level of heterozygosity (0.000 to 0.043) was detected in *C. gloeosporioides* isolates; but CG22 marker detected high heterozygosity of 0.865. Nine SSR loci had no heterozygosity while six displayed less than 0.01 heterozygosity. The expected heterozygosity ranged from 0.042 to 0.554 (Table 7.3). The Hardy-Weinberg exact test for all populations revealed that 21 loci (95.65%) exhibited significant deviation from HWE corrected for multiple comparisons (P<0.001), having levels of heterozygosity less than expected. Two loci (CG22 and CG23) did not show significant departure from HWE, and CG22 had a level of heterozygosity higher than expected. The Fisher's exact test showed that 60% of the pairwise combinations had significant genotypic linkage disequilibrium using Bonferroni correction. Given that most of the test isolates of *C. gloeosporioides* used in this study were assumed to be obtained from asexual populations, these results were not surprising.

Table 7.3. Diversity indices of the microsatellite loci used in the study.

SSR loci	MAF	Na	N_E	R_S	R_P	GD	H_O	H_E	PIC
CG1	0.91	3	1.1	1.46	0.04	0.160	0.000	0.055	0.153
CG2	0.89	3	1.1	1.25	0.25	0.200	0.000	0.048	0.186
CG3	0.72	5	1.5	2.75	0.11	0.452	0.012	0.319	0.427
CG4	0.50	5	2.1	2.86	0.26	0.588	0.043	0.507	0.506
CG6	0.92	5	1.2	1.73	0.73	0.151	0.000	0.118	0.149
CG7	0.85	6	1.3	2.22	0.74	0.277	0.012	0.205	0.268
CG9	0.85	7	1.2	2.26	0.98	0.274	0.000	0.135	0.260
CG11	0.64	8	1.9	3.03	1.07	0.537	0.037	0.431	0.494
CG14	0.83	4	1.3	2.24	0.03	0.299	0.006	0.199	0.285
CG16	0.46	5	2.2	2.72	0.28	0.611	0.006	0.541	0.533
CG18	0.89	5	1.1	1.93	0.39	0.198	0.006	0.088	0.189
CG20	0.49	4	1.7	2.24	0.24	0.630	0.018	0.407	0.561
CG21	0.43	5	2.0	2.57	0.26	0.623	0.018	0.495	0.546
CG22	0.44	6	2.3	2.89	0.56	0.626	0.865	0.554	0.552
CG23	0.88	5	1.0	1.55	0.55	0.219	0.000	0.042	0.202
CG27	0.44	5	1.8	2.39	0.39	0.636	0.000	0.436	0.561
CG28	0.75	5	1.4	2.34	0.47	0.417	0.006	0.241	0.386
CG29	0.94	4	1.1	1.48	0.48	0.106	0.000	0.099	0.104
CG30	0.41	6	2.0	2.75	0.75	0.664	0.006	0.471	0.597
CG32	0.49	5	2.2	2.82	0.17	0.599	0.025	0.529	0.520
CG33	0.83	7	1.2	2.21	0.98	0.296	0.006	0.125	0.277
CG36	0.86	6	1.1	1.93	0.93	0.252	0.000	0.103	0.237
CG37	0.49	4	1.6	2.25	0.25	0.620	0.000	0.363	0.547
Mean	0.69	5.13	1.54	2.26	0.48	0.410	0.046	0.283	0.371

MAF = major allele frequency; Na = number of alleles; N_E = number of effective alleles; R_S = allelic richness; R_P = private allelic richness; GD = gene diversity; H_O= observed heterozygosity; H_E= expected heterozygosity; and PIC = polymorphism information content

7.4.2. Population genetic diversity

The genetic diversity indices for the four *C. gloeosporioides* populations are summarized in Table 7.4. The number of different alleles (Na), private alleles (N_P) and effective alleles (N_E) averaged across all loci ranged from 1.70 to 3.26, 0.043 to 1.261, and 1.38 to 1.85, respectively for the four populations (northwest, central, southwest and south Ethiopia). The south Ethiopia (SE) population had the highest while the northwest Ethiopia (NWE) population had the lowest Na, N_P and N_E values. Similarly, the SE (R_S = 3.16) and NWE (R_S = 1.62) populations had the highest and the lowest allelic richness over all pairs of loci,

respectively. Private allelic richness was also the least in NWE (R_P = 0.04) and the highest in SE (R_P = 1.24) populations. Average observed heterozygosity (H_O) was in the range of 0.046 to 0.058, with a mean of 0.052 across all loci. Gene diversity was the lowest in NWE population (H_E = 0.209) and the highest in SE population (H_E = 0.403), and its value averaged over all populations and loci was 0.283 (SE = 0.022). The percentage of polymorphic loci (PL) ranged from 60.87% (NWE) to 100% (SE), with an average of 82.61% (Table 7.4).

Table 7.4. Summary of population diversity indices (average of 23 loci).

Population[a]	**Na**	**N_P**	**N_E**	**R_S**	**R_P**	**PL**	**H_O**	**H_E**	**I**
CE	2.04	0.130	1.42	1.87	0.13	73.91	0.058	0.234	0.368
NEW	1.70	0.043	1.38	1.62	0.04	60.87	0.048	0.209	0.311
SE	3.26	1.261	1.85	3.16	1.24	100	0.055	0.403	0.725
SWE	2.52	0.522	1.52	2.37	0.49	95.65	0.046	0.287	0.491
Overall	2.38	0.489	1.54	2.26	0.48	82.61	0.052	0.283	0.474

Na = number of different alleles; N_P = number of private alleles; N_E = number of effective alleles; R_S = allelic richness; R_P = private allelic richness; PL = percentage of polymorphic loci; H_O = average observed heterozygosity, H_E = expected heterozygosity or gene diversity, and I = Shannon's Information Index

[a] CE = central Ethiopia, NWE = northwest Ethiopia, SE = south Ethiopia, and SWE = southwest Ethiopia

7.4.3. Population genetic structure and gene flow

The analysis of molecular variance results showed that 84% of the total variation was due to differences among isolates within populations; 11% was due to heterozygosity within isolates; and the variation among populations accounted only 5% of the total variation (Table 7.5). The genetic differentiation among populations (F_{ST} = 0.049 at $P < 0.001$) was significant as indicated by the randomization test. Pairwise F_{ST} values of the genetic distance among all populations were significant ($P < 0.01$) (Table 7.6). The average estimate of N_m was 4.8.

Table 7.5. Analysis of molecular variance among and within populations, and within individuals of *C. gloeosporioides* populations based on 23 SSR loci.

Source of variation	Degree of freedom	Sum of squares	Mean squares	Estimated variance	Variation (%)	P value
Among Populations	3	83.428	27.809	0.238	5	< 0.001
Among Individuals	159	1367.517	8.601	4.033	84	< 0.001
Within Individuals	163	87.000	0.534	0.534	11	< 0.001
Total	325	1537.945	4.732			

Table 7.6. Pairwise genetic distance based on F_{ST} matrix, a measure of divergence among the *C. gloeosporioides* populations.

Population	CE	NWE	SE
NEW	0.062**		
SE	0.022*	0.056**	
SWE	0.056**	0.067**	0.027*

* significant at $P<0.01$; ** significant at $P< 0.001$

The unweighted NJ dendrogram grouped the 163 isolates of the four populations into three major clusters (Fig 7.1). Of the 163 isolates, 119, 42 and 2 isolates were grouped together in Cluster I, II and III, respectively. Overall topology of the dendrogram indicated the presence of three lineages in *C. gloeosporioides* complex associated with citrus leaf and fruit disease in Ethiopia. Several subgroups were observed for populations indicating genetic variability within and among isolates in each population. In terms of locations-specific alleles among the isolates, 17 SSR loci (CG2, CG4, CG6, CG7, CG9, CG11, CG16, CG18, CG20, CG22, Cg23, CG27, CG29, CG30, CG33, CG36 and CG37) showed unique alleles for the isolates from SE, 12 SSR marker (CG6, CG7, CG9, CG11, CG21, CG22, CG23, CG28, CG29, CG30, CG33 and CG36) displayed unique alleles for the isolates from SWE, three SSR markers (CG23, CG27 and CG32) detected unique alleles for the isolates from CE, and only one SSR marker (CG11) detected a unique allele for the isolates from NWE.

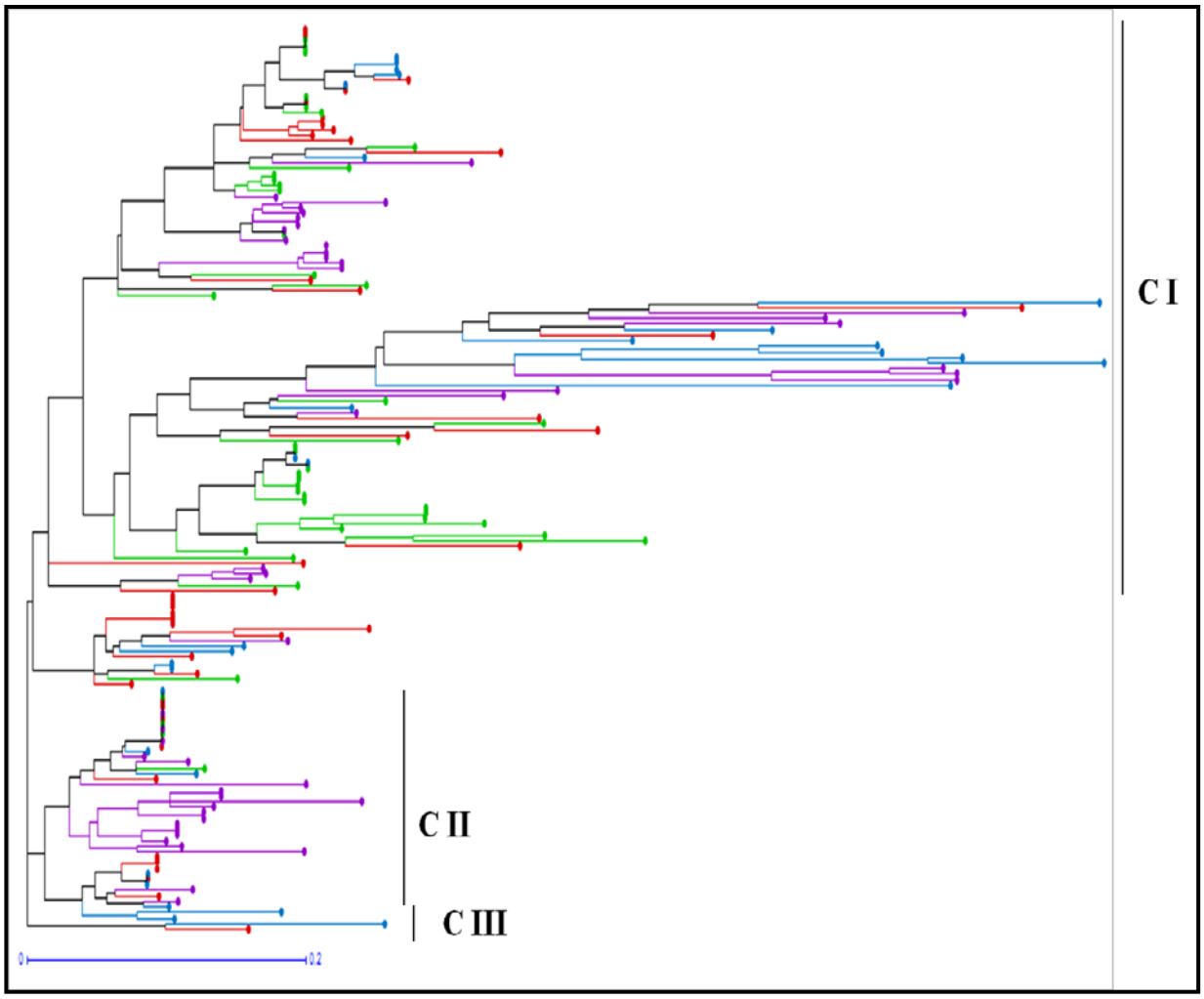

Fig 7.1. Unweighted Neighbor-joining tree using the simple matching similarity coefficient based on 23 microsatellite markers for the 163 isolates of *C. gloeosporioides* isolated from citrus in Ethiopia. The tree shows the clustering pattern of isolates from the four *C. gloeosporioides* populations. The populations are color coded as follows: central Ethiopia (*red*), northwest Ethiopia (*green*), south Ethiopia (*blue*) and southwest Ethiopia (*violet*).

The pattern of clustering was similar to the Principal coordinates analysis (PCoA) based on the 23 microsatellite loci (Fig 7.2). Percentages of variation explained by the first 3 axes were 36.16%, 11.6% and 8.68%, respectively. Evanno *et al.* (2005) method on STRUCTURE outputs predicted $K = 3$ to be the most likely number of clusters (Fig 7.3).

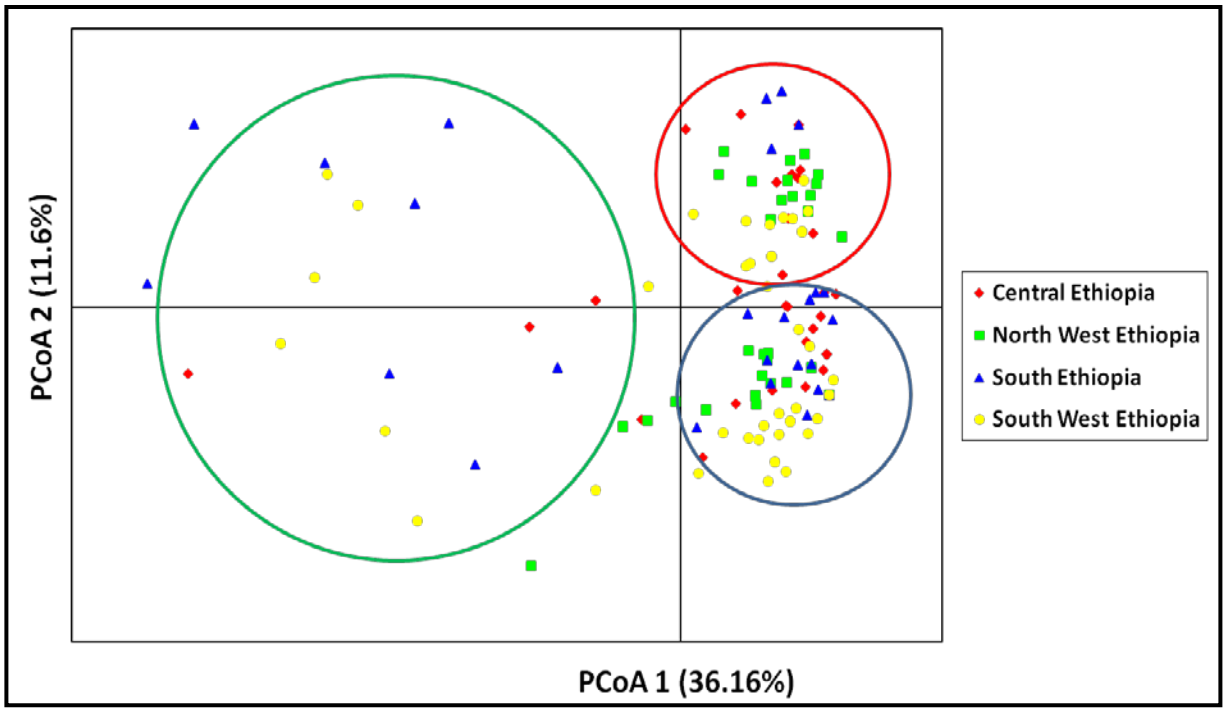

Fig 7.2. Principal coordinates analysis (PCoA) bi-plot showing the clustering of the 163 *C. gloeosporioides* isolates based on 23 microsatellite loci. The four populations are color coded as follows: central Ethiopia (*red*), northwest Ethiopia (*green*), south Ethiopia (*blue*) and southwest Ethiopia (*yellow*).

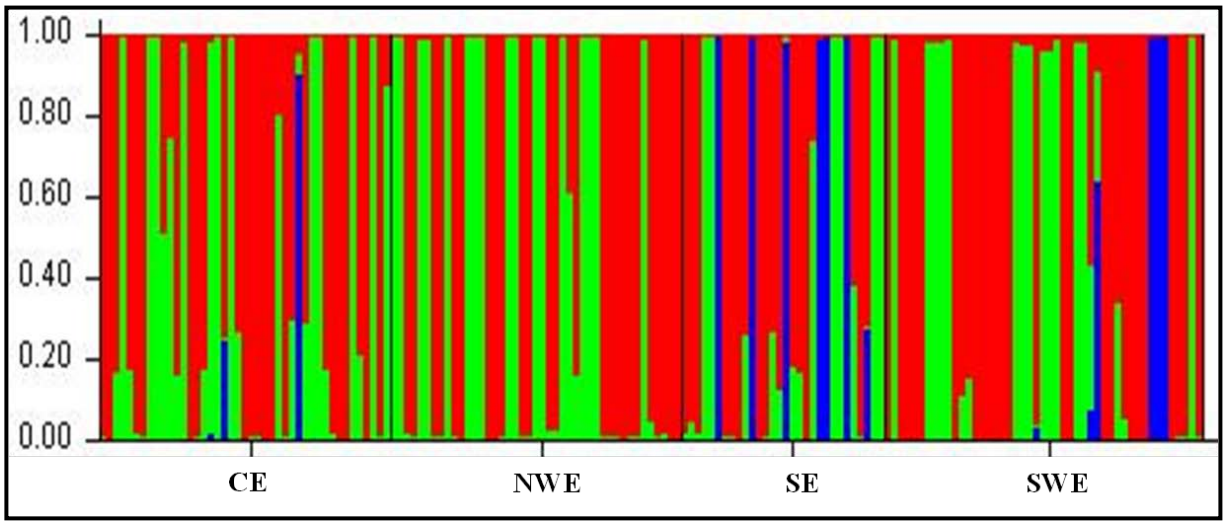

Fig 7.3. Bayesian model-based estimation of population structure (*K*= 3) for 163 *C. gloeosporioides* isolates in four pre-determined populations (x-axis): central Ethiopia (CE), northwest Ethiopia (NWE), south Ethiopia (SE) and southwest Ethiopia (SWE). Each group is separated by a black vertical line. Numbers in the y-axis show coefficient of membership/assignment.

7.5. DISCUSSION

The present study is the first report that used SSR markers for genetic diversity and population structure study of *C. gloeosporioides* isolates from citrus. Although a large number of hosts are affected by *Colletotrichum* worldwide, SSR markers are available only for a few *Colletotrichum* spp. such as *C. capsici*, associated with chili pepper anthracnose disease (Ranathunge *et al.*, 2009), and *C. acutatum*, the causal agent of post-bloom fruit drop on citrus (Ciampi *et al.*, 2011). Information on pathogen genetic diversity and population structure on temporal and spatial scales are important to understand the potential of pathogen populations to spread and overcome host resistance (McDonald and Linde, 2002; Ranathunge *et al.*, 2009). Areas of high biodiversity may serve as a source for the emergence of new genotypes with novel biological characteristics, including changes in pathogen fitness or resistance to certain fungicides (Rampersad, 2013).

In this study, 23 polymorphic SSR markers were used for assessing the genetic diversity of *C. gloeosporioides* isolates from citrus. The polymorphisms detected by these SSR markers were in the range of slightly informative to highly informative. They displayed allelic diversity among the isolates, with 3 to 8 alleles per locus, and the PIC values ranged from 0.104 to 0.597. The PIC provides an estimate of the discriminatory power of a locus by taking into account the number and the relative frequencies of the alleles (Marulanda *et al.*, 2014). All the 23 loci displayed differences for observed heterozygosity, with loci CG22 showing the highest H_O at 0.865 and several loci with the lowest value at zero. Higher number of private alleles was observed in the isolates from the south Ethiopia. Private alleles, or alleles that are unique to certain species or geographic area, are useful in comparing diversity between species or population (Mahmodi *et al.*, 2014). Genetic diversity in other *Colletotrichum* spp. were also reported after studied using various genetic markers. Ranathunge *et al.* (2009) analyzed the genetic diversity of *C. capsici* isolates using 27 sequence-tagged microsatellite markers and found the highest gene diversity of 0.857 at a locus with up to 18 alleles among all the isolates and the differentiation ranged from 0.05 to 0.45. However, Ciampi *et al.* (2011) reported that SSR markers showed better discriminatory power in comparison to the

commonly used markers such as the ITS, the intron 2 of the glyceraldehyde-3-phosphate dehydrogenase gene (G3PD), and the glutamine synthase intron 2 (GS) to estimate genetic diversity in *C. acutatum*. The authors found from 3 to 6 alleles per locus, and heterozygosity ranging from 0.093 to 0.590 across loci using nine polymorphic SSR markers.

The AMOVA results of the present study indicated that the highest percentage of variation (84%) was within populations of *C. gloeosporioides* isolates. However, the gene diversity observed among the Ethiopian *C. gloeosporioides* populations was low. This might be attributed to the recent introduction of the fungus, probably of a few genotypes. Detection of high regional genetic diversity within the south Ethiopian population can be explained by the earlier introduction of the disease (in late 1980s) in the region (Yimenu, 1993). Similarly, the diversity was also higher within the southwest population where the disease was reported in 1990 (Eshetu, 1999). The low genetic diversity in the populations from central and northwest regions can be explained by recent introduction of the disease in these regions (Yigzaw and Gelelbelu, 2002; Mohammed, 2007). There are various findings that support the argument that areas with recent introduction of pathogens are likely to have low genetic diversity compared to areas where the pathogen introduction happened long ago. It was stated that in older population, sufficient time has passed to allow mutational events to introduce new genetic variants and for genetic drift to increase the frequencies of these alleles to quantitative levels (Rampersad, 2013). McDonald (1997) indicated that isolates located at or near the center of origin of the species would have higher level of gene diversity than isolates at other locations because the original population is older. Unique pathogen genotypes may also occupy particular geographical areas that may be associated with host coevolution and adaptation (Cannon *et al.*, 2008). Weeds *et al.* (2003) demonstrated that genetic diversity of *C. gloeosporioides* isolates is high where native or naturalized host species occur compared with locations where the host species has been recently introduced. In addition to age of the population establishment, differences may also occur due to environmental conditions, geography, and differences in alternative host species diversity that may have a role in generating variability within populations (Burdon and Silk, 1997).

In theory, there are various factors that can affect genetic diversity of an organism (Bennett *et al.*, 2005). Mutation, population gene flow, and sexual and asexual recombination are the main mechanisms by which genetic diversity can be generated in populations of pathogenic microorganisms (Marulanda *et al.*, 2014). Pathogens showing sexual reproduction pose a greater risk as the reproductive pattern leads to inbreeding, and new genotypes with high virulence can emerge during the sexual cycle (McDonald and Linde, 2002). However, this does not mean that gene diversity is influenced only by sexual reproduction because clonally reproducing fungi may also show as many alleles as those that undergo recombination (McDonald, 1997). This might be due to the fact that diversity in fungal populations can arise from transposable elements and mitotic reciprocal translocation events (Kistler and Miao, 1992). Genetic diversity can also result from accumulation of mutations that can create variations within a species or population (Milgroom, 1996). Rampersad (2013) suggested that high genetic diversity may be a function of population size.

An insight into the structure of *C. gloeosporioides* populations from different locations is valuable in enhancing the understanding of the biology of the pathogen and potentially identify adaptive genotypic isolates of the species. The *C. gloeosporioides* isolates from the four geographic regions of Ethiopia are closely related to one another as reflected by the high genetic identity among populations. Population genetic analyses supported subdivision of the Ethiopian *C. gloeosporioides* isolates into three inferred sub-populations. STRUCTURE analysis, PCoA, and the unweighted Neighbor-joining algorithm indicated admixture among the three populations. Geographical separation of isolates into distinctly isolated subpopulations was not observed as evidenced by admixture among isolates from the different regions. The relatively low F_{ST} value (0.049) between the *C. gloeosporioides* populations evaluated in this study indicated low differentiation among the groups that might be attributed to gene flow among regions, which is reflected by the high migration rate (N_m = 4.8) estimates. A low degree of differentiation in populations of *C. gloeosporioides* shown in this study may be attributed to the dispersal of the clonal inoculum over long distances that may allow for pathogen spread in citrus-growing areas in Ethiopia. The citrus fruit produced in the south and southwest parts of Ethiopia are transported to the central areas for marketing while

those produced in the northwest are consumed in the same region. The wind direction in Ethiopia is generally from northeast to southwest during the dry season and *vice versa* during the rainy season. These may cause migration and gene flow between populations that resulted in admixture among isolates from the different geographic origin. The pathogen is cosmopolitan in distribution, and conidia are known to be disseminated over long distances by wind (Farr *et al.*, 2006) and via the movement of infected fruits (Freeman *et al.*, 2013). Abang *et al.* (2006) reported that *C. gloeosporioides* infecting yam in Nigeria inferred by 51 microsatellite loci was described as a single population with low intra-population differentiation, high genetic diversity, and evidence of high gene flow. Conversely, there was significant and high genetic differentiation and gene diversity of subpopulations of *C. gloeosporioides* infecting strawberry in the United States based on 40 RAPD or microsatellite markers (Urena-Padilla *et al.*, 2002).

Pathogen population divergence may occur as a result of genetic drift and local adaptation to increase relative fitness in local environments (Kawecki and Ebert, 2004). McDermott and McDonald (1993) indicated that if N_m is greater than one, there will be little differentiation among populations and migration is more important than genetic drift. Similarly, Wright (1951) stated that the movement of as few as one individual per generation is sufficient to prevent significant divergence between populations. Mechanisms that enable gene flow may act randomly and may be a result of a combination of anthropogenic activities, such as movement and exchange of infected plant material, the process of extinction and recolonization (balance between genetic drift and migration), and alternate hosts outside of the growing season that may allow certain genotypes to persist and undergo expansion in the field (McDermott and McDonald, 1993; Milgroom, 1996; McDonald, 1997; McDonald and Linde, 2002; Milgroom and Peever, 2003). For asexually reproducing fungi such as *C. gloeosporioides*, identification of population subdivision within a particular geographic area can be associated with the epidemiology of the disease, such as sources of inoculum and host or tissue specificity (Urena-Padilla *et al.*, 2002; Milgroom and Peever, 2003).

The current molecular analysis study as expressed in the parameters like genetic diversity of isolates and gene flow estimates supported previous reports on the location of disease introduction (Eshetu, 1997) into the country, and its spread route in to other parts of the country like southwest, central, and northwest Ethiopia (Eshetu, 1999; Yigzaw and Gelelbelu, 2002; Kassahun *et al.*, 2006; Mohammed, 2007). This again consolidates the need for a strong internal quarantine system to contain the spread of this and other plant pathogens.

In conclusion, the microsatellite markers developed and used in this study were useful to comprehend the genetic diversity and population structure of *C. gloeosporioides* isolates from citrus growing regions of Ethiopia. Despite regional differences, the observed genetic diversity in all four populations was lower than expected suggesting inter-regional exchanges of planting materials and dispersal of inoculum among the regions. Information generated in this study could be useful in understanding the pathogen biology and provide basis for other studies on disease development, host-pathogen interaction, and developing disease management strategies including development and use of resistant citrus varieties for citrus leaf and fruit spot. The SSR markers developed and used in this study could be useful to characterize *C. gloeosporioides* isolates that infect other fruit crops.

REFERENCES

Abang, M.M., S. Winter, H.D. Mignouna, K.R. Green and R. Asiedu. 2003. Molecular taxonomic, epidemiological and population genetic approaches to understanding yam anthracnose disease. *African Journal of Biotechnology*, 2: 486-496.

Abang, M.M., R. Asiedu, P. Hoffmann, G.A. Wolf, H.D. Mignouna and S. Winter. 2006. Pathogenic and genetic variability among *Colletotrichum gloeosporioides* isolates from different yam hosts in the agroecological zones in Nigeria. *Journal of Phytopathology*, 154:51-61.

Adaskaveg, J.E., and H. Forster. 2000. Occurrence and management of anthracnose epidemics caused by *Colletotrichum* species on tree fruit crops in California. pp. 317-336. In: D.

Prusky, S. Freeman and M.B. Dickman (eds.), *Colletotrichum*: Host Specificity, Pathology and Host-Pathogen Interaction. APS Press, St. Paul, MN, USA.

Afanador-Kafuri, L., D. Minz, M. Maymon and S. Freeman. 2003. Characterization of *Colletotrichum* isolates from tamarillo, *Passiflora*, and mango in Colombia and identification of a unique species from the genus. *Phytopathology*, 93:579-587.

Aiello, D., R. Carrieri, V. Guarnaccia, A. Vitale, E. Lahoz and G. Polizzi. 2015. Characterization and pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* causing preharvest disease on *Citrus sinensis* in Italy. *Journal of Phytopathology*, 163:168-177.

Anderson, J.A., G.A. Churchill, J.E. Autrique, S.D. Tanksley and M.E. Sorrels. 1993. Optimizing parental selection for genetic linkage maps. *Genome*, 36:181-186.

Bankevich, A., S. Nurk, D. Antipo, A.A. Gurevich, M. Dvorkin, N. Kulikov, V.M. Lesin, S.I. Nikolenko, S. Pham, A.D. Prjibelski, A.V. Pyshkin, N. Vyahhi, G. Tesler, M.A. Alekseyev and P.A. Pevzner. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. *Journal of Computational Biology*, 19:455-477.

Bayraktar, H., F.S. Dolar and M. Tor. 2007. Determination of genetic diversity within *Ascochyta rabiei* (Pass.) Labr., the cause of ascochyta blight of chickpea in Turkey. *Journal of Plant Pathology*, 89:341-347

Belayneh Admassu, W. Friedt and F. Ordon. 2010. Genetic characterization of *Puccinia graminis f.sp. tritici* populations from Ethiopia by SSRs. *Journal of Phytopathology*, 158:806-812.

Benali, S., B. Mohamed, H.J. Eddine and C. Neema. 2011. Advances of molecular markers application in plant pathology research. *European Journal of Scientific Research*, 50:110-123.

Bennett, R.S., M.G. Milgroom and G.C. Bergstrom. 2005. Population structure of seedborne *Phaeosphaeria nodorum* on New York wheat. *Phytopathology*, 95:300-305.

Benson, G. 1999. Tandem repeats finder: a program to analyze DNA sequences. *Nucleic Acid Research*, 27:573-580.

Botstein, D., R.L. White, M. Skolnick and R.W. Davis. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *American Journal of Human Genetics*, 32:314-331.

Brouwer, D.J. and T.C. Osborn. 1999. A molecular marker linkage map of tetraploid alfalfa (*Medicago sativa* L.). *Theoretical and Applied Genetics*, 99:1194-1200.

Buddie, A.G., A.G. Martínez-Culebras, P. Buddie, P.D. Bridge, M.D. Garcia, A. Querol, P.F. Cannon and E. Monte. 1999. Molecular characterization of *Colletotrichum* strains derived from strawberry. *Mycological Research*, 103:385-394.

Burdon, J.J. and J. Silk. 1997. Sources and patterns of diversity in plant pathogenic fungi. *Phytopathology*, 87:664-669.

Cai, Z., G. Li, C. Lin, T. Shi, L. Zhai, Y. Chen and G. Huang. 2013. Identifying pathogenicity genes in the rubber tree anthracnose fungus *Colletotrichum gloeosporioides* through random insertional mutagenesis. *Microbiological Research*, 68:340-350.

Cannon, P.F., P.D. Bridge and E. Monte. 2000. Linking the past, present, and future of *Colletotrichum* systematics. pp. 1-20. In: D. Prusky, S. Freeman and M. Dickman (eds.), *Colletotrichum*: Host specificity, Pathology and Host-pathogen interaction. APS Press, St. Paul, MN, USA.

Cannon, P.F., A.G. Buddie and P.D. Bridge. 2008. The typification of *Colletotrichum gloeosporioides*. *Mycotaxon*, 104:189-204.

Capote, N., A.M. Pastrana, A. Aguado and P. Sanchez-Torres. 2012. Molecular tools for detection of plant pathogenic fungi and fungicide resistance. pp. 151-202. In: C.J. Cumagun (ed.), Plant Pathology. InTech, Rijeka, Croatia.

Carvalho, F.M.S., R.P. Leite Jr. and B. Ueno. 2000. Pathogenic characterization of *Colletotrichum* spp. associated with apple diseases in southern Brazil. *Fitopatologia Brasileira*, 25:72-78.

Ciampi, M.B., C. Baldauf, B.B.Z. Vigna, A.P. Souza, M.B. Sposito and L. Amorim. 2011. Isolation and characterization of microsatellite loci in *Colletotrichum acutatum*, the causal agent of post-bloom fruit drop on citrus. *Conservation Genetics Resources*, 3: 651-654.

Cox, M.P., D.A. Peterson and P.J. Biggs. 2010. SolexaQA: At-a-glance quality assessment of Illumina second-generation sequencing data. *BMC Bioinformatics*, 11:485.

Da Maia, L.C., D.A. Palmieri, V.Q. de Souza, M.M. Kopp, F.I.F. de Carvalho and A.C. de Oliveira. 2008. SSR Locator: tool for simple sequence repeat discovery integrated with primer design and PCR simulation. *International Journal of Plant Genomics*, 2008:412696. doi:10.1155/2008/412696

Dutech, C., J. Enjalbert, E. Fournier, F. Delmotte, B. Barres, J. Carlier, D. Tharreau and T. Giraud. 2007. Challenges of microsatellite isolation in fungi. *Fungal Genetics and Biology*, 44:933-949.

Earl, D.A. and B.M. vonHoldt. 2012. STRUCTURE HARVESTER; a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, 4:359-361.

Edwards, A., A. Civitello, H.A. Hammond and C.T. Caskey. 1991. DNA typing and genetic mapping with trimeric and tetrameric tandem repeats. *American Journal of Human Genetics*, 49:746-756.

Ellegren, H. 2004. Microsatellites: Simple sequences with complex evolution. *Nature Review Genetics*, 5:435-445.

Eshetu Derso. 1999. Occurrence, prevalence and control methods of *Phaeoramularia* leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 54:225-232.

Eshetu Derso. 1997. Leaf and fruit spot: a new disease of citrus in Ethiopia. pp. 215-223. In: Proceedings of the Seventh Annual Conference, 27-28 April 1995, Crop Science Society of Ethiopia, Addis Ababa.

Evanno, G., S. Regnaut and J. Goudet. 2005. Detecting the number of clusters of individuals using the software STRUCTURE: a simulation study. *Molecular Ecology*, 14:2611-2620.

Falush, D., M. Stephens and J.K. Pritchard. 2003. Inference of population structure using multilocus genotype data: linked loci and correlated allele frequencies. *Genetics*, 164:1567-1587.

Farr, D.F., M.C. Aime, A.Y. Rossman and M.E. Palm. 2006. Species of *Colletotrichum* on Agavaceae. *Mycological Research*, 110:1395-1408.

Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39:783-791.

Freeman, S. 2000. Genetic diversity and host specificity of *Colletotrichum* species on various hosts. pp. 131-144. In: D. Prusky, S. Freeman, and M.B. Dickman (eds.), *Colletotrichum*: Host Specificity, Pathology, and Host-pathogen Interaction. APS Press, St. Paul, MN, USA.

Freeman, S., S. Horowitz-Brown, L. Afanador-Kafuri, M. Maymon and D. Minz. 2013. *Colletotrichum*: host specificity and pathogenicity on selected tropical and subtropical crops. *Acta Horticulturae*, 975:209-216. DOI: 10.17660/ActaHortic.2013.975.22. http://dx.doi.org/10.17660/ActaHortic.2013.975.22

Gan, P., K. Ikeda, H. Irieda, M. Narusaka, R.J. O'Connell, Y. Narusaka, Y. Takano, Y. Kubo and K. Shirasu. 2013. Comparative genomic and transcriptomic analyses reveal the hemibiotrophic stage shift of *Colletotrichum* fungi. *New Phytologist*, 197:1236-1249.

Ghimire, S.R., N.D. Charlton, J.D. Bell, Y.L. Krishnamurthy and K.D. Craven. 2011. Biodiversity of fungal endophyte communities inhabiting switch grass (*Panicum virgatum* L.) growing in the native tall grass prairie of northern Oklahoma. *Fungal Diversity*, 47:19-27.

Hancock, J.M. 1995. The contribution of slippage-like processes to genome evolution. *Journal of Molecular Evolution*, 41:1038-1047.

Halkett, F., J.-C. Simon and F. Balloux. 2005. Tackling the population genetics of clonal and partially clonal organisms. *Trends in Ecology and Evolution*, 20:194-201.

Hernandez-Delgado, S., M.H. Reyes-Valdes, R. Rosales-Serna and N. Mayek-Perez. 2009. Molecular markers associated with resistance to *Macrophomina phaseolina* (Tassi) Goid in common bean. *Journal of Plant Pathology*, 91:163-170.

Hochberg, Y. 1988. A sharper Bonferroni procedure for multiple tests of significance. *Biometrika*, 75:800-803.

Hulbert, S.H. 1971. The nonconcept of species diversity: a critique and alternative parameters. *Ecology*, 52:577-586.

Hyde, K.D., L. Cai, P.F. Cannon, J.A. Crouch, P.W. Crous, U. Damm, P.H. Goodwin, H. Chen, P.R. Johnston, E.B.G. Jones, Z.Y. Liu, E.H.C. McKenzie, J. Moriwaki, P.

Noireung, S.R. Penny cook, L.H. Pfenning, H. Prihastuti, T. Sato, R.G. Shivas, Y.P. Tan, P.W.J. Taylor, B.S. Weir, Y.L. Yang, and J.Z. Zhang. 2009. *Colletotrichum* – names in current use. *Fungal Diversity*, 39:147-182.

Jana, T., T.R. Sharma and N. Singh. 2005. SSR-based detection of genetic variability in the charcoal root rot pathogen *Macrophomina phaseolina*. *Mycological Research*, 109:81-86.

Jarne, P. and P.J.L. Lagoda. 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution*, 11:424-429.

Jin, L. and R. Chakraborty. 1994. Estimation of genetic distance and coefficient of gene diversity from single-probe multilocus DNA fingerprinting data. *Molecular Biology and Evolution*, 11:120-127.

Kalendar, R., D. Lee and A.H. Schulman. 2011. Java web tools for PCR, in silico PCR, and oligonucleotide assembly and analysis. *Genomics*, 98:137-144.

Kalinowski, S.T. 2005. HP-Rare 1.0: a computer program for performing rarefaction on measures of allelic diversity. *Molecular Ecology Notes*, 5:187-189.

Karaoglu, H., C.M.Y. Lee and W. Meyer. 2005. Survey of simple sequence repeats in completed fungal genomes. *Molecular Biology and Evolution*, 22:639-649.

Kassahun Tessega, Temam Hussien and P.K. Sakhuja. 2006. Management of *Phaeoramularia* fruit and leaf spot disease of citrus in Ethiopia. *Agricultura Tropica et Subtropica*, 39:242-248.

Katti, M.V., P.K. Ranjekar and V.S. Gupta. 2001. Differential distribution of simple sequence repeats in eukaryotic genome sequences. *Molecular Biology and Evolution*, 18:1161-1167.

Kawecki, T.J. and D. Ebert. 2004. Conceptual issues in local adaptation. *Ecology Letters*, 7:1225-1241.

Kiran Babu, T., R. Sharma, H.D. Upadhyaya, P.N. Reddy, S. Deshpande, S. Senthilvel, N.D.R.K. Sarma and R.P. Thakur. 2013. Evaluation of genetic diversity in *Magnaporthe grisea* populations adapted to finger millet using simple sequence repeats (SSRs) markers. *Physiological and Molecular Plant Pathology*, 84:10-18.

Kistler, H.C. and V.P.W. Miao. 1992. New modes of genetic change in filamentous fungi. *Annual Review of Phytopathology*, 30:131-152.

Knapik, E.W., A. Goodman, M. Ekker, M. Chevrette, J. Delgado, S. Neuhauss, N. Shimoda, W. Driever, M.C. Fishman and H.J. Jacob. 1998. A microsatellite genetic linkage map for zebrafish (*Danio rerio*). *Nature Genetics*, 18:338-343.

Levinson, G. and G.A. Gutman. 1987. Slipped-strand misspairing: a major mechanism for DNA sequence evolution. *Molecular Biology and Evolution*, 4:203-2021.

Litt, M. and J.A. Luty. 1989. A hypervariable microsatellite revealed by in vitro amplification of dinucleotide repeat within the cardiac muscle actin gene. *American Journal of Human Genetics*, 44:397-401.

Liu, K. and S.V. Muse. 2005. PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics*, 21:2128-2129.

Luikart, G., P.R. England, D. Tallmon, S. Jordan and P. Taberlet. 2003. The power and promise of population genomics: from genotyping to genome typing. *Nature Review Genetics*, 4:981-994.

Mahmodi, F., J.B. Kadir, A. Puteh, S.S. Pourdad, A. Nasehi and N. Soleimani. 2014. Genetic diversity and differentiation of *Colletotrichum* spp. isolates associated with Leguminasae using multigene loci, RAPD and ISSR. *Plant Pathology Journal*, 30:10-24.

Martin, M.P. and F. Garcia-Figueres. 1999. *Colletotrichum acutatum* and *C. gloeosporioides* caused anthracnose on olives. *European Journal of Plant Pathology*, 105: 733-745.

Marulanda, M.L., A.M. Lopez, L. Isaza and P. Lopez. 2014. Microsatellite isolation and characterization for *Colletotrichum* spp., causal agent of anthracnose in Andean blackberry. *Genetics and Molecular Research*, 13:7673-7685.

Matsuoka, Y., S.E. Mitchell, S. Kresovich, M. Goodman and J. Doebley. 2002. Microsatellites in *Zea* variability, patterns of mutations, and use for evolutionary studies. *Theoretical and Applied Genetics*, 104:436-450.

McDermott, J.M. and B.A. McDonald. 1993. Gene flow in plant pathosystems. *Annual Review of Phytopathology*, 31:353-373.

McDonald, B.A. 1997. The population genetics of fungi: tools and techniques. *Phytopathology*, 87:448-453.

McDonald, B.A. and C. Linde. 2002. Pathogen population genetics, evolutionary potential and durable resistance. *Annual Review of Phytopathology*, 40:349-379.

Milgroom, M.G. 1996. Recombination of multilocus structure of fungal populations. *Annual Review of Phytopathology*, 34:457-477.

Milgroom, M.G. and T.L. Peever. 2003. Population biology of plant pathogens; the synthesis of plant disease epidemiology and population genetics. *Plant Disease*, 87:608-617.

Mohammed Yesuf. 2007. Distribution and management of Phaeoramularia leaf and fruit spot disease of citrus in Ethiopia. *Fruits*, 62:99-106.

Morgante, M. and A.M. Olivieri. 1993. PCR-amplified microsatellites as markers in plant genetics. *The Plant Journal*, 3:175-182.

Morgante, M., H. Hanafey and W. Powell. 2002. Microsatellites are preferentially associated with non-repetitive DNA in plant genome. *Nature Genetics*, 30:194-200.

Nei, M. 1987. Molecular evolutionary genetics. Columbia University Press, NY, USA.

Page, R.D.M. 2001. TreeView 1.6.6. http://taxonomy.zoology.gla.ac.uk/rod/rod.html

Peakall, R. and P.E. Smouse. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research - an update. *Bioinformatics*, 28:2537-2539.

Peixoto-Junior, R.F., S. Creste, M.G.A. Landell, D.S. Nunes, A. Sanguino, M.F. Campos, R. Vencovsky, E.V. Tambarussi and A. Figueira. 2014. Genetic diversity among *Puccinia melanocephala* isolates from Brazil assessed using simple sequence repeat markers. *Genetics and Molecular Research*, 13:7852-7863.

Perrier, X., A. Flori and F. Bonnet. 2003. Data analysis methods. pp. 43-76. In: P. Hamon, M. Sequin, X. Perrier and J.C. Glaszmann (eds.), Genetic Diversity of Cultivated Tropical Plants. Enfield, Science Publishers, Montpellier. http://darwin.cirad.fr.

Powell, W., G.C. Machray and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. *Trends in Plant Science*, 1:215-222.

Pritchard, J.K., M. Stephens and P. Donnelly. 2000. Inference of population structure using multilocus genotype data. *Genetics*, 155:945-959

Ramos, A.P., Z. Merali, P. Talhinhas, S. Sreenivasaprasad and H. Oliveira. 2006. Molecular and morphological characterization of *Colletotrichum* species involved in citrus anthracnose in Portugal. *Bulletin OILB/SROP*, 29(3): 317-326.

Rampersad, S.N., D. Perez-Brito, C. Torres-Calzada, R. Tapia-Tussell and C.V.F. Carrington. 2013. Genetic structure of *Colletotrichum gloeosporioides* sensu lato isolates infecting papaya inferred by multilocus ISSR markers. *Phytopathology*, 103:182-189.

Ranathunge, N.P., R. Ford and P.W.J. Taylor. 2009. Development and optimization of sequence-tagged microsatellite site markers to detect genetic diversity within *Colletotrichum capsici*, a causal agent of chili pepper anthracnose disease. *Molecular Ecology Resources*, 9:1175-1179.

Rizatto, S., C.E. de Araujo Batista, M.M. Bajay, M.S. Sigrist, M.F. Ito, M. Monteiro, M.M. Cavallari, J.B. Pinheiro and M.I. Zucchi. 2010. A new set of microsatellite markers for the genetic characterization of *Ceratocystis fimbriata*, an economically important plant pathogen. *Conservation Genetics Resources*, 2:55-58.

Rousset, F. 2008. GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources*, 8:103-106.

Rozen, S. and H.J. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. pp. 365-368. In: S. Krawetz Misener (ed.), Bioinformatics Methods and Protocols: Methods in Molecular Biology. Humana Press, Totowa, NJ.

Schaad, N.W., R.D. Frederick, J. Shaw, W.L. Schneider, R. Hickson, M.D. Petrillo and D.G. Luster. 2003. Advances in molecular based diagnostics in meeting crop biosecurity and phytosanitary issues. *Annual Review of Phytopathology*, 41:305-324.

Schlotterer, C. and D. Tautz. 1992. Slippage synthesis of simple sequence DNA. *Nucleic Acids Research*, 20:211-215.

Schlotterer, C. 2000. Evolutionary dynamics of microsatellite DNA. *Chromosoma*, 109:365-371.

Schuler, G.D., M.S. Boguski, E.A. Stewart, L.D. Stein, G. Gyapay, K. Rice, *et al*. 1996. A gene map of the human genome. *Science*, 274: 540-546.

Seifu Gebre-Mariam. 2003. Status of commercial fruit production in Ethiopia. Ethiopian Agricultural Research Organization, Addis Ababa.

Sharopova, N. 2008. Plant simple sequence repeats: distribution, variation and effects on gene expression. *Genome*, 51:79-90.

Sisay Bekele Mekbib. 2007. Identification of citrus (*Citrus sinensis*) postharvest pathogens from Ethiopia and their control. PhD thesis, University of Pretoria, Pretoria, South Africa.

Smith, B.J. and L. Black. 1990. Morphological, cultural, and pathogenic variation among *Colletotrichum* species isolated from strawberry. *Plant Disease*, 74:69-76.

Sokal, R.R. and C.D. Michener. 1958. A statistical method for evaluating systematic relationships. *University of Kansas Scientific Bulletin*, 28:1409-1438.

Sreenivasaprasad, S. and P. Talhinhas. 2005. Genotypic and phenotypic diversity in *Colletotrichum acutatum*, a cosmopolitan pathogen causing anthracnose on a wide range of hosts. *Molecular Plant Pathology*, 6:361-378.

Sutton, B.C. 1992. The genus *Glomerella* and its anamorph *Colletotrichum*. pp. 1-26. In: J.A. Bailey and M.J. Jeger (eds.), *Colletotrichum*: Biology, Pathology and Control. CAB international, Wallingford, UK.

Szabo, L.J. 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus, *Puccinia graminis*. *Molecular Ecology Notes*, 7:92-94.

Szabo, L.J. and J.A. Kolmer. 2007. Development of simple sequence repeat markers for the plant pathogenic rust fungus *Puccinia triticina*. *Molecular Ecology Notes*, 7:708-710.

Tautz, D. and M. Renz. 1984. Simple sequences are ubiquitous repetitive components of eukaryotic genomes. *Nucleic Acids Research*, 12:4127-4138.

Tautz, D. 1989. Hypervariability of simple sequences as a general source for polymorphic DNA markers. *Nucleic Acids Research*, 17:6463-6471.

Temnykh, S., G. DeClerck, A. Lukashova, L. Lipovich, S. Cartinhour and S. McCouch. 2001. Computational and experimental analysis of microsatellites in rice (*Oryza sativa* L.): Frequency, length variation, transposon associations, and genetic marker potential. *Genome Research*, 11:1441-1452.

Thiel, T., W. Michalek, R.K. Varshney and A. Graner. 2003. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theoretical and Applied Genetics*, 106: 411-422.

Timmer, L.W. and G.E. Brown. 2000. Biology and control of anthracnose diseases of citrus. pp. 300-316. In: D. Prusky, S. Freeman and M.B. Dickman (eds.), *Colletotrichum*: Host Specificity, Pathology, and Host-Pathogen Interaction. APS Press, St. Paul, MN, USA.

Toth, G., Z. Gaspari and J. Jurka. 2000. Microsatellites in different eukaryotic genomes: survey and analysis. *Genome Research*, 10:967-981.

Urena-Padilla, A.R., S.J. MacKenzie, B.W. Bowen and D.E. Legard. 2002. Etiology and population genetics of *Colletotrichum* spp. causing crown and fruit rot of strawberry. *Phytopathology*, 92:1245-1252.

Weber, J.L. 1990. Informativeness of human (dC-dA)n. (dG-dT) n polymorphisms. *Genomics*, 7:524-530.

Weber, J.L. and C. Wong. 1993. Mutation of human short tandem repeats. *Human Molecular Genetics*, 2:1123-1118.

Weeds, P.L., S. Chakraborty, C.D. Fernandes, M.J. d'A. Charchar, C.R. Ramesh, Y. Kexian and Segenet Kelemu. 2003. Genetic diversity in *Colletotrichum gloeosporioides* from *Stylosanthes* spp. at centers of origin and utilization. *Phytopathology*, 93:176-185.

Weir, B.S. 1996. Genetic data analysis II: methods for discrete population genetic data. Sinauer Associates, Inc. Sunderland, Massachusetts.

Weising, K., R.G. Atkinson and R.C. Gardner. 1995. Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. *PCR Methods and Applications*, 4:249-255.

Winton, L.M., L. Krohn and R.H. Leiner. 2007. Microsatellite markers for *Sclerotinia subarctica* nom. prov., a new vegetable pathogen of the High North. *Molecular Ecology Notes*, 7:1077-1079.

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics*, 15:323-354.

Xie, L., J. Zhang, Y. Wan and D. Hu. 2010. Identification of *Colletotrichum* spp. isolated from strawberry in Zhejiang Province and Shanghai City, China. *Journal of Zhejiang University-Science B*, 11: 61-70.

Xu, X., M. Peng and Z. Fang. 2000. The direction of microsatellite mutations is dependent upon allele length. *Nature Genetics*, 24:396-399.

Yigzaw Dessalegn and Gelelbelu Girma. 2002. *Phaeoramularia angolensis*: a citrus disease in Northwest Ethiopia. *AgriTopia*, 17(1):12-13. [Review of Plant Pathology 81(9), September 2002, abstract 8614, p. 1226].

Yimenu J. 1993. Preliminary survey report on leaf spot disease of citrus in Sidamo. Coffee and Tea Authority, Addis Ababa, Ethiopia.

Zane, L., L. Bargelloni and T. Patarnello. 2002. Strategies for microsatellite isolation: a review. *Molecular Ecology*, 11:1-16.

8. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

8.1. Summary

Citrus is one of the major fruit crops that have been grown in Ethiopia for several decades. The major commercial citrus species that are cultivated include sweet orange, mandarin, lime and lemon, and to some extend grapefruits. Citrus are produced by both smallholder and commercial farmers. Commercial farms are mainly located in the central rift valley and the eastern parts of Ethiopia contributing about 46% to the total production; whereas small-scale production is scattered throughout the country contributing the rest of the citrus fruits. Large portion of citrus fruits are consumed locally as fresh fruit, juice and marmalade. Sweet orange and lime fruits are exported to different countries.

In Ethiopia, citrus is seriously threatened by various pathogenic diseases resulting in the declining of its production and productivity. Among the major fungal diseases reported to affect citrus, leaf and fruit spot disease has been causing a serious damage in many citrus producing areas. In some areas, complete citrus fruit yield losses were reported. The disease was first observed in the southern Ethiopia in 1988. Later, similar disease symptoms were reported in the southwest, central and the northwest parts of the country. In 1993, the causal pathogen was reported as *Phaeoramularia angolensis* based on cultural and morphological characteristics. However, cultural and morphological characters are highly variable depending upon experimental conditions and host. Moreover, the variability in pathogenicity of isolates of the same pathogen make identification of pathogens unreliable. To avoid such confusion, DNA-based technologies have been extensively employed in plant pathology. Nucleotide sequence information for different loci have been used as reliable technique to identify pathogen species. Besides, information on genetic diversity of a pathogen and its population structure is vital for devising and implementing effective disease management strategies.

Despite the importance of leaf and fruit spot disease in Ethiopia, there are very limited information available on the etiology of the causal pathogen and epidemiology of the disease. Therefore, the objectives of the present study were to assess the distribution, incidence and severity of leaf and fruit spot disease of citrus, to identify the fungal pathogens associated with leaf and fruit spot disease, to analyze the phylogenetic relationships of the pathogens, to discover genome wide microsatellite loci, to develop simple sequence repeat markers, and to characterize the genetic diversity and population structure of the pathogen causing the disease.

Surveys and laboratory works were conducted to assess the distribution, incidence and severity of leaf and fruit spot disease of citrus, and to characterize the causal pathogen under laboratory conditions using cultural, morphological, pathogenicity and molecular features. Surveys were conducted on forty-nine citrus orchards in the major citrus growing areas of Ethiopia. Disease prevalence, incidence and severity on citrus leaves and intact fruits were determined by randomly taking citrus trees diagonally across the field in each orchard. During the surveys, the current citrus production and management status were also assessed using questionnaire and field observations. Infected citrus leaves and fruits samples collected from various orchards were surface sterilized and isolated on water agar and potato dextrose agar media. Pure cultures were prepared using single spore or hyphal tip for each fungal isolate. The colony growth and growth rate was measured at daily basis, while colony color and density were assessed by visual observation. Each fungal isolate culture was also evaluated for conidial and mycelial morphology by the help of stereomicroscope. Pathogenicity test for each isolate was conducted using citrus detached leaves following the standard procedure.

The survey results indicated that leaf and fruit spot disease is a serious threat to citrus production in the country. The disease caused severe leaf and fruit damages which rendered significant defoliation and fruit drop. In some areas, complete loss of fruit yield was recorded. The disease has widely distributed in most citrus producing areas with high rainfalls. Citrus orchards in the south and southwest were more severely affected by the disease than those in the central and northwest parts of the country. Disease incidences and severities were high in Jimma town, Abeshege, Aleta Wendo, Kebena, Mana, Gomma, Ginbo and Debre Werk

districts. In the present study, the disease severity varied among different citrus species and locations. Disease severity was higher on sweet orange and mandarin. The disease attack was more severe on fruits than leaves. The questionnaire assessment and field observations indicated that various citrus species and varieties were produced by smallholder and commercial farmers. The assessment also showed that diseases and insect pests, poor agronomic and irrigation practices, and shortage of adapted high yielding varieties were the major citrus production constraints in the country. It has been observed that commercial citrus orchards practice field sanitation, pruning, irrigation, and fertilizer and pesticide applications. However, most of the orchards of the smallholders were not well managed.

The fungal isolates produced colonies with compact, medium or sparse density. The average daily colony growth rate ranged from 0.04 to 2.3 cm. Some isolates were very slow-growing, whereas most cultures had characteristic fast-growing compact aerial mycelia. The colonies varied from white to dark gray in color. The majority of the isolates produced circular, wooly or cottony colonies with pale brown or grayish white color. Most isolates produces hyaline, ovoid to oblong, slightly curved or dumbbell shaped conidia. Pathogenicity of fungal isolated were employed on detached leaves of different citrus cultivars, and necrotic lesions were developed for most of the isolates. Based on cultural, morphological and pathogenic characters, several fungal pathogens were identified that could be associated with leaf and fruit spot disease of citrus.

The identity and multilocus phylogenetic relationships of the fungal isolates were also further analyzed using three sets of universal primers that span internal transcribed spacers, portion of long subunit region of the nuclear ribosomal DNA and partial actin gene sequences. The isolates were identified as *Alternaria*, *Cladosporium*, *Cercospora*, *Colletotrichum*, *Mycosphaerella*, *Penicillium*, *Podospora*, *Phoma*, *Pseudocercospora angolensis*, and uncultured fungal species. The majority of the fungal isolates (81%) belonged to *C. gloeosporioides* species complex. These findings provide information on the causal pathogen of leaf and fruit spot disease of citrus in Ethiopia and suggest the need for in-depth studies to determine the role of *C. gloesporioides* species complex in leaf and fruit spot disease

epidemiology. The information will also be useful in developing disease management measures against *C. gloesporioides*.

The distribution and frequency of microsatellite loci in *C. gloeosporioides* genome were analyzed by generating pair-end reads from *C. gloeosporioides* isolate using the high-throughput Illumina sequencing platform, and reads were de novo assembled into a draft genome. The results showed that a genome-wide microsatellite database of 5030 microsatellite motifs were identified at 4506 loci, including 274 compound loci. Of these motifs, 4756 were perfect motifs. Trinucleotide repeats were the most frequent; whereas penta- and hexanucleotide motifs were the least abundant. The number of motifs decreased as the number of the repeats increased. Among the mononucleotide microsatellites, A/T repeats were more abundant than G/C repeats in the *C. gloeosporioides* genome. In penta- and hexanucleotide repeats, GC-rich motifs were predominant. These findings contributed to a detailed characterization of microsatellite loci in *C. gloeosporioides* genome.

Simple sequence repeat markers were developed from the draft assembled whole genome sequences of *C. gloeosporioides*. Fifty simple sequence repeat markers across the genome were screened using thirteen geographically representative *C. gloeosporioides* isolates. Forty-six loci were successfully amplified and yielded scorable single amplicons. Of these, twenty-one markers showed polymorphism and demonstrated allele diversity among the test isolates. The data and information presented here increase the genomic resources available in *C. gloeosporioides* by adding a large set of designed microsatellite primers. The present approach was successful for microsatellite discovery and development of SSR markers for the genetic analysis of *C. gloeosporioides* populations from citrus in Ethiopia.

Twenty three polymorphic simple sequence repeat (microsatellite) markers were used to analyze the genetic diversity and population structure of 163 isolates of *C. gloeosporioides* from four different geographic regions of Ethiopia. These loci produced a total of 118 alleles. The polymorphic information content values ranged from 0.104 to 0.597 with an average of 0.371. The average observed heterozygosity across all loci varied from 0.046 to 0.058. The

gene diversity among the loci ranged from 0.106 to 0.664. Despite regional differences, the observed genetic diversity in all four populations was lower than expected suggesting inter-regional exchanges of planting materials and dispersal of inoculum among the regions. There was low genetic differentiation in the total populations (F_{ST} = 0.049) as evidenced by high level of gene flow estimate (N_m = 4.8 per generation) among populations. Analysis of molecular variance showed 85% of the total variation within populations and only 5% among populations. The results showed that Ethiopian *C. gloeosporioides* populations are generally characterized by a low level of genetic diversity. Unweighted Neighbor-joining and Bayesian model-based population structure analyses grouped these 163 isolates into three major groups. However, the clusters were not according to the geographic origin of the isolates. In conclusion, the microsatellite markers developed and used in this study were useful to comprehend the genetic diversity and population structure of *C. gloeosporioides* isolates from citrus growing regions of Ethiopia. Information obtained from this study could be useful as a base for other studies on disease development, host-pathogen interaction, and developing disease management strategies. The informative SSR markers developed this study could be used to characterize *C. gloeosporioides* isolates that infect other fruit crops.

8.2. Conclusions

The surveys conducted and the assessment results (Chapter 3) showed that the major constraints associated with citrus production were shortage of improved varieties, lack of technical know-how, knowledge and skill of agronomic and irrigation practices, and disease and insect pest problems. Among diseases, citrus leaf and fruit spot was found to be one of the major constraints in many of the orchards surveyed. The disease caused severe leaf and fruit damages which rendered significant defoliation and fruit drop. The disease widely distributed in areas with high rainfall. Citrus orchards in the south and southwest were more severely affected by the disease than those in the central and northwest parts. Disease severity varied among different citrus species and locations. Disease severity was higher on sweet orange and mandarin than lemon and lime. In some areas, complete loss of fruit yield was recorded. The disease was also more severe on fruits than leaves.

A total of 223 fungal isolates recovered from symptomatic citrus leaf and fruit tissues were identified and characterized based on their pathogenic, colonial, morphological and molecular characters (Chapters 4 and 5). Most of the cultures grew fast. Isolates produced colonies with different density. Colonies varied from white to dark gray in color. Most fungal isolates produced conidia, but varied in size and shape. Many isolates totally failed to sporulate. The mycelium of most of the cultures was hyaline, septated and branched. Based on cultural and morphological characters, the isolates were belonged to several fungal species. Most of the isolates were not found identical to *P. angolensis*. Pathogenicity tests were assayed on apparently healthy detached leaves of different citrus cultivars. Most of the isolates caused foliar necrotic lesions on inoculated citrus cultivars. Isolates were consistently recovered from symptomatic leaf tissues. Many fungal isolates caused the death of the entire leaf area.

The identity and phylogenetic relationships of the isolates were also analyzed using three sets of universal primers that targeted the ITS, LSU and ACT loci. Among the 223 isolates, 201 fungal DNA samples were successfully amplified and sequenced. Isolates were identified as *Alternaria*, *Cladosporium*, *Cercospora*, *Colletotrichum*, *Mycosphaerella*, *Penicillium*,

Podospora, *Phoma*, *P. angolensis*, and uncultured fungus species. About 81% of the isolates were belonged to *C. gloeosporioides*. Isolates of the *Colletotrichum* spp. were pathogenic and were associated with leaf and fruit spot disease of citrus in Ethiopia. The phylogenetic analyses resulted in the delineation of four main clades. Most of the isolates clustered in the first main clade that consisted of *C. gloeosporioides*, *C. boninense*, *Podospora* spp. and one undescribed fungus species. The second clade contained only *Penicillium* species. *P. angolensis*, *Cercospora* and *Mycosphaerella* spp. belonged to the third main clade. The fourth main clade consisted of *Cladosporium*, *Alternaria* and *Phoma* spp. The results demonstrated that cultural, morphological and pathogenic characters supported by molecular analyses are reliable and useful approaches for pathogen species identification and analyzing their phylogenetic relationships. The findings also suggest that disease management strategies need to focus on *C. gloeosporioides* spp.

As described in Chapter 6, genome sequences of *C. gloeosporioides* was successfully generated using high-throughput Illumina sequencing platform, and used in microsatellite mining. Several thousands of mono- to hexanucleotide microsatellite motifs were identified. About 94.5% of them were perfect motifs. Trinucleotide repeats were the most frequent followed by mono- and dinucleotides. Penta- and hexanucleotide motifs were the least abundant. The number of motifs decreased as the number of the repeats increased. A/T repeats were more abundant than G/C repeats in the *C. gloeosporioides* genome. GC-rich motifs were predominant in penta- and hexanucleotide repeats,. However, the occurrence of microsatellites in *C. gloeosporioides* genome was comparatively less frequent than in other eukaryotic genomes. These results contributed to a detail characterization of genome wide microsatellite loci in *C. gloeosporioides*. Fifty SSR loci across the genome were also screened using thirteen geographically representative *C. gloeosporioides* isolates. Twenty-one markers showed polymorphism and demonstrated allele diversity among the test isolates. The data and information obtained in this study increased the genomic resources available in *C. gloeosporioides* by adding a large set of designed microsatellite primers. The present approach was successful for microsatellite discovery and development of SSR markers for the genetic analysis of *C. gloeosporioides* populations from citrus in Ethiopia.

Twenty-three SSR markers were used for assaying the genetic diversity of four populations of *C. gloeosporioides* (Chapter 7). The polymorphisms detected by these markers were in the range of slightly to highly informative. The markers displayed allelic diversity among the isolates. Higher number of private alleles was observed in the isolates from the south Ethiopia. The gene diversity observed among the *C. gloeosporioides* populations was low. Despite regional differences, the observed genetic diversity in all four populations was lower than expected. There was low genetic differentiation in the total populations and high level of gene flow estimate among populations. The analysis of molecular variance showed that 85% of the total variation was within populations. Isolates were clustered into three major groups, but the groupings were not according to their geographic origin. The SSR markers developed and used in this study were useful to comprehend the genetic diversity and population structure of *C. gloeosporioides* isolates. Information generated in this study could provide a base for other studies on disease development, host-pathogen interaction, and developing disease management strategies including resistance breeding, biocontrol and integrated disease management practices for the control of the pathogen causing leaf and fruit spot disease of citrus in Ethiopia. The informative SSR markers developed in the present study could be used to characterize *C. gloeosporioides* isolates that infect other fruit crops.

8.3. Recommendations

- Cultural, morphological and pathogenic characters should be supported by molecular analyses data for reliable identification of pathogen species complexes.
- Multilocus sequence analyses should be used for species identification and delimitation.
- Disease control measures against leaf and fruit spot disease of citrus in Ethiopia should focus on *C. gloeosporioides*.
- High-throughput next generation sequencing technologies should be applied to future genetic and genomic studies of *C. gloeosporioides*.
- The informative SSR markers developed in this study may be useful to characterize other *Colletotrichum* spp. complexes from various crops.
- Strategic interventions should be applied to address citrus production constraints in Ethiopia through repeated and continuous practical trainings and technology demonstration to farmers and extension agents on fruit crops nursery and orchard management, soil fertility and irrigation, and integrated disease management practices.
- To manage leaf and fruit spot disease of citrus in Ethiopia:
  - Producers should improve the soil fertility status of citrus orchards, practice field sanitation measures to reduce inoculum source, and apply relatively safer fungicides.
  - The government should put in place a regulatory system on the movement of citrus planting materials and/or fruits to limit the disease spread to new, unaffected areas.
  - The research system should give emphasis to develop tolerant/resistant cultivars, biocontrol and integrated disease management practices.

APPENDICES

Appendix Table 3.1. Survey questionnaire on general assessment of citrus orchards in major citrus producing areas in Ethiopia

Interviewer_____Date_____
Region _____ Zone _____District _____
Farm/Orchard Site Name _____
Farm/Orchard Name _____

I. GEOGRAPHICAL DATA

1. What are the average daily summer and winter temperatures?

S/N	Period	Average daily temperature (degree Celsius)	Remark
1.1	Summer day		
1.2	Summer night		
1.3	Winter day		
1.4	Winter night		

2. What are the water sources used in the farm/orchard?

S/N	Water source	Mark as "X"	Remark
2.1	Rain		
2.2	River		
2.3	Lake		
2.4	Ground water		
2.5	Harvested water		
2.6	Two or more of the above		

3. If irrigated, how often do you irrigate your farm/orchard?

_____

4. What is the average annual rainfall (mm) in the farm/orchard area?

_____

5. What is the altitude (elevation) of the farm/orchard in meter above sea level?

_____

6. What is the average humidity (%) of the farm/orchard?

_____

II. HISTORICAL BACKGROUND OF THE CITRUS FARM/ORCHARD

7. What is the ownership status of the citrus farm/orchard?

S/N	Ownership status	Mark as "X"	Remark
7.1	Government		
7.2	Private (Association)		
7.3	Private (Individual)		

8. How big is the farm/orchard in hectares?

_____

9. Which citrus species, scion varieties and stock cultivars are produced on the farm/ orchard? Can you name and put them in order of importance in terms of area coverage?

S/N	Citrus species	Scion variety	Rootstock cultivar	Area coverage (ha /or %)	Remark
9.1					
9.2					
9.3					
9.4					
9.5					
9.6					
9.7					
9.8					
9.9					
9.10					

10. From where did you obtain or purchase the planting materials?

S/N	Source category	Name of the source	Remark
10.1	Certified growers		
10.2	Local growers		
10.3	Any other sources		

11. How was the citrus planting materials produced?

S/N	Means of planting material produced	Mark as "X"	Remark
11.1	By seedling		
11.2	By grafting (budding)		
11.3	By both		

12. How old is the farm/orchard?

_____

13. Are there any other crops growing in or around the citrus farm/orchard?
Yes _____ No _____

14. If your answer for question number 13 is 'yes', which crops are they?

_____

_____

15. What is the soil type of the farm/orchard?

_____

16. Have you determined the nutrient status of the soil?
Yes _____ No _____

17. If your answer for question number 16 is ‘yes’, are there deficient nutrients identified? List their names.

_____

_____

18. If your answer for question number 16 is ‘no’, how did you manage disease and/or disorders associated with nutrient deficiencies?

_____

_____

19. Do you fertilize the citrus farm/orchard?
Yes _____ No _____

20. If your answer for question number 19 is ‘yes’, what type of fertilizer do you apply? What amount and how often do you apply fertilizer to the farm/orchard annually?

S/N	Type of fertilizer	Amount per ha	Frequency of application	Remark
20.1				
20.2				
20.3				
20.4				

III. DISEASE AND INSECT EPIDEMIOLOGY AND MANAGEMENT PRACTICES

21. Do you have disease problem on your citrus trees?
Yes _____ No _____

22. Which type of infection is most common in the farm/orchard?

S/N	Type of infection	Rank by level of damage	Remark
22.1	Root infection		
22.2	Stem infection		
22.3	Twigs infection		
22.4	Leaf infection		
22.5	Fruit infection		

23. Which cause of infection is most prevalent? Put in order (1-5) of their importance.

S/N	Cause of infection	Ranking	Remark
23.1	Fungi		
23.2	Bacteria		
23.3	Viruses		
23.4	Nematodes		
23.5	Insect problems		
23.6	Others (specify)		

24. If the disease has a microbial origin, which type? Can you name or describe the type of disease and its pathogen in order of importance?

S/N	Type of disease	Causal organism	Ranking	Remark
24.1				
24.2				
24.3				
24.4				
24.5				

25. If nematodes are present, which type? Mention by name according to their importance.
    25.1 _____
    25.2 _____
    25.3 _____

26. If insects are important, what type of insects? Write the names in order of importance.
    26.1 _____
    26.2 _____
    26.3 _____
    26.4 _____
    26.5 _____

27. Referring to question number 23, when do you think the fungal infections start to appear on the farm/orchard?

S/N	Type of fungal infection	Stage of the crop				Remark
		Seedling	Flowering	Fruiting	Ripening	
27.1						
27.2						
27.3						
27.4						
27.5						

28. Referring to question number 23, when do you think the respective bacterial infections start to appear on the farm/orchard?

S/N	Type of bacterial infection	Stage of the crop				Remark
		Seedling	Flowering	Fruiting	Ripening	
28.1						
28.2						
28.3						
28.4						
28.5						

29. Referring to question number 23, when do you think the respective viral infections start to appear on the farm/orchard?

S/N	Type of viral infection	Stage of the crop				Remark
		Seedling	Flowering	Fruiting	Ripening	
29.1						
29.2						
29.3						
29.4						
29.5						

30. Referring to question number 23, when do you think the respective nematode infections start to appear on the farm/orchard?

S/N	Type of nematode infection	Stage of the crop				Remark
		Seedling	Flowering	Fruiting	Ripening	
30.1						
30.2						
30.3						

31. Referring to question number 25, when do you think insect problems start to appear on the farm/orchard?

S/N	Type of insect	Stage of the crop				Remark
		Seedling	Flowering	Fruiting	Ripening	
31.1						
31.2						
31.3						
31.4						
31.5						

32. How do you control diseases? Explain your experience on the farm/orchard.

_____

_____

_____

33. Do you apply pesticides on your farm/orchard?
Yes _____ No _____

34. Referring to question number 33, if you have applied pesticides, mention their names and application involved.

S/N	Name of the commercial pesticide	What concentration is being applied?	When do you start spraying pesticide?	How often do you spray during the growing season?	What is the type of disease pathogen controlled?
34.1					

34.2					
34.3					
34.4					
34.5					

General Remark: _____

_____

_____

_____

_____

_____

_____

Appendix Table 3.2. Diseases and insect pests recorded, and use of pesticides in citrus orchards in 2012 and 2013 in Ethiopia.

Citrus Orchards	Diseases and/or Disorders Observed Based on Field Symptoms	Major Insect Pests Recorded	Chemical Pesticides Use
Bebeka	Citrus leaf and fruit spot disease, Lichens, Nutrient imbalance, decline	Leaf miner, Red scale	No application
Nura Era	Citrus Canker; Sudden death/Tristeza, *Phytophthora* rot, Dieback, Nutrient imbalance, Water stress	Woolly white fly ("Sukarie"), Med fruit fly, Leaf miner, Red scale, Citrus thrips, Cottony cushion scale, Termite	Ultracide, Diazinon, Diazol, White mineral oil
Merti	Anthracnose, Dieback, Nutrient imbalance	Leaf miner, Cottony cushion scale	Ultracide, Diazinon, Diazol, White mineral oil
Abadeshka-Jeju	Anthracnose, Nutrient imbalance, Water stress	Leaf miner, Woolly white fly, Red scale, Citrus thrips	Same as in Merti
Erer	Tristeza, Greening, Nutrient imbalance, Unknown disease	Leaf miner, Citrus thrips	Kocide 101, Ridomil MZ, Ridomil 5G, Bayleton, Mancozeb; Selecron, Suprathion, Endosulfan, Karate, Diazinon
Fetuli	Tristeza, Exocortis viroid, *Phytophthora*	Leaf miner	Same as in Erer
Gota	Tristeza, Exocortis viroid, Nutrient imbalance, Water stress	Leaf miner, Citrus thrips	Same as in Erer
Hurso	*Phytophthora*, Tristeza, Greening, Nutritional imbalance	Red scale, Leaf miner, Citrus thrips, Bud mite	Mancozeb, Diazinon, Methidathion, Sumithion
Ziway	Decline/Dieback, Nutrient imbalance, Salinity	Cottony cushion scale, Leaf miner, Citrus thrips, Med fruit fly	Karate
Shewarobit	Greening, Dieback, *Phytophthora* disease	Red scale	Mancozeb, Bayleton
Tibila (Tifhste Genet)	Nutrient imbalance	Red scale, Woolly white fly, Fruit fly, False codling moth, Leaf miner, Thrips, Mealy bug, Cottony cushion scale	Diazinon, Confider
Gibe	Citrus leaf and fruit spot disease, Canker, Dieback, Gummosis, Tristeza, Greening, Melanose, Nutrient imbalance	Leaf miner, Scales (red and black), Citrus thrips, Orange dog, Med fruit fly	Ultracide, Karate
Guangua (Dilla area)	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner	No application
Bikolo	Citrus leaf and fruit spot disease, Nutrient imbalance	Red scale	Kocide

Appendix Table 3.2. Continued.

Citrus Orchards	Diseases and/or Disorders Observed Based on Field Symptoms	Major Insect Pests Recorded	Chemical Pesticides Use
Chagni	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner	Daconil, Kocide
Finote Selam	Citrus leaf and fruit spot disease	Leaf miner, Red scale, Cottony cushion scale	Bayleton
Dejen (Kurar)	Deformation on graft union, Fruit drop, Water stress	Red scale	No application
Harbu (South Wello)	Dieback	Woolly white fly	No application
Melkassa	Dieback, Tristeza, Greening, Citrus Canker, Anthracnose, Fruit rot, Sooty mold, Nutrient imbalance	Red scale, Leaf miner, Cottony cushion scale, Citrus thrips, Med fruit fly	Dimethoate, Karate, Diazinon 60
Jarre (Hayk)	Spot-like disease, Water stress	Leaf miner, Red scale	No application
Jimma City	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner, Red scale	No application
Tony Farm (Dire Dawa)	Tristeza, Exocortis viroid, Greening, *Phytophthora*/ Nematodes, Fruit rot, Nutritional imbalance	Termite, Med fruit fly, Leaf miner, Red scale	Methidathion, Sumithion
Ethioflora (Adami Tulu)	*Phytophthora*, Gummosis, Graft incompatibility, Nutrient imbalance	Leaf miner, Med fruit fly	Diazinon, Thiodan
Woldiya	No disease	Red scale	No data
Koka	Decline, Nutrient imbalance	Leaf miner	No data
Aleta Wendo	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner	No application
Abeshege (Welkite area)	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner, Red scale	Daconil, Kocide
Kebena	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner	No application
Ginbo	Citrus leaf and fruit spot disease, Lichens, Greening	Leaf miner, Red scale	No application

Appendix Table 3.2. Continued.

Citrus Orchards	Diseases and/or Disorders Observed Based on Field Symptoms	Major Insect Pests Recorded	Chemical Pesticides Use
Lado (Lake Abaya area)	Citrus leaf and fruit spot disease, Sooty mold, Melanose, Nutrient imbalance	Leaf miner, Red scale, Citrus thrips	No application
Senbo	Citrus leaf and fruit spot disease, Nutrient imbalance	Red scale, Leaf miner, Cottony cushion scale	No application
Agaro	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner, Red scales	No application
Yebu	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner, Red scales	No application
Goro (Wolliso area)	Citrus leaf and fruit spot disease, Nutrient imbalance	Leaf miner	No application
Harbu Tropical Fruits Propagator Farmers Association	Dieback	Woolly white fly, Leaf miner	No data

Appendix Table 4.1. List of fungal isolates obtained from infected leaves and fruits from major citrus growing areas of Ethiopia

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
1	ETHCTR001	MMGOL21	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
2	ETHCTR002	AKROF1	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Fruit
3	ETHCTR003	GGKOF1	SNNP	Gurage	Geta	Kuante/Kebul	Sweet Orange	Fruit
4	ETHCTR004	GAJOF	SNNP	Gurage	Abeshege	Rumuga	Sweet Orange	Fruit
5	ETHCTR005	GAJOL3	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
6	ETHCTR006	WDDOL1	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
7	ETHCTR007	GAHOF1	SNNP	Gurage	Abeshege	Holie	Sweet Orange	Fruit
8	ETHCTR008	BCPAHL	SNNP	Bench Maji	Debre Werk	Bebeka	Alternate Host	Leaf
9	ETHCTR009	GMDOL1	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
10	ETHCTR010	SWFSOL3	Oromia	Southwest Shewa	Wollisso	Fodu Gora	Sour Orange	Leaf
11	ETHCTR011	GAJOL1	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
12	ETHCTR012	KTMOL21	Oromia	Jimma	Shebe Senbo	Kishe-Kosta	Sweet Orange	Leaf
13	ETHCTR013	GAJOL2	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
14	ETHCTR014	GAJOL5	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
15	ETHCTR015	GAJOL4	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
16	ETHCTR016	SWFSOL1	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sour Orange	Leaf
17	ETHCTR017	SWFSOF1	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sour Orange	Fruit
18	ETHCTR018	SWFOL1	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sweet Orange	Leaf
19	ETHCTR019	SWFSOL2	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sour Orange	Leaf
20	ETHCTR020	GAHOF2	SNNP	Gurage	Abeshege	Holie	Sweet Orange	Leaf
21	ETHCTR021	WDDOL2	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
22	ETHCTR022	WDDOL3	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
23	ETHCTR023	WDDOL4	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
24	ETHCTR024	GCEOL1	SNNP	Gurage	Cheha	Chifangira	Sweet Orange	Leaf
25	ETHCTR025	GCEOL2	SNNP	Gurage	Cheha	Chifangira	Sweet Orange	Leaf
26	ETHCTR026	GCEOL31	SNNP	Gurage	Cheha	Sisena Mitia	Sweet Orange	Leaf
27	ETHCTR027	GCEOL32	SNNP	Gurage	Cheha	Sisena Mitia	Sweet Orange	Leaf
28	ETHCTR028	GCEOL4	SNNP	Gurage	Cheha	Sisena Mitia	Sweet Orange	Leaf
29	ETHCTR029	GCEOL5	SNNP	Gurage	Cheha	Sisena Mitia	Sweet Orange	Leaf
30	ETHCTR030	GCEOL6	SNNP	Gurage	Cheha	Sisena Mitia	Sweet Orange	Leaf
31	ETHCTR031	WDDOL5	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
32	ETHCTR032	WBAOL2	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Leaf

Appendix Table 4.1. Continued.

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
33	ETHCTR033	WBAOF1	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Fruit
34	ETHCTR034	WBAOF2	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Fruit
35	ETHCTR035	ACLL	Amhara	Awi	Guangua	Chagni	Lime (Bears)	Leaf
36	ETHCTR036	ACTL	Amhara	Awi	Guangua	Chagni	Tangor/Tangelo	Leaf
37	ETHCTR037	GOF	Oromia	Jimma	Sekoru	Gibe Unit Two	Sweet Orange	Fruit
38	ETHCTR038	GML	Oromia	Jimma	Sekoru	Gibe Unit Two	Mandarin	Leaf
39	ETHCTR039	AGOL21	Oromia	Borena	Abaya	Guangua	Sweet Orange	Leaf
40	ETHCTR040	ACOF21	Amhara	Awi	Guangua	Chagni	Sweet Orange (Jaffa)	Fruit
41	ETHCTR041	WGFML1	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
42	ETHCTR042	WGFOL21	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
43	ETHCTR043	WGFOF41	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
44	ETHCTR044	WGFOF51	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (Jaffa)	Fruit
45	ETHCTR045	GBAOL1	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
46	ETHCTR046	GBAOF	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Fruit
47	ETHCTR047	ABDOL	Oromia	Southwest Shewa	Gorro	Adami Wedessa	Sweet Orange	Leaf
48	ETHCTR048	AGSOL1	Oromia	Borena	Abaya	Guangua	Sour Orange	Leaf
49	ETHCTR049	ENAOL11	Oromia	Jimma	Gomma	Elbu	Sweet Orange	Leaf
50	ETHCTR050	ALSOL1	Oromia	Borena	Abaya	Lado	Sour Orange	Leaf
51	ETHCTR051	AGOF11	Oromia	Borena	Abaya	Guangua	Sweet Orange	Fruit
52	ETHCTR052	GFHOL2	Oromia	Jimma	Gomma	Genji Elbu	Sweet Orange	Leaf
53	ETHCTR053	JFWOL21	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
54	ETHCTR054	ENAOF1	Oromia	Jimma	Gomma	Elbu	Sweet Orange	Fruit
55	ETHCTR055	GMAOL11	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
56	ETHCTR056	AGOL11	Oromia	Borena	Abaya	Guangua	Sweet Orange	Leaf
57	ETHCTR057	AGOL12	Oromia	Borena	Abaya	Guangua	Sweet Orange	Leaf
58	ETHCTR058	JFWOF1	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Fruit
59	ETHCTR059	ACOL21	Amhara	Awi	Guangua	Chagni	Sweet Orange (Hamlin)	Leaf
60	ETHCTR060	ACOL22	Amhara	Awi	Guangua	Chagni	Sweet Orange (Hamlin)	Leaf
61	ETHCTR061	WGFOF42	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
62	ETHCTR062	WGFOF43	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
63	ETHCTR063	AGOL23	Oromia	Borena	Abaya	Guangua	Sweet Orange	Leaf
64	ETHCTR064	AGOF22	Oromia	Borena	Abaya	Guangua	Sweet Orange	Fruit

Appendix Table 4.1. Continued.

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
65	ETHCTR065	AKROF2	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Fruit
66	ETHCTR066	AKROF3	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Fruit
67	ETHCTR067	BCPOF2	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Fruit
68	ETHCTR068	BCPOL1	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
69	ETHCTR069	BCPOL2	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
70	ETHCTR070	AKROL1	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
71	ETHCTR071	AKROL2	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
72	ETHCTR072	WGFMF11	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Fruit
73	ETHCTR073	WGFMF12	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Fruit
74	ETHCTR074	AWOCOL21	SNNP	Sidama	Aleta Wendo	Omacho Chawa	Sweet Orange	Leaf
75	ETHCTR075	AWOCOL22	SNNP	Sidama	Aleta Wendo	Omacho Chawa	Sweet Orange	Leaf
76	ETHCTR076	ACOL1	Amhara	Awi	Guangua	Chagni	Sweet Orange (OV)	Leaf
77	ETHCTR077	ACOL32	Amhara	Awi	Guangua	Chagni	Sweet Orange (Jaffa)	Leaf
78	ETHCTR078	GATOF2	SNNP	Gurage	Abeshege	Tawela	Sweet Orange	Fruit
79	ETHCTR079	GGKOF2	SNNP	Gurage	Geta	Kuante/Kebul	Sweet Orange	Fruit
80	ETHCTR080	MSZLL	SNNP	Kaffa	Ginbo	Megenagna	Lime	Leaf
81	ETHCTR081	JAHOF1	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Fruit
82	ETHCTR082	JAHOF2	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Fruit
83	ETHCTR083	MMGOL22	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
84	ETHCTR084	MMGOL31	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
85	ETHCTR085	MMGOL32	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
86	ETHCTR086	KAFOL	Oromia	Jimma	Shebe Senbo	Kishe-Kosta	Sweet Orange	Leaf
87	ETHCTR087	WGFOL11	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
88	ETHCTR088	WGFOL12	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
89	ETHCTR089	GMAOF1	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Fruit
90	ETHCTR090	WGFOL22	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
91	ETHCTR091	WGFOL23	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
92	ETHCTR092	ACOF11	Amhara	Awi	Guangua	Chagni	Sweet Orange (OV)	Fruit
93	ETHCTR093	ACOF12	Amhara	Awi	Guangua	Chagni	Sweet Orange (OV)	Fruit
94	ETHCTR094	AGOL22	Oromia	Borena	Abaya	Guangua	Sweet Orange	Leaf
95	ETHCTR095	ACOL31	Amhara	Awi	Guangua	Chagni	Sweet Orange (Jaffa)	Leaf
96	ETHCTR096	KTMOL22	Oromia	Jimma	Shebe Senbo	Kishe-Kosta	Sweet Orange	Leaf

Appendix Table 4.1. Continued.

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
97	ETHCTR097	AGOF12	Oromia	Borena	Abaya	Guangua	Sweet Orange	Fruit
98	ETHCTR098	WBAOL1	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Leaf
99	ETHCTR099	ALLL1	Oromia	Borena	Abaya	Lado	Lime	Leaf
100	ETHCTR100	KBSSOL	Oromia	Jimma	Gomma	Koye Seja	Sour Orange	Leaf
101	ETHCTR101	LTMB1	SNNP	Gurage	Abeshege	Layignaw Tatessa	Sweet Orange	Leaf
102	ETHCTR102	ALLL2	Oromia	Borena	Abaya	Lado	Lime	Leaf
103	ETHCTR103	ALSOL2	Oromia	Borena	Abaya	Lado	Sour Orange	Leaf
104	ETHCTR104	AKSOL2	Oromia	Jimma	Gomma	Agaro 01	Sweet Orange	Leaf
105	ETHCTR105	ENAOL12	Oromia	Jimma	Gomma	Elbu	Sweet Orange	Leaf
106	ETHCTR106	ENAOL2	Oromia	Jimma	Gomma	Elbu	Sweet Orange	Leaf
107	ETHCTR107	ENAOF2	Oromia	Jimma	Gomma	Elbu	Sweet Orange	Fruit
108	ETHCTR108	WGFOL41	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
109	ETHCTR109	WGFOL42	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
110	ETHCTR110	WGFOL3	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
111	ETHCTR111	WGFOF52	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (Jaffa)	Fruit
112	ETHCTR112	LTMB2	SNNP	Gurage	Abeshege	Layignaw Tatessa	Sweet Orange	Leaf
113	ETHCTR113	LTMK	SNNP	Gurage	Abeshege	Layignaw Tatessa	Sweet Orange	Leaf
114	ETHCTR114	SWJOL	Amhara	South Wello	Tehuledere	Hayk/Jarre	Sweet Orange	Leaf
115	ETHCTR115	AWOCOL11	SNNP	Sidama	Aleta Wendo	Omacho Chawa	Sweet Orange	Leaf
116	ETHCTR116	AWOCOL12	SNNP	Sidama	Aleta Wendo	Omacho Chawa	Sweet Orange	Leaf
117	ETHCTR117	GMDOL2	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
118	ETHCTR118	WGFMF21	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Fruit
119	ETHCTR119	WGFMF22	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Fruit
120	ETHCTR120	WGFMF31	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Fruit
121	ETHCTR121	WGFMF32	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Fruit
122	ETHCTR122	BAAOL1	SNNP	Kaffa	Ginbo	Balewold	Sweet Orange	Leaf
123	ETHCTR123	BAAOL21	SNNP	Kaffa	Ginbo	Balewold	Sweet Orange	Leaf
124	ETHCTR124	BAAOL22	SNNP	Kaffa	Ginbo	Balewold	Sweet Orange	Leaf
125	ETHCTR125	GMAOF2	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Fruit
126	ETHCTR126	ACOF22	Amhara	Awi	Guangua	Chagni	Sweet Orange (Jaffa)	Fruit
127	ETHCTR127	KTMOL11	Oromia	Jimma	Shebe Senbo	Kishe-Kosta	Sweet Orange	Leaf
128	ETHCTR128	KTMOL12	Oromia	Jimma	Shebe Senbo	Kishe-Kosta	Sweet Orange	Leaf

Appendix Table 4.1. Continued.

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
129	ETHCTR129	BCPOL61	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
130	ETHCTR130	BCPOL62	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
131	ETHCTR131	BCPOL7	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
132	ETHCTR132	GATOF11	SNNP	Gurage	Abeshege	Tawela	Sweet Orange	Fruit
133	ETHCTR133	GATOF12	SNNP	Gurage	Abeshege	Tawela	Sweet Orange	Fruit
134	ETHCTR134	JFWOL11	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
135	ETHCTR135	JFWOL12	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
136	ETHCTR136	JFWOL22	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
137	ETHCTR137	JFWOF2	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Fruit
138	ETHCTR138	JFWOF3	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Fruit
139	ETHCTR139	BCPOL31	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
140	ETHCTR140	BCPOL32	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
141	ETHCTR141	BCPOL33	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
142	ETHCTR142	BCPOL41	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
143	ETHCTR143	BCPOL42	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
144	ETHCTR144	BCPOL43	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
145	ETHCTR145	WGFML33	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
146	ETHCTR146	GFHOL1	Oromia	Jimma	Gomma	Genji Elbu	Sweet Orange	Leaf
147	ETHCTR147	GMAOL23	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
148	ETHCTR148	JAHOL1	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
149	ETHCTR149	JAHOL2	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
150	ETHCTR150	JAHOL3	SNNP	Gurage	Abeshege	Jejeba and Gasorie	Sweet Orange	Leaf
151	ETHCTR151	BCPOF11	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Fruit
152	ETHCTR152	BCPOF12	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Fruit
153	ETHCTR153	BCPOF13	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Fruit
154	ETHCTR154	WGFOF21	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
155	ETHCTR155	AGSOL2	Oromia	Borena	Abaya	Guangua	Sour Orange	Leaf
156	ETHCTR156	AGOF21	Oromia	Borena	Abaya	Guangua	Sweet Orange	Fruit
157	ETHCTR157	BCPOL51	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
158	ETHCTR158	BCPOL52	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
159	ETHCTR159	BCPOL53	SNNP	Bench Maji	Debre Werk	Bebeka	Sweet Orange	Leaf
160	ETHCTR160	AKSOL1	Oromia	Jimma	Gomma	Agaro 01	Sweet Orange	Leaf

Appendix Table 4.1. Continued.

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
161	ETHCTR161	ACOL4	Amhara	Awi	Guangua	Chagni	Sweet Orange	Leaf
162	ETHCTR162	WGFOF33	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (Jaffa)	Fruit
163	ETHCTR163	WGFOL51	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
164	ETHCTR164	WGFOL52	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
165	ETHCTR165	WGFOL53	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
166	ETHCTR166	WBAOF3	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Fruit
167	ETHCTR167	WBAOF4	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Fruit
168	ETHCTR168	WBAOF5	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Fruit
169	ETHCTR169	WBAOF6	SNNP	Wolayita	Boloso Sore	Areka	Sweet Orange	Fruit
170	ETHCTR170	MMGOL11	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
171	ETHCTR171	MMGOL12	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
172	ETHCTR172	MMGOL13	SNNP	Kaffa	Ginbo	Megenagna	Sweet Orange	Leaf
173	ETHCTR173	GMAOL12	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
174	ETHCTR174	GMAOL22	Oromia	Jimma	Mana	Gube Bosoka	Sweet Orange	Leaf
175	ETHCTR175	WGFML21	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
176	ETHCTR176	WGFML22	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
177	ETHCTR177	WGFML23	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
178	ETHCTR178	WGFML24	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
179	ETHCTR179	WGFML31	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
180	ETHCTR180	WGFML32	Amhara	West Gojjam	Jabitehnan	Finote Selam	Mandarin	Leaf
181	ETHCTR181	WGFOF11	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
182	ETHCTR182	WGFOF12	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
183	ETHCTR183	WGFOF22	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
184	ETHCTR184	WGFOF31	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (Jaffa)	Fruit
185	ETHCTR185	WGFOF32	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (Jaffa)	Fruit
186	ETHCTR186	WGFOF61	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
187	ETHCTR187	GCEOL7	SNNP	Gurage	Cheha	Sisena Mitia	Sweet Orange	Leaf
188	ETHCTR188	GGKOF3	SNNP	Gurage	Geta	Kuante/Kebul	Sweet Orange	Fruit
189	ETHCTR189	WDDOL6	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
190	ETHCTR190	WDDOL7	SNNP	Wolayita	Damot Pulasa	Denba Galie	Sweet Orange	Leaf
191	ETHCTR191	WGFOL61	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf
192	ETHCTR192	WGFOL62	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Leaf

Appendix Table 4.1. Continued.

No.	Isolate No.	Isolate Code	Region	Zone	District	Kebele	Crop species	Sampled plant part
193	ETHCTR193	WGFOF62	Amhara	West Gojjam	Jabitehnan	Finote Selam	Sweet Orange (WN)	Fruit
194	ETHCTR194	GAHOF3	SNNP	Gurage	Abeshege	Holie	Sweet Orange	Leaf
195	ETHCTR195	GAHOF4	SNNP	Gurage	Abeshege	Holie	Sweet Orange	Leaf
196	ETHCTR196	SWFSOL4	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sour Orange	Leaf
197	ETHCTR197	SWFSOL5	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sour Orange	Leaf
198	ETHCTR198	SWFSOF2	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sour Orange	Fruit
199	ETHCTR199	SWFOL2	Oromia	Southwest Shewa	Wolisso	Fodu Gora	Sweet Orange	Leaf
200	ETHCTR200	SWGOL1	Oromia	Southwest Shewa	Goro	Goro	Sweet Orange	Leaf
201	ETHCTR201	SWGOL2	Oromia	Southwest Shewa	Goro	Goro	Sweet Orange	Leaf
202	ETHCTR202	SWGOL3	Oromia	Southwest Shewa	Goro	Goro	Sweet Orange	Leaf
203	ETHCTR203	GAJOL1	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
204	ETHCTR204	GAJOL2	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
205	ETHCTR205	GAJOL3	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
206	ETHCTR206	GAJOL4	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
207	ETHCTR207	GAJOL5	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
208	ETHCTR208	GAJOL6	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
209	ETHCTR209	GAJOL7	SNNP	Gurage	Abeshege	Jejeba	Sweet Orange	Leaf
210	ETHCTR210	GKAOL1	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
211	ETHCTR211	GKAOL2	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
212	ETHCTR212	GKAOL3	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
213	ETHCTR213	GKAOL4	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
214	ETHCTR214	GKAOL5	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
215	ETHCTR215	GKAOL6	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
216	ETHCTR216	GKAOL7	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
217	ETHCTR217	GKAOL8	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
218	ETHCTR218	GKAOL9	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
219	ETHCTR219	GKAOL10	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
220	ETHCTR220	GKAOL11	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
221	ETHCTR221	GKAOL12	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
222	ETHCTR222	GKAOL13	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf
223	ETHCTR223	GKAOL14	SNNP	Gurage	Kebena	Aregita	Sweet Orange	Leaf

Appendix Table 5.1. Isolates of *Colletotrichum* species used for multilocus phylogenetic study and their origin in Ethiopia.

Isolate	Host plant	Plant part	Region	District	Collection Site
ETHCTR001	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Megenagna
ETHCTR002	*Citrus sinensis*	Fruit	Central Ethiopia	Kebena	Aregita
ETHCTR003	*Citrus sinensis*	Fruit	Central Ethiopia	Geta	Kebul
ETHCTR004	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Rumuga
ETHCTR006	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR007	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Holie
ETHCTR008	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR009	*Citrus sinensis*	Leaf	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR012	*Citrus sinensis*	Leaf	Southwest Ethiopia	Shebe Senbo	Kishe-Kosta
ETHCTR013	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Jejeba
ETHCTR014	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Jejeba
ETHCTR016	*Citrus aurantium*	Leaf	Central Ethiopia	Wolliso	Fodu Gora
ETHCTR017	*Citrus aurantium*	Fruit	Central Ethiopia	Wolliso	Fodu Gora
ETHCTR018	*Citrus sinensis*	Leaf	Central Ethiopia	Wolliso	Fodu Gora
ETHCTR019	*Citrus aurantium*	Leaf	Central Ethiopia	Wolliso	Fodu Gora
ETHCTR020	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Holie
ETHCTR021	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR022	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR023	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR024	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Chifangira
ETHCTR025	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Chifangira
ETHCTR026	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Sisena Mitia
ETHCTR027	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Sisena Mitia
ETHCTR028	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Sisena Mitia
ETHCTR029	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Sisena Mitia
ETHCTR031	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR032	*Citrus sinensis*	Leaf	South Ethiopia	Boloso Sore	Areka
ETHCTR033	*Citrus sinensis*	Fruit	South Ethiopia	Boloso Sore	Areka
ETHCTR034	*Citrus sinensis*	Fruit	South Ethiopia	Boloso Sore	Areka
ETHCTR037	*Citrus sinensis*	Fruit	Central Ethiopia	Sekoru	Gibe
ETHCTR038	*Citrus reticulate*	Leaf	Central Ethiopia	Sekoru	Gibe
ETHCTR039	*Citrus sinensis*	Leaf	South Ethiopia	Abaya	Guangua
ETHCTR041	*Citrus reticulate*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam

Appendix Table 5.1. Continued.

Isolate	Host plant	Plant part	Region	District	Collection Site
ETHCTR042	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR043	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR044	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR045	*Citrus sinensis*	Leaf	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR046	*Citrus sinensis*	Fruit	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR047	*Citrus sinensis*	Leaf	Central Ethiopia	Gorro	Adami Wedessa
ETHCTR048	*Citrus aurantium*	Leaf	South Ethiopia	Abaya	Guangua
ETHCTR049	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR050	*Citrus aurantium*	Leaf	South Ethiopia	Abaya	Lado
ETHCTR051	*Citrus sinensis*	Fruit	South Ethiopia	Abaya	Guangua
ETHCTR052	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR053	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Gasorie
ETHCTR054	*Citrus sinensis*	Fruit	Southwest Ethiopia	Gomma	Agaro
ETHCTR055	*Citrus sinensis*	Leaf	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR056	*Citrus sinensis*	Leaf	South Ethiopia	Abaya	Guangua
ETHCTR057	*Citrus sinensis*	Leaf	South Ethiopia	Abaya	Guangua
ETHCTR058	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Gasorie
ETHCTR059	*Citrus sinensis*	Leaf	Northwest Ethiopia	Guangua	Chagni
ETHCTR060	*Citrus sinensis*	Leaf	Northwest Ethiopia	Guangua	Chagni
ETHCTR061	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR062	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR063	*Citrus sinensis*	Leaf	South Ethiopia	Abaya	Guangua
ETHCTR064	*Citrus sinensis*	Fruit	South Ethiopia	Abaya	Guangua
ETHCTR065	*Citrus sinensis*	Fruit	Central Ethiopia	Kebena	Aregita
ETHCTR066	*Citrus sinensis*	Fruit	Central Ethiopia	Kebena	Aregita
ETHCTR067	*Citrus sinensis*	Fruit	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR068	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR069	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR070	*Citrus sinensis*	Leaf	Central Ethiopia	Kebena	Aregita
ETHCTR071	*Citrus sinensis*	Leaf	Central Ethiopia	Kebena	Aregita
ETHCTR072	*Citrus reticulate*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR073	*Citrus reticulate*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR074	*Citrus sinensis*	Leaf	South Ethiopia	Aleta Wendo	Omacho Chawa
ETHCTR075	*Citrus sinensis*	Leaf	South Ethiopia	Aleta Wendo	Omacho Chawa

Appendix Table 5.1. Continued.

Isolate	Host plant	Plant part	Region	District	Collection Site
ETHCTR077	*Citrus sinensis*	Leaf	Northwest Ethiopia	Guangua	Chagni
ETHCTR078	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Tawela
ETHCTR081	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Gasorie
ETHCTR082	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Gasorie
ETHCTR084	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Megenagna
ETHCTR085	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Megenagna
ETHCTR086	*Citrus sinensis*	Leaf	Southwest Ethiopia	Shebe Senbo	Kishe-Kosta
ETHCTR088	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR089	*Citrus sinensis*	Fruit	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR090	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR091	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR092	*Citrus sinensis*	Fruit	Northwest Ethiopia	Guangua	Chagni
ETHCTR093	*Citrus sinensis*	Fruit	Northwest Ethiopia	Guangua	Chagni
ETHCTR094	*Citrus sinensis*	Leaf	South Ethiopia	Abaya	Guangua
ETHCTR095	*Citrus sinensis*	Leaf	Northwest Ethiopia	Guangua	Chagni
ETHCTR096	*Citrus sinensis*	Leaf	Southwest Ethiopia	Shebe Senbo	Kishe-Kosta
ETHCTR097	*Citrus sinensis*	Fruit	South Ethiopia	Abaya	Guangua
ETHCTR098	*Citrus sinensis*	Leaf	South Ethiopia	Boloso Sore	Areka
ETHCTR101	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Layignaw Tatessa
ETHCTR103	*Citrus aurantium*	Leaf	South Ethiopia	Abaya	Lado
ETHCTR104	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR105	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR106	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR107	*Citrus sinensis*	Fruit	Southwest Ethiopia	Gomma	Agaro
ETHCTR108	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR109	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR110	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR111	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR112	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Layignaw Tatessa
ETHCTR113	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Layignaw Tatessa
ETHCTR114	*Citrus sinensis*	Leaf	North Central	Tehuledere	Hayk/Jarre
ETHCTR115	*Citrus sinensis*	Leaf	South Ethiopia	Aleta Wendo	Omacho Chawa
ETHCTR116	*Citrus sinensis*	Leaf	South Ethiopia	Aleta Wendo	Omacho Chawa
ETHCTR117	*Citrus sinensis*	Leaf	Southwest Ethiopia	Mana	Gube Bosoka

Appendix Table 5.1. Continued.

Isolate	Host plant	Plant part	Region	District	Collection Site
ETHCTR118	*Citrus reticulate*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR119	*Citrus reticulate*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR120	*Citrus reticulate*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR121	*Citrus reticulate*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR122	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Balewold
ETHCTR123	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Balewold
ETHCTR124	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Balewold
ETHCTR127	*Citrus sinensis*	Leaf	Southwest Ethiopia	Shebe Senbo	Kishe-Kosta
ETHCTR128	*Citrus sinensis*	Leaf	Southwest Ethiopia	Shebe Senbo	Kishe-Kosta
ETHCTR129	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR130	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR131	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR132	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Tawela
ETHCTR133	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Tawela
ETHCTR134	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Gasorie
ETHCTR136	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Gasorie
ETHCTR137	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Gasorie
ETHCTR138	*Citrus sinensis*	Fruit	Central Ethiopia	Abeshege	Gasorie
ETHCTR139	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR140	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR141	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR142	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR143	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR146	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR148	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Gasorie
ETHCTR151	*Citrus sinensis*	Fruit	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR152	*Citrus sinensis*	Fruit	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR153	*Citrus sinensis*	Fruit	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR154	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR156	*Citrus sinensis*	Fruit	South Ethiopia	Abaya	Guangua
ETHCTR157	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR158	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR159	*Citrus sinensis*	Leaf	Southwest Ethiopia	Debre Werk	Bebeka
ETHCTR160	*Citrus sinensis*	Leaf	Southwest Ethiopia	Gomma	Agaro
ETHCTR161	*Citrus sinensis*	Leaf	Northwest Ethiopia	Guangua	Chagni

Appendix Table 5.1. Continued.

Isolate	Host plant	Plant part	Region	District	Collection Site
ETHCTR162	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR163	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR164	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR165	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR166	*Citrus sinensis*	Fruit	South Ethiopia	Boloso Sore	Areka
ETHCTR167	*Citrus sinensis*	Fruit	South Ethiopia	Boloso Sore	Areka
ETHCTR169	*Citrus sinensis*	Fruit	South Ethiopia	Boloso Sore	Areka
ETHCTR170	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Megenagna
ETHCTR172	*Citrus sinensis*	Leaf	Southwest Ethiopia	Ginbo	Megenagna
ETHCTR173	*Citrus sinensis*	Leaf	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR174	*Citrus sinensis*	Leaf	Southwest Ethiopia	Mana	Gube Bosoka
ETHCTR175	*Citrus reticulata*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR176	*Citrus reticulata*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR178	*Citrus reticulata*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR179	*Citrus reticulata*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR180	*Citrus reticulata*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR181	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR182	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR183	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR184	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR185	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR186	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR187	*Citrus sinensis*	Leaf	Central Ethiopia	Cheha	Sisena Mitia
ETHCTR188	*Citrus sinensis*	Fruit	Central Ethiopia	Geta	Kebul
ETHCTR189	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR190	*Citrus sinensis*	Leaf	South Ethiopia	Damot Pulasa	Denba Galie
ETHCTR192	*Citrus sinensis*	Leaf	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR193	*Citrus sinensis*	Fruit	Northwest Ethiopia	Jabitehnan	Finote Selam
ETHCTR194	*Citrus sinensis*	Leaf	Central Ethiopia	Abeshege	Holie
ETHCTR197	*Citrus aurantium*	Leaf	Central Ethiopia	Wolliso	Fodu Gora
ETHCTR198	*Citrus aurantium*	Fruit	Central Ethiopia	Wolliso	Fodu Gora

Appendix Table 5.2. Reference *Colletotrichum* isolates with collection details and GenBank accession numbers.

Species	Accession number[a]	Host	Country	GenBank number[b]			Reference
				ITS	LSU	ACT	
C. aenigma	ICMP 18608, LC0038, C1253.4	*Persea americana*	Israel	NR_120140	JN940409	JX009443	Cai 2011; Weir et al. 2012
C. aotearoa	C1252.9, AR2802, ICMP 18532	*Kunzea ericoides*, Pueraria , *Vitex lucens*	New Zealand, USA	JX010198	DQ286187	JX009544	Farr et al., 2006; Weir et al. 2012
C. asianum	C1187, LC0036, CPC 20981	*Mangifera indica*, unknown, Fruits	Australia, Thailand, Brazil	JX010192	JN940407	KC566879	Cai 2011; Weir et al. 2012; Braganca 2013 (unpublished data)
C. boninense	CBS 128547, ICMP 10338	*Camellia* sp.	New Zealand	JQ005159	DQ286169	JQ005507	Farr et al. 2006; Damm et al. 2012
C. fructicola	ICMP 12568, LC0032, CMM3811	*Persea americana*, unknown, *Mangifera indica*	Australia, Thailand, Brazil	JX010166	JN940418	KC702919	Cai 2011; Weir et al. 2012; Vieira et al. 2013
C. gloeosporioides	OCAC24, LC0553, C1254.3	*Elettaria cardamomum*, unknown, *Citrus* sp.	India, China, USA	KJ813602	JN940414	JX009494	Cai 2011; Weir et al. 2012; Chowdappa & Chethana 2014 (unpublished data)
C. gloeosporioides	GM62-L03, PP143, CPC 20904	*Annona muricata*; Fruits	Colombia, Brazil	KC512137	FJ890371	KC566853	Gazis & Chaverri 2010 (unpublished data); Braganca 2013 (unpublished data); Alvarez et al. 2014
C. gloeosporioides	Strain 8, GJS01-199, CBS 953.97	Olive, *Citrus sinensis*, Theobroma	Italy, Cameroon	JN121209	DQ286177	GQ856782	Farr et al. 2006; Yang et al. 2010 (unpublished data); Faedda et al. 2011
C. gloeosporioides	CK13b7, AR4031, CBS 131329	*Citrus limon*, Fruits	Cameroon, Brazil	JX436791	AY539807	KC566856	Berner et al. 2004 (unpublished data); Braganca 2013 (unpublished data); Douanla-Meli et al. 2013 (unpublished data)

Appendix Table 5.2. Continued.

Species	Accession number[a]	Host	Country	GenBank number[b]			Reference
				ITS	LSU	ACT	
C. gloeosporioides	M2P3D7, CBS 122687, C1014.6	Soybean, *Leucospermum* sp., *Citrus* sp.	Brazil, South Africa, New Zealand	JX258787	EU552111	JX009462	Marincowitz et al. 2008 (unpublished data); Cnossen-Fassoni et al. 2012 (unpublished data); Weir et al. 2012
C. karstii	OCAC4, CBS 102667, BRIP:28443a	*Elettaria cardamomum*; Passiflora; *Mangifera indica*	India, New Zealand, Australia	KJ813595	DQ286173	JQ005551	Farr et al. 2006; Damm et al. 2012; Chowdappa & Chethana 2014 (unpublished data)
C. siamense	ICMP 12567 , FAU 553, CBS 114054/BPI 747978 C1315.2	*Persea americana*, Fragaria, *Coffea arabica*	Australia , USA, Thailand	JX010250	AF543786	JX009518	Farr et al. 2006; Weir et al. 2012
Glomerella cingulata	ICMP 10646, AR 2799	*Camellia sasanqua*, *Pueraria lobata*	USA	JX010225	DQ286193	JX009563	Farra et al. 2006; Weir et al. 2012
C. acutatum (outgroup)	MEP1323, CBS:126521	Vaccinium, Anemone F1 hybrid	New Zealand, Netherlands	DQ286124	DQ286125	JQ949687	Farr et al. 2006; Damm et al. 2012

[a] BPI: The U.S. National Fungus Collections; BRIP: Plant Pathology Herbarium, Department of Primary Industries, Queensland, Australia; CBS: Culture collection of the Centraalbureau voor Schimmelcultures, Fungal Biodiversity Centre, Utrecht, The Netherlands; CMM: Culture Collection of Phytopathogenic Fungi Prof. Maria Menezes (Colecao de Culturas de Fungos Fitopatogenicos Prof. Maria Menezes), Brazil; CPC: Culture collection of Pedro Crous, housed at CBS; FAU: Florida Atlantic University, Harbor Branch Marine Microbial Database, Boca Raton, Florida; ICMP: International Collection of Microorganisms from Plants; GJS: Gary J. Samuels searchable database;
[b] ITS: internal transcribed spacers and intervening 5.8S nrDNA; LSU: partial long subunit of nrDNA gene; ACT: partial actin gene.

Appendix Table 6.1. Primers designed for the amplification of microsatellite loci for *C. gloeosporioides*.

Locus[a]	Forward Primer Sequences (5'- 3')	Size	Tm[b] Forward	GC %	Reverse Primer Sequences (5'- 3')	Size	Tm Reverse	GC %	Estimated Product Size (bp)	Repeat Motifs
CG1	CAAGCAGTCTTTCTGGTCTT	20	51.3	45	AAAACAACTTCTCTCGTCCA	20	50.5	40	129	(TG)6
CG2	TCACCTTCACTCACACTTGA	20	51.9	45	CTACTTCGAGACAAGCACG	19	51.4	52.6	200	(CT)6
CG3	GGGTTTTCTCATTCTCAACA	20	49.1	40	CGACATGATCCATAGCAAG	19	48.9	47.4	249	(AC)6
CG4	AACTCAAGATCAAGAGCAGC	20	51.4	45	ATGTACAGACGCTCACACAA	20	52.9	45	158	(TG)6
CG5	GCTCGTACCTACGCAGTAAT	20	58	50	TCATCATGGACAATCATCAC	20	48.7	40	216	(AC)6
CG6	AGAGCAAGACAGGTGGAATA	20	51.4	45	ATCCCTGACTGCATAAACC	19	49.9	47.4	223	(AC)9
CG7	ATCTCCAGAGAGAACACAGC	20	52.3	50	GAGACCTCACGGAATTGAC	19	50.8	52.6	161	(TG)7
CG8	CTGCATATCAACCAGCACTA	20	50.9	45	AAAACAACAAGGACGACAAG	20	50.4	40	156	(GC)6
CG9	GTCTTGATGCTGAAGTCCAC	20	52.2	50	CACTCCTTCATAGAACACCC	20	50.8	50	222	(TG)6
CG10	GAGAAGGCTGACGAAGAAG	19	50.9	52.6	GACGGCTTCCGCAACTAC	18	53.8	61.1	213	(GC)6
CG11	CAGTGAAGATAGGGAAGCAG	20	50.9	50	ACCACTCAGCGTATGAGAAA	20	52.2	45	119	(GT)8
CG12	AGACACATCGAAGATGGAAT	20	49.8	40	TGCCAGAATGTAGTTGTGAA	20	50.5	40	248	(TG)10
CG13	GAGGCAATTGAACTCACACT	20	51.6	45	GAAGTACACCAAGTGCAGGT	20	53.4	50	247	(CT)6
CG14	ACATGACATCAAACCAGCTT	20	51.2	40	CTCTTGACCCGATGTTCTAT	20	49.9	45	171	(TC)7
CG15	GTTTGCATATCCGAGTGC	18	49.2	50	ACATCCCAGTCACGTTTTAC	20	51.4	45	236	(TC)7
CG16	CCATTCTTTGTACTGGTCGT	20	51.1	45	GACATCAGACATCCATCCTC	20	50.7	50	193	(TG)6
CG17	TATACCAGTCCCCTCAACTG	20	51.2	50	GATCCAGAGTCTCTTATCGC	20	50.6	50	246	(GA)7
CG18	TCCAGACGGATAGCTTACAC	20	52.1	50	GAGGTATTGCGTCCACTAAG	20	51.4	50	200	(CT)7
CG19	AATATCCAAGCCAACTGATG	20	49	40	TGGAGATCTTTACAATCGCT	20	49.8	40	216	(CA)6
CG20	CATAGTCCGTCCAGTCTCAT	20	51.8	50	CTAATGAAAAGTCGTGGAGC	20	50.2	45	234	(GA)8
CG21	GTCTCACTCAGTCTCAAGCC	20	53.3	55	AACACAGTCTGAGAGGCAAT	20	52.4	45	229	(AT)9
CG22	CTTCGAGTCACCTCTTCAAC	20	51.6	50	CAGAGTGGTAAAGGTGGTGT	20	52.7	50	239	(AC)7
CG23	TATTAGATCCCGACCTTGTG	20	49.6	45	ATCCTGGTCACCATAATCC	19	48.8	47.4	176	(GA)6
CG24	GTATGCGACCTTACGCTTC	19	51.8	52.6	TTGACGGGAGACTCTAATTG	20	50.2	45	220	(TC)6
CG25	AAGAGCCTCCTCTCGGTAT	19	52	52.6	AAGTATTTGTCGCCATCAAC	20	50	40	235	(AG)8

Appendix Table 6.1. Continued.

Locus[a]	Forward Primer Sequences (5'- 3')	Size	Tm[b] Forward	GC %	Reverse Primer Sequences (5'- 3')	Size	Tm Reverse	GC %	Estimated Product Size (bp)	Repeat Motifs
CG26	CGCATCTTGGATTTCTATTC	20	47.9	40	TTTCCTCCATCTCAACATTC	20	48.5	40	237	(GT)8
CG27	CCTGTTGATCCATGATGTAA	20	48.3	40	GAAAGGCTGACTTGTGAACT	20	51.6	45	128	(GT)6
CG28	CATATCTCTTCGTACCTCGC	20	51.1	50	GGTTTGTTGTCTGCTTCTCT	20	51.6	45	168	(AG)8
CG29	TTTCAACTACATCCCACCTC	20	50.3	45	GTATTTGAGGCTGAAGCAAG	20	50.4	45	70	(AC)7
CG30	CGTCATTTTCTGGATTCACT	20	49.3	40	ATCCATTGGGCTGTCCAT	18	50.7	50	158	(GT)9
CG31	CAGGATATATTGGACCATGC	20	49	45	CTACTTTTACCGCACACACA	20	51.8	45	158	(TG)10
CG32	TTGTTAGCATCGTGAGTCAG	20	51.3	45	GCAGTTGATTGAGCAGTACA	20	51.8	45	213	(AG)10
CG33	GGCATCTATGGACTAGCAGA	20	51.8	50	TCATACACCAAAGCTTCCTC	20	50.7	45	233	(GC)6
CG34	GGGACTCTCTCTCTTTTCGT	20	52	50	GTGGTGGAAAATCTGTCCTA	20	50.3	45	225	(TA)6
CG35	TAAGTCGGGTAATGAATGGT	20	49.3	40	TGGTGCTCTTCTTACCTACC	20	51.9	50	250	(GA)6
CG36	CCACTCAATTCAATGACAGA	20	48.9	40	TGAGAGAGTTGTGTCCATCA	20	51.4	45	227	(AC)7
CG37	TTATATGCCCCATACTCACC	20	49.5	45	GGGTCATCTTACACCGTTAC	20	51.3	50	234	(CA)8
CG38	TTCTCTTGGAGCTAGACGAC	20	52.1	50	AGTCATTGACGTGTATGTGC	20	51.9	45	224	(CT)6
CG39	ATAAATCAGGTCGTCTGCAT	20	50.2	40	TAAGAGTGGAAAAGAGCCAA	20	49.8	40	226	(GT)7
CG40	GTCTTGACGTTGGGAAAAT	19	48.9	42.1	TTGAACAGAGCATTATGACG	20	49.5	40	242	(CA)8
CG41	ACGATTGAGTTCTGAAAGGA	20	50	40	AATGTACTCCGTTCCGCC	18	52.2	55.6	96	(GA)6
CG42	GACTGACGGTGTTGTTCC	18	50.9	55.6	GACTTGGAGTGAAGGGAGAT	20	51.8	50	237	(TC)7
CG43	AGTTGCTGTAGAACCACCAC	20	53.4	50	GAGACCGAGACGTTGAGAG	19	52.5	57.9	131	(GA)6
CG44	TCCATCGTCATATTTCCTTC	20	48.2	40	TTCATGCGTTAGTCAGTTTG	20	50	40	188	(TC)7
CG45	GGCACCGATAAGATTTTGTA	20	49.1	40	ACTGGGTCTAACTCGAAACA	20	51.7	45	158	(CG)6
CG46	AGAGACTCAACAGGCATTGT	20	52.4	45	CTAATCACGAGACCCAACAT	20	50.6	45	242	(TC)6
CG47	GGAGTCGAGCACACTACTAGA	21	54.3	52.4	CGAATCATCGATAGGCTTAC	20	49.3	45	228	(GA)6
CG48	TGATGGAGACGTACACTTGA	20	51.6	45	GACACCATGCAGAGAAACTT	20	51.6	45	179	(GA)7
CG49	AACTGTATCCACCAGAGCC	19	51.7	52.6	TAACTCATCATCGACAGCAG	20	50.7	45	149	(GC)6
CG50	AACCACTCCAACAACCAC	18	50	50	CAAGTACTCCTGAAACCCAG	20	51.1	50	136	(AG)6

[a] CG12, CG24, CD38 and CG 41 did not amplify. [b] Tm = Melting temperature

Appendix Table 7.1. Isolates of *C. gloeosporioides* used for genetic diversity analysis and their geographic origin.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR001	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR002	Central Ethiopia	Kebena	*Citrus sinensis*	Fruit	22-Mar-13	8.279 N 37.790 E
ETHCTR003	Central Ethiopia	Geta	*C. sinensis*	Fruit	26-May-14	7.876 N 38.034 E
ETHCTR004	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	27-May-14	8.268 N 37.741 E
ETHCTR006	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	25-May-14	7.035 N 37.914 E
ETHCTR007	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	27-May-14	8.285 N 37.661 E
ETHCTR008	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR009	Southwest Ethiopia	Mana	*C. sinensis*	Leaf	19-Mar-13	7.756 N 36.776 E
ETHCTR012	Southwest Ethiopia	Shebe Senbo	*C. sinensis*	Leaf	20-Mar-13	7.506 N 36.514 E
ETHCTR013	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	22-Mar-13	8.268 N 37.741 E
ETHCTR014	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	27-May-14	8.268 N 37.741 E
ETHCTR016	Central Ethiopia	Wolliso	*C. aurantium*	Leaf	28-May-14	8.533 N 37.967 E
ETHCTR017	Central Ethiopia	Wolliso	*C. aurantium*	Fruit	28-May-14	8.533 N 37.967 E
ETHCTR018	Central Ethiopia	Wolliso	*C. sinensis*	Leaf	28-May-14	8.533 N 37.967 E
ETHCTR019	Central Ethiopia	Wolliso	*C. aurantium*	Leaf	28-May-14	8.533 N 37.967 E
ETHCTR020	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	27-May-14	8.285 N 37.661 E
ETHCTR021	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	25-May-14	7.035 N 37.914 E
ETHCTR022	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	25-May-14	7.035 N 37.914 E
ETHCTR023	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	25-May-14	7.043 N 37.908 E
ETHCTR024	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.170 N 37.868 E
ETHCTR025	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.170 N 37.868 E
ETHCTR026	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.182 N 37.809 E
ETHCTR027	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.182 N 37.809 E
ETHCTR028	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.182 N 37.809 E

Appendix Table 7.1. Continued.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR029	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.182 N 37.809 E
ETHCTR031	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	25-May-14	7.043 N 37.908 E
ETHCTR032	South Ethiopia	Boloso Sore	*C. sinensis*	Leaf	25-May-14	7.096 N 37.709 E
ETHCTR033	South Ethiopia	Boloso Sore	*C. sinensis*	Fruit	25-May-14	7.096 N 37.709 E
ETHCTR034	South Ethiopia	Boloso Sore	*C. sinensis*	Fruit	25-May-14	7.096 N 37.709 E
ETHCTR037	Central Ethiopia	Sekoru	*C. sinensis*	Fruit	19-Jun-12	8.248 N 37.540 E
ETHCTR038	Central Ethiopia	Sekoru	*C. reticulata*	Leaf	19-Jun-12	8.248 N 37.540 E
ETHCTR039	South Ethiopia	Abaya	*C. sinensis*	Leaf	13-Feb-13	6.417 N 38.308 E
ETHCTR041	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR042	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR043	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR044	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR045	Southwest Ethiopia	Mana	*C. sinensis*	Leaf	21-Mar-13	7.756 N 36.776 E
ETHCTR046	Southwest Ethiopia	Mana	*C. sinensis*	Fruit	21-Mar-13	7.756 N 36.776 E
ETHCTR047	Central Ethiopia	Gorro	*C. sinensis*	Leaf	22-Mar-13	8.403 N 37.870 E
ETHCTR048	South Ethiopia	Abaya	*C. aurantium*	Leaf	13-Feb-13	6.417 N 38.308 E
ETHCTR049	Southwest Ethiopia	Gomma	*C. sinensis*	Leaf	21-Mar-13	7.850 N 36.583 E
ETHCTR050	South Ethiopia	Abaya	*C. aurantium*	Leaf	13-Feb-13	6.433 N 37.883 E
ETHCTR051	South Ethiopia	Abaya	*C. sinensis*	Fruit	13-Feb-13	6.417 N 38.308 E
ETHCTR052	Southwest Ethiopia	Gomma	*C. sinensis*	Leaf	21-Mar-13	7.850 N 36.583 E
ETHCTR053	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	22-Mar-13	8.266 N 37.725 E
ETHCTR054	Southwest Ethiopia	Gomma	*C. sinensis*	Fruit	21-Mar-13	7.850 N 36.583 E
ETHCTR055	Southwest Ethiopia	Mana	*C. sinensis*	Leaf	21-Mar-13	7.756 N 36.776 E
ETHCTR056	South Ethiopia	Abaya	*C. sinensis*	Leaf	13-Feb-13	6.417 N 38.308 E

Appendix Table 7.1. Continued.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR057	South Ethiopia	Abaya	*C. sinensis*	Leaf	13-Feb-13	6.417 N 38.308 E
ETHCTR058	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.266 N 37.725 E
ETHCTR059	Northwest Ethiopia	Guangua	*C. sinensis*	Leaf	26-May-13	10.950 N 36.500 E
ETHCTR060	Northwest Ethiopia	Guangua	*C. sinensis*	Leaf	26-May-13	10.950 N 36.500 E
ETHCTR061	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR062	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR063	South Ethiopia	Abaya	*C. sinensis*	Leaf	13-Feb-13	6.417 N 38.308 E
ETHCTR064	South Ethiopia	Abaya	*C. sinensis*	Fruit	13-Feb-13	6.417 N 38.308 E
ETHCTR065	Central Ethiopia	Kebena	*C. sinensis*	Fruit	22-Mar-13	8.279 N 37.790 E
ETHCTR066	Central Ethiopia	Kebena	*C. sinensis*	Fruit	22-Mar-13	8.279 N 37.790 E
ETHCTR067	Southwest Ethiopia	Debre Werk	*C. sinensis*	Fruit	19-Mar-13	6.904 N 35.593 E
ETHCTR068	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR069	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR070	Central Ethiopia	Kebena	*C. sinensis*	Leaf	22-Mar-13	8.279 N 37.790 E
ETHCTR071	Central Ethiopia	Kebena	*C. sinensis*	Leaf	22-Mar-13	8.279 N 37.790 E
ETHCTR072	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR073	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR074	South Ethiopia	Aleta Wendo	*C. sinensis*	Leaf	14-Feb-13	6.600 N 38.417 E
ETHCTR075	South Ethiopia	Aleta Wendo	*C. sinensis*	Leaf	14-Feb-13	6.600 N 38.417 E
ETHCTR077	Northwest Ethiopia	Guangua	*C. sinensis*	Leaf	26-May-13	10.950 N 36.500 E
ETHCTR078	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	27-May-14	8.280 N 37.692 E
ETHCTR081	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.266 N 37.725 E
ETHCTR082	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.266 N 37.725 E
ETHCTR084	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E

Appendix Table 7.1. Continued.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR085	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR086	Southwest Ethiopia	Shebe Senbo	*C. sinensis*	Leaf	20-Mar-13	7.506 N 36.514 E
ETHCTR088	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR089	Southwest Ethiopia	Mana	*C. sinensis*	Fruit	21-Mar-13	7.756 N 36.776 E
ETHCTR090	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR091	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR092	Northwest Ethiopia	Guangua	*C. sinensis*	Fruit	26-May-13	10.950 N 36.500 E
ETHCTR093	Northwest Ethiopia	Guangua	*C. sinensis*	Fruit	26-May-13	10.950 N 36.500 E
ETHCTR094	South Ethiopia	Abaya	*C. sinensis*	Leaf	13-Feb-13	6.417 N 38.308 E
ETHCTR095	Northwest Ethiopia	Guangua	*C. sinensis*	Leaf	26-May-13	10.950 N 36.500 E
ETHCTR096	Southwest Ethiopia	Shebe Senbo	*C. sinensis*	Leaf	20-Mar-13	7.506 N 36.514 E
ETHCTR097	South Ethiopia	Abaya	*C. sinensis*	Fruit	13-Feb-13	6.417 N 38.308 E
ETHCTR098	South Ethiopia	Boloso Sore	*C. sinensis*	Leaf	26-May-14	7.096 N 37.709 E
ETHCTR101	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	20-Jun-12	8.283 N 37.783 E
ETHCTR103	South Ethiopia	Abaya	*C. aurantium*	Leaf	13-Feb-13	6.433 N 37.883 E
ETHCTR104	Southwest Ethiopia	Gomma	*C. sinensis*	Leaf	21-Mar-13	7.850 N 36.583 E
ETHCTR105	Southwest Ethiopia	Gomma	*C. sinensis*	Leaf	21-Mar-13	7.850 N 36.583 E
ETHCTR106	Southwest Ethiopia	Gomma	*C. sinensis*	Leaf	21-Mar-13	7.850 N 36.583 E
ETHCTR107	Southwest Ethiopia	Gomma	*C. sinensis*	Fruit	21-Mar-13	7.850 N 36.583 E
ETHCTR108	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR109	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR110	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR111	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR113	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	20-Jun-12	8.283 N 37.783 E

Appendix Table 7.1. Continued.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR115	South Ethiopia	Aleta Wendo	*C. sinensis*	Leaf	14-Feb-13	6.600 N 38.417 E
ETHCTR116	South Ethiopia	Aleta Wendo	*C. sinensis*	Leaf	14-Feb-13	6.600 N 38.417 E
ETHCTR117	Southwest Ethiopia	Mana	*C. sinensis*	Leaf	21-Mar-13	7.756 N 36.776 E
ETHCTR118	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR119	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR120	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR121	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR122	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR123	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR124	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR127	Southwest Ethiopia	Shebe Senbo	*C. sinensis*	Leaf	20-Mar-13	7.506 N 36.514 E
ETHCTR128	Southwest Ethiopia	Shebe Senbo	*C. sinensis*	Leaf	20-Mar-13	7.506 N 36.514 E
ETHCTR129	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR130	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR131	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR132	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.280 N 37.692 E
ETHCTR133	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.280 N 37.692 E
ETHCTR134	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	22-Mar-13	8.266 N 37.725 E
ETHCTR136	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	22-Mar-13	8.266 N 37.725 E
ETHCTR137	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.266 N 37.725 E
ETHCTR138	Central Ethiopia	Abeshege	*C. sinensis*	Fruit	22-Mar-13	8.266 N 37.725 E
ETHCTR140	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR141	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR142	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E

Appendix Table 7.1. Continued.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR143	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR148	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	22-Mar-13	8.266 N 37.725 E
ETHCTR151	Southwest Ethiopia	Debre Werk	*C. sinensis*	Fruit	19-Mar-13	6.904 N 35.593 E
ETHCTR152	Southwest Ethiopia	Debre Werk	*C. sinensis*	Fruit	19-Mar-13	6.904 N 35.593 E
ETHCTR153	Southwest Ethiopia	Debre Werk	*C. sinensis*	Fruit	19-Mar-13	6.904 N 35.593 E
ETHCTR154	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR156	South Ethiopia	Abaya	*C. sinensis*	Fruit	13-Feb-13	6.417 N 38.308 E
ETHCTR157	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR158	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR159	Southwest Ethiopia	Debre Werk	*C. sinensis*	Leaf	19-Mar-13	6.904 N 35.593 E
ETHCTR160	Southwest Ethiopia	Gomma	*C. sinensis*	Leaf	21-Mar-13	7.850 N 36.583 E
ETHCTR161	Northwest Ethiopia	Guangua	*C. sinensis*	Leaf	26-May-13	10.950 N 36.500 E
ETHCTR162	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR163	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR164	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR165	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR166	South Ethiopia	Boloso Sore	*C. sinensis*	Fruit	26-May-14	7.096 N 37.709 E
ETHCTR167	South Ethiopia	Boloso Sore	*C. sinensis*	Fruit	26-May-14	7.096 N 37.709 E
ETHCTR169	South Ethiopia	Boloso Sore	*C. sinensis*	Fruit	26-May-14	7.096 N 37.709 E
ETHCTR170	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR172	Southwest Ethiopia	Ginbo	*C. sinensis*	Leaf	20-Mar-13	7.333 N 36.167 E
ETHCTR173	Southwest Ethiopia	Mana	*C. sinensis*	Leaf	21-Mar-13	7.756 N 36.776 E
ETHCTR174	Southwest Ethiopia	Mana	*C. sinensis*	Leaf	21-Mar-13	7.756 N 36.776 E
ETHCTR175	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Leaf	27-May-13	10.700 N 37.267 E

Appendix Table 7.1. Continued.

Isolate Code	Geographic Region	District	Host	Plant Part	Date of Collection	Latitude-Longitude
ETHCTR178	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR179	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR180	Northwest Ethiopia	Jabitehnan	*C. reticulata*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR181	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR182	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR183	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR184	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR185	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR186	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR187	Central Ethiopia	Cheha	*C. sinensis*	Leaf	26-May-14	8.182 N 37.809 E
ETHCTR188	Central Ethiopia	Geta	*C. sinensis*	Fruit	26-May-14	7.876 N 38.034 E
ETHCTR189	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	26-May-14	7.043 N 37.908 E
ETHCTR190	South Ethiopia	Damot Pulasa	*C. sinensis*	Leaf	26-May-14	7.043 N 37.908 E
ETHCTR192	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Leaf	27-May-13	10.700 N 37.267 E
ETHCTR193	Northwest Ethiopia	Jabitehnan	*C. sinensis*	Fruit	27-May-13	10.700 N 37.267 E
ETHCTR194	Central Ethiopia	Abeshege	*C. sinensis*	Leaf	27-May-14	8.285 N 37.661 E
ETHCTR197	Central Ethiopia	Wolliso	*C. aurantium*	Leaf	28-May-14	8.533 N 37.967 E
ETHCTR198	Central Ethiopia	Wolliso	*C. aurantium*	Fruit	28-May-14	8.533 N 37.967 E