Title Molecular genetic characterization of fungal isolates representing biogeographic diversity in the colletotrichum-bean pathosystem

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Molecular Genetic Characterization of Fungal Isolates Representing Biogeographic Diversity in the *Colletotrichum*-Bean Pathosystem

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2013

ABSTRACT

Colletotrichum lindemuthianum is a pathogen of *Phaseolus vulgaris* (common bean) causing anthracnose disease and poses a threat to food security. The aim of the study was to advance understanding of genotype-phenotype-environmental interactions in *Colletotrichum spp.* through biomolecular approaches including multilocus molecular phylogenetic analysis, AP-PCR and morphological diversity assessment. Following initial screening five loci were selected for further investigation including ribosomal RNA gene block internal transcribed spacer (ITS), tubulin (TUB), glyceraldehyde phosphate dehydrogenase, glutamine synthetase, and the mating type gene. Study included 18 *Colletotrichum* isolates representing wide biogeographic diversity. Two isolates were identified as *C. gloeosporioides* and *C. truncatum*, which are not commonly known bean pathogens and this needs further research. The TUB marker was the most conserved amongst the *C. lindemuthianum* isolates. Universal marker ITS distinguished 5 haplotypes; concatenated sequence data provided the highest resolution with 7 haplotypes. AP-PCR differentiated between 5-9 haplotypes and

appeared more suitable for local population monitoring purposes. Variability in growth rate, sporulation and colony morphology was observed among the *Colletotrichum* spp. isolates. The study would serve as a platform for genome sequencing based studies into environmental change adaptation in *Colletotrichum* spp. particularly *C. lindemuthianum* using isolates representing historical and contemporary populations.

ACKNOWLEDGEMENTS

I would like to thank my Dos Prof S. Sreenivasaprasad for sharing his invaluable experience and knowledge with me. His perceptive guidance, support and patience enabled me to carry out this research. I can only aspire to live up to his standards. I also wanted to thank Riccardo Baroncelli for his help with bioinformatics analysis, technical stuff at University of Bedfordshire and Warwick HRI, Wellesbourne at University of Warwick, UK and Professors Sreenivasaprasad and Eric Holub for supplying the *Colletotrichum* isolates used in this study.

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LIST OF ABBREVIATIONS

µl-Microlitre

µm-Micrometer

µM-Micromolar

ABI-Applied Biosystems

ACTF- Actin Forward Primer

ACTR- Actin Reverse Primer

ACT-Actin

AFLP-Amplified Fragment Length Polymorphism Analysis

AM- Mycorrhizae arbuscular

AMS-Accelerator Mass Spectrometry

AMT-*Agrobacterium*-mediated transformation

AP-PCR-Arbitrary-primed PCR

ATCC- American Type Culture Collection

ATP- Adenosine Triphosphate

B.P. -Before Present set at 1950

BLAST- Basic Local Alignment Search Tool

bp-Base Pairs

CABI-Centre for Agriculture and Biosciences International

CAL-Calmodulin-1

CHS-Chitin Synthase-1

CHSF- Chitin Synthase-1 Forward Primer

CHSR- Chitin Synthase-1 Reverse Primer

CIAT- International Center for Tropical Agriculture

CL1-Calmodulin Forward Primer

CL2-Calmodulin Reverse Primer

ddNTPs -2', 3'-Dideoxy Derivatives

dNTPs-Deoxynucleotides

e.g.- *exempli gratia,* for example

EcM- Mycorrhizae Ectomycorrhizal

EDTA- Ethylenediaminetetraacetic acid

ER- Endoplasmic Reticulum

et al.- *et alii,* and others

Et Br-Ethidium Bromide

f.sp.- *formae speciales*

FAO- Food and Agriculture Organization of the United Nations

GAPDH-Glyceraldehyde-3-Phosphate Dehydrogenase

Gb-Gigabases

GBIF- Global Biodiversity Information Facility

GDF- Glyceraldehyde-3-Phosphate Dehydrogenase Forward Primer

GDR- Glyceraldehyde-3-Phosphate Reverse Primer

GFG-Gene-for-Gene

GFP- Green Fluorescent Protein

GS-Glutamine Synthase

GSF- Glutamine Synthase Forward Primer

GSR- Glutamine Synthetase Reverse Primer

GTP- Guanosine Triphosphate

HGP-Human Genome Project

His3- Histone 3

HIS3F- Histone 3 Forward Primer

HIS3R- Histone 3 Reverse Primer

HMG- High Mobility Group Box

HMGCLF-Specific MAT1-2-1 Forward Primer

HMGCLR-Specific MAT1-2-1 Reverse Primer

HMGDF-Degenerate MAT1-2-1 Forward Primer

HMGDR-Degenerate MAT1-2-1 Reverse Primer

HT-Haplotype

IPCC-Intergovernmental Panel on Climate Change

ITS1-ITS Forward Primer

ITS4-ITS Reverse Primer

ITS-Internal Transcribed Spacer

LRR- Leucine Rich Repeat

LSU-Large Subunit

MAPK-Mitogen-Activated Protein Kinase

MAT1-1-Idiomorph of Mating Type Gene

MAT1-2-1-Idiomorph of Mating Type Gene-part of HMG box

MAT1-Mating Type Gene

Mb-Megabases

ml- Millilitre

mln-million

m-meter

mm-millimeter

MSA-Multiple sequence alignment

MWM-Molecular Weight Marker

N50-the length of a contig for which the cumulative number of contings of the same size represents 50% of total genome size

NBS-LRR-Nucleotide-Binding Site-Leucine Rich Repeat

NCBI- National Center for Biotechnology Information

ng-nanograms

NGS-Next-Generation Sequencing

nm-Nanometer

No.-Number

PCA-Principle Component Analysis

PCR-Polymerase Chain Reaction

PDA-Potato dextrose agar

PDB-Potato dextrose broth

PL-Endo-Pectin Lyase

ProMed-Program for Monitoring Emerging Diseases

R/S-Resistance/Susceptibility

RAPD-Rapid Amplified Polymorphic DNA

rcf- Relative Centrifugal Force

RFLP-Restriction Fragment Length Polymorphism

RNAi -RNA Interference

rpm- Rotations Per Minute

SAR-Systemic Acquired Induced Resistance

SNPs-Single Nucleotide Polymorphisms

sp.-*specie*

spp.-*species*

SSRs-Simple Sequence Repeats

SSU-Small Subunit

STRs-Short Tandem Repeats

TEF-Translation Elongation Factor 1 Alpha Subunit

TIGR-Venter from the Institute for Genomic Research

TUB5- β-Tubulin Forward Primer

TUB6- β-Tubulin Reverse Primer

TUB-β-Tubulin

UV-Ultra Violet

VNTRs-Variable Number Tandem Repeats

V-Volts

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CHAPTER 1: INTRODUCTION

1.1. **Hypothesis/Aim of the Research**

Hypothesis of the proposed research is that environmental changes influence adaptive evolution reflected by the relationship between the DNA sequence variation and the biogeographic diversity of the *Colletotrichum* isolates. The aim is to generate new knowledge and resources to advance understanding of genotype-phenotype-environmental interactions in *Colletotrichum* spp.

1.1.1. Objectives

1) Identify markers suitable for multilocus genotyping of *Colletotrichum lindemuthianum* isolates.

2) Multilocus phylogenetic analysis of a set of *Colletotrichum* species isolates displaying biogeographic diversity.

3) Comparative analysis of multilocus phylogenetics and amplified fragment length polymorphism (AP-PCR) approaches.

4) Gain an understanding of the differences in the growth, morphology and sporulation of the *Colletotrichum* spp. isolates.

1.2. Fungal Diversity

Fungi are diverse, heterotrophic eukaryotic organisms that play very important ecological and economical roles. Saprophytes use dead and decaying matter as a source of nutrition making them one of the most potent natural recyclers in the world. Parasitic fungi attack plants, humans and animals and even bacteria. They not only are a threat to immunocompromised people causing infections in hospital environment, but also leading to massive crop losses every year. Recent reports state that fungi affecting maize, rice and wheat alone costs global economy

$60mln, while 125 mln tonnes of five top food sources including the above plus soybean and potatoes are damaged every year (Fisher *et al*., 2012).

Previous studies claimed there are 1.5mln of fungal species on Earth (Hawksworth, 1991). The main reason for underestimation of the number of organisms was the existence of 'cryptic species', that came from homogenous groups having virtually same morphology and physiology; however, they may differ greatly at molecular level (Hibbett and Donoghue, 1996). The latest technology cantered around molecular techniques and high-throughput DNA sequencing aka Next-Generation Sequencing (NGS) allowed rapid discoveries of new organisms. More recently, ~97,330 species of fungi have been described (Kirk *et al*., 2008) while the new estimate of the overall number of existing species is 5.1mln (Blackwell, 2011). Novel molecular methods have led to vast improvement of fungal classification providing knowledge about their genetic diversity and evolutionary relationships including in the genus *Colletotrichum* (Riccardo Baroncelli *et al*, Unpublished).

There are many types of studies regarding the species concept as outlined by Endler (1989) including amongst others taxonomy and evolutionary type of studies. The modern concept of species considers its morphological, biological, ecological and phylogenetic characteristics. The latest approach to phylogenetics is Genealogical Concordance Phylogenetic Species Recognition that entails bioinformatic analysis (Taylor *et al.*, 2000) and it became a leading method for fungal systems (Giraud *et al*., 2008). Speciation is a process where one species is divided into two or more new ones as a part of on-going evolutionary development, adaptation and a source of biodiversity (Cracraft, 1983). Speciation is usually considered in allopatric terms where two groups undergo genetic drift due to the geographic barrier (Mayr, 1963).

Cryptic species, defined as one or more species described as a single species, have posed problems in taxonomy for the past centuries. However, current technology including molecular phylogenetics utilizing DNA sequence comparison can differentiate morphologically and physiologically identical entities (Bickford *et al*., 2007).

Due to those advances *Colletotrichum* phylogenetics have evolved in recent years. The name of the species complex would generally refer to the originally identified species e.g. *boninense*. There are 9 major species complexes or clades within the *Colletotrichum* genus (Cannon *et al*., 2012).

1.3. *Colletotrichum* Genus

1.3.1. *Ascomycota - Sordariomycetes*

Colletotrichum genus belongs to *Ascomycota - Sordariomycetes* and includes endophytes, pathogens, mycoparasites and saprobes (Zhang *et al*., 2006). One of the *Sordariomycetes* is *Fusarium* genus containing many economically important fungi including *Fusarium graminearum* –a causative agent of head blight affecting cereal particularly barley and wheat (Goswami and Kistler, 2004). *Neurospora* (order *Sordariales*) contains *N. crassa*- a saprotrophic fungus that became a model organism equivalent to *Drosophila*. Its haploid life cycle allowed to carry out many genetic studies including discovery of gene silencing mechanism (Davis and Perkins, 2002).

1.3.2. Biological and Pathological Diversity

Colletotrichum is a mainly asexual genus with the sexual morph referred to as *Glomerella*. There is still a lot of confusion regarding taxonomy of the *Colletotrichum* genus (Cannon *et al*., 2000; Hyde *et al*., 2009). However, most of these issues were addressed by current *Colletotrichum* research (Cannon *et al*., 2012). *Colletotrichum* species are ubiquitous endophytes meaning they can invade their plant host without causing an apparent disease at some stage in their life cycle. (Redman *et al*., 2001). *Colletotrichum* spp. affect many crop plants and ornamentals in the world including legumes, grasses (sorghum), yucca, coffee beans, cereals (e.g. maize), sugar cane, and many fruits and vegetables (Broad Institute, 2010). More pressures are imposed on farmers from tropical and subtropical countries (Tu, 1992a).

Colletotrichum species are a causative agent of anthracnose spots and blight on a wide range of plant hosts (causing chlorosis where lack of chlorophyll lead to browning plant tissue and necrosis) as well as few other major diseases specific to the host including: red rot of sugar cane infected with *C. falcatum* Went, coffee berry disease caused by *C. kahawae* (Fig 1.1.), and brown blotch of cowpea by *C. truncatum* (Fig 1.2.) (Lenné 2002; Dean *et al*. 2013). *C. acutatum* is a causative agent of root rot/necrosis on strawberry (Mertely and Peres, 2005), while *C. gloeosporioides* and *C. fragariae* cause crown rot of strawberries (Peres and MacKenzie, 2007). Avocado and almond are affected by *C. gloeosporioides* (Penzig) Penzig et Sacc where the avocado is associated with postharvest fruit rot while the latter becomes apparent in young fruit (Prusky and Keen, 1993; Striem *et al*., 1989). Postbloom fruit drop of citrus is caused by *C. acutatum*, while *C. gloeosporioides* causes postharvest anthracnose of the same fruit (Zulfiqar *et al*., 1996). Mango anthracnose is mainly caused by *C. gloeosporioides* (Jeffries *et al*., 1990; Prusky and Keen, 1993) and few minor pathogens including *C. asianum* (Lima *et al*., 2013). *C. lagenarium* is a causative agent of anthracnose fruit rot affecting watermelon, muskmelon, cantaloupe, cucumber and more (Prusky, 1996).

Research by Redman *et al*. (1999) showed that a single gene disruption is able to transform the pathogenic *Glomerella magna* affecting *Citrullus lanatus* into non-pathogenic strain. They were trying to establish why some pathogenic *Colletotrichum* fungi can also express mutualism and commensalism providing the benefits for host plant including: biotic and abiotic stress tolerance, and enhanced growth (Redman *et al*., 2001). Researchers concluded that this type of interactions are dependent on plant's genotype (Rodriguez and Redman, 2008). *Colletotrichum* spp. are mainly pathogenic, however, there are examples of mutualism when exposed to non-disease hosts e.g. *C. gloeosporioides* pathogenic to strawberry provided drought resistance to its non-disease host watermelon (Redman *et al*., 2001).

Many species from this genus also proved to be excellent models for studies surrounding e.g. fungal-plant interactions, nutrition, and host resistance (Tu, 1992b; Talhinhas and Sreenivasaprasad, 2005; Perfect *et al*., 1999).

Fig 1.1. Green coffee berry affected by *C. kahawee* (Silva *et al*., 2006)

Fig 1.2. Brown blotch on soybeans caused by *C. truncatum* (Yorinori J. T., EcoPort, available at: www.ecoport.org accessed)

C. lindemuthianum was a break-through organism when the definition of host specificity and race were recognised (Barrus, 1911). *Colletotrichum* affecting

beans served as model organisms for research on phytoalexins-antimicrobial chemicals (Kuc, 1972).

There are still a lot of unanswered questions surrounding shift between biotrophy and necrotrophy in *Colletotrichum* spp.; however, recent advances in genomics research is expected to address them.

1.3.3. Colletotrichum- Major Clades and Clusters

Recent studies on *Colletotrichum* phylogenetics have resolved a lot of confusions regarding taxonomy and nomenclature (Cannon *et al*., 2012). Online resources like Q-bank (http://www.q-bank.eu/) solved problems regarding the application of *C. lindemuthianum* name. It also provides current information on *Colletotrichum* spp. based on multilocus phylogenetic analysis (Q-bank).

Early studies based on the ribosomal RNA gene block internal transcribed spacer (ITS) region sequence provided an understanding of the genetic diversity and phylogenetic relationships amongst various species in the *Colletotrichum* genus (e.g. Sreenivasaprasad *et al*., 1996). The DNA barcoding was first applied to *Colletotrichum* based on ITS1 sequence polymorphism allowing to differentiate between various *Colletotrichum* spp. and strains within *C. gloeosporioides* (Mills *et al*., 1992; Sreenivasaprasad *et al*., 1992). ITS is continuously used by researchers in *Colletotrichum* phylogenetics (Xie *et al*., 2010; Yang *et al*., 2011; Crouch and Tomaso-Peterson, 2012). This discovery led to fast progress of molecular phylogenetics in *Colletotrichum* genus. ITS sequence was coupled with LSU and resolved 27 strains within 13 different species (Sherriff *et al*., 1994). Further research included combined sequences ITS1 and 2 of 18 *Colletotrichum* species, which formed six phylogenetic groups non-congruent with spore morphology results (Sreenivasaprasad *et al*., 1996). This was followed by studies on *C. acutatum* that involved use of β-tubulin and histone markers (Talhinhas *et al*., 2002) as well as glyceraldehyde-phosphate dehydrogenase and glutamine synthetase (Guerber *et al*., 2003). More recent phylogenetic studies on *Colletotrichum* spp. associated with herbaceous hosts using the above as well as actin and chitin synthase-1 markers resolved 20 clades including 12 that were

formerly identified as *C. dematium* (Damm *et al*., 2009). Latest research also includes calmodulin (Yang *et al*., 2009), MAT1-2, and SOD2 markers (Crouch and Tomaso-Peterson, 2012). More information on the multilocus phylogenetic analysis is contained in section 1.4.3.

Recent phylogenetic studies of *Colletotrichum* species carried out by Cannon *et al*. (2012) revealed 9 large clades and few minor clusters with potentially separate origins (Fig 1.3.). The *acutatum*, *gloeosporioides* and *boninense* clades are the largest in the genus. *C. acutatum* clade consists of 30 species with the two most important subclades. First *C. acutatum sensu stricto* made of 21 species containing *C. floriniae* and the second one containg 9 organisms including among others *C. salicis*. *C. orchidophilum* is a separate sister taxon clade (Cannon *et al*., 2012).

C. dematium clade contains 6 species with *C. spinaciae* and *C. circinans* being most economically significant (Washington *et al*., 2006; Kim *et al.*, 2008).

The *C. destructivum* complex entails few economically important species: *C. higginsianum*, *C. fascum* and *C. destructivum*. Out of the three, *C. higginsianum* appears to have highest scientific value due to genome sequencing studies as well as host-pathogen research using model plant *Arabidopsis thaliana* (O'Connell *et al.*, 2012; Kleeman *et al*., 2012). *C. destructivum* is a monopyletic taxon meaning all of the species within this clade have a common ancestor (O'Connell *et al*., 2012).

C. gloeosporioides clade includes 22 species with two principal subclades *C. kahawee* and *C. musae* (Weir *et al*., 2012). As with many other taxons within *Colletotrichum* genus, subclades are not well differentiated based only on ITS sequence, and further multilocus analysis is required (Cannon *et al*., 2012). *C. boninense* is a sister taxon of *C. gloeosporioides* and contains 17 species.

C. graminicola taxon consists of 13 species with 2 subclades: *C. graminicola* and *C. cereale* each represented by a single species and both being grass pathogens (Cannon *et al*., 2012).

The *orbiculare* clade is a sister taxon to all other *Colletotrichum* clades and contains *C.lindemuthianum, C. trifolii, C.malvarum* and *C. orbiculare* (Liu *et al*., 2007; Young *et al*., 2009). All members of the group are characterised by straight, short and wide conidia and small appressorium (Sutton, 1980). There was a lot of confusion in the past about the *C. lindemuthianum* classification due to high differentiation level in spore morphology. Cannon *et al*. (2000) and Mordue (1971) characterized them as long and narrow of various sizes, while Sutton (1980) reported short, wide and spherical conidia, which are universally considered typical of *orbiculare* complex (Bain and Essary, 1906). *C. lindemuthianum* is a common bean pathogen from Fabaceae (*Leguminosae*) family; however, some organisms from the *C. gloeosporioides* taxon also affect these types of plants leading to misidentifications (Cannon *et al*., 2012).

Recent study on *C. orbiculare* phylogenetics using multilocus molecular phylogenetic analysis revealed nine clades out of which four were previously known: *C. lindemuthianum, C. malvarum, C. orbiculare* and *C. trifolii*. There were four new species identified *C. bidentis, C. sidae, C. spinosum* and *C. tebeestii.* There were two clades recognized within the *C. lindemuthianum* referred to as 1 and 2, however, there was not enough evidence to split the groups into separate species due to common origins, similar morphology and host preference (Damm *et al*., 2013). There is still a lot of uncertainty regarding the orbiculare complex. However, *C. lindemuthianum* has been epitypified (Liu *et al*., 2013). The purpose of epitype is to find a representative of particular species that comply with the original characterization of an organism while fitting with modern taxonomy and nomenclature principles (Cannon *et al*., 2008).

The remaining two clades include: *spethianum* and *truncatum*- sister to *gloeosporioides* and *boninense* taxons (Cannon *et al*., 2012). Outside the clades are species that do not fit the phylogenetic tree that includes *C. coccodes*, which became more economically significant due to recent outbursts of infections on tomatoes and potatoes (Anon, 1998, cited by: Lees and Hilton, 2003).

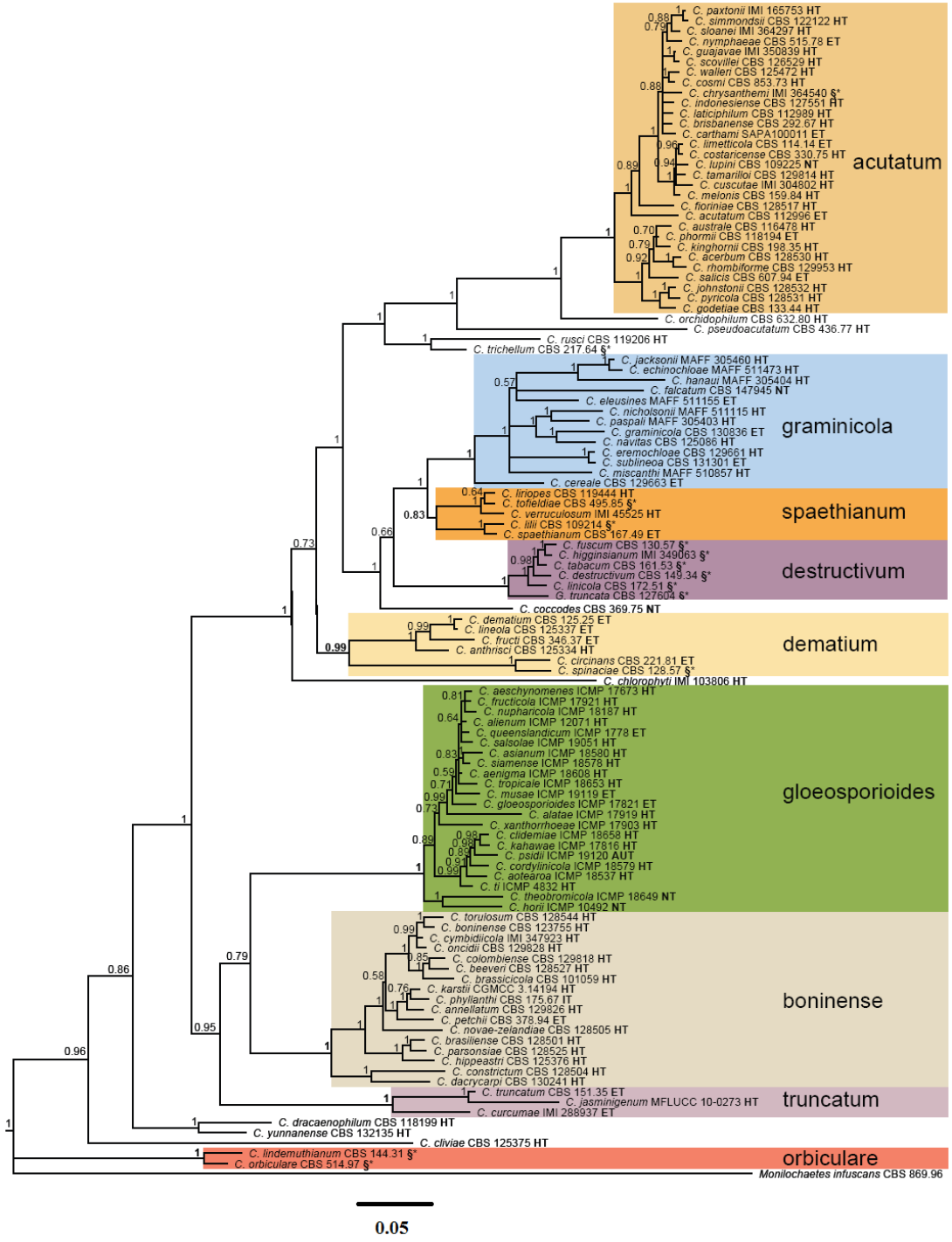


Fig 1.3. Phylogenetic Tree Illustrating the Colletotrichum Genus Based on Bayesian analysis of Concatenated Multiple Sequence Alignment of CHS-1, ACT and ITS Sequences (Cannon *et al*., 2012).

1.4. Infection Mechanisms of *Colletotrichum* Species

Fungi in general obtain their nutrients through two different modes. Biotrophs acquire their nutrients from living cells. Necrotrophic fungi kill the host plant tissue and obtain their nutrients from decaying matter (Lewis, 1973). *C. lindemuthianum* is a hemibiotroph, an organism that initially exhibits biotrophy and later switches to necrotrophy. This mode of action is common amongst various pathogens that cause anthracnose diseases (Luttrell, 1974).

All members of *Colletotrichum* genus go through similar infection pathway initially: adherence of conidia to the plant surface, germination, and formation of germ tubes that lead to development of appresoria. Intracellular hemibiotrophy is marked by swelling of infection peg giving rise to formation of infection vesicle and ultimately primary hyphae that permeate through epidermal and mesophyll cells. Colonization of living cells is asymptomatic (biotrophic stage) followed by development of thin secondary hyphae indicating necrotrophic phase where the host plant is killed (O'Connell and Bailey, 1991). Examples of hemibiotrophs include: *C. lindemuthianum* (O'Connell and Bailey, 1991), *C. graminicola* (*Zea mays:* Politis and Wheeler, 1973), *C. truncatum* (*Pisum sativum*: Uronu, 1989), and *C. orbiculare* (*Cucumis sativus*). Many *Colletotrichum* species go through biotrophy phase without any growth manifestation (Cerkauskas, 1988; Tiffany, 1951).

Subcuticular intramural pathogens like *C. capsici* on cowpea (*Vigna unguiculata*: Tu, 1992b; Pring *et al*., 1995) are characterized by development of hyphae under the cuticle within the walls of epidermal cells hence the initial growth stages remain asymptomatic (Tu, 1992b). Second stage involves development of necrothrophic secondary hyphae (Mendgen and Lesemann, 1991).

The organism that uses both modes of infection pathways: intracellular hemibiotrophy and subcuticular intramural process is *C. gloeosporioides* when colonising *Citrus* spp. (Brown, 1977) and *Stylosanthes* spp. (Ogle *et al*., 1990) depending on the available conditions.

C. lindemuthianum is a causative agent of anthracnose in common bean (*Phaseolus vulgaris* L., Fig 1.4.) grown mainly in tropical and subtropical countries (Paula Jr *et al*., 2008).

Fig 1.4. Anthracnose pod lesions on beans (*Phaseolus vulgaris*) (Biddle and McKeow, 2007)

Currently, there is not enough evidence how the fungus switches its nutritional modes of action. It appears cell wall degrading enzymes, particularly endo-pectin lyase (PL) are responsible for development of anthracnose lesions, tissue maceration and electrolyte leakage (Wijesundera, 1984; Wijesundera *et al*., 1989).

1.5. *Colletotrichum lindemuthianum*

1.5.1. Geographical Distribution

C. lindemuthianum occurs in Central and South America, Europe and Africa, South and South East Asia and Australasia in temperate and tropical climates. Most of geographical locations occupied by *C. lindemuthianum* were recorded by

CABI (Centre for Agriculture and Biosciences International) (Fig 1.5.). The countries of occurrence not recorded by CABI but included in work of Ansari *et al*. (2004) are amongst others: Bolivia, Tanzania, Argentina, Dominican Republic, Columbia, and Peru.

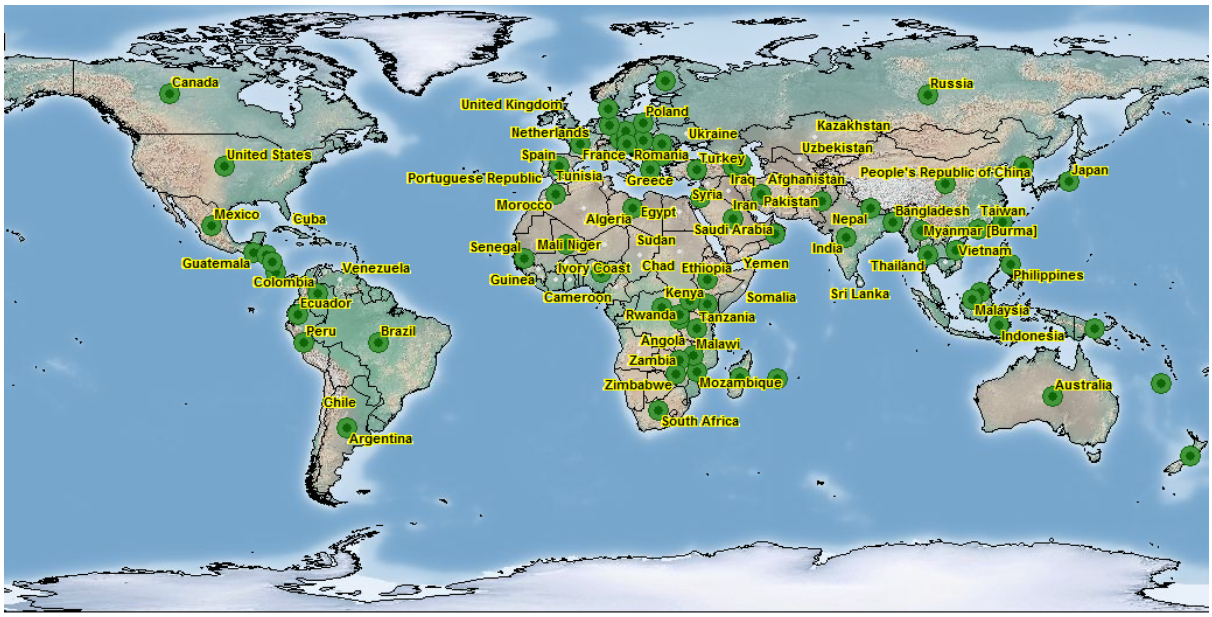


Fig 1.5. Geographical Occurrences of *C. lindemuthianum* reported by CABI (http://www.plantwise.org)

1.5.2. Pathogenic Variation

There is no clearly defined International Race Designation and a Host Differential Set for *C. lindemuthianum* – bean system. Race classification process is based on observation of virulence towards a particular set of common bean cultivars.

Differentiation of *C. lindemuthianum* races using Greek letters was first introduced by Barrus that described races alpha and beta in 1911 and 1918 respectively. This was followed by discoveries of gamma (Burkholder, 1923), delta (Andrus and Wade, 1942), epsilon (Blondet, 1962, cited by: Thomazella *et al.*, 2002), lambda (Hubbeling, 1961; 1974, cited by: Thomazella *et al*., 2002) and Ebnet race also designated as kappa race (Hoffman *et al*., 1974, cited by: Thomazella *et al*., 2002). The reactions of differential cultivars when exposed to specific *C. lindemuthianum* isolates were reported by Bannerot (1965, cited by:

Thomazella *et al*., 2002) and Charrier and Bannerot (1970, cited by: Thomazella *et al*., 2002) using 3 cultivars: Windusa, Dark Red Kidney and Kaboon (Fig 1.6., A). Krüger *et al*., (1977) introduced the Cornell 49-242 cultivar containing the '*Are*' resistance gene that differentiated kappa race (Fig 1.6., B). Currently, races designated with Greek letters constitute race groups as they have been further divided into races labelled with Arabic numerals using other differential cultivars e.g. Michelite, Perry Marrow (Krüger *et al*., 1977). This not only indicates inconsistent structure of race/differential set for *C. lindemuthianum* on beans but also suggests that it is a dynamic process with new race discoveries along with migration of already identified ones.

A) Differential host reaction to races of *Colletotrichum lindemuthianum*.

Bean cultivars	Host reaction of each race[1]				
	alpha	beta	gamma	delta	epsilon
Widusa	S	R	R	S	R
Dark Red Kidney	R	S	S	S	R
Kaboon	R	R	S	R	R

[1] R = resistant; S = susceptible.

B) Differential host reaction to races of *Colletotrichum lindemuthianum*.

Bean cultivars	Host reaction of each race[1]		
	delta	kappa	lambda
Dark Red Kidney	S	S	S
Kaboon	R	R	MS
Cornell 49–242	R	S	R

[1] R = resistant; S = susceptible; MS = moderately susceptible.

Fig 1.6. Dataset Produced by Krüger *et al*. (1977) Demonstrating the Race Differentiation of *C. lindemuthianum* Races Denominated with Greek Letters*

*A) Shows the results generated by Bannerot (1965, cited by: Thomazella *et al*., 2002) and Charrier and Bannerot (1970, cited by: Thomazella *et al*., 2002); B) Presents the results generated by Krüger *et al*. (1977) that differentiated kappa race using Cornell 49-242 cultivar.

The cultivar/pathogen reactions are determined using a scoring system, which often leads to misinterpretations and wrong labelling of the race/organisms (Ansari *et al*., 2004). Field isolates have to be separated and subcultured into monoconidial cultures that ensure homogeneity (Casela and Fredriksen, 1994). Subsequently, races are identified on the basis of reaction to specific variety: either susceptible or resistant. However, genetic fingerprinting methods and use of molecular markers can help in further clarification and/or validation of the race designation process.

The gene-for-gene (GFG) model first introduced by Flor (1971) claims that for each resistance gene in host plant there is a corresponding avirulence gene in the pathogen. This phenomenon driven by reciprocal selection leads to high genetic diversity especially in wild populations (Thompson and Burden, 1992; Geffroy *et al*., 1999).

Molecular studies have shed some light on the genetic basis of plant resistance, which uncovered a multialleic gene cluster (Crute and Pink, 1996). Vast majority of those genes have nucleotide-binding site-leucine rich repeat (NBS-LRR) protein structure (Hammond-Kosack and Jones, 1997).

Higher genetic diversity was observed amongst Central American races based on rapid amplified polymorphic DNA (RAPD) and restriction fragment length polymorphism (RFLP) studies. Nevertheless, RAPD (Alzate-Martin *et al*., 1999) and isoenzyme analysis (Fabre *et al*., 1995) did not point out the relationship between the country of origin and molecular diversity of the related isolates. This was further analysed using an AFLP-based approach (Ansari *et al*., 2004).

1.5.3. Phaseolus vulgaris and other Hosts of C. lindemuthianum

Despite the fact that isolates for this study were collected only from *Phaseolus vulgaris* it is important to note the other host plants affected by *C. lindemuthianum* (Table 1.1.).

Table 1.1. List of Major and Minor Host Plants Affected by *C. lindemuthianum*

Latin Name	*Common Name*	*Host Importance*	*References*
Cajanus cajan	*pigeon pea*	Major	International Agricultural Research Centres, 2014
Canavalia ensiformis	*gotani bean*	Minor	International Agricultural Research Centres, 2014
Dolichos sp.	Range of species	Minor	Lenne, 1990
Glycine max	*soyabean*	Minor	Royal Botanic Gardens, Kew, 2014
Lablab purpureus	*hyacinth bean, countrybean*	Major	Zhuang, 2001; Manjunath *et al*., 2013
Lens culinaris subsp. culinaris	*lentil*	Minor	The International Society for Molecular Plant-Microbe Interactions,1996
Lotus corniculatus	*bird's-foot trefoil*	Minor	Mulenko *et al.*, 2008
Phaseolus acutifolius	*tepary bean*		Royal Botanic Gardens, Kew, 2014
Phaseolus coccineus	*runner bean*	Minor	Mahuku *et al*., 2002
Phaseolus lunatus	*lima bean*		Balhorn, 2011
Phaseolus polyanthus	*polyanthus beans*		Mahuku *et al*., 2002
Pisum sativum	*pea*	Minor	Royal Botanic Gardens, Kew, 2014
Vicia faba	*faba bean, broad bean*	Minor	Zhuang 2005; Mohammed, 2013

Vigna mungo	*black gram*	Minor	Basandrai *et al.*, 1999
Vigna radiata	*mung bean*	Minor	Mohammed, 2013
Vigna sinensis* ssp. *sesquipedalis	*asparagus bean*	Major	Pande and Rao, 1998; Royal Botanic Gardens, Kew 2014; Mohammed, 2013
Vigna unguiculata	*cowpea*	Major	Wong and Thrower, 1978; Royal Botanic Gardens, Kew, 2014

*Information obtained from Plantwise (2014).

The major host of *C. lindemuthianum* is *P. vulgaris* (common bean) and the history of domestication and agricultural intensification of this plant has a crucial role in understanding the evolutionary processes, that in turn can relate to the evolution of *C. lindemuthianum* and development of genetic groups.

Archaeological evidence from Mexico and Peru based on radiocarbon dating shows that *Phaseolus* is around 10, 000 years old. However, the accelerator mass spectrometry (AMS) provided a different estimate. It indicates that *P. vulgaris* started to be cultivated in Mexico 2500 B.P. (Before Present set at 1950) and 4400 B.P. in Peru. Common bean is the most prevalent crop of all *Phaseolus* group members (Lynch and Kaplan, 1999; Hart *et al*., 2002). It is consumed by over half a billion people in the world predominantly in Latin America. Mendel, Johannsen and Sax discovered and demonstrated the genetics and inheritance theory using beans (Gepts, 2001). It grows rapidly at temperatures around 22-30ºC and the crop is ready for harvesting within 4-8 weeks. Common bean is mostly propagated by seeds. Highest yields are in Europe estimated for 1.5 t/ha (Brink and Belay, 2006).

1.5.4. Common Bean (Phaseolus vulgaris) Anthracnose

Bean anthracnose (Fig 1.4.) is caused by *C. lindemuthianum* (Sacc & Magn.) Br. & Cav. found ubiquitously around the world (Fig 1.5.). Disease is particularly problematic on snap and dry beans including navy beans, kidney beans and pinto

(Sherf, 1986). It appears as black spots with reddish/brown outline. In humid environment the anthracnose spots acquire pinky/creamy pigment, while in dry conditions they become brown. The spore masses are formed from conidia emerging from acervuli. Disease can be transmitted from infected plant debris and disseminated through wind currents, water splash, insects, animals, and clothing. The optimal temperature for fungal growth is 20-25ºC. Post-harvest rotting is also a common issue (Snowdon, 2010).

The first record of bean anthracnose was by Lindemuth dating at 1875 followed by more comprehensive description few years later made by Saccardo. Pathogen has the most favourable conditions in temperate climate rather than tropics, which is reflected by the crop losses. Free moisture, humidity, frequent rains, wind and cooler environment supports faster growth and spreading of *C. lindemuthianum* (Sharma, 2004).

First signs of infection appear on bottom part of the leaf and petioles (attaching leaf to the stem), which later spreads onto the upper part and onto stem, leaf veins and hypocotyl (stem under cotyledons). Stem colonisation can often weaken the stem to the point when they fall under the wind (Zaumeyer and Rex, 1958).

The perfect state of *C. lindemuthianum* is known as *G. lindemuthianum*. The disease is both seed-borne and soil-borne. Use of seeds free of contamination, crop rotation, spraying, avoidance of contact with wet plants and use of anthracnose resistant cultivars are amongst the most commonly used practices against the disease. Fungicides have proven to be ineffective (Schwartz and Hall, 2005).

Future prospects involve wide use of molecular markers, cloning and transformation techniques along with high density linkage mapping in order to improve the germplasm of common bean and help with the improvement of existing anthracnose resistant cultivars (Kole, 2007).

1.6. Molecular Characterization of *Colletotrichum* spp. and Adaptive Markers

1.6.1. Molecular Markers Related to Adaptation

Microsatellites also known as simple sequence repeats (SSRs), short tandem repeats (STRs), and variable number tandem repeats (VNTRs) are repetitive stretches of DNA variable in number between individuals making them useful markers for genetic fingerprinting/barcoding. Microsatellites can also indirectly indicate the SNPs (Single Nucleotide Polymorphisms) density (Griffiths *et al*., 2008a).

Another method used for genetic barcoding is amplified fragment length polymorphism (AFLP) technology which is considered superior to microsatellite approach for genetic barcoding. AFLP provided much higher resolution and reproducibility than microsatellites especially when using large number of isolates (Vos *et al*., 1995).

Arbitrary-primed PCR (AP-PCR) is a technique for the detection of AFLPs. AP-PCR can be used to illustrate the relationships between organisms and support sequencing data in taxonomic and phylogenetic studies. It has an advantage of rapid data generation for a large number of isolates (Caetano-Anolles, 1993).

SNPs proved very useful in the identification of adaptive divergence of closely related populations and species (Renaut *et al*., 2010). SNP is a sequence variation between closely related species/isolates within their genome. SNPs usually occur in parts of non-coding DNA and constitute around 1 % of whole genome for common and 0.5 % for rarer ones. The SNP density relates to level of genetic recombination and mutation as an adaptive response to environmental factors (Dale *et al*., 2008). SNPs may help to locate the genes under positive selection as it was demonstrated on *Picea glauca* (white spruce). Sequencing-based approaches including Next Generation Sequencing enables the discovery of SNPs on a large scale (Pavy *et al*., 2006).

1.6.2. Molecular Approaches to Phylogenetics and Value of Multilocus Markers

In fungal molecular phylogenetic studies based on DNA sequences, the term homology is commonly and routinely used in describing and discussing the relatedness of various isolates belonging to the same or different species. The term homology, in this context is widely used in the literature to provide a quantitative estimate of the level of DNA sequence similarity between two or more isolates (Damm *et al*., 2012a; Guerber *et al*. 2003)

The ITS sequence analysis can be very useful in the preliminary identification of *Colletotrichum* species (Sreenivasaprasad *et al*., 1996). Unfortunately, there is a lot of misinterpreted data deposited in the GenBank with sequences given a wrong species name (Cai *et al*., 2009). ITS is highly conserved and therefore cannot provide enough resolution that would differentiate between taxa and multilocus analysis has proven to be much more effective (Crouch *et al*., 2009). Despite that, ITS has been pointed out as a universal marker, mainly due to the amount of ITS sequence data available in open access databases (Schoch *et al*., 2011).

There are other popular diagnostic markers used depending on the fungal species, e.g. translation elongation factor 1alpha subunit (TEF) gene has been used successfully with *Fusarium* genus (Mulè *et al*., 2004), while beta-tubulin (TUB2) and calmodulin (CAL) have been applied well with *Aspergillus* and *Penicillium* (Samson *et al*., 2007; Peterson, 2008; Houbraken *et al*., 2011). In terms of *Colletotrichum*, analysis based purely on ITS sequence data is useful in resolving major clades, but lacks resolution at higher order level. Combined TUB2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) markers resolved all 29 sub-clades within *C. acutatum* clade (Cannon *et al*., 2012). More information about the history of molecular characterization of *Colletotrichum* spp. as well as current methods are included in section “1.3.3. *Colletotrichum*- Major Clades and Clusters”.

1.6.3. Markers Used for Characterization of Colletotrichum spp. in this Study

Mainly based on previous research, a number of markers were selected for this study to characterise a set of *Colletotrichum* isolates displaying biogeographic diversity (e.g. Damm *et al*., 2009, 2012a,b; Yang *et al*., 2012).

Internal Transcribed (ITS) is a non-coding part of DNA situated between two genes encoding structural components of ribosomal RNAs: small subunit (SSU) 18S rRNA and large subunit (LSU) 28S rRNA. ITS 1 and ITS 2 are partitioned by 5.8S rRNA gene (Baldwin, 1992).

Glyceraldehyde-3-phosphate (GAPDH) is an enzyme used in 6th step of glycolysis pathway. More recently it has also been proven that it initiates transcription and induces apoptosis (Tarze *et al*., 2007). Research on *Candida albicans* showed that can also act as virulence factor (Gozalbo *et al*., 1998).

Glutamine synthetase (GS) is responsible for catalysis of ammonia and glutamate yielding glutamine (Liaw and Eisenberg, 1994). Evidence indicates that GS has pathogenic value in bacteria. It is involved in cell wall resistance in *Mycobacterium bovis* (Chandra *et al*., 2010).

Beta-tubulin (TUB2) amplified with TUB5 and TUB6 primers designed by Talhinhas *et al*. (2002). B-tubulin is a monomeric globular protein that along with α-tubulin makes up the heterodimer tubulin - a building block of microfilaments (Kuznetsov *et al.*, 2013).

Part of histone 3 (His3) gene was amplified using primers HIS3F and HIS3R designed by Glass and Donaldson (1995). Histone 3 is one of five histone proteins involved in DNA packaging forming ‘beads on the string’ structure (Griffiths *et al*., 2008b).

Actin (ACT) gene fragment was amplified using primers ACT-512F and ACT-783R designed by Carbone and Kohn (1999). Actin is a highly conserved globular protein playing crucial role in cell processes by formation of polymerised microfilaments and facilitating amongst others: cell morphogenesis, cytokinesis,

motility, and organelle movement (Walker and Garrill, 2006; Dominguez and Holmes, 2011)

Chitin synthase1 (CHS) is an enzyme that maintains chitin levels during cytokinesis stage in cell division (Silverman *et al*., 1988; Shaw *et al*., 1991).

Calmodulin-1 (CAL) is a calcium binding receptor molecule with EF-hand motif. CAL is one of 20 calmodulin proteins and plays a role in signal transduction pathways, cell growth and cycle regulation (Stevens, 1983).

The MAT1-2-1 fragment of conserved mating type locus HMG box. MAT1 has two idiomorphs/alleles: MAT1-1 and MAT1-2 (Turgeon, 1998; Coppin *et al*., 1997). Heterothallic ascomycetes possess either one of the two alleles but not both, while homothallic have a pair (Coppin *et al*., 1997). Members of *Glomerella*, a sexual morph of *Colletotrichum* are currently known to contain only the MAT1-2 allele (Vaillancourt *et al*., 2000) unlike the vast majority of filamentous ascomycetes.

1.7. Sequencing – Developments and Novel Approaches

1.7.1. History of Genome Sequencing

Bacteriophage fX174 (5,386 bp) was the first full genome to be sequenced by Fred Sanger and his colleagues in 1977 (Fleischmann *et al*., 1995). It required preparation of genomic library of DNA fragments each cloned into a viral vector and taken up by host organisms like *Escherichia coli* or *S. cerevisiae* followed by sequencing (Sanger *et al*., 1977). Sequencing of bacteriophage (lambda) at 48,502 bp was performed using shotgun cloning method (Sanger *et al*., 1982). In 1989, the smallpox virus was a pioneer genome sequenced using automated platform (Massung *et al*., 1993). The first free living organism to be sequenced to everyone's surprise was *Haemophilus influenzae* in 1995 led by Craig Venter from the Institute for Genomic Research (TIGR) (Fleischmann *et al*., 1995; Venter *et al*., 2004). The sequencing of *Saccharomyces cerevisiae* was set up by Andre Goffeau in 1989 resulting in completion of 12.5 Mb genome (Johnston,

2003; Levy, 1994). This success inspired the Human Genome Project (HGP) established in 1990, while the first draft of 3,000-Mb Human Genome was submitted in 2005 (Sawicki *et al*., 1993; Griffiths *et al*., 2008a). With the progression of sequencing technologies and major historical events was the enormous expansion of sequencing database (Fig 1.7.) as reported by NCBI authorities (Hutchison, 2007).

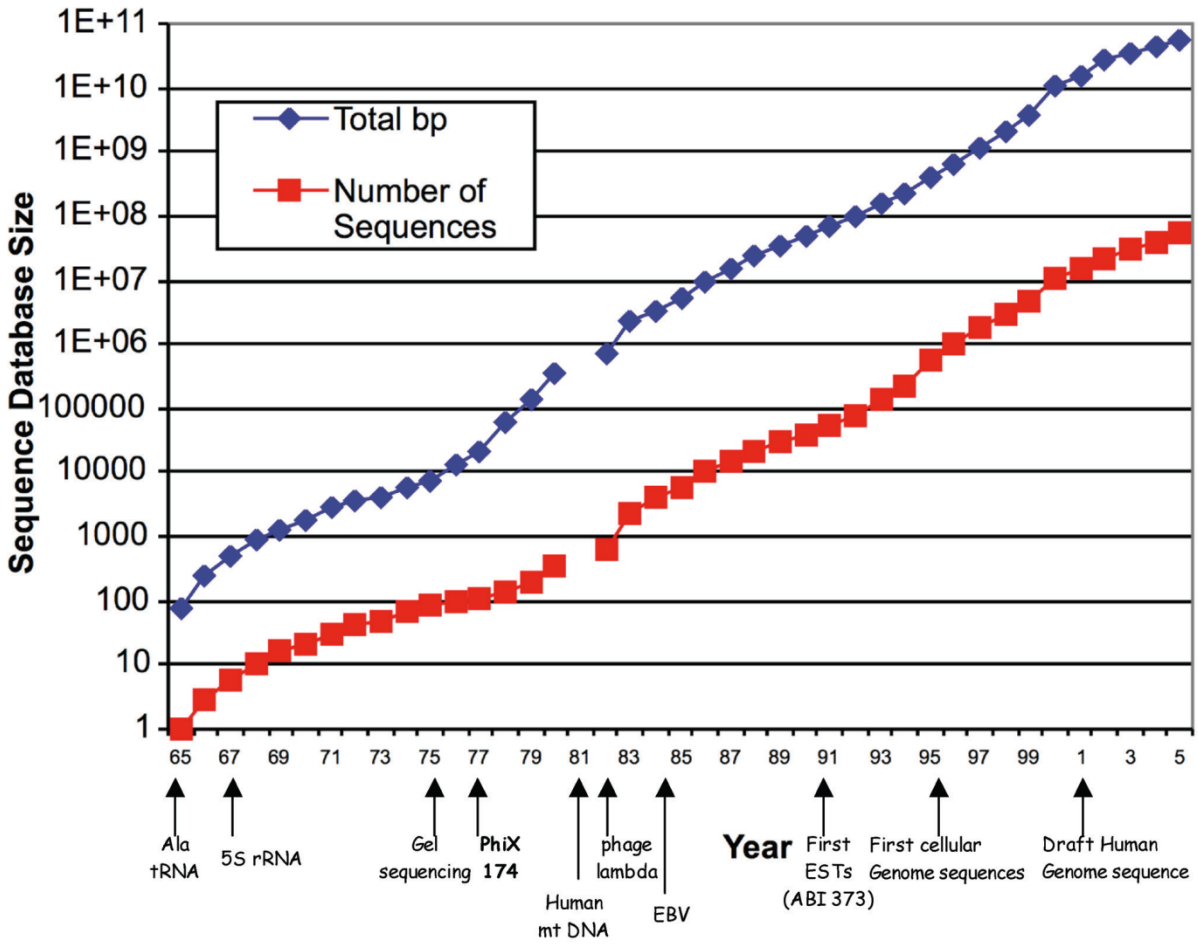


Fig 1.7. The Time Scale Illustrating the Growth of Sequencing Database in Relation to Major Sequencing Events* (Hutchison, 2007).

*According to Hutchison (2007) the statistical data covering the size of the database before 1981 was retrieved from Dayhoff (1981) and after 1981 it was based on NCBI information (http://www.ncbi.nlm.nih.gov/Genbank/).

1.7.2. Sanger Sequencing

Sanger sequencing was developed by Frederick Sanger and colleagues in 1977 (Sanger and Coulson, 1975; Sanger *et al*., 1977). Along with the method developed by Maxam and Gilbert, it is considered the first generation sequencing technology (Maxam and Gilbert, 1977). Sanger became the primary method of sequencing till the beginning of the Millenium when it was replaced by Next Generation platforms (Schuster, 2008). Currently, NGS is cheaper and much more accessible enabling large-scale genome sequencing studies. However, Sanger's method remained the preferable sequencing method for smaller scale projects (Morozova and Marra, 2008).

Sanger 's method is also referred to as dideoxy sequencing in which the deoxynucleotides (dNTPs) are replaced by 2', 3'-dideoxy derivatives (ddNTPs) that lack the 'OH'-group leading to termination of the reaction (Dale *et al*., 2008). Subsequently, products of the reaction are separated on polyacrylamide gels for sequence reads. Alternatively ddNTPs can be labelled with fluorescent dyes and separated using capillary electrophoresis (Janitz, 2011).

This laborious system was replaced by Applied Biosystems (ABI) capable of producing 96 kb data in single three-hour run (Ewing and Green, 1998). Nowadays 96-capillary machine can provide 0.5 Mb of sequence data per day (Janitz, 2011).

1.7.3. Next Generation Sequencing (NGS)

Over the last three – five years, NGS has become one of the principal approaches used by molecular geneticists among other researchers. Genome sequencing allows screening the organism in an attempt to find highly variable regions with potential susceptibility to adaptation. These markers are associated with functional genetic variation. NGS also enables studies at the transcriptome level allowing identification of genes expressed under particular conditions (Angeloni *et al.*, 2010). Neutral markers like microsatellites and amplified fragment length polymorphism are widely used to characterize population gene flow, density, size and genetic drift (Foll *et al*., 2010). However, neutral markers

are not fully adequate in defining adaptation processes (Allendorf *et al*. 2010). Furthermore, NGS tools applied at the population level are required to illustrate the gene activity in relation to habitat fragmentation, inbreeding depression, and environmental change (Primmer, 2009; Avise, 2010).

Recent developments in sequencing technologies termed Next Generation Sequencing (NGS) has revolutionised genome level analysis of biosystems. It was the platform developed by 454 Life Sciences Corporation (now Roche Applied Science) that changed the face of NGS. It dramatically reduced the time and cost of DNA sequencing (e.g. 25 mln bases in one 4-hour run) while providing accuracy of 99% or higher (Margulies *et al*., 2005) utilizing pyrosequencing chemistry (Nyren *et al*., 1993). In parallel, technological improvements from capillary systems limited to only 96 samples (Schuster, 2008) to picolitre plate-based solid phase systems led to the publication of complete Neantheral genome (Green *et al*., 2010).

The SOLiD system developed by Applied Biosystems follows the principles of the sequencing by ligation technology (Morozova and Marra, 2008). Due to shorter read lengths, compared to the 454 methodology, this method is more suitable for resequencing projects rather than *de novo* sequencing (Dale *et al*., 2008)

The Illumina-Solexa is a sequencing by synthesis method also referred to as bridge amplification sequencing (Morozova and Marra, 2008). Illumina/Solexa system provides shorter sequence reads when compared with other NGS platforms (Bentley, 2006). Currently available sequencing technologies from Illumina-Solexa are HiSeq, MiSeq and Genome Analyzer IIx systems. The sequencing chemistry behind them is the same; however, there are certain technical differences that make them more applicable for different research investigations. For example, MiSeq is promoted to have the broadest range of applications including RNA sequencing and ChIP-Seq (http://www.illumina.com).

Technological advances in NGS also required parallel developments in computational analysis of the huge amounts of data for *de novo* assembly of the

genome, resequencing and other applications including transcriptomics (Baker, 2012). For example, Velvet is a *de novo* assembler specifically designed for the short sequence reads generated by NGS platforms (Young, 2009). SOAPdenovo (Li *et al*., 2010), ABySS (Simpson *et al*., 2009) and ALLPATHS (Butler *et al*., 2008) are some alternatives to Velvet. Similarly, genome annotation and gene prediction areas required the development of software such as Augustus (Stanke *et al*., 2004) and GeneMark (Lukashin and Borodovsky, 1998) applicable for eukaryotic genomes.

1.7.4. Colletotrichum and NGS Technology-Current Status

Building on the NGS technologies, there are at least four *Colletotrichum* genome sequences available in the public domain: *C. higginsianum* (O'Connell *et al*., 2012), *C. graminicola* (O'Connell *et al*., 2012), *C. orbiculare* (Gan *et al*., 2013) and *C. gloeosporioides* (Gan *et al*., 2013). Further, genome sequencing and assembly of a selected set of *C. acutatum* strains is on-going through joint research (Baroncelli, Thon and Sreenivasaprasad, pers.com.). *C. higginsianum* host range includes the model system *Arabidopsis thaliana* and many cruciferous crops (Kleemann *et al*., 2012); while *C. graminicola* is virtually confined to maize-*Zea mays*. Genomes of both species were of similar size: 57.4 Mb for *C. graminicola* and 53.4 Mb for *C. higginsianum*. Fugal genomes encode a range of biomolecules like secondary metabolites e.g. polyketides, small secreted peptides, toxins and carbohydrate-active enzymes that are linked to pathogenicity and host specificity. Recent genome sequencing studies of *C. higginsianum* and *C. graminicola* recorded relatively high numbers of these virulence factors in both species, however, an expansion of secondary metabolism effectors, peptidases transporters and other secreted proteins has been reported in *C. higginsianum* (O'Connell *et al*., 2012). Another *Colletotrichum* sequencing project completed involved two economically significant fungal pathogens: *C. orbiculare*- primarily linked to cucurbits and *Nicotiana benthamiana*, and *C. gloeosporioides* with a wide host range. *C. orbiculare* genome size was 88.3Mb, much larger compared to other *Colletotrichum* species including *C. gloeosporioides* at 55.6 Mb (Gan *et al*., 2013).

Genome sequence of an isolate of *C. acutatum sensu lato* (*C. fioriniae*) has just been released (Baroncelli *et al*., 2014). Many more genomes from *Colletotrichum* genus are pending publication e.g. from within *C. acutatum sensu lato* species complex including *C. simmondsii* (Riccardo Baroncelli, unpublished).

CHAPTER 2: MATERIALS AND METHODS

2.1. Fungal isolates, culture media and conditions

2.1.1. Isolates

In this study, 18 isolates previously identified as *C. lindemuthianum* and all associated with common bean anthracnose were used (Table 2.1). Isolates 771 and 449 were used as out-groups where appropriate.

Table 2.1. Details of *Colletotrichum spp.* isolates+ Characterised in this Study

Species	Serial No.	ATCC No.	Code	Race	Host Name	Origin
C. lindemuthianum	701	_	3157B	gamma	*Phaseolus vulgaris*	Tanzania
C. lindemuthianum	776	_	UPS9	gamma-2(20)	*Phaseolus vulgaris*	France
C. lindemuthianum	216	62984	_	beta-1	*Phaseolus vulgaris*	Europe
C. lindemuthianum	832	_	CRS 73-1-1-M	_	*Phaseolus vulgaris*	Costa Rica
C. lindemuthianum	779	_	H433	_	-	Europe
C. lindemuthianum	29	_	20780	kappa	*Phaseolus vulgaris*	Europe
C. lindemuthianum	45	_	_	_	*Phaseolus vulgaris*	UK
C. lindemuthianum	206	_	20884	alpha	*Phaseolus vulgaris*	Europe
C. lindemuthianum	217	_	10283	delta	*Phaseolus vulgaris*	Europe
C. lindemuthianum	219	_	20186	iota	*Phaseolus vulgaris*	Europe
C. lindemuthianum	428	_	20380	lambda	*Phaseolus vulgaris*	-
C. lindemuthianum	533	_	P1-I4	_	*Phaseolus vulgaris*	Malawi
C. lindemuthianum	560	_	_	_	*Phaseolus vulgaris*	USA
C. lindemuthianum	693	_	2860	31, kappa	*Phaseolus vulgaris*	Brazil
C. lindemuthianum	694	_	2862	137, epsilon	*Phaseolus vulgaris*	Colombia
C. lindemuthianum	814	_	CRP 7-4-1-M	_	*Phaseolus vulgaris*	Costa Rica
C. lindemuthianum*	771	_	C11G-01	_	*Phaseolus vulgaris*	China
C. lindemuthianum*	449	_	1	_	*Phaseolus vulgaris*	Pakistan

+All isolates were obtained from the collection maintained at Warwick HRI, Wellesbourne, University of Warwick, UK and University of Bedfordshire, UK by Professors Eric Holub and Sreenivasaprasad, respectively.

* 771 and 449 were identified as *C. gloeosporioides* and *C. truncatum*, respectively in this study based on multilocus sequence data

- Indicates details not available

2.1.2. Colletotrichum culturing

Potato dextrose agar (PDA) and potato dextrose broth were used for routine culturing and in the growth experiments of *Colletotrichum* isolates following manufacturer's directions. Solutions were autoclaved at 121°C.

Each Petri dish (Sarstedt, UK) was dispensed with 20-25ml of PDA in the laminar flow bench. Plates were inoculated with isolates in a microbiological safety cabinet (MSC) using sterile inoculation loops.

Microfuge tubes were filled with 1ml of PDB and inoculated with mycelial material from PDA plates minimising the amount of agar transferred to ensure efficient DNA extraction. Adequate care was taken to maintain aseptic conditions, and the genetic integrity of the isolates.

2.1.3. Growth conditions

Generally, *Colletotrichum* isolates were grown at 25°C for periods between 10-14days. For the experiments involving the observation of the growth, *Colletotrichum* sp. isolates were maintained at 20 and 25°C.

2.1.4. Preparation of stock cultures for storage

Water stock cultures were prepared for storage of the *Colletotrichum* sp. isolates. Universal tubes with approx. 15 ml sterile water were prepared. Agar blocks (~0.7mm square) from fresh cultures (~7 -10 days old) were transferred to the tubes, which were maintained at room temperature.

2.1.5. Monitoring the growth of Colletotrichum isolates

The growth was measured in mm and recorded every 1-5 days for 16 days (cultures incubated at 20°C) and 14 days (cultures incubated at 25°C). There were 8 measurements taken from the plate (Fig 2.1) in order to calculate average values for each isolate. Inoculations on PDA plates were prepared using cork borer 8 mm in diameter to ensure the comparable results.

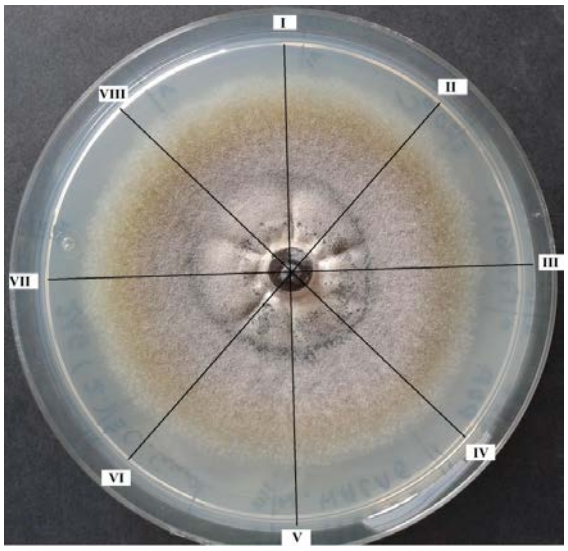


Fig 2.1 The Diagram Illustrating the Manner in which Measurements were Taken for Growth Monitoring.

2.1.6. Microscopic observation of cultures/sporulation.

Observation of fungal cultures to assess the level of sporulation was performed using a compound microscope. Fungal material mounted on slides was stained with lactophenol cotton blue dye to check for sporulation at required magnifications.

2.2. DNA Extraction

2.2.1. Chelex-based method

Microcentrifuge tubes containing 3 to 5 day-old fungal cultures were centrifuged at maximum speed (14,680rpm=20,238rcf) for 5-7min. Supernatant

was removed and cultures were washed twice each using 500µl of sterile water. Tubes were centrifuged for 1-2min at max speed. Supernatant was removed. Subsequently, near equal amounts of sand and chelex were added to fungal material in a 1:1:1 ratio. Afterwards 300-500µl of molecularly sterile water was added to the tube depending on the volume of the components. Autoclaved plastic micropestle was used to grind the mycelium with sand and chelex. Separate pestle was used for each isolate to avoid cross-contamination. Centrifugation was repeated at max speed for 5-7 min and the supernatant was collected into a fresh 1.5ml eppendorf tube. The supernatant containing the genomic DNA was stored at -20 C till further use.

2.2.2. Column-based method for multilocus sequencing work

GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich) was used for DNA extractions for multilocus sequencing purposes. Sigma protocol was followed as indicated by the manufacturer with omission of the first step (Appendix I). Hot block was set for 65ºC 100µl of sterile water (Sigma-Aldrich) warmed up at 65ºC on a hot block was used for eluting the DNA for each sample.

2.2.3. DNA extraction method for genome sequencing

The DNeasy Plant Mini Kit (Qiagen) was used for the extraction of DNA for the genome sequencing processes. The mycelial material was prepared as below, and was used for the genomic DNA extraction according to manufacturer's protocol (Appendix II).

The cultures were grown in 20 ml beakers filled with a thin layer of PDB. Minimal amount of liquid medium was used to provide optimal surface area for fungal growth under aerobic conditions. Inoculum comprised of small pieces of mycelial material, with minimal carry over of agar, cut from fresh culture. Cultures were incubated at 25ºC for 3-5days; then the mycelial mat was removed and washed twice with sterile water. The mat was placed on filter paper and excess moisture was removed. Subsequently, fungal material was wrapped in 3 layers of aluminium foil and frozen in dry ice. Appropriate amount of the frozen

material was used for DNA extraction according to Qiagen protocol (Appendix III).

2.3. PCR reactions, conditions and primer sequences

2.3.1. Preparation of 100 µM stock and 20 µM working stock of primers

According to the supplier's instructions (SIGMA) specified quantity of sterile water was added to freezedried primers under laminar flow bench to prepare the 100 µM stocks. Tubes were tapped and inverted repeatedly to ensure the content is mixed. To prepare 20 µM working stock, 20µl of the stock primer (100 µM) was taken in a 1.5ml eppendorf tube and 80 µl of sterile water were added. Tubes were inverted few times to mix the content and centrifuged for 1 min at max speed.

2.3.2. Preparation of 20 µl and 50 µl PCR reactions

BioMix Red (Bioline, UK) is a pre-mixed and pre-optimized 2X PCR solution using *Taq* DNA polymerase. The reagent contains dye and loading buffer for convenient use. The 20 µl reactions required: 1µl of DNA, 1µl of forward primer, 1µl of reverse primer, 7µl of sterile water and 10 µl of BioMix Red. For the 50 µl reactions, all reagents were scaled-up to 2 µl of DNA, 2.5 µl forward primer, 2.5 µl reverse primer, 18 µl of water and 25 µl of BioMix Red. Thin-walled flat cap 200 ul tubes (Sigma-Aldrich) were used for the assembly of PCR reactions. The PCR reactions were run using a thermal cycler with a heated lid (Bio-rad).

2.3.3. Preparation of Arbitrary-Primed PCR

Reaction contents were generally same as for standard PCR (details above); however, only 1ul of a single AP-PCR primer was added to the mix and adjusted accordingly with sterile water. Final volume of reaction was 20 µl. Later 10 µl was loaded on 1.5 % agarose gel and electrophoresed at 80V.

2.3.4. PCR conditions

The PCR conditions for amplification of ITS region using primers ITS1 and ITS4 were according to standard protocol (Table 2.2.).

Table 2.2. PCR Conditions for the Amplification of the ITS Region

Process	Temperature (°C)	Time (min)	Cycle No.
Initial Denaturation	95	3	1
Denaturation	94	1	35X
Annealing	60	1	
Extension	72	1	
Final Extension	72	5	1

All other loci used in multilocus phylogenetic analysis were amplified following the same PCR conditions as below (Table 2.3.) with only the annealing temperature changing for each primer set (Table 2.4.).

Table 2.3. PCR Conditions for Other Loci Used for Multilocus Phylogenetic Analysis.

Process	Temperature (°C)	Time (min)	Cycle No.
Initial Denaturation	94	5	1
Denaturation	94	0.5	40X
Annealing	Varied; see Table 3	0.5	
Extension	72	0.5	
Final Extension	72	7	1

Table 2.4. Annealing Temperatures Used with Various Primer Sets for Different Loci.

Primer Set	Annealing Temperatures (°C)	Final Temp. Setting (°C)
ACTF/ACTR	55,63	63
CHSF/CHSR	52,55	55
CL1/CL2	55,57	57
HIS3F/HIS3R	64,65	65
GDF1/GDR1	52,57	57
GSF/GSR	52,61,63	63
TUB5/TUB6	69	69
HMGDF/HMGDR	61	61

2.3.5. Primer sets used for multilocus sequencing

Various primer sets were identified from the literature and applied to *Colletotrichum spp.* in this study. The full sequence, name of the amplified locus, expected amplicon size and the source are listed (Table 2.5).

Table 2.5. List of Primer Sets Used in the Study for Various Loci with Full Sequence Information and Amplicon Size

Name of the primers	Primer Sequences (5'-3')	Locus/Gene	Expected Size of the fragment	Ref.
GDF1/ GDR1	Forward primer GDF1: GCCGTCAACGAC CCCTTCATTGA Reverse primer GDR1: GGGTGGAGTCGT ACTTGAGCATGT	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	~115bp (*C.lindemuthianum*) , ~200bp (*C. gloeosporioides*)	(Liu *et al.*,2007;) Guerber *et al.*, 2003)

GSF1/ GSR1	Forward primer GSF1: ATGGCCGAGTAC ATCTGG Reverse primer GSR1: GAACCGTCGAAG TTCCAC	Glutamine synthetase (GS)	~930bp (*C. lindemuthianum*), ~820bp (*C. gloeosporioides*)	(Liu *et al*.,2007;) Guerber *et al*., 2003)
ITS1/ ITS4	Forward primer ITS1: TCCGTAGGTGAA CCTGCGG Reverse primer ITS4: TCCTCCGCTTATT GATATGC	Internal transcribed spacer	~500bp	(Innis *et al*.,1990)
ACTF/ ACTR	Forward primer ACT-512F: ATGTGCAAGGCC GGTTTCGC Reverse primer ACT-783R: TACGAGTCCTTCT GGCCCAT	Actin	~230bp	(Carbone and Kohn, 1999)
TUB5/ TUB6	Forward primer TUB5: GGTAACCAGATT GGTGCTGCCTT Reverse primer TUB6: GCAGTCGCAGCC CTCAGCCT	β-Tubulin	~430bp (*C.lindemuthianum*) , ~450bp (*C. gloeosporioides*)	Talhinhas *et al*., 2005
CHSF/ CHSR	Forward primer CHS-79 F: TGGGGCAAGGAT GCTTGGAAGAAG	chitin synthase1	~250bp	(Carbone and Kohn, 1999)

	Reverse primer CHS-354 R: TGGAAGAACCAT CTGTGAGAGTTG			
HIS3F/ HIS3R	Forward primer CYLH3F: AGGTCCACTGGT GGCAAG Reverse primer CYLH3R: AGCTGGATGTCCT TGGACTG	H3-1a and H3-1b parts of histone 1	~370bp	(Crous *et al*., 2004)
CL1/ CL2	Forward primer CL1: GARTWCAAGGAGG CCTTCTC Reverse primer CL2: TTTTTGCATCATGA GTTGGAC	Calmodulin	~650bp	(Johnston and Jones, 1997)

For the mating-type locus MAT1-2-1, two primer sets of primers were tested (Table 2.6); one set specific to *C. lindemuthianum* and one degenerate set designed for use with various *Colletotrichum spp*. (Garcia-Serano *et al*., 2008).

Table 2.6. Primers Tested for the Amplification of the Mating-Type Locus (MAT1-2-1)**

Name of the primers	Primer Sequences (5'-3')	For amplification of:	Used with	Size of PCR product (bp)	Ref.
HMGD	Degenerate: Forward primer HMGDF: CCYCGYCCYCCY AAYGCNTAYAT Reverse primer HMGDR: CGNGGRTTRTARC GRTARTNRGG	MAT1-2-1****	*C. gloeosporioides, Colletotrichum spp.*	~200bp	Garcia-Serano *et al.*, 2008
HMGCL**	Specific: Forward primer HMGCLF: CATGCCGCAGTAA AGCAAAT Reverse primer HMGCLR: ATCATCAGACGTT CTTTGTG	MAT1-2-1	*C. lindemuthianum*	~150bp	Garcia-Serano *et al.*, 2008

*MAT1-2-1 is more variable part of HMG box (mating-type gene).

**Data not shown in the thesis as the primers were amplifying the same fragment of DNA.

2.3.6. Arbitrary Primed PCR (AP-PCR) Conditions

A set of 10 AP-PCR primers (Table 2.7.) were identified from the literature (Talhinhas *et al*., 2002; Talhilans *et al*., 2005; Freeman *et al*., 2000b) and were tested for preliminary screening of all isolates at the annealing temperatures recommended in the source. The general AP-PCR temperature setting along with other details are listed in Table 2.8.

Table 2.7. Sequence Data and Annealing Temperature of AP-PCR Primers

Primer	Sequence (5'-3')	Annealing Temp. (°C)	Species Used in Original Study	Reference
(TGTC)4	TGTCTGTCTGTCTGTC	48	*C. acutatum, C.gloeosporioides* and *Colletotrichum* from almond fruit	Freeman *et al*., 2000a
(ACTG)4	ACTGACTGACTGACTG	48	*C. fragariae C. acutatum, C. gloeosporioides*	Freeman *et al*., 2000b
(GACAC)3	GACACGACACGACAC	48	As above	Freeman *et al*., 2000a,b
(GACA)4	GACAGACAGACAGACA	48	As above	Freeman *et al*., 2000a,b
(CAG)5	CAGCAGCAGCAGCAG	60	As above	Freeman *et al*., 2000a, b
(TCC)5	TCCTCCTCCTCCTCC	60	*Colletotrichum spp.*	Talhinhas *et al*., 2002;
			C.acutatum, C.gloeosporioides	Talhinhas *et al*., 2005

(GAC)5	GACGACGACGACGAC	60	As indicated above	Talhinhas *et al.*, 2005
(CAC)5	CACCACCACCACCAC	60	As indicated above	Talhinhas *et al.*, 2005
(GACG)4	GACGGACGGACGGACG	65	As indicated above	Talhinhas *et al.*, 2005
(GCA)5	GCAGCAGCAGCAGCA	65	As indicated above	Talhinhas *et al.*, 2005

Table 2.8. Conditions Used for Arbitrary Primed PCR *

Process	Temperature (°C)	Time (min)	Cycle No.
Initial Denaturation	95	5	1
Denaturation	94	1	30X
Annealing	Varied; see Table 3.4.	2	
Extension	72	2	
Final Extension	72	5	1

* Conditions as recommended in Talhinhas *et al.*, 2002.

2.4. Agarose gel electrophoresis

2.4.1. Preparation of Tris-Acetate-EDTA electrophoresis buffer

The stock 50 X Tris-Acetate-EDTA buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA – pH 8.4; Fisher, UK) was diluted in Milli-Q water for preparation of 1 X concentration of the solution used both for preparing the agarose gel and the running buffer in the electrophoresis tank.

2.4.2. Preparation of 1 % (w/v) agarose gel and electrophoresis

Agarose powder (Sigma-Aldrich) was melted in 1 X Tris-Acetate-EDTA buffer (1g/100 ml). The gels were routinely electrophoresed at 80 V for 45 min in a horizontal gel system (BIO-RAD).

2.4.3. Staining with Ethidium Bromide (Et Br) and visualization under UV light

To aid the visualization of DNA bands, 5µl of Et Br (10 mg/ml in H_2 O, Sigma-Aldrich,) was added to every 100 ml of the agarose gel . Adequate health and safey precautions were taken in handling, and disposal of the Et Br stained gels and the buffer; Et Br stock was stored at room temperature.

2.4.4. Molecular weight marker

2.4.4.1. For amplicon size and concentration estimation

2. 4.4.1. Easy Ladder I (Bioline, UK) was used to estimate the size of the amplicons, and also to provide an approximate estimate of the DNA concentration through comparison of the fluorescence level of the bands on the gel. This particular ladder produces 5 bands on the gel allowing the determination of the size between 100-2000bp with each band at 50 ng (Fig 2.2.). The reagent was kept at -20°C.

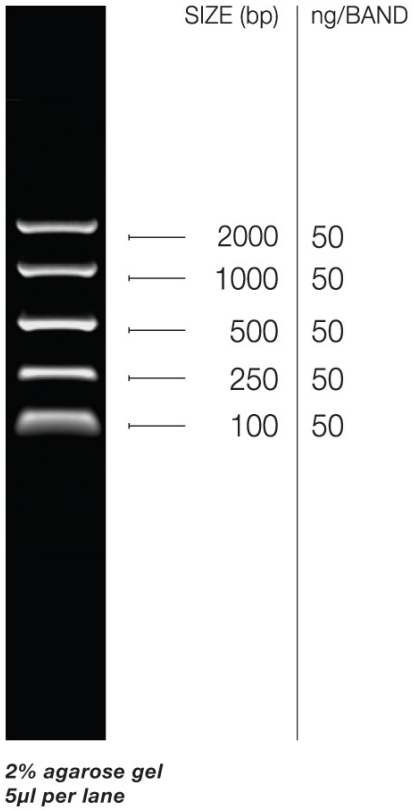


Fig 2.2 Easy Ladder I (Bioline, http://www.bioline.com)

2.4.4.2. For assessing the quality and quantity of genomic DNA

The Lambda DNA is a linear double-stranded temperate *E. coli* bacteriophage of 48,502 bp size. Lambda DNA was used for assessing the genomic DNA of two *C. lindemuthianum* isolates: 216 and 776. Stock lambda DNA (0.3 µg/µl), was diluted 10-fold to 30 ng/µl in order to prepare a working stock. Lambda DNA was loaded on 0.7 % agarose gels run at 60 V for 120 min. Final loading volume was 10µl. While only 1µl of *C. lindemuthianum* genomic DNA was loaded on gel (made up to 10µl with 9µl of water), four different concentrations of Lambda DNA were selected: 30 ng (1µl), 60 ng (2µl), 90 ng (3µl), and 120 ng (4µl).

2.5. Purification of PCR products

QIAquick PCR Purification Kit was used for clean-up of PCR products to remove primer dimers, enzyme, and other components present in PCR. The process was followed according to the manufacturer's recommendations (Appendix III).

2.6. Preparation of DNA Samples for Sequencing

According to the recommendations provided by the Cambridge Sequencing Facility (Appendix IV) 20 ng per 100 base pairs of a PCR fragment was prepared in 10µl water. The preliminary fragment size and concentration estimation were made using the molecular weight marker Easy Ladder I referred to earlier. Calculations regarding the amount of DNA and water dilutions were made for each sample as explained with an example below:

A PCR amplicon of 600 bp would require 120 ng (at 20 ng per 100 bases). By observing the level of brightness of the band on the gel and comparing it to the MWM (loaded at 30 ng and 50 ng concentration on the gel) the concentration of the PCR product was estimated, e.g. 1µl equals of 30 ng. Therefore 4 µl (equal 120 ng) were mixed with sterile water to make up a total volume of 10 µl. DNA samples were mixed throughly, centrifuged and sealed with parafilm for shipment to the sequencing facility.

2.7. DNA sequencing

Sequence data was generated through automated Sanger sequencing using ABI Applied Biosystems 3730xl DNA Analyser technology based on capillary electrophoresis as discussed in Introduction Chapter.

2.8. Multilocus phylogenetic analysis- bioinformatics and software

2.8.1. Opening and analysing the sequence files/data

Geospiza, a free software, was downloaded and used to allow DNA sequence viewing, reverse complementary sequence and generate the FASTA sequence file for further analysis.

2.8.2. BLAST- Basic Local Alignment Search Tool

BLAST (protein blast, nucleotide blast) was used for database searches using both accession numbers and FASTA sequences to find sequence homologies with other organisms as well as to identify the isolates. BLAST results/sequence data was used for multiple sequence alignments.

2.8.3. ClustalW2- Multiple Sequence Alignment software

Multiple sequence alignment (MSA) with ClustalW2 was used for preliminary comparison of sequence data, allowing to position and identify the differences like SNPs. MSAs were then used for further phylogenetic analysis using Geneious software.

2.8.4. Phylogenetic Analysis

Geneious is a software that allows BLAST searches, multiple sequence alignment, gene prediction and annotation of RNA and DNA sequence data. It also enables phylogenetic analysis including: bootstrapping, maximum likelihood analysis, Bayesian analysis (MrBayes), tree building and editing (www.geneious.com). Geneious was used to carry out a range of bioinformatics/phylogenetic analysis of the multilocus sequence data generated in this study.

2.9. Preparation of DNA for genome sequencing

2.9.1. Assessment of DNA quality and quantity using NanoDrop

NanoDrop technology was used for assessment of concentration and purity of DNA (Appendix V).

2.9.2. Validation of size of genomic DNA fragments and concentration

The size and concentration of genomic DNA was further estimated using uncut lambda DNA as molecular marker (section 2.4.4.2. in Materials and Methods).

CHAPTER 3: RESULTS

PART I. Screening of Nine Loci for Multilocus Phylogenetic Analysis and their Propriety for *Colletotrichum spp*.

Initial screening of the nine selected loci (ITS, ACT, TUB, CL, CYLH, HMG, GD, GS, and CHS) was performed using 6 isolates originally identified as *C. lindemuthianum* (216, 701, 771, 776, 779, and 832; Table 2.1.). This was done in order to assess the resolution of the chosen markers based on the level of conservation within the genome, their potential in species identification, and in determining the genetic diversity and relationships within and between species in relation to the biogeographic diversity.

3.1. Species Identification of *Colletotrichum* Isolates

Using ITS sequence data (Appendix VI) generated for each isolate (Table 2.1). BLAST search on NCBI database was performed to validate the species identity of the isolates. During the first part of the investigation, isolate 771 was identified as *C. gloeosporioides*. Similarly, during the second part, isolate 449 was identified as *C. truncatum*. Sequence data for *C. truncatum* was generated only for the five loci that were most useful: ITS, TUB, GD, GS and HMG. However, in order to provide a comparative view of the variation in the amplicon size, data for *C. truncatum* was added to the gel images, where available.

3.2. Standardization of PCR Conditions for Each Locus Used in Multilocus Phylogenetic Analysis

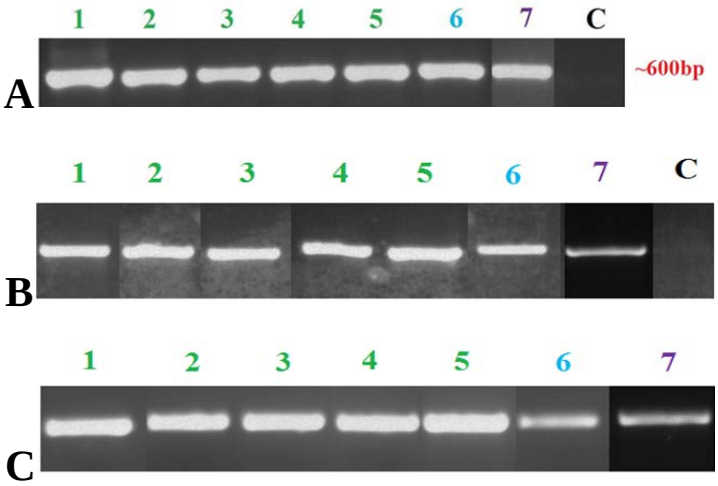
The PCR conditions were standardized for each locus by assessing the banding pattern on the gel (Table 3.1. and 3.2.). The aim was to obtain clean single band of expected size on the gel with no non-specific amplification and minimal amount of primer dimers.

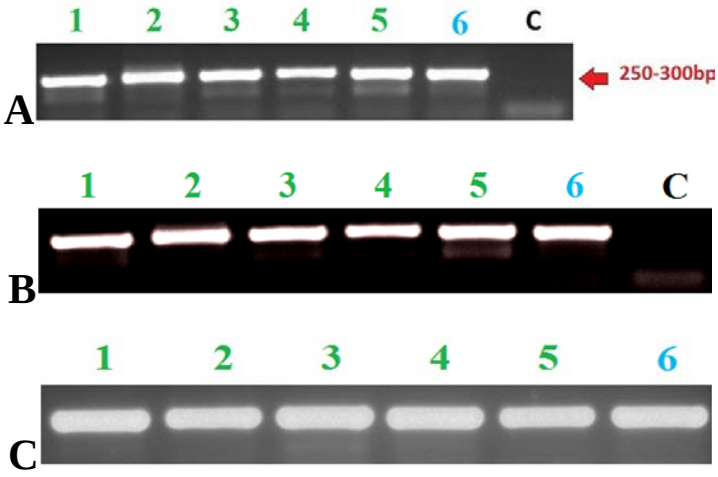
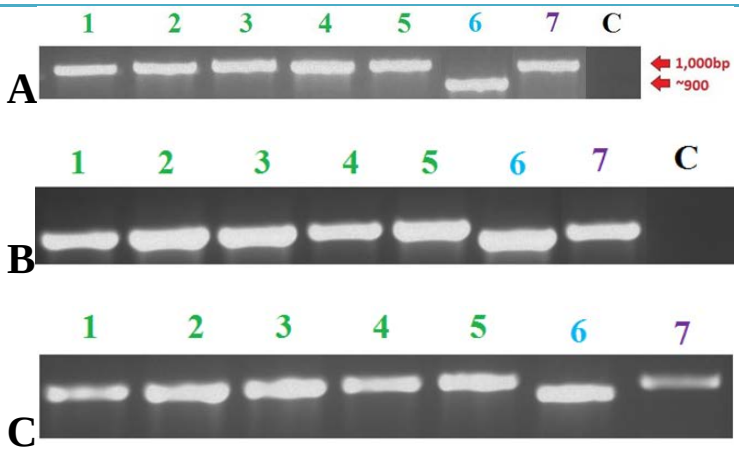
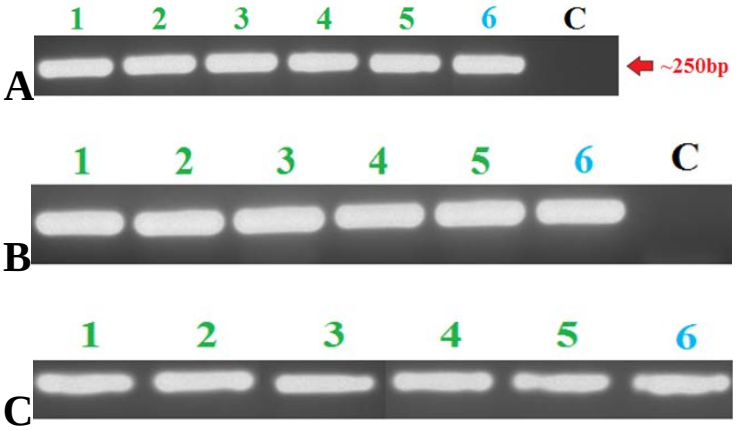
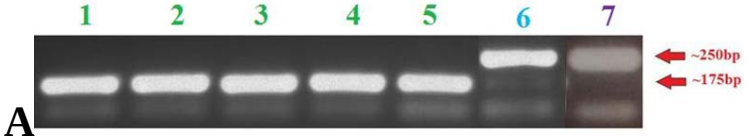
Table 3.1. Details of Labelling to Figures used in Standardization of PCR Conditions for Each Locus Used in Multilocus Phylogenetic Analysis*

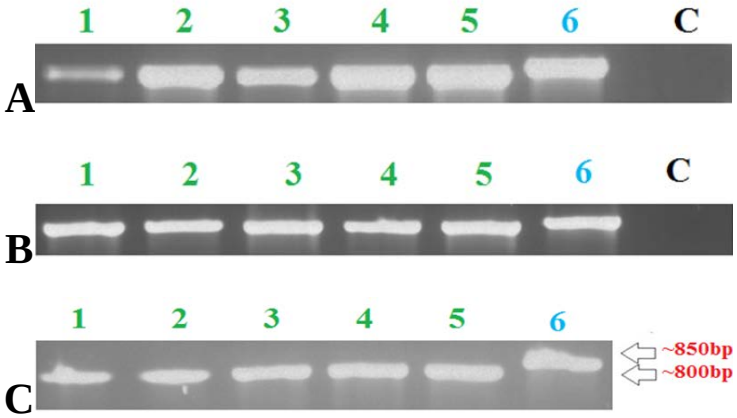
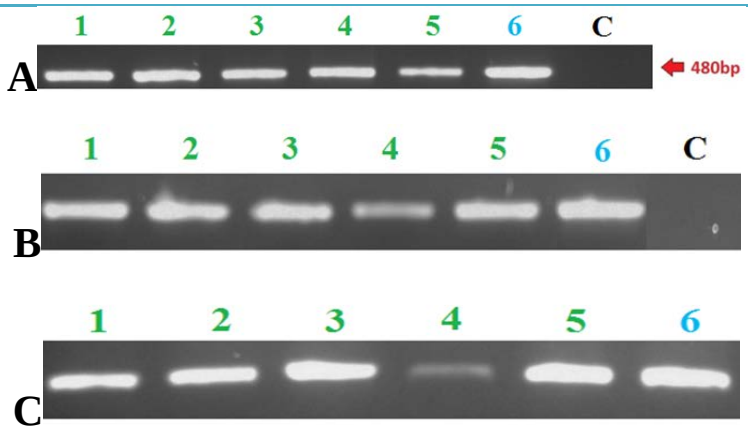
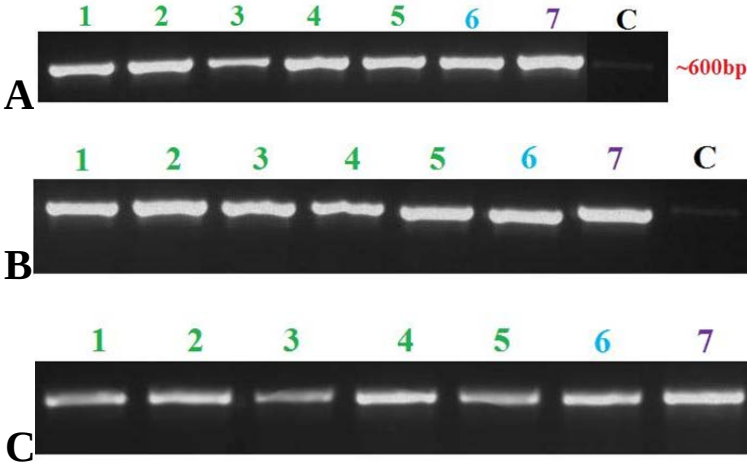
Number on Picture	Isolate Code	Species
1	701	*C. lindemuthianum*
2	216	*C. lindemuthianum*
3	776	*C. lindemuthianum*
4	779	*C. lindemuthianum*
5	832	*C. lindemuthianum*
6	771	*C. gloeosporioides*
7	449	*C. truncatum*
C	**Control** (No DNA)	**C**
	A-20µl reactions	
	B-50µl reactions	
	C-cleanup products	

*The number on the pictures are linked to the codes of the isolates (Table 2.1.).

Table 3.2. Results of Amplification of Multiple Loci in *Colletotrichum* Isolates.

Locus	Gel Pictures	Amplified at (°C)	Details
ITS		60	There were no visible non-specific bands or contamination.

CHS		52	Non-specific banding especially noticeable in sample 5 was minimised during the scale-up and the purification.
GS		61	Difference in the size of the amplicon in sample 6 that was ~100 bp smaller than the rest of the PCR products.
ACT		63	No non-specific binding.
GD/ GAPDH		57	PCR product was ~175 bp for *C. lindemuthianum* samples and ~250 bp for *C. gloeosporioides* and *C. truncatum* isolates. Low

			low level non-specific binding.
CAL		57	No non-specific binding.
HIS3		65	As above.
TUB		69	As above. Low contamination of control.

HMG	61	No non-specific binding.

*Table 3.1. represents the labelling legend to the pictures.

Summary of the amplicon sizes for various loci is presented in Table 3.7.

3.3. Multilocus Phylogenetic Analysis

3.3.1. Determination of Amplicon Structure and Position using Reference Colletotrichum spp. Genomes

Sequence data for one isolate *C. lindemuthianum* 216 was selected for the deciphering structure of the sequenced gene and to generate the schematic representation. Each locus used in multilocus phylogenetic analysis was mapped against the reference high homology gene found on NCBI database. Two sequences were aligned using ClustalW2 and the structure of gene was generated using Geneious software. The two sequences were then assembled against the reference genome of *C. orbiculare* MAFF 240442 (reference assembly provided by Riccardo Baroncelli, Warwick University). Attempts to align the sequence data against other *Colletotrichum* genomes (e.g. *C. higginsianum*, *C.graminicola*) were not successful potential due to high divergence. The only genome that was suitable for reference was *C. orbiculare* that shares the same clade as *C. lindemuthianum*. Gene structure images were generated using Geneious version 6.1, Biomatters (www.geneious.com).

Table 3.3. The Labelling Legend to the Fig 3.1.-3.9.*

Feature on the Diagram	Purpose
Top Scale	Illustration of the position of given gene within the reference *C. orbiculare* genome.
Grey/Coloured Bar below	Represents consensus sequence where dark grey/black areas represent the *C. orbiculare* genome and coloured regions depict variables.
Green/Khaki Bar	Green bar refers to the level of homology between the sequences where khaki refer to one strand and bright green region to both strands.
Coloured/Black Bar Next to Reference Gene with Scale above	Illustrates the length and/or structure of reference gene. Colours represent nucleotides within sequence: A (red), T (green), C (blue), and G (yellow).
Red/Pink Bar (ITS Sequence)	Illustrates the structure of the amplified ITS region where red highlights the small fragment of 18S rRNA, 5.8S rRNA and fragment of 28S rRNA, while pink shows 2 blocks of the ITS RNA.
Purple Bar	Illustrates the Blast Hit between two sequences.
Yellow Bar	Represents codons.
Grey Bar	Shows exons.
Line Bar	Represents introns.
Green Bar	Represents full gene/sequence.
Red Bar	Shows mRNA.
Bottom Grey Bars	Represent the *C. orbiculare* genome and the gene sequence generated in the study. Gaps refer to deletions and black/coloured regions are representing variable data.

*Single diagram may not contain all features.

The ITS structure (Fig 3.1.) shows the 5.8S unit 153 bp in length with ITS on each side 164 and 165 bp long. Part of the 28S unit was amplified at 57bp along with the 18S on the other side amplified at 30 bp. The BLAST Hit was from 1,990,386 bp to 1,989,882 bp on the genome contig/scaffold.

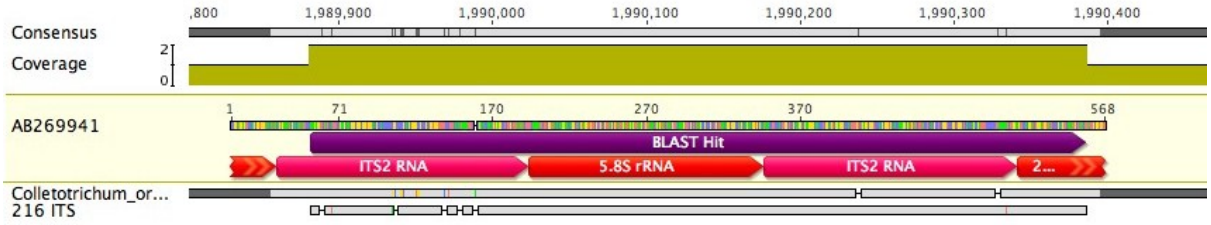


Fig 3.1. *C. lindemuthianum* Ribosomal RNA Gene Block ITS (Internal Transcribed Spacer) Region Structure Mapped Against Reference Sequence and *C. orbiculare* Genome

The actin (ACT) gene fragment amplified was 251 bp (Fig 3.2.) while the reference actin gene (JQ005842) was 250 bases long containing 3 exons spanning the reference gene sequence from 22nd base to the 273rd base. The amplicon also contained 14 bp of further sequence past the ACT gene, but was missing the first 31 bp of the exon 1. The amplified fragment contained a small part of exon 1(6 bp), full sequence of exon 2 (31 bp) and a part of exon 3 (21bp) giving a total of 89 bp of coding sequence and long stretches of two intron sequences. The BLAST hit with the *C. orbiculare* genome contig was positioned between 340,520 bp and 340,737 bp.

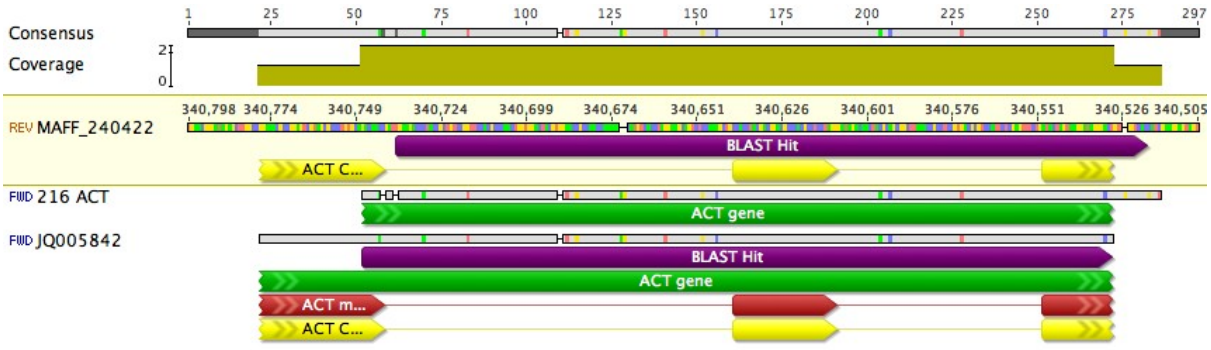


Fig 3.2. *C. lindemuthianum* Actin Gene Fragment (ACT) Sequence Mapped against the Reference Sequence and *C. orbiculare* Genome

The calmodulin (CAL) gene amplified was 648 bp (Fig 3.3.) and alignment against the reference calmodulin gene sequence revealed the coverage of small part of 2nd intron (14 bp) and full part of exons 3 (16 bp), 4 (126 bp), 5 (74 bp), 6 (138 bp) and part of 7 (17 bp). The amplicon was positioned between 463 and 1,111 bases within the reference gene (CTU15993). The reference CL gene comprised of at least 7 exons and 6 introns. The CL amplicon was located between 18,726 and 19,373 bases within *C. orbiculare* genome contig with the BLAST hit 646 bp long.

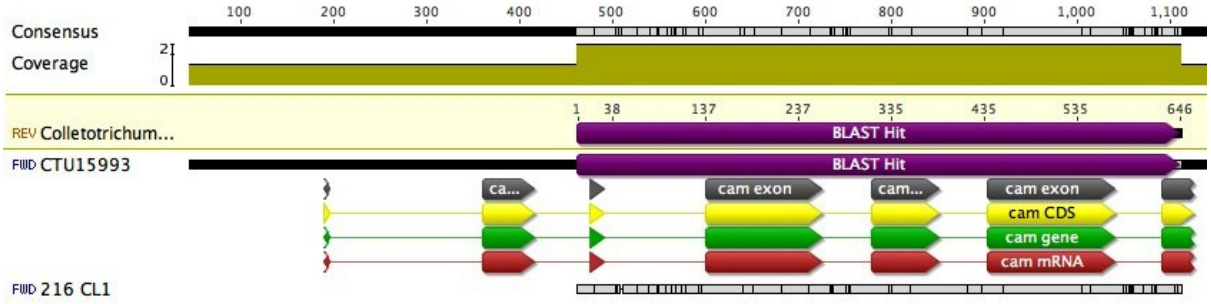


Fig 3.3. *C. lindemuthianum* Calmodulin (CAL) Gene Structure Mapped against Reference Sequence and *C. orbiculare* Genome

The histone 3 gene fragment sequenced was ~370 bp long (Fig 3.4.). The sequence was first aligned against the reference gene from NCBI (JX546768) in order to reveal its structure. The reference gene was 413 bp long with two exons 186 bp and 167 bp, respectively separated by an intron of 61 bp. The amplicon was spanning parts of the exon 1 (142 bp of the full 186 bp) and full exon 2. The BLAST Hit within the *C. orbiculare* was 370 bp long and located between 60,382 and 60,751 bases on the genome contig.

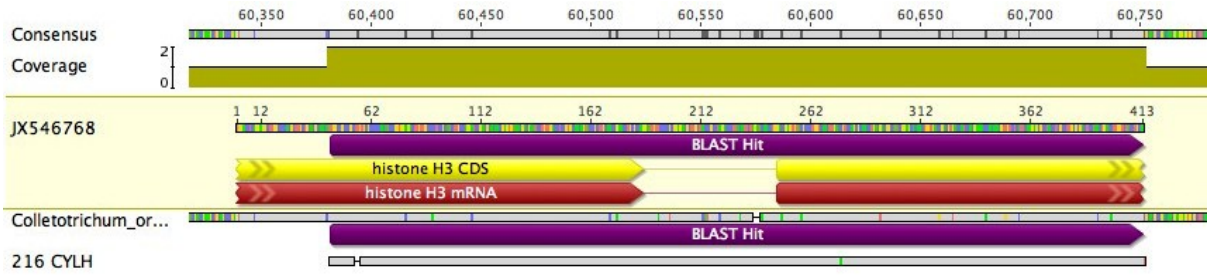


Fig 3.4. *C. lindemuthianum* Histone 3 (HIS3) Gene Structure Mapped against Reference Sequence and *C. orbiculare* Genome

The glutamine synthetase (GS) sequence appeared to be a large intron within the glutamine synthase gene amplified as a 933 bp fragment (Fig 3.5.). The BLAST Hit within *C. orbiculare* genome was 910 bp long and positioned between 302,392 to 303,301 bp. The reference glutamine synthase intron gene (DQ792886) was 907 bp. The amplicon was fully covering the sequence data from both sources with additional 4 bases and 22 bases at the 5' and the 3', respectively.

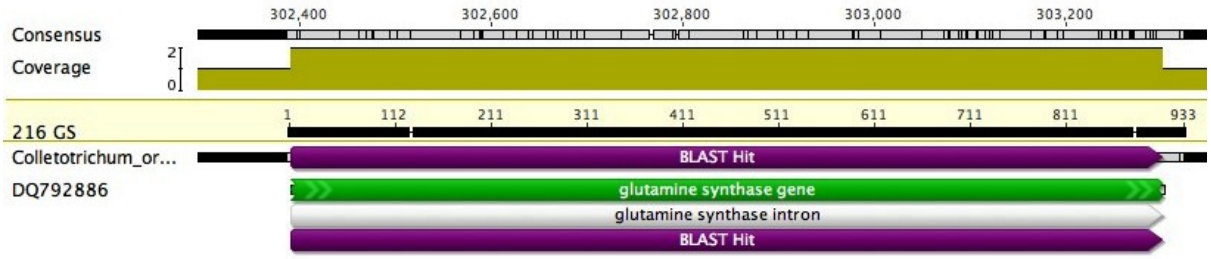


Fig 3.5. Structure of the *C. lindemuthianum* Glutamine Synthetase Gene (GS) Amplicon Mapped against Reference Sequence and *C. orbiculare* Genome

The glyceraldehyde-3-phosphate dehydrogenase (GD) sequence was the shortest in the multilocus sequence analysis at only 115 bp (Fig 3.6.). The amplicon mainly spanned the intron between exons 1 and 2 and the 5' part of exon 2. The GD/GAPDH gene is built of 2 intervals of coding sequence of 129 bp and 885 bp and a total sequence 2188 bp. The amplicon covered 84 bp of the intron separating the two exons and 28 bp of the 2nd exon. The BLAST Hit for the reference *C. orbiculare* genome was significantly lower at only 66 bp positioned between 545,091 and 545,156 bp on the genome contig and covering the 3' part of intron (37 bp) and the 5'part of exon 2 (28 bp).

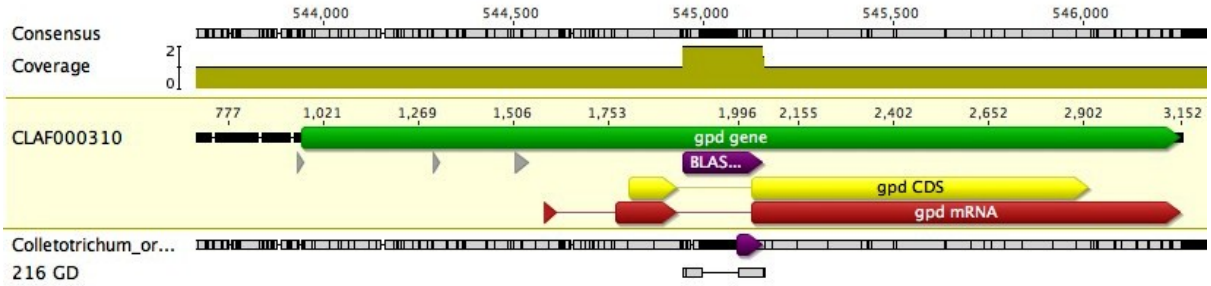


Fig 3.6. Structure of the *C. lindemuthianum* Glyceraldehyde-3-Phosphate Dehydrogenase Gene (GD/GAPDH) Amplicon Mapped against Reference Sequence and *C. orbiculare* Genome

The beta-tubulin gene (TUB) fragment sequence was 437 bp (Fig 3.7.). The reference sequence contained a full beta-tubulin gene (JQ005863) at 485 bp in length spanning 4 exons and 5 introns. The sequenced amplicon stretched over the intervals 3, 4 and 5. The BLAST Hit with the reference gene was 328 bp long from the 158 to 485 bases. The amplicon also contained ~ 110 bp of DNA sequence following the TUB gene. The BLAST Hit with the *C. orbiculare* genome was the same size as the amplicon at 437 bp long and was spanning the genome contig from the 146,705 to 147,141 bases.

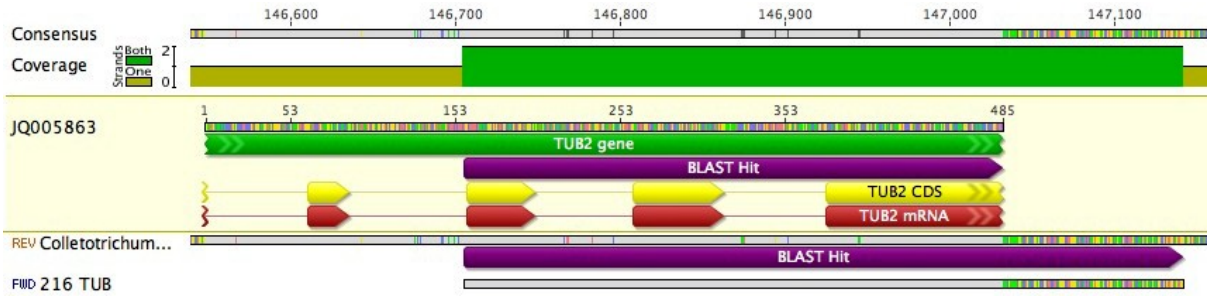


Fig 3.7. *C. lindemuthianum* Beta-Tubulin (TUB) Gene Structure Mapped against Reference Sequence and *C. orbiculare* Genome

The amplified chitin synthase 1 (CHS-1) gene (Fig 3.8.) was 248 bp in length with BLAST Hit within *C. orbiculare* genome of 245 bp and positioned at 1,683,116 to 1,682,872 bp of the genome contig/scaffold. The amplified region contained only the coding sequence and was a partial sequence of CHS-1 gene. The reference gene (Acc.no: JX546660) was larger at 298 bp but was still only a

partial sequence of the CHS-1. The amplicon overlapped from the 53 to 298 bases of the reference sequence.

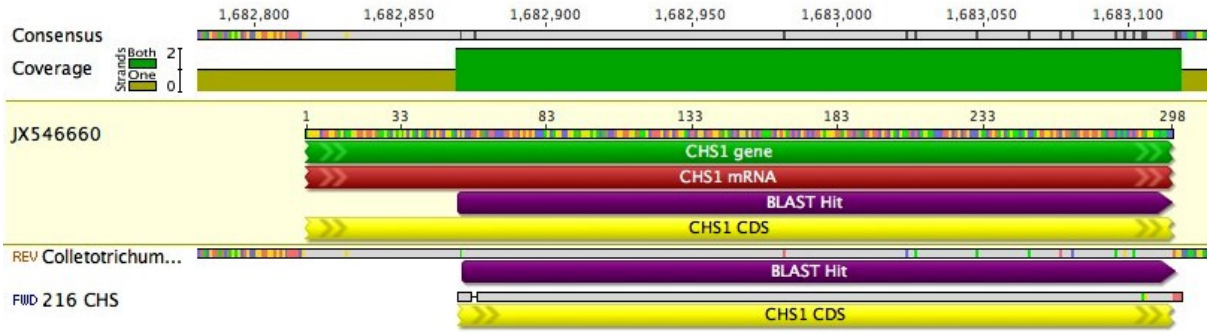


Fig 3.8. *C. lindemuthianum* Chitin Synthase-1 (CHS-1) Gene Structure Mapped against Reference Sequence and *C. orbiculare* Genome

The amplified mating type gene/high mobility group domain (HMG) DNA fragment spanned the MAT1-2-1 fragment of HMG box (Fig 3.9.). The amplicon size was 212 bp covered by 207 bp BLAST Hit from 499,859 to 500,065 bases within the *C. orbiculare* genome contig. The full MAT1-2-1 sequence contains 4 intervals of coding DNA sequence (CDS) with the amplicon spanning parts of interval 3 and 4 including the intervening non-coding sequence.

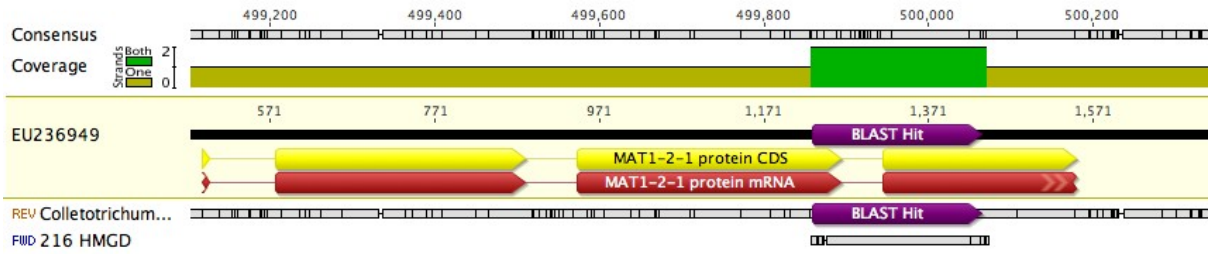


Fig 3.9. *C. lindemuthianum* mating type gene/high mobility group domain (MAT1-2-1 Gene/HMG domain) Structure Mapped against Reference Sequence and *C. orbiculare* Genome

3.3.2. Example of Multiple Sequence Alignment for Selected Colletotrichum Isolates of GAPDH Sequence Data

Sequence data was aligned using ClustalW2 multiple sequence alignment tool available on Geneious and presented in text view (Fig 3.10.). Initially, Geospiza (Finch TV, Appendix VIII) freeware was used for opening the raw sequence trace data and exporting the FASTA files used for the alignment. An example of ClustalW2 multiple sequence alignment is contained in Fig 3.10., while the rest of the alignment files are presented in Appendix VI. The alignment is presented in blocks of 60 bases; the scale above the alignment shows 60 bases at 10 base intervals. On the left side isolate codes are shown running in the same order across the alignment. Gaps (-) introduced by the algorithm to optimise the alignment indicate indels (insertions/deletions). More detailed information on the sequence homology/ divergence is presented in Table 3.5. and Table 3.6. where % values were calculated based on the number of variable nucleotides within the multiple sequence alignment.

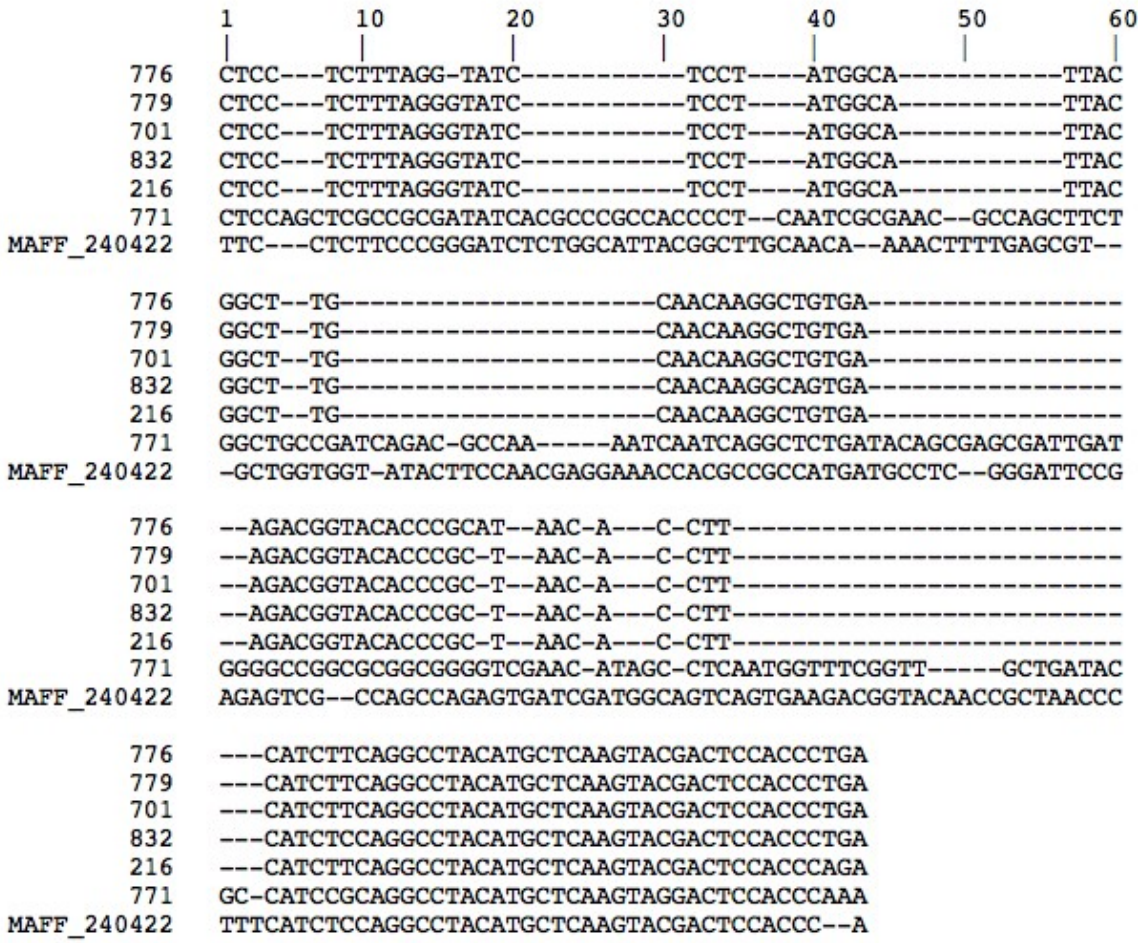


Fig 3.10. Multiple Sequence Alignment of Glyceraldehyde-3-Phosphate Dehydrogenase (GD/GAPDH) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

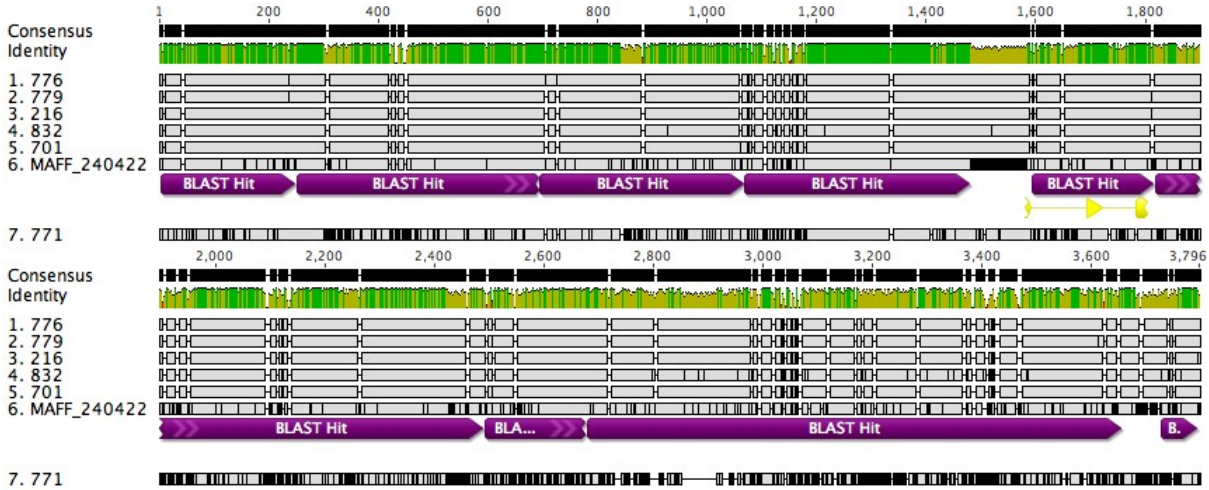


Fig 3.11. Diagrammatic Representation of the Multiple Sequence Alignment Concatenated Sequence Data of the Nine Loci of *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence Generated with Geneious*

*The scale on top shows the size of the sequence running from 1 to 3,796 bp at 200 bp intervals. The top and bottom blocks shows first and second part of the sequence respectively. The black block refers to the consensus sequence with the gaps linked to deletions. The green block below illustrates the level of homology where light green patches represent lower level of homology and the bright green indicate more conserved regions. Level of similarity is also demonstrated by the height of the graph. Refer to labelling legend Table 3.3.

Diagrammatic representation of the concatenated alignment (Fig. 3.11.) provides an overview comparison of sequences. Concatenated sequence data revealed 90.2 - 90.3% homology between *C. lindemuthianum* isolates and *C. orbiculare* MAFF 240422; while *C. gloeosporioides* was within 69.0 - 71.5% range. Similarity values amongst *C. lindemuthianum* isolates varied from 99.0 - 99.9% (Table 3.5. and 3.6.)

3.3.3. Generation of Phylogenetic Trees for the Nine Loci Used for Multilocus Phylogenetic Analysis Based on Multiple Sequence Alignments

The multiple sequence alignments for each locus provided the means for generation of phylogenetic trees (Fig 3.12.-3.20.) illustrating the evolutionary distances between the 6 *Colletotrichum* isolates. Trees were generated using

Bayesian analysis adopting the Jukes and Cantor (1969) model. The bootstrap support values (generated for 10, 000 replicates) varied between 25 - 75% depending on the locus. The *C. gloeosporioides* 771 isolate was used as outgroup. Table 3.4. contains legend for figures 3.12.-3.21. used in this section.

Table 3.4. Labelling Legend to Fig 3.12.-3.21.*

Isolate	Species	Colour
771	*C. gloeosporioides*	Blue
JQ005778; MAFF_240422	*C. orbiculare*	Orange
832	*C. lindemuthianum*	Green
701	*C. lindemuthianum*	Green
776	*C. lindemuthianum*	Green
779	*C. lindemuthianum*	Green
216	*C. lindemuthianum*	Green

*Species are highlighted with different colours.

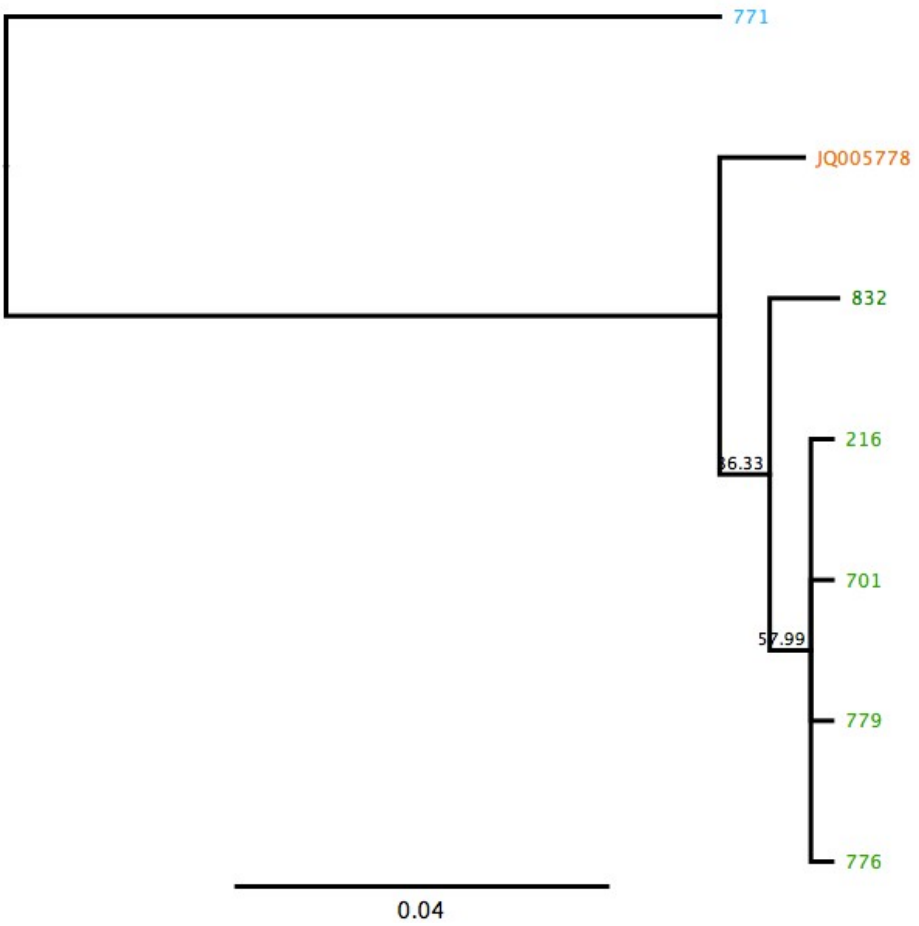


Fig 3.12. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Ribosomal RNA Gene Block Internal Transcribed Spacer (ITS) Region (see Table 3.4 for legend)

While generating the dendrogram/phylogenetic tree based on ITS sequences (Fig 3.12.), JQ005778 sequence of *C. orbiculare* from NCBI was used instead of the MAFF_240422 sequence to optimise the analysis. The ITS data was able resolve the *gloeosporioides* and *orbiculare* clades from the *C. lindemuthianum* isolates. The closer relationship between *C. lindemuthianum* isolates and *C. orbiculare* is illustrated by the common ancestral branching. *C. lindemuthianum* isolates showed 100 % homology apart from the isolate 832 which is separated from the rest as a different haplotype with 99.4% (Appendix VII Table 1.).

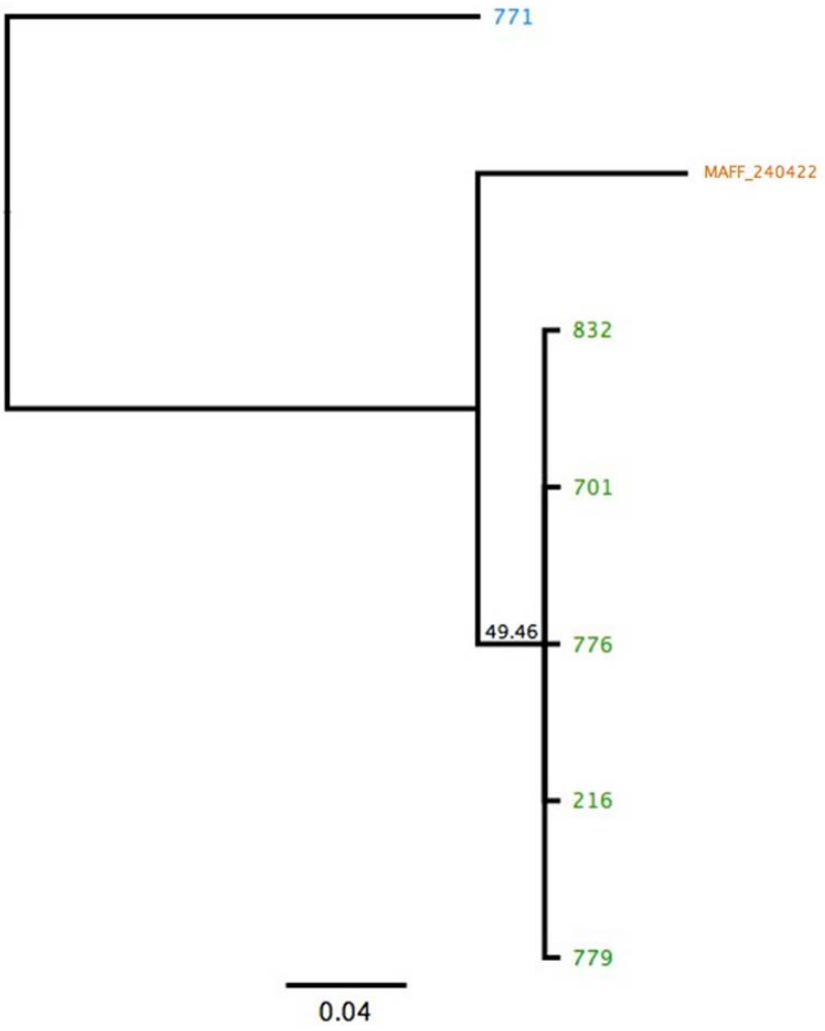


Fig 3.13. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Actin (ACT) Gene (see Table 3.4 for legend)

The ACT gene was one of the highly conserved molecular phylogenetic markers, which can be observed from the tree (Fig 3.13.). All five *C. lindemuthianum* isolates were grouped together although isolate 832 at 99.6 % homology represented a separate haplotype from the others (Table 3.5.). Isolates 701 and 776 had a single ambiguous base within their sequence; despite sequencing of the samples with the reverse primer, the ambiguities could not be resolved.

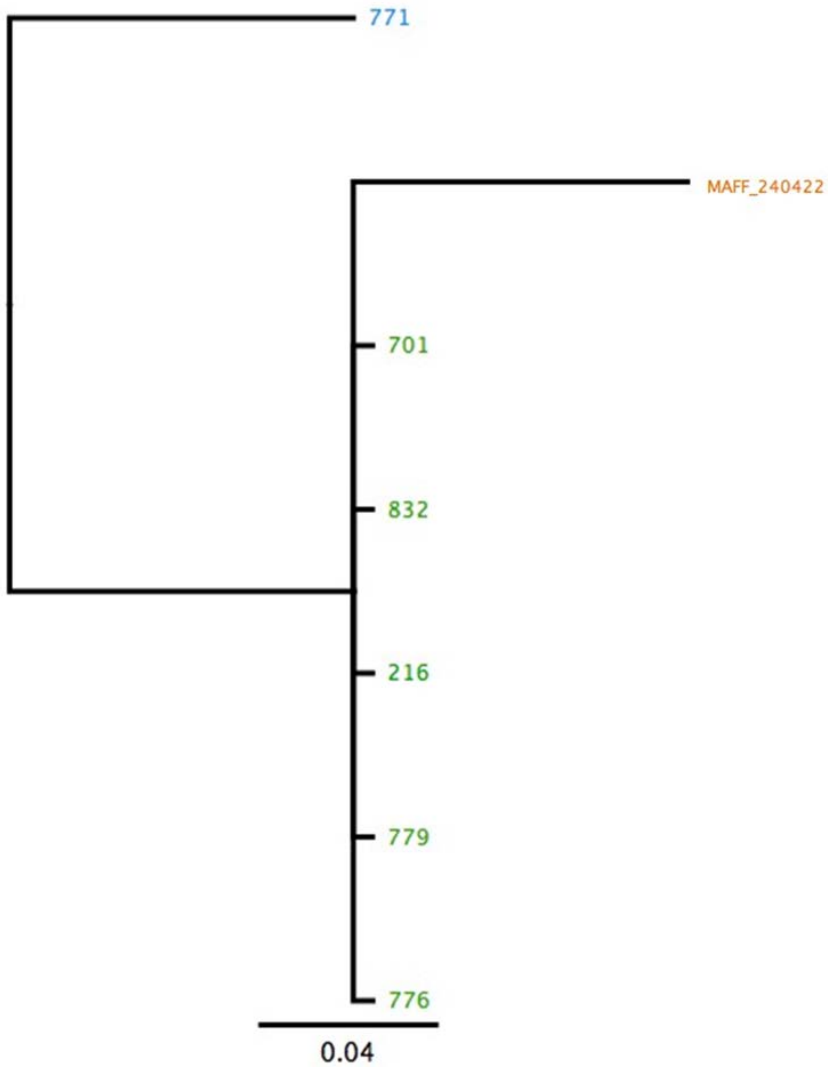


Fig 3.14. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Chitin Synthase-1 (CHS) Gene

The CHS sequence data could not resolve the *C. orbiculare* and *C. lindemuthianum* species complex and the branches were collapsed (Fig 3.14.). Within *C. lindemuthianum* two haplotypes could be distinguished based on the sequence data: 216, 701 and 832 represented first haplotype at 100% homology; 776 and 779 were assigned to 2nd haplotype with 99.6% homology to the first haplotype (Table 3.5.).

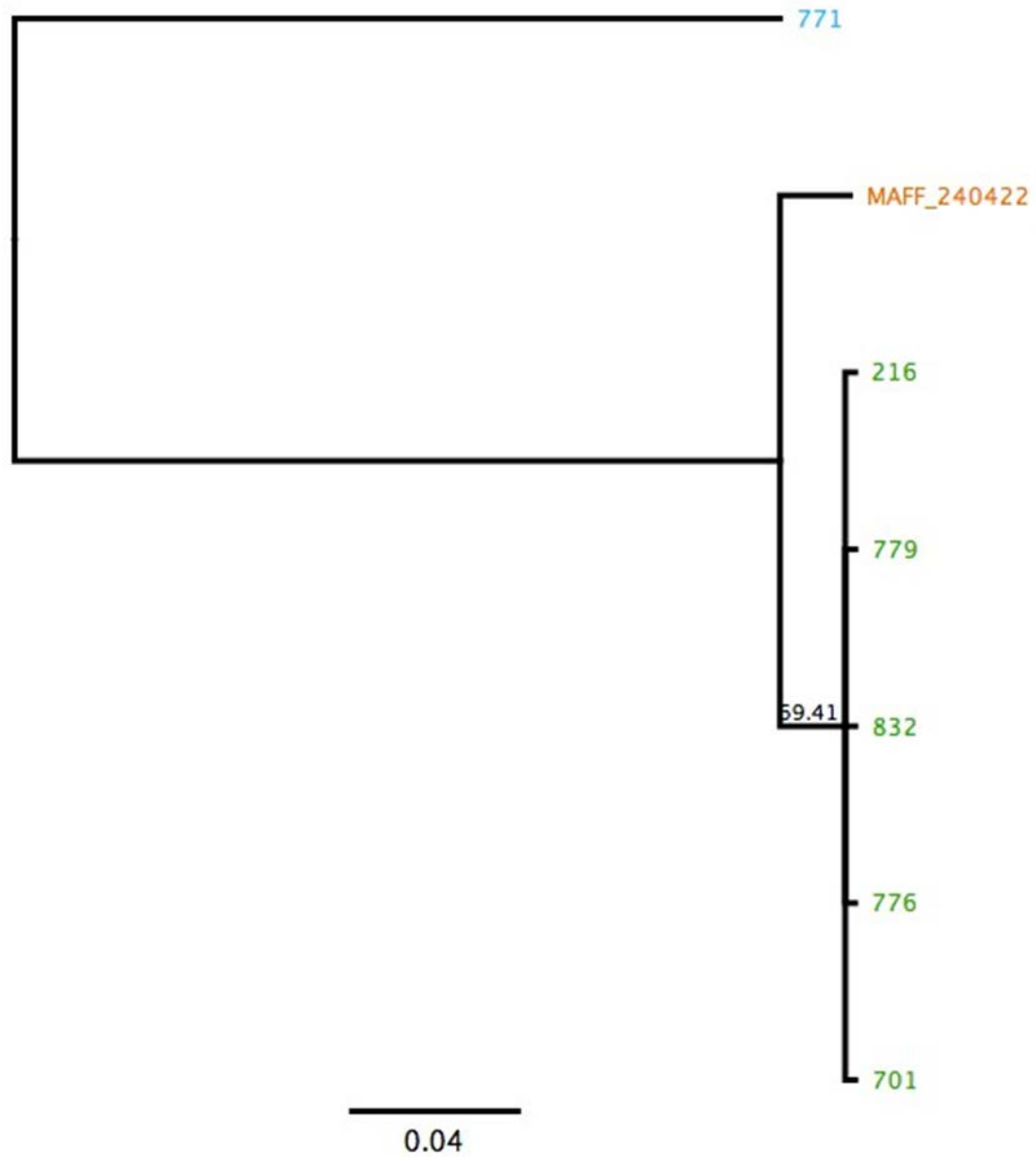


Fig 3.15. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Beta-Tubulin (TUB) Gene

The TUB as well as CL were the most conserved amongst the various loci tested with 100% homology among the *C. lindemuthianum* isolates (Table 3.5 and Appendix VII Table 4.). However, the *C. lindemuthianum* isolates were differentiated from the orbiculare clade (Fig 3.15. and 3.16. respectively).

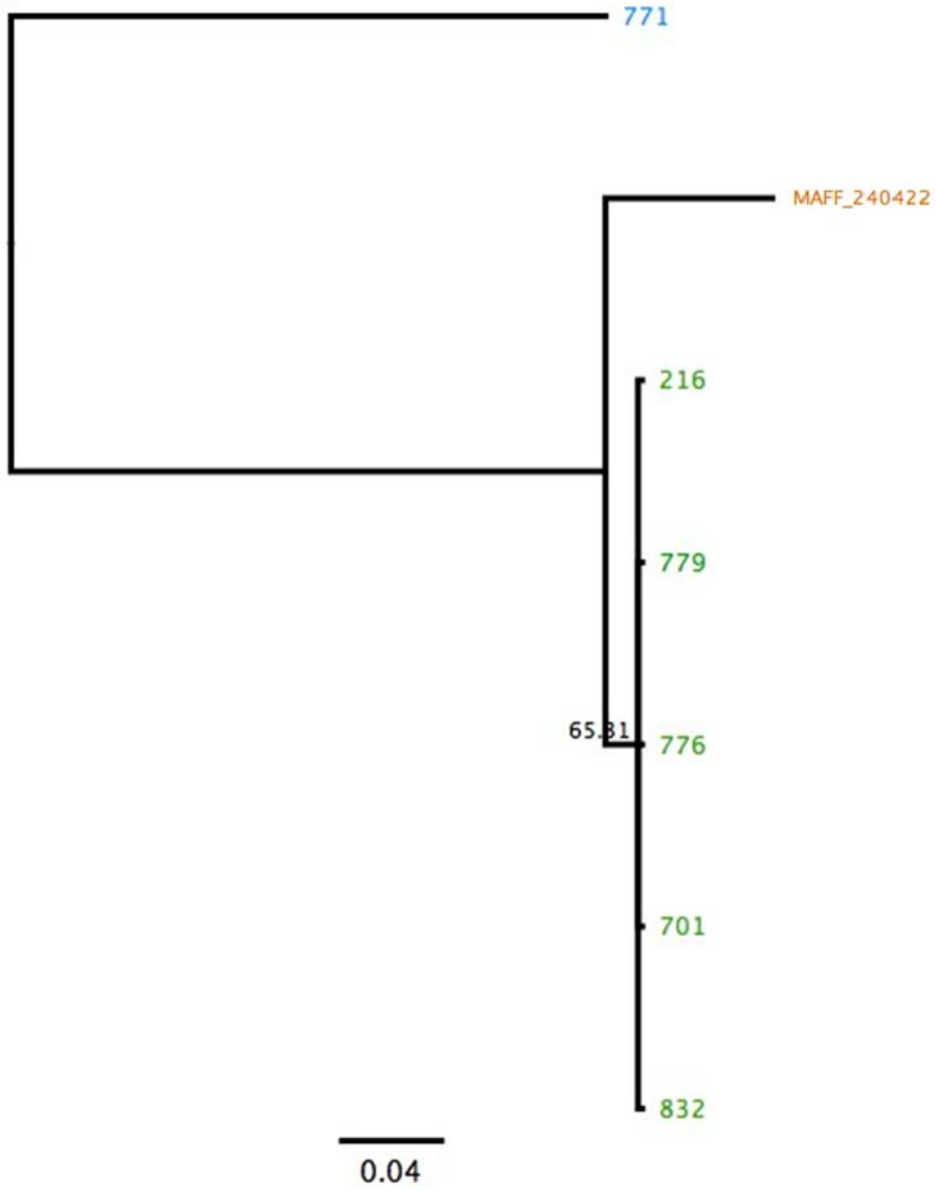


Fig 3.16. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Calmodulin (CAL) Gene

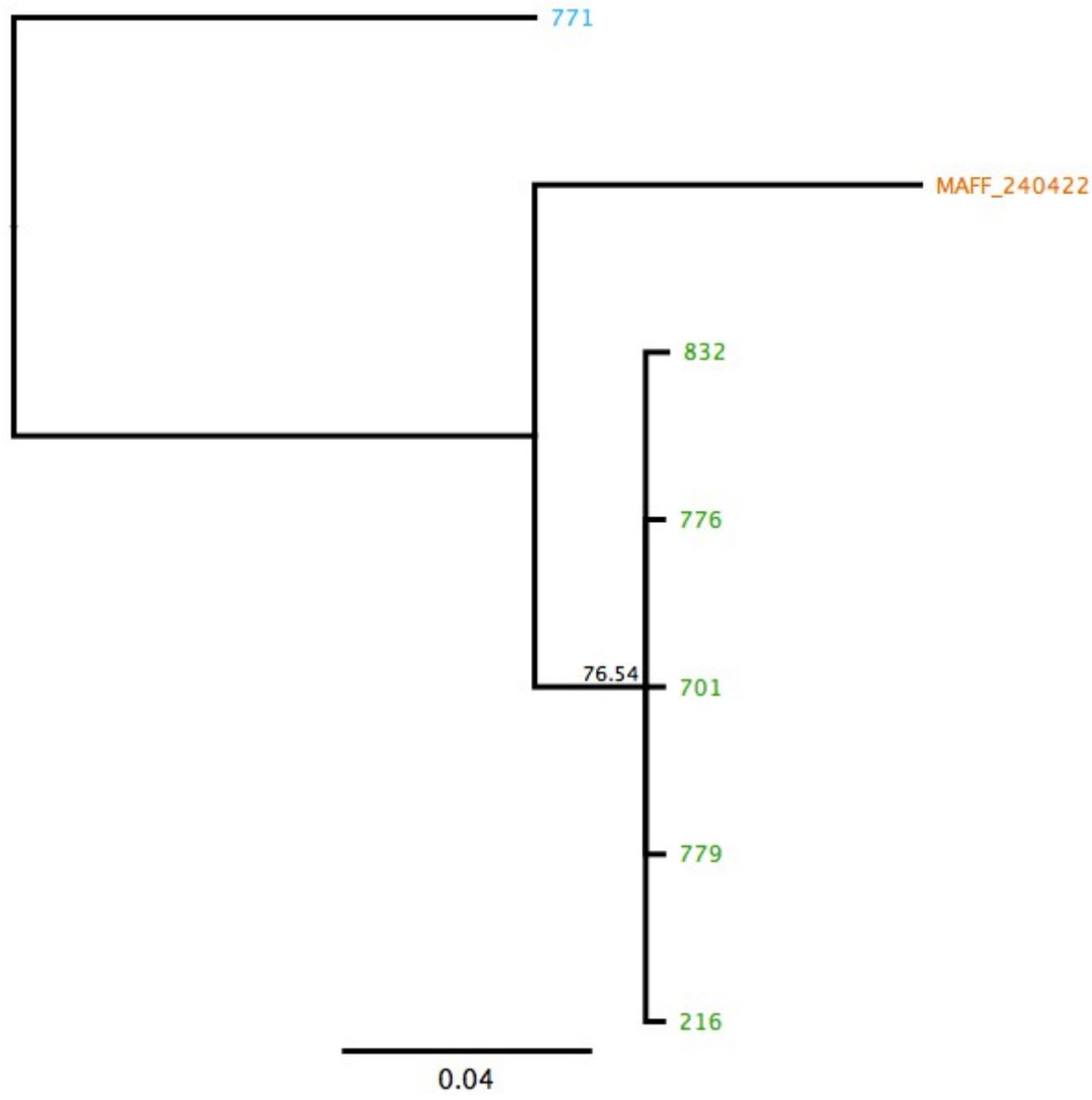


Fig 3.17. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Histone 3 Gene

The histone gene sequence revealed 4 haplotypes within the *C. lindemuthianum* species complex. Isolates 216 and 779 with 100 % homology represented a haplotype; whilst 701, 776 and 832 each represented an individual haplotype (Table 3.5.). The *C. lindemuthianum* isolates were well differentiated from the *orbiculare* clade with bootstrap value at 76.54% (Fig 3.17.).

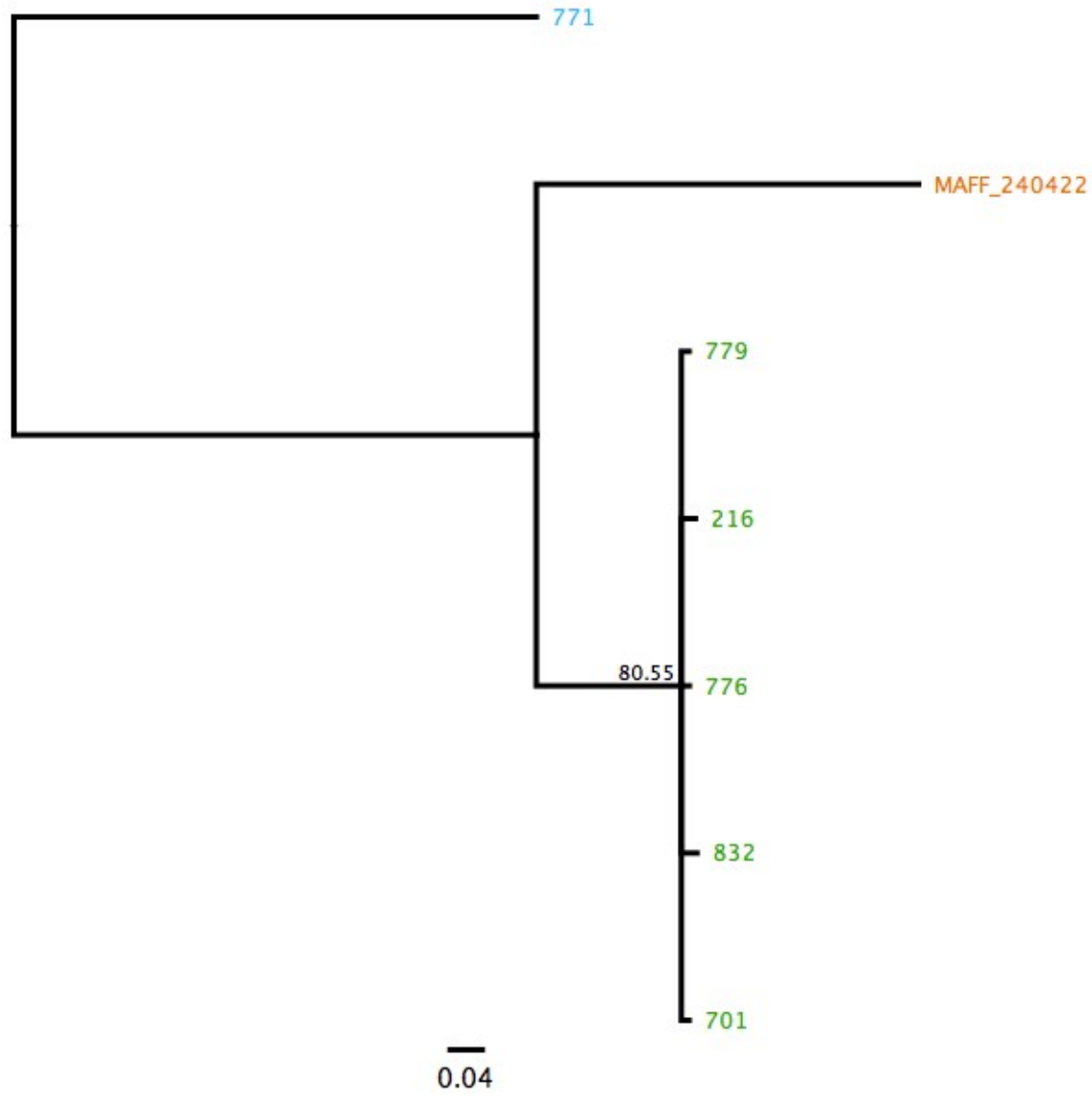


Fig 3.18. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Glyceraldehyde-3-Phosphate Dehydrogenase (GD/GAPDH) Gene

The GAPDH sequence also revealed 4 haplotypes within the *C. lindemuthianum* species. Isolates 216, 701,779, and 776 at 100% homology represented a haplotype; whilst and 832 was an individual haplotype (Appendix VII Table 2). The *C. lindemuthianum* species complex was well differentiated from/within the *orbiculare* clade despite low number of isolates with high bootstrap values at 80.55% (Fig 3.18.).

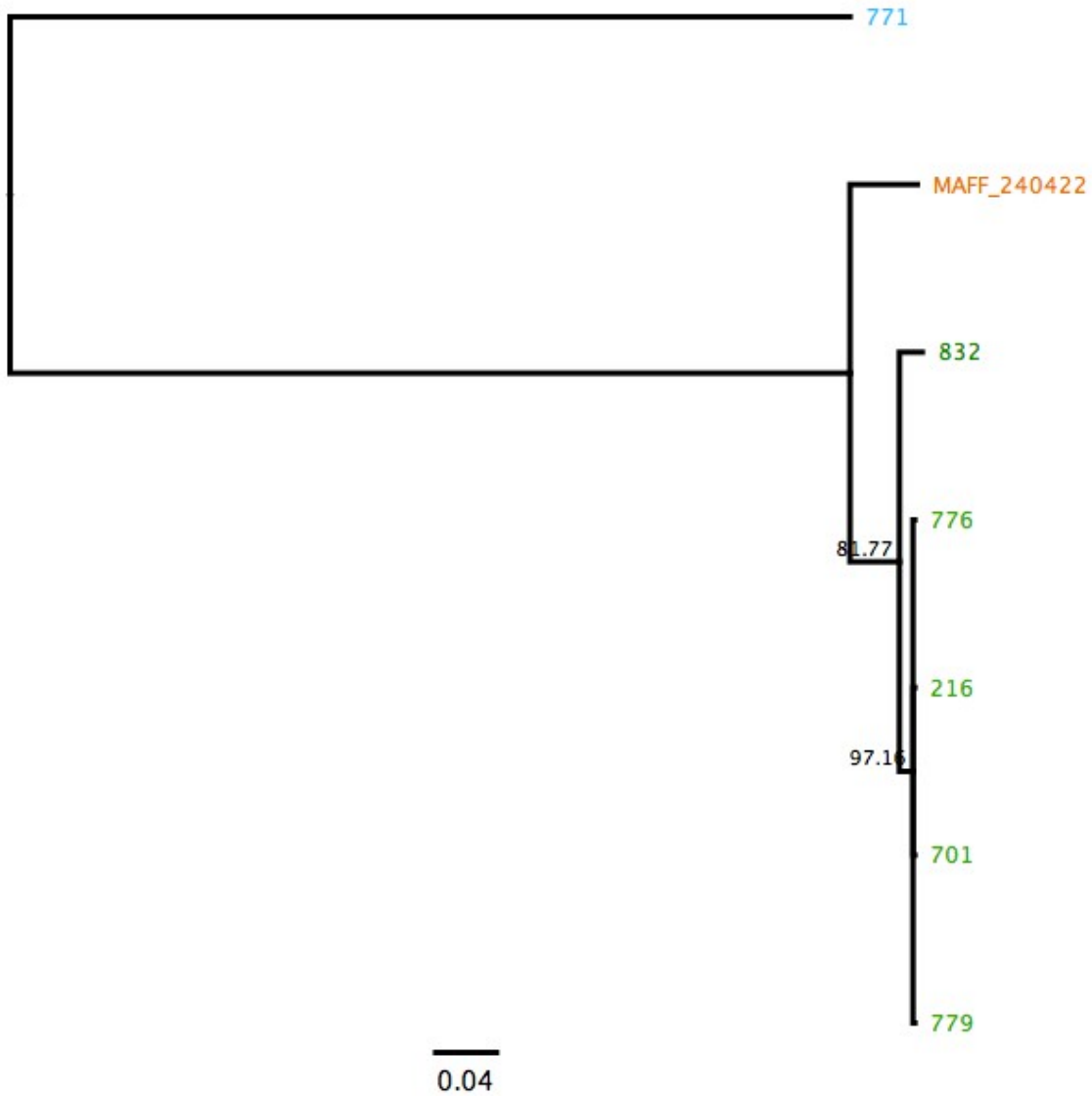


Fig 3.19. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Glutamine Synthetase (GS) Gene

The GS gene was moderately conserved (Fig 3.19.) and two haplotypes were distinguished represented by 832 at 97.4% homology to 216, 701, 776 and 779 had 100% homology amongst them (Appendix VII Table 3.). The separation of the 832 isolate representing a separate genetic group was supported at bootstrap value 81.77%. The rest of *C. lindemuthianum* isolates were grouped together with bootstrap support value 97.16%.

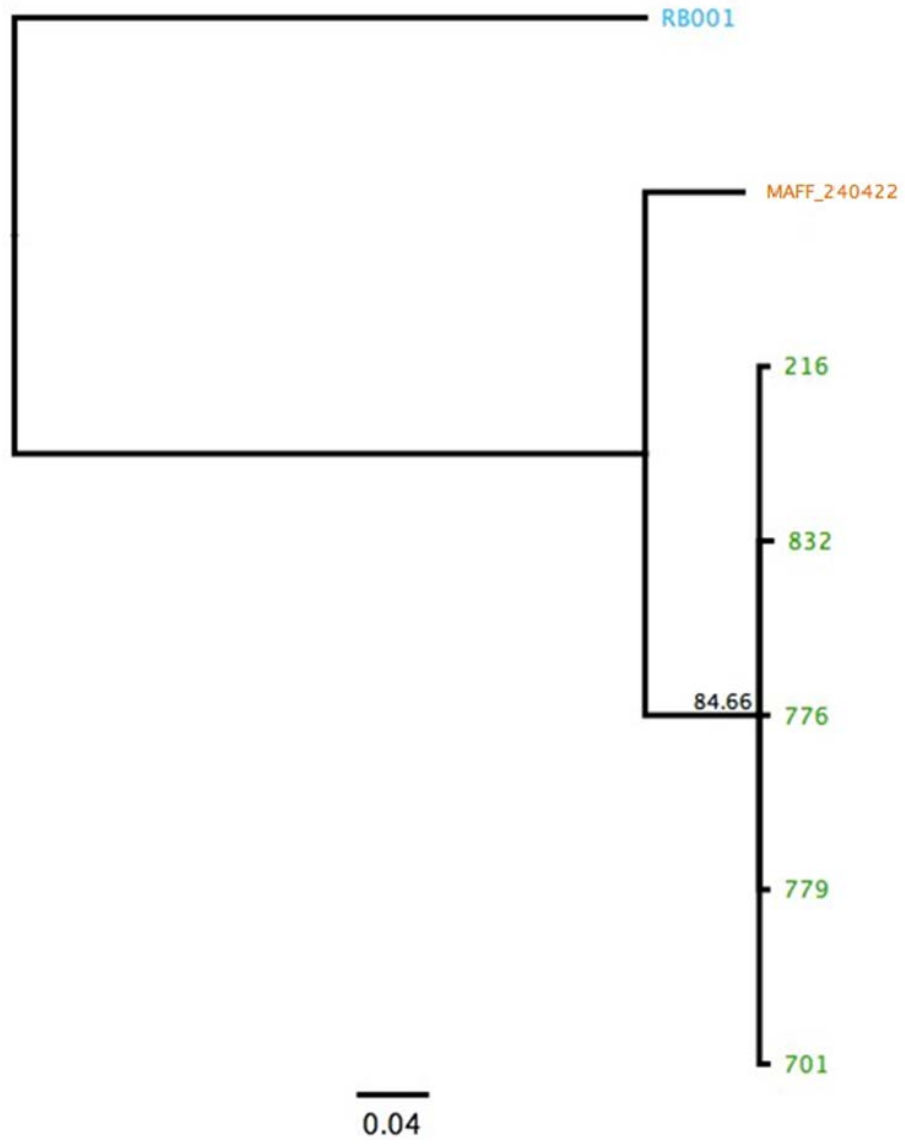


Fig 3.20. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Mating Type Gene/High Mobility Group Domain.

The HMG gene was the molecular marker linked to the reproductive biology of the fungi and it differentiated the *C. lindemuthianum* isolates within the tree at 84.66% bootstrap support value (Fig 3.20.). One haplotype was represented by isolates 216 and 776, a 2nd haplotype by isolates 701 and 779, and a 3rd haplotype by isolate 832 (Appendix VII Table 5).

3.3.4. Generation of Consensus Tree Using Concatenated Multiple Sequence Alignment for All Nine Loci

The consensus tree (Fig 3.21.) was built based on concatenated multiple sequence alignment containing sequence data generated for all nine loci. The tree revealed the close evolutionary relationships between isolates within *C. lindemuthianum* species. Isolates 779, 776, 701 and 216 were recognized as one genetic group with bootstrap value 99.98 % within which isolates 776 and 779 were identified as a subgroup resolved at bootstrap value of 98.24 %. Isolate 832 was separated as representing a separate genetic group with bootstrap value of 99.98 %. The closer relationship between *C. orbiculare* and *C.lindemuthianum* is well reflected by the tree and the *C. gloeosporioides* remains distinctly separated from the *orbiculare* clade.

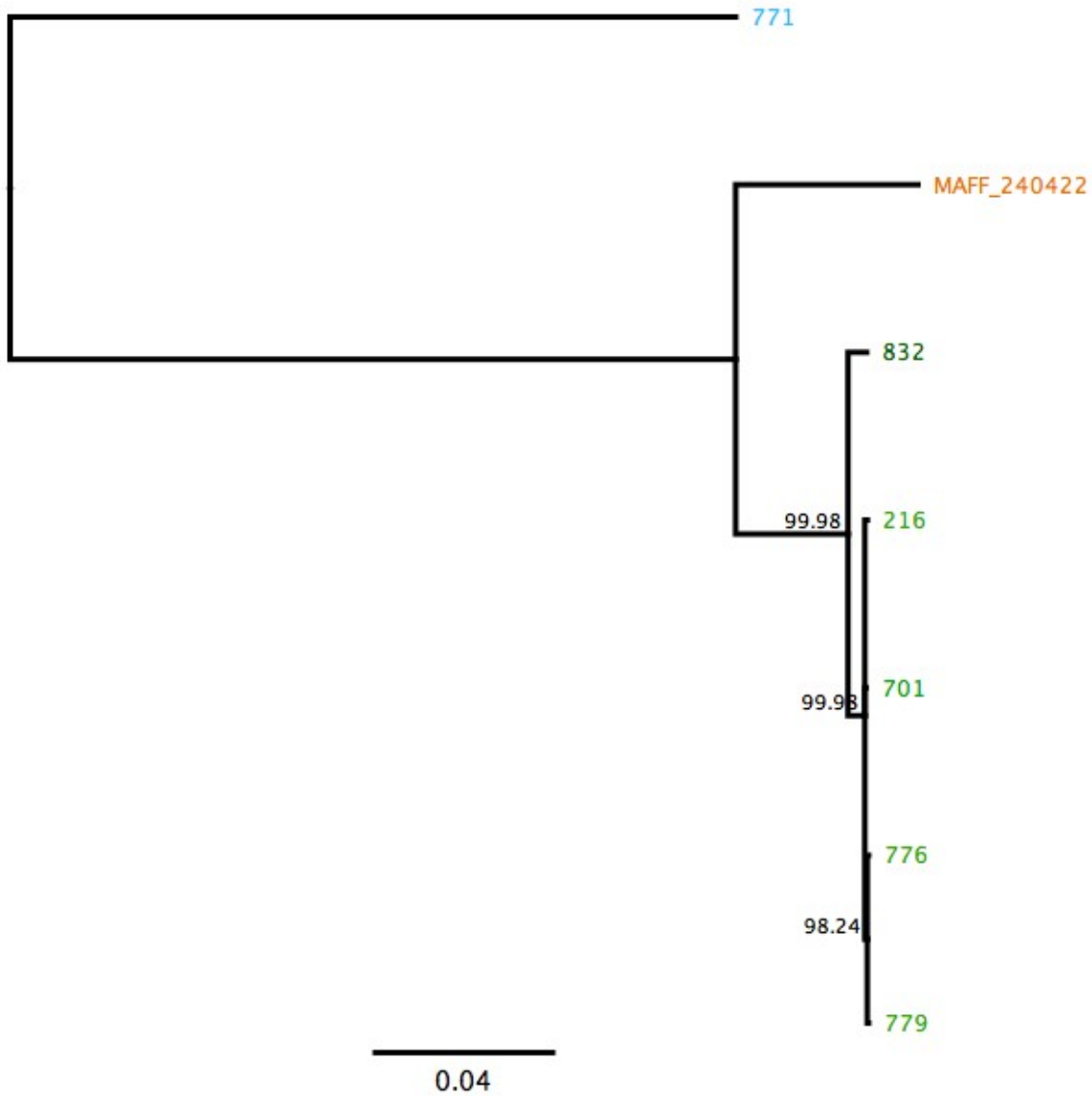


Fig 3.21. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis using Jukes and Cantor (1969) Model Based on Concatenated Multiple Sequence Alignment (including: ITS, ACT, CHS, HIS3, TUB, GS, GAPDH, CAL, and HMG)*

*Bootstrap support values (10, 000 replicates) above 90% are shown at the nodes.

3.3.5. The Resolution of the Loci Based on Sequence Variation and Haplotype Identification

Homology and divergence values were calculated using the sequence data generated from nine different loci for the six *Colletotrichum* isolates. Data for four loci and concatenated alignment is shown in Table 3.5.and 3.6. respectively while the rest of the molecular markers datasets are presented in Appendix VII Table 1-6. The values were obtained based on the generation of multiple sequence alignment, and provide an overview of the level of resolution of the markers and their ability in differentiating the haplotypes within *C. lindemuthianum* species. For examples, the GD marker had the highest resolution within the *C. lindemuthianum* species that distinguished four haplotypes. Markers CAL and TUB were the most conserved with 100% similarity amongst all *C. lindemuthianum* isolates (Table 3.5. and Appendix VII Table 4).

Table 3.5. Sequence Homology and Divergence between *Colletotrichum* Isolates Based on Sequence Data from Four Different Loci

CHS Sequence* Homology and Divergence Between *Colletotrichum* Isolates (%)							
Isolates	216	701	776	779	832	MAFF	771
216	-	0	0.4	0.4	0	7.2	10.4
701	100.0	-	0.4	0.4	0	7.2	10.4
776	99.6	99.6	-	0	0.4	6.8	10.8
779	99.6	99.6	100.0	-	0.4	6.8	10.8
832	100.0	100.0	99.6	99.6	-	7.2	10.4
MAFF	92.8	92.8	93.2	93.2	92.8	-	13.2
771	89.6	89.6	89.2	89.2	89.6	86.8	-

*Chitin synthase-1 gene sequence.

ACT Sequence* Homology and Divergence Between *Colletotrichum* Isolates (%)							
Isolates	216	701	776	779	832	MAFF	771
216	-	0.4	0.4	0	0.4	8.5	20.4
701	99.6	-	0	0.4	0	8.9	20.9
776	99.6	100.0	-	0.4	0	8.9	20.9
779	100.0	99.6	99.6	-	0.4	8.5	20.4

832	99.6	100.0	100.0	99.6	-	8.9	20.9
MAFF	91.5	91.1	91.1	91.5	91.1	-	24.4
771	79.6	79.1	79.1	79.6	79.1	75.6	-

*Actin gene sequence.

CAL Sequence* Homology and Divergence Between *Colletotrichum* Isolates (%)							
Isolates	216	701	776	779	832	MAFF	771
216	-	0	0	0	0	7.7	30.3
701	100.0	-	0	0	0	7.7	30.3
776	100.0	100.0	-	0	0	7.7	30.3
779	100.0	100.0	100.0	-	0	7.7	30.3
832	100.0	100.0	100.0	100.0	-	7.7	30.3
MAFF	92.3	92.3	92.3	92.3	92.3	-	32.4
771	69.7	69.7	69.7	69.7	69.7	67.6	-

*Calmodulin gene sequence

HIS3 Sequence Homology and Divergence Between *Colletotrichum* Isolates (%)							
Isolates	216	701	776	779	832	MAFF	771
216	-	0.3	0.5	0	0.3	8.0	13.8
701	99.7	-	0.8	0.3	0.5	8.3	13.6
776	99.5	99.2	-	0.5	0.8	8.0	14.3
779	100.0	99.7	99.5	-	0.3	8.0	13.8
832	99.7	99.5	99.2	99.7	-	7.8	13.6
MAFF	92.0	91.7	92.0	92.0	92.2	-	16.4
771	86.2	86.4	85.7	86.2	86.4	83.6	-

*Histone 3 gene sequence.

+*C. orbiculare* isolate MAFF_240422 was referred in the Table 3.5. as MAFF.

High mobility group domain/mating type locus gene sequence (HMG) primers were used to amplify all 6 *Colletotrichum* isolates. However, they did not yield an amplicon with the 771 isolate despite the fact that this primer pair was degenerate and designed for *Colletotrichum spp*. Therefore NCBI database was searched for *C. gloeosporioides* sequence data for the same region; the closest BLAST hit was represented by sequence RB001 which was included for the comparative analysis in this study (Appendix VII Table 5).

Based on the concatenated multiple sequence alignment for all molecular markers the total homology and divergence values were calculated (Table 3.6.) illustrating the comprehensive relationships between the six *Colletotrichum* isolates used.

Table 3.6. Concatenated Sequence Homology and Divergence between *Colletotrichum* Isolates (%)

Isolates	216	701	776	779	832	MAFF	771
216	-	0.1	0.2	0.1	0.9	9.7	28.5
701	99.9	-	0.2	0.1	0.9	9.7	28.5
776	99.8	99.8	-	0.2	1.0	9.7	28.6
779	99.9	99.9	99.8	-	0.9	9.7	28.5
832	99.1	99.1	99.0	99.1	-	9.8	28.6
MAFF*	90.3	90.3	90.3	90.3	90.2	-	31.0
771	71.5	71.5	71.4	71.5	71.4	69.0	-

The difference in the sequence value was generally due to indels (insertion/deletion), however in case of *C. gloeosporioides* GD/GAPDH sequence a much larger fragment was amplified (Table 3.7.). Amplicon was 115 bp long in original sequence in the case *of C. lindemuthianum* isolates, while it was 205 bp for 771 isolate. However, the data was reduced to only 98 bp for GAPDH marker in order to align all *Colletotrichum* isolates. This type of variation is also observed for GS DNA fragment where *C. lindemuthianum* isolates range from 871-875 while *C. gloeosporioides* amplicon is much shorter at only 759 bp.

Table 3.7. Amplicon Size of Each Locus for *Colletotrichum* Isolates (bp)*

Isolate / Locus	216	701	776	779	832	771
ITS	500	500	500	500	500	511
CHS	248	248	248	248	248	250
ACT	232	231	231	232	231	229
CL	648	648	648	648	648	673
HIS	371	372	373	371	371	373
GS	871	871	871	871	875	759
GD/GAPDH	98	98	99	98	98	98
TUB	437	437	437	437	437	449
HMG	200	201	200	201	200	172**

*Raw sequence data was edited to optimize the alignment..

**Data obtained from NCBI Accession No: RB001

CHAPTER 3

PART II. Results of Multilocus Phylogenetic Analysis of *Colletotrichum* Isolates

Based on the results from Part I, five loci ranging from conserved to highly variable such as ITS, TUB, GD, GS and HMG were selected for the multilocus phylogenetic analysis of 18 *Colletotrichum* isolates including the six used in the intial screening (Table 2.1.).

3.4. Multilocus Molecular Phylogenetic Analysis

3.4.1. Assembling Multiple Sequence Alignment from Colletotrichum Sequence Data for 5 Selected Markers/Loci

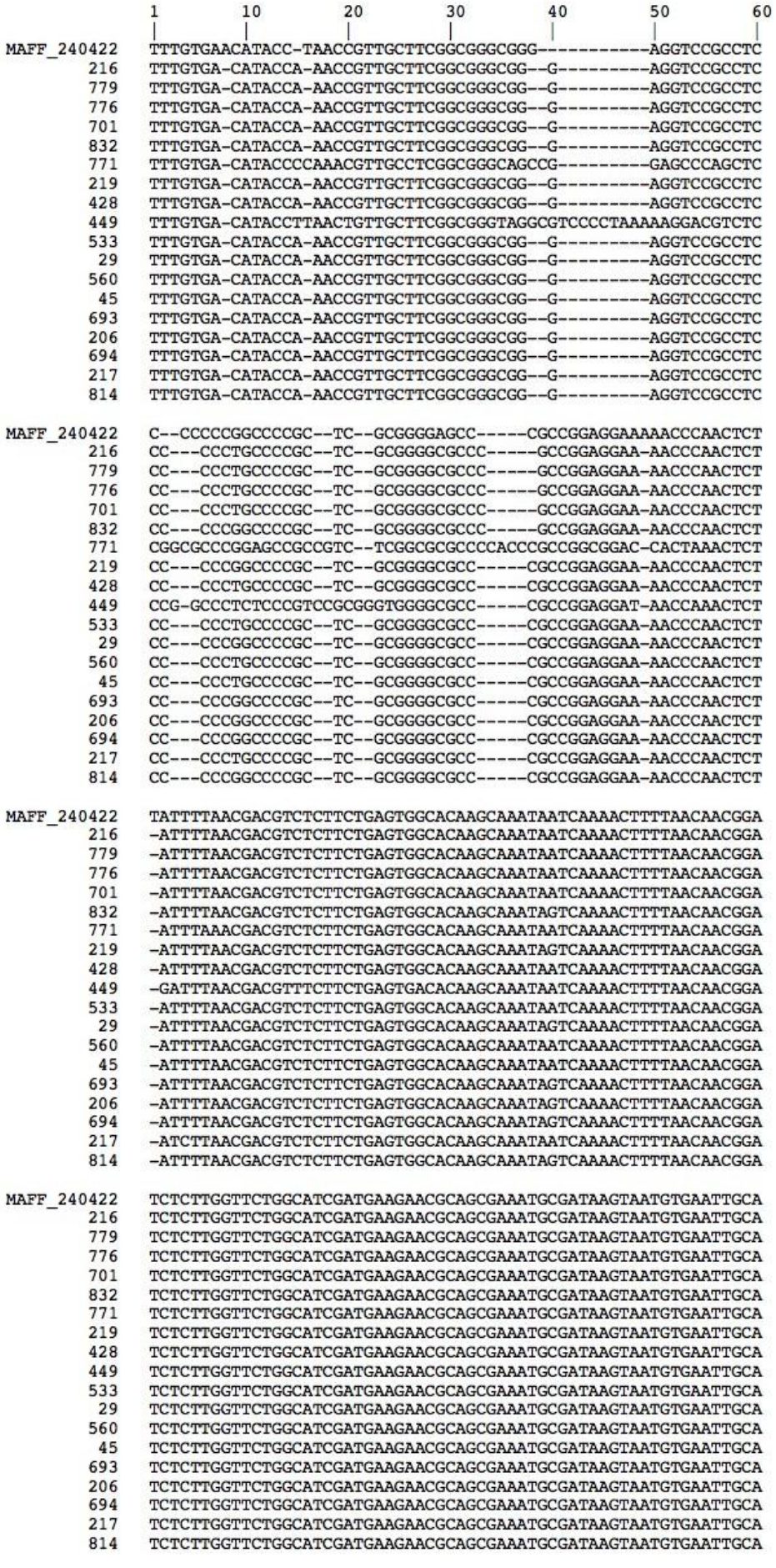
Fasta files generated from the ABI trace data files were opened with Geneious and aligned using ClustalW for each molecular marker/locus (see Fasta files in Appendix VI). Sample illustrating the Geneious alignment output (Fig. 3.22.) contains all *Colletotrichum* sequence data generated for GD locus against the *C. orbiculare* isolate MAFF_240442 sequence. The most variable sequences generated for *C. lindemuthianum* were expressed by 3 isolates: 694, 814 and 832 due to two nucleotide substitutions at 33rd base where ‘T’ was replaced by ‘A’ and at 69th base where ‘T’ was substituted for ‘C’; the ‘A’ insertion at the 55th base position of the isolate 776 requires further validation.

Fig 3.22. Sample of GAPDH Alignment View Generated by Geneious*

*The top scale shows the number of bases within the sequence in 10 bp intervals. Consensus identity is the sequence generated by Geneious based on compared isolates sequence information. Nucleotides are colour coded. Numbers 1-16 represent *C. lindemuthianum* isolates followed by *C. orbiculare* (MAFF_240422), *C. gloeosporioides,* and *C truncatum* respectively.

Multiple sequence alignments for all *Colletotrichum* isolates was generated for ITS, TUB, GS, GAPDH and HMG (Fig 3.23.-3.27.) loci. The percentage homology/divergence values calculated based on the aligned sequence data are presented in Appendix VII Tables 1-6 based on the number of variable nucleotides.

MrBayes was used for the creation of phylogenetic trees based on maximum likelihood analysis of the multiple sequence alignment which is then analysed using Marcov chain Monte Carlo method for calculation of the posterior probabilities distribution of the multiple phylogenetic trees (Huelsenbeck and Ronquist, 2001) and to identify a consensus tree illustrating the most optimal representation of phylogenetic relationships (Mau *et al*., 1999).


1 10 20 30 40 50 60
MAFF_240422 TTTGTGAACATACC-TAACCGTTGCTTCGGCGGGCGGG----------AGGTCCGCCTC
216 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
779 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
776 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
701 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
832 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
771 TTTGTGA-CATACCCCAAACGTTGCCTCGGCGGGCAGCCG---------GAGCCCAGCTC
219 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
428 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
449 TTTGTGA-CATACCTTAACTGTTGCTTCGGCGGGTAGGCGTCCCCTAAAAAGGACGTCTC
533 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
29 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
560 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
45 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
693 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
206 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
694 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
217 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
814 TTTGTGA-CATACCA-AACCGTTGCTTCGGCGGGCGG--G---------AGGTCCGCCTC
MAFF_240422 C--CCCCCGGCCCCGC--TC--GCGGGGAGCC-----CGCCGGAGGAAAAACCCAACTCT
216 CC---CCCTGCCCCGC--TC--GCGGGGCGCCC-----GCCGGAGGAA-AACCCAACTCT
779 CC---CCCTGCCCCGC--TC--GCGGGGCGCCC-----GCCGGAGGAA-AACCCAACTCT
776 CC---CCCTGCCCCGC--TC--GCGGGGCGCCC-----GCCGGAGGAA-AACCCAACTCT
701 CC---CCCTGCCCCGC--TC--GCGGGGCGCCC-----GCCGGAGGAA-AACCCAACTCT
832 CC---CCCGGCCCCGC--TC--GCGGGGCGCCC-----GCCGGAGGAA-AACCCAACTCT
771 CGGCGCCCGGAGCCGCCGTC--TCGGCGCGCCCCACCCGCCGGCGGAC-CACTAAACTCT
219 CC---CCCGGCCCCGC--TC--GCGGGGCGCC-----CGCCGGAGGAA-AACCCAACTCT
428 CC---CCCTGCCCCGC--TC--GCGGGGCGCC-----CGCCGGAGGAA-AACCCAACTCT
449 CCG-GCCCTCTCCCGTCCGCGGGTGGGGCGCC-----CGCCGGAGGAT-AACCAAACTCT
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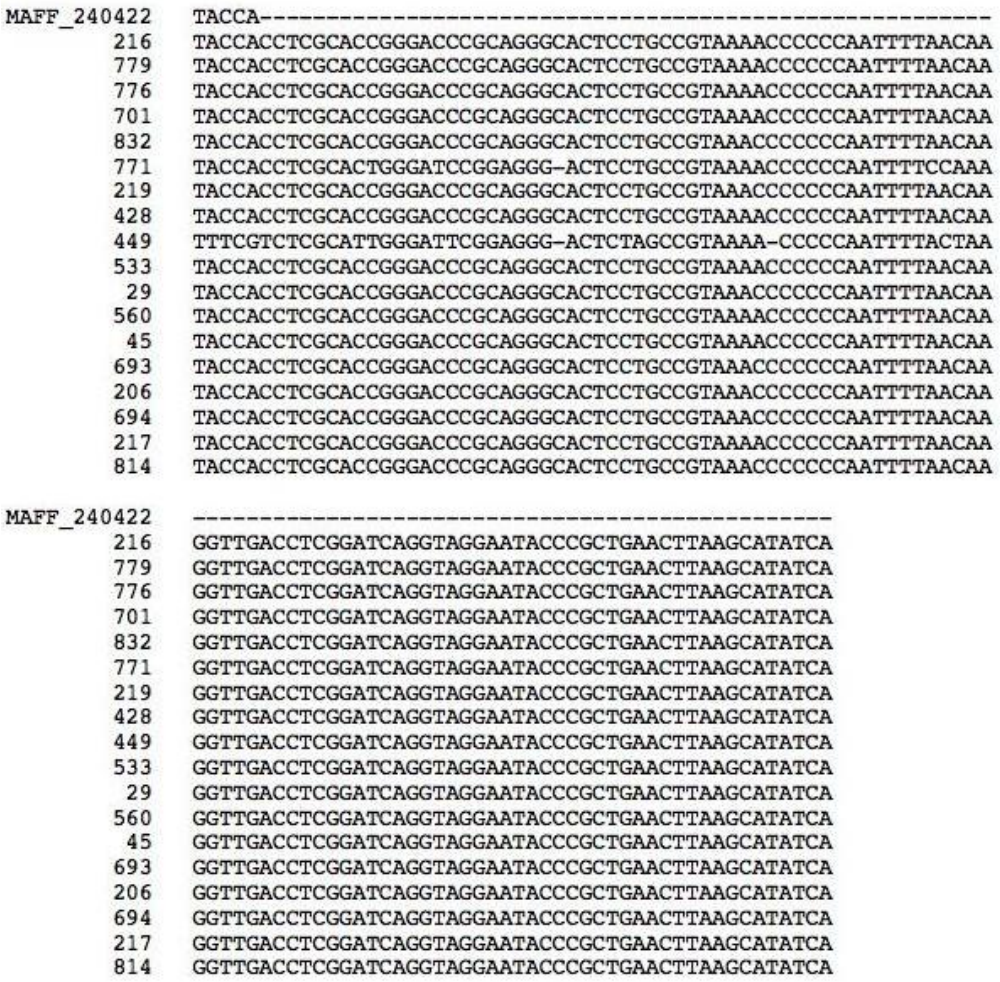
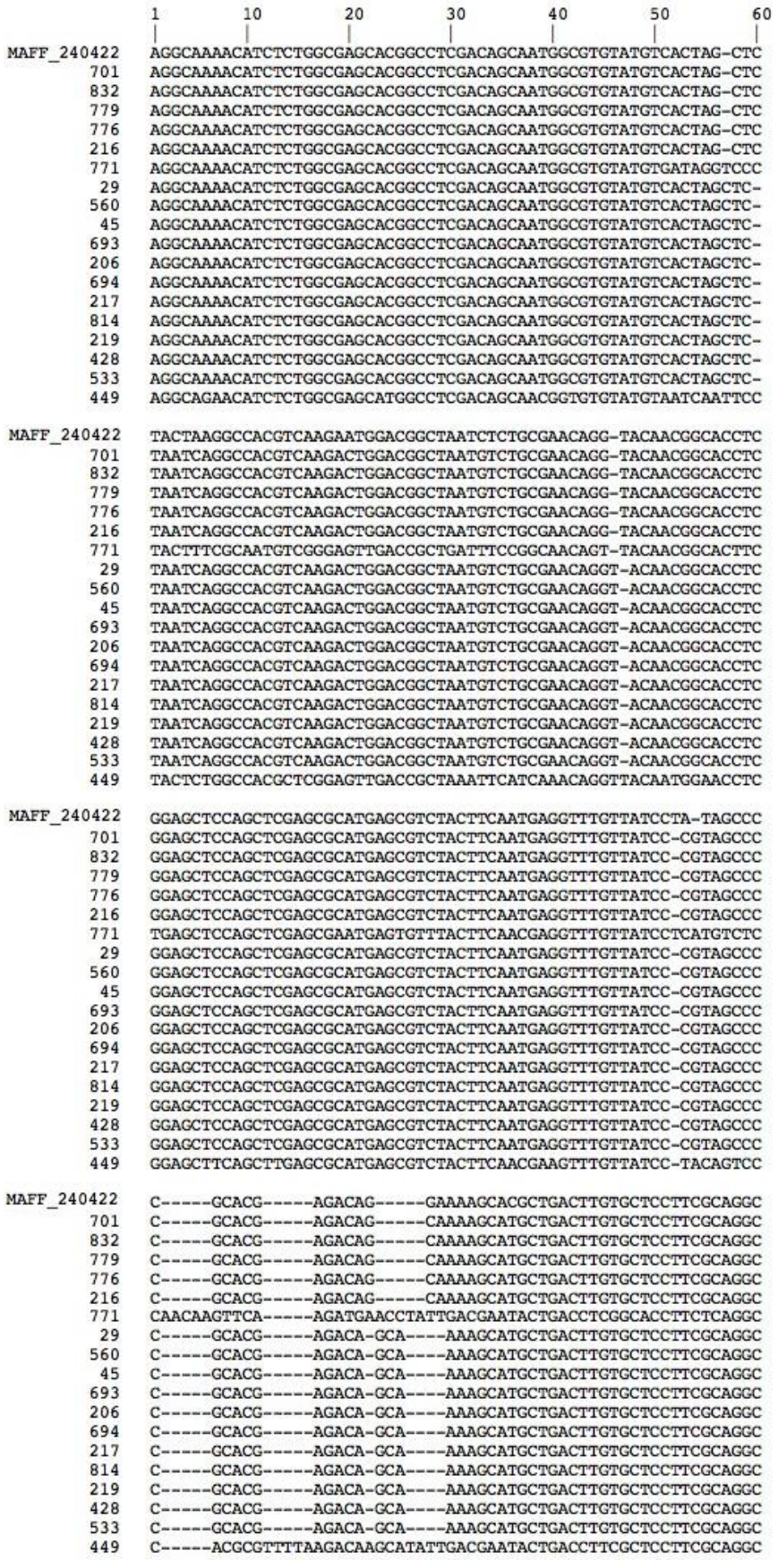


Fig 3.23. Multiple Sequence Alignment of Ribosomal RNA Gene Block Internal Transcribed Spacer (ITS) Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.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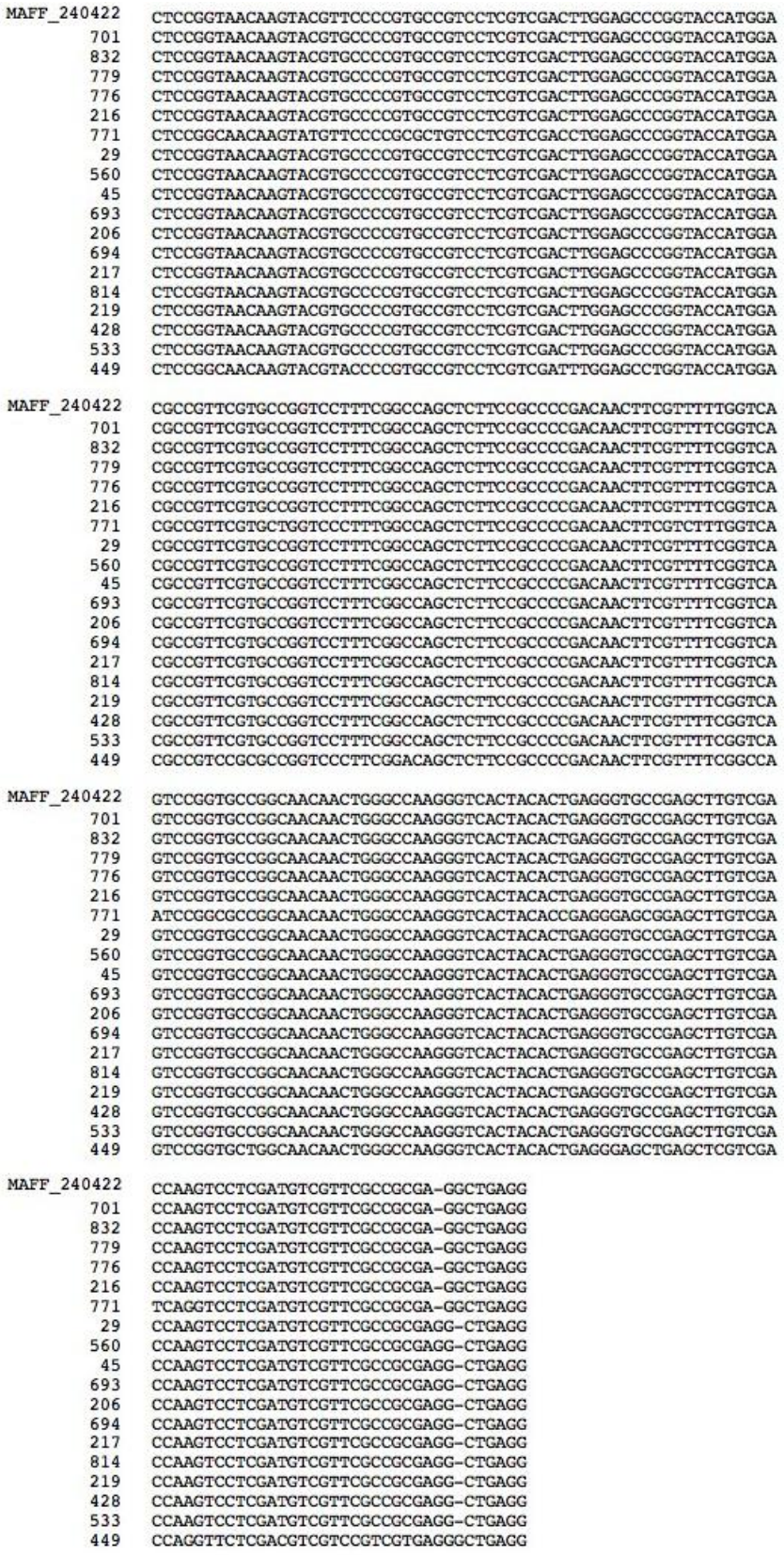


Fig 3.24 Multiple Sequence Alignment of Beta-Tubulin (TUB) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

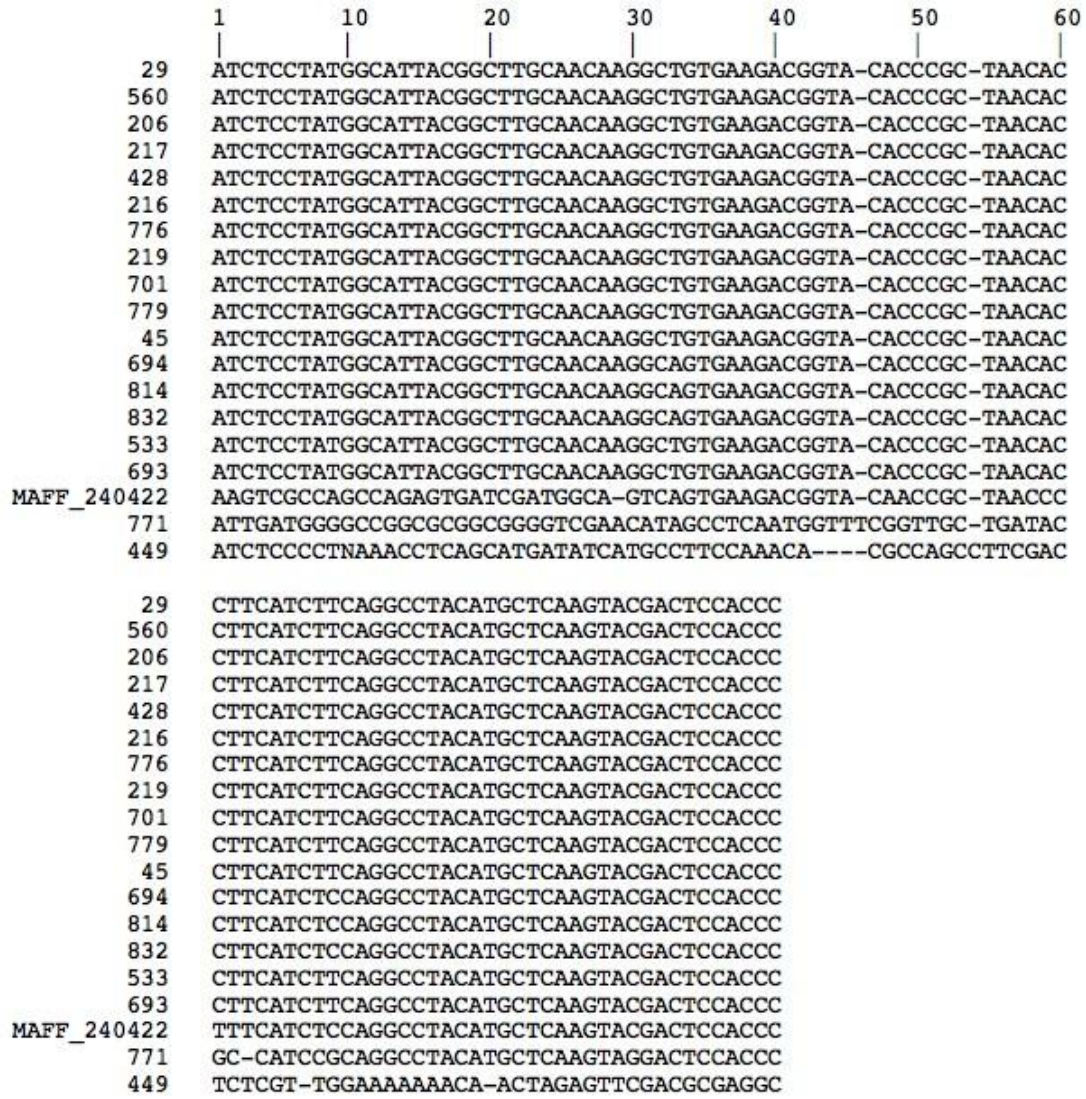


Fig 3.25. Multiple Sequence Alignment of Glyceraldehyde-3-Phosphate Dehydrogenase (GD/GAPDH) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

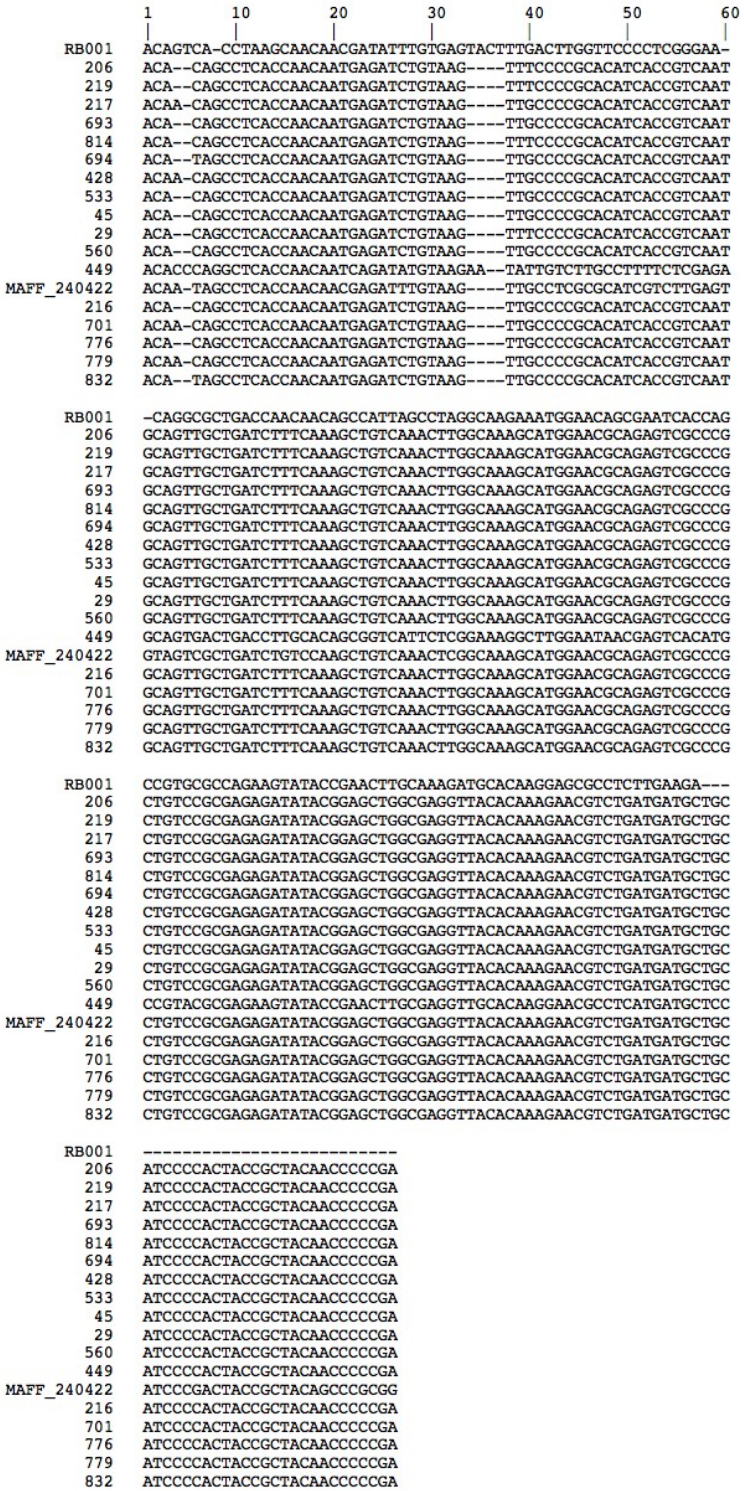
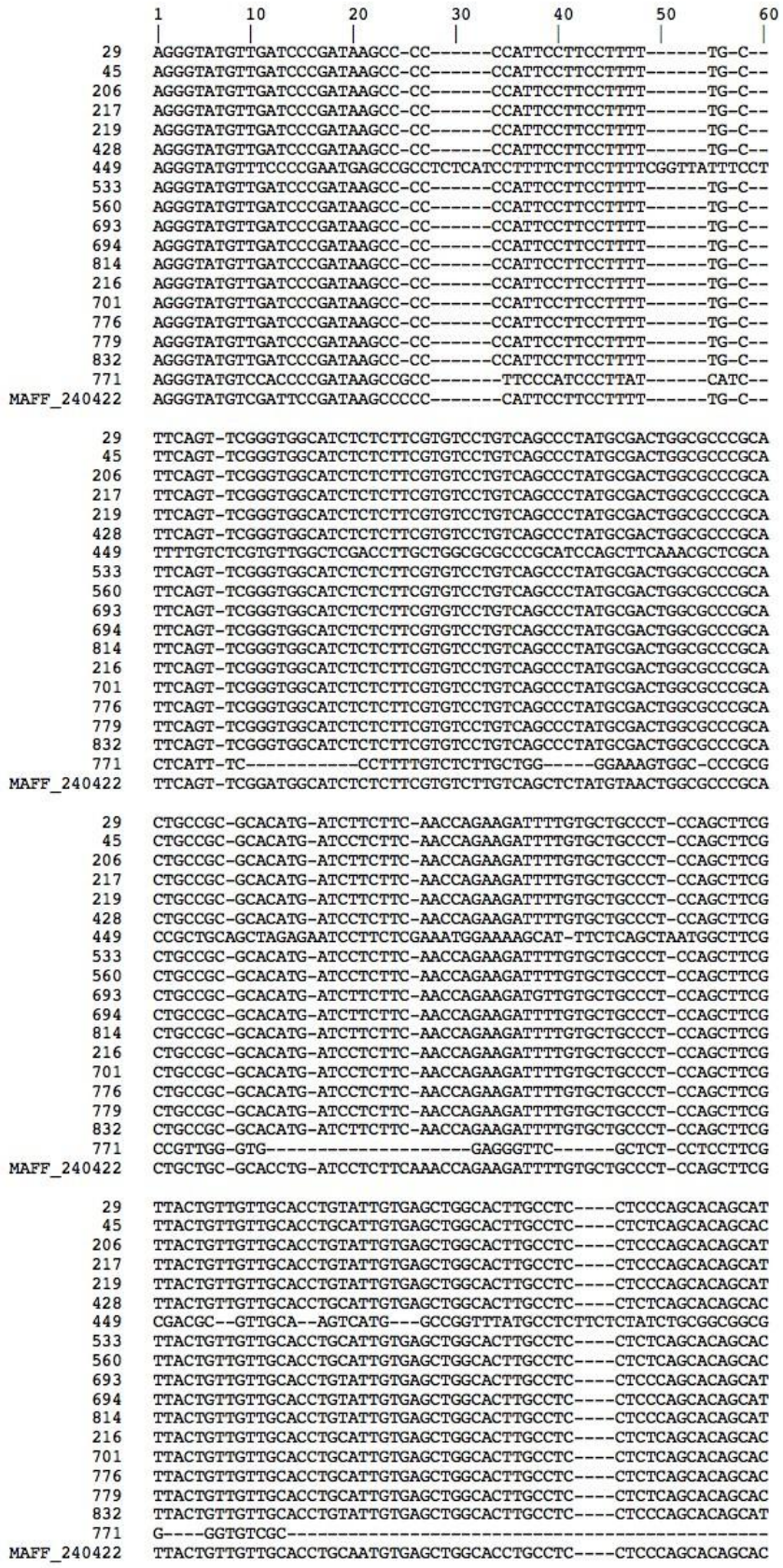


Fig 3.26. Mating Type Gene/High Mobility Group domain (HMG) Multiple Sequence Alignment of Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.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449 CGACGC--GTTGCA--AGTCATG---GCCGGTTTATGCCTCTTCTCTATCTGCGGCGGCG
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         814   GACGAACA------ACAC---CGTC------AT-GTCCCCACCGGCGA-TGGGCCCCAGG
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         832   --CCCAGGAA------CCCGATGAGACAATTGGAGAAGCTCCGCTTGAGCATCGTCAACT
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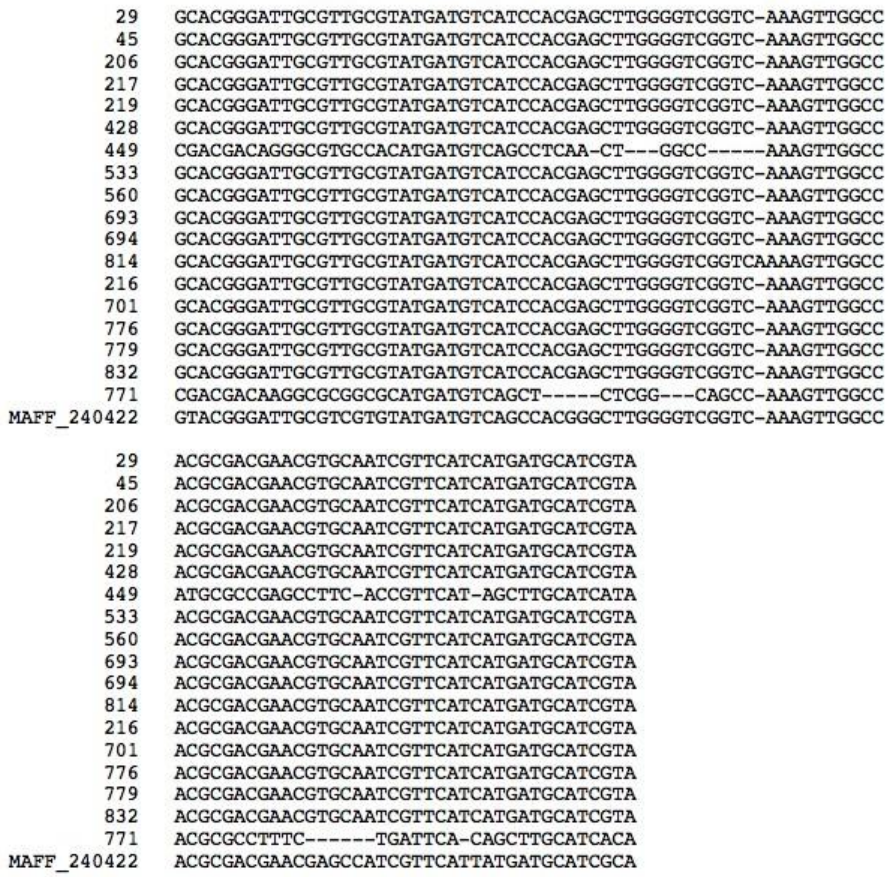


Fig 3.27. Multiple Sequence Alignment of Glutamine Synthetase (GS) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence

Due to the large number of characters and high resolution of the GS marker, Appendix IX contains nucleotide substitutions identified in each of the two genetic groups recognized through GS sequence data using multiple sequence analysis (Fig 3.27.).

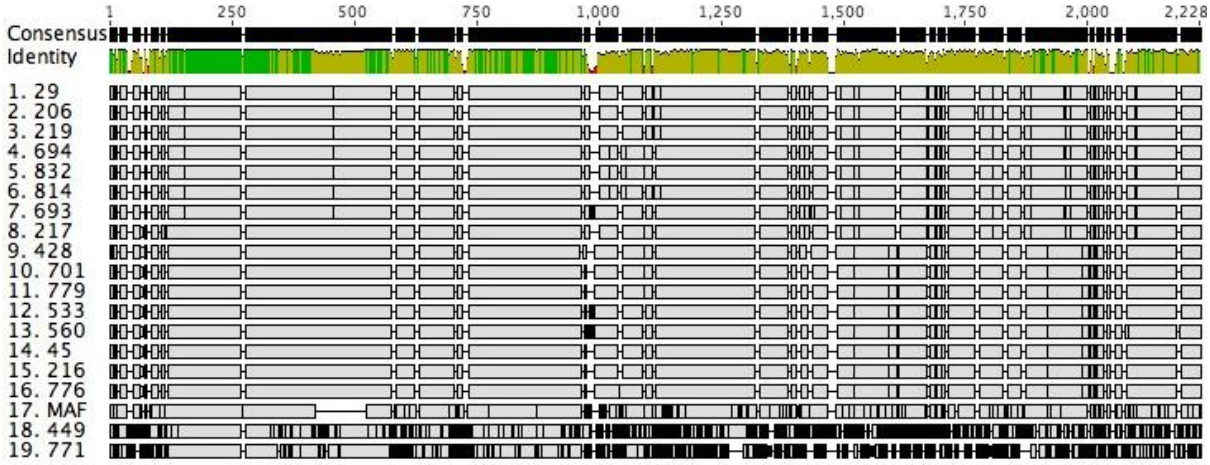


Fig 3.28. Concatenated Sequence Alignment for *Colletotrichum* Isolates Generated with ClustalW2 and Visualized with Geneious*****

*The scale on top shows the size of the sequence from 1 to 2,228 bp at 250 bp intervals. The higher the variation from the consensus sequence within given region, the darker that area appears (e.g. most noticeable in 771 and 449 isolates). Refer to description under Fig 3.28.

The concatenated alignment (Fig 3.28.) was performed for 18 *Colletotrichum* isolates (Table 2.1.) based on the sequence data generated for ITS, TUB, GAPDH, GS and HMG loci. The highest level of divergence from the *C. lindemuthianum* isolates was observed in *C. gloeosporioides* ranging from 36.7-37.0 %, while *C. truncatum* ranged from 31.8-32.4%. *C. lindemuthianum* isolates showed similarity between 88.3-89.1% to *C. orbiculare*, while divergence ranged from 10.9 to 11.7% (Appendix VII Table 6).

3.4.2. Generation of Phylogenetic Trees for the Five Selected Loci

The multiple sequence alignments provided the means for development of phylogenetic trees illustrating the evolutionary distances between the 18 *Colletotrichum* spp. isolates (Table 2.1.). Trees were prepared using Bayesian analysis adopting the Jukes and Cantor (1969) model. Refer to Table 3.4. for general labelling information. The bootstrap support values (generated for 10,000 replicates) ranged from 25 to 75 % depending on the locus. The *C. gloeosporioides* 771 isolate was used as an outgroup.

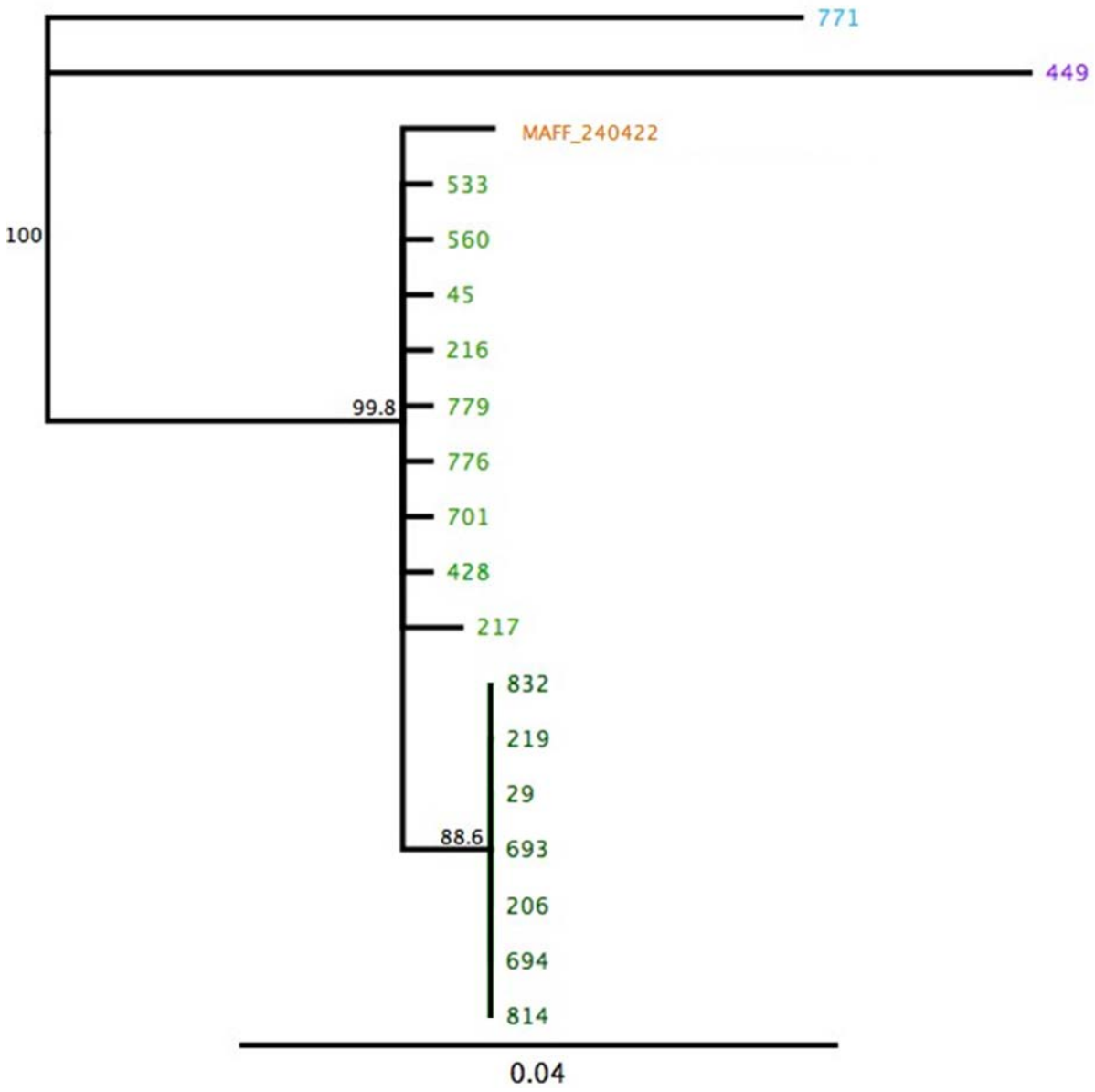


Fig 3.29. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Ribosomal RNA Gene Block Internal Transcribed Spacer (ITS) Region.

C. lindemuthianum isolates were separated into two clear clusters of seven and nine isolates each with high bootstrap values (88.6 – 99.8 %). *C. orbiculare* reference isolate was positioned within the larger cluster, although *C. gloeosporioides* and *C. truncatum* were well resolved with 100 % bootstrap support (Fig. 3.29.).

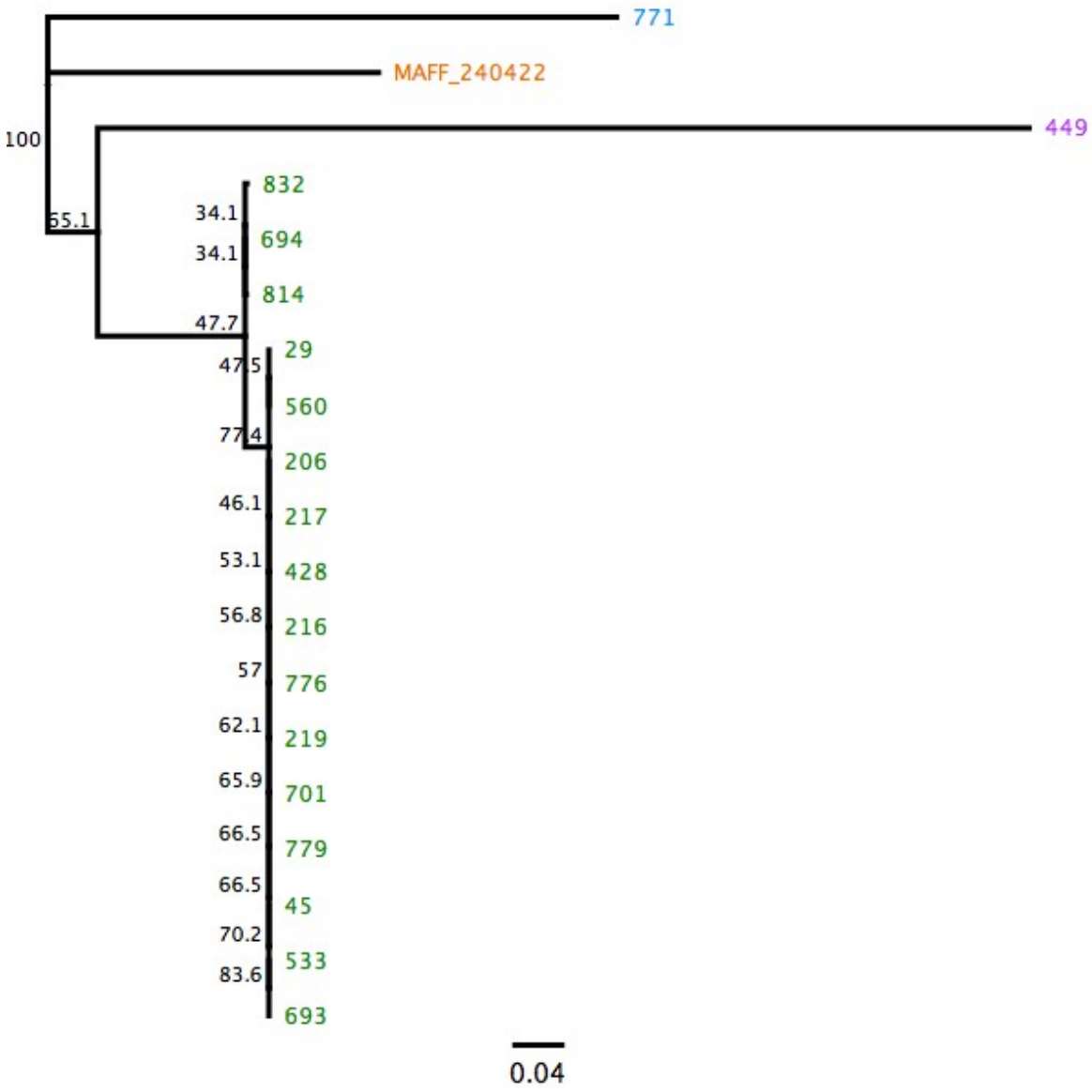


Fig 3.30. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Glyceraldehyde-3-Phosphate Dehydrogenase (GD/GAPDH) Gene.

Based on the glyceraldehyde-3-phosphate dehydrogenase (GD/GAPDH) sequence data *C. lindemuthianum* isolates were clustered into two main groups of 3 and 13 isolates (Fig 3.30.) each represented by a distinct haplotype at 55.1% bootstrap value despite *C. truncatum* being 50.3% divergent from the *C. lindemuthianum* isolates (Appendix VII Table 2; Table 3.8.). The overall tree topology was not optimal with this locus as *C. orbiculare* was positioned between *C. gloeosporioides* and *C. truncatum* and not close to *C. lindemuthianum*.

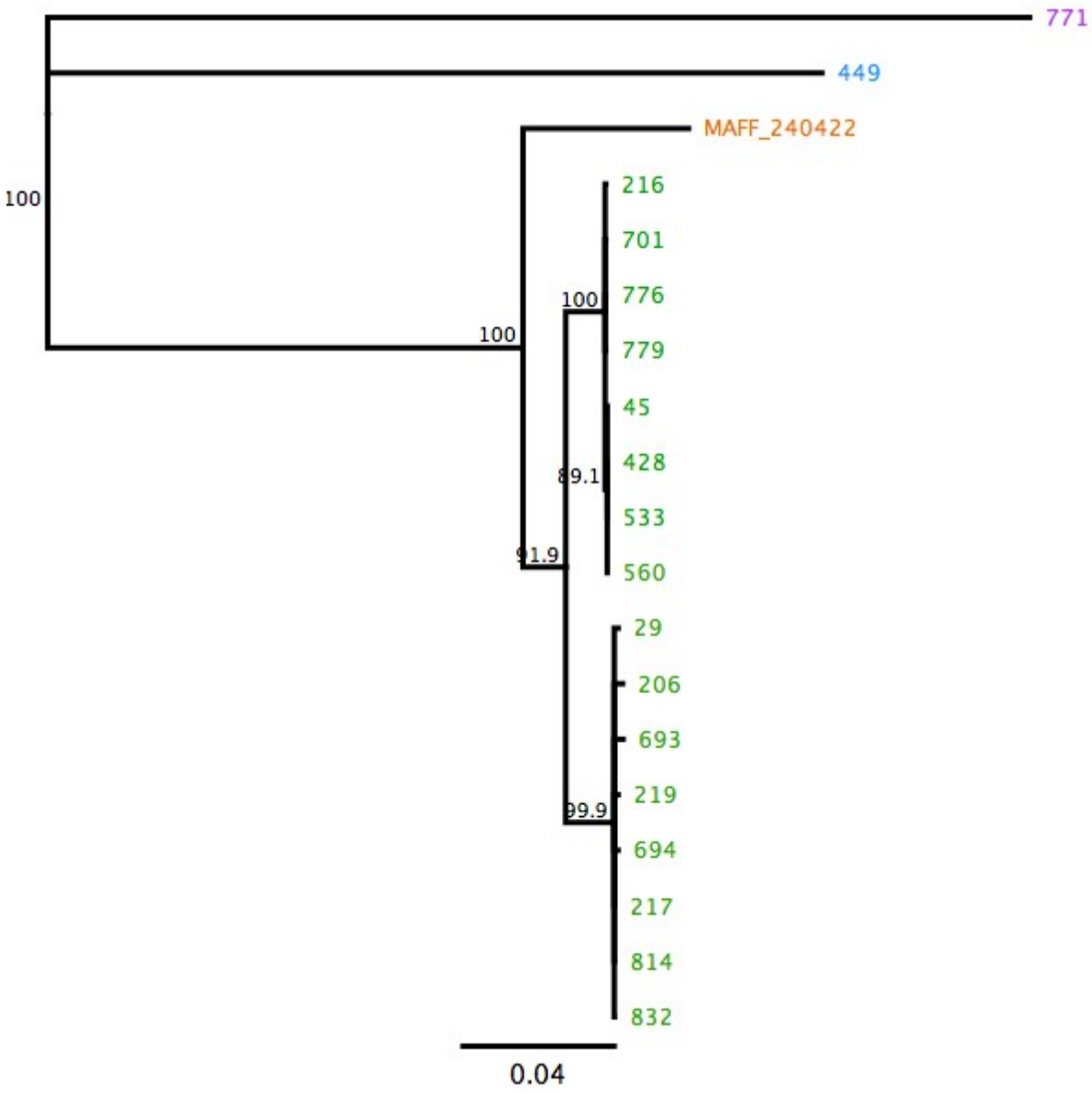


Fig 3.31. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Glutamine Synthetase (GS) Gene.

GS locus provided a well resolved phylogenetic tree distinguishing three genetic clusters within *C. lindemuthianum* species supported by high bootstrap values (Fig 3.31.) linked to various haplotypes. *C. lindemuthianum, C. orbiculare, C. truncatum* and *C. gloeosporioides* relationships were clearly displayed with 100 and 99.9 % bootstrap values.

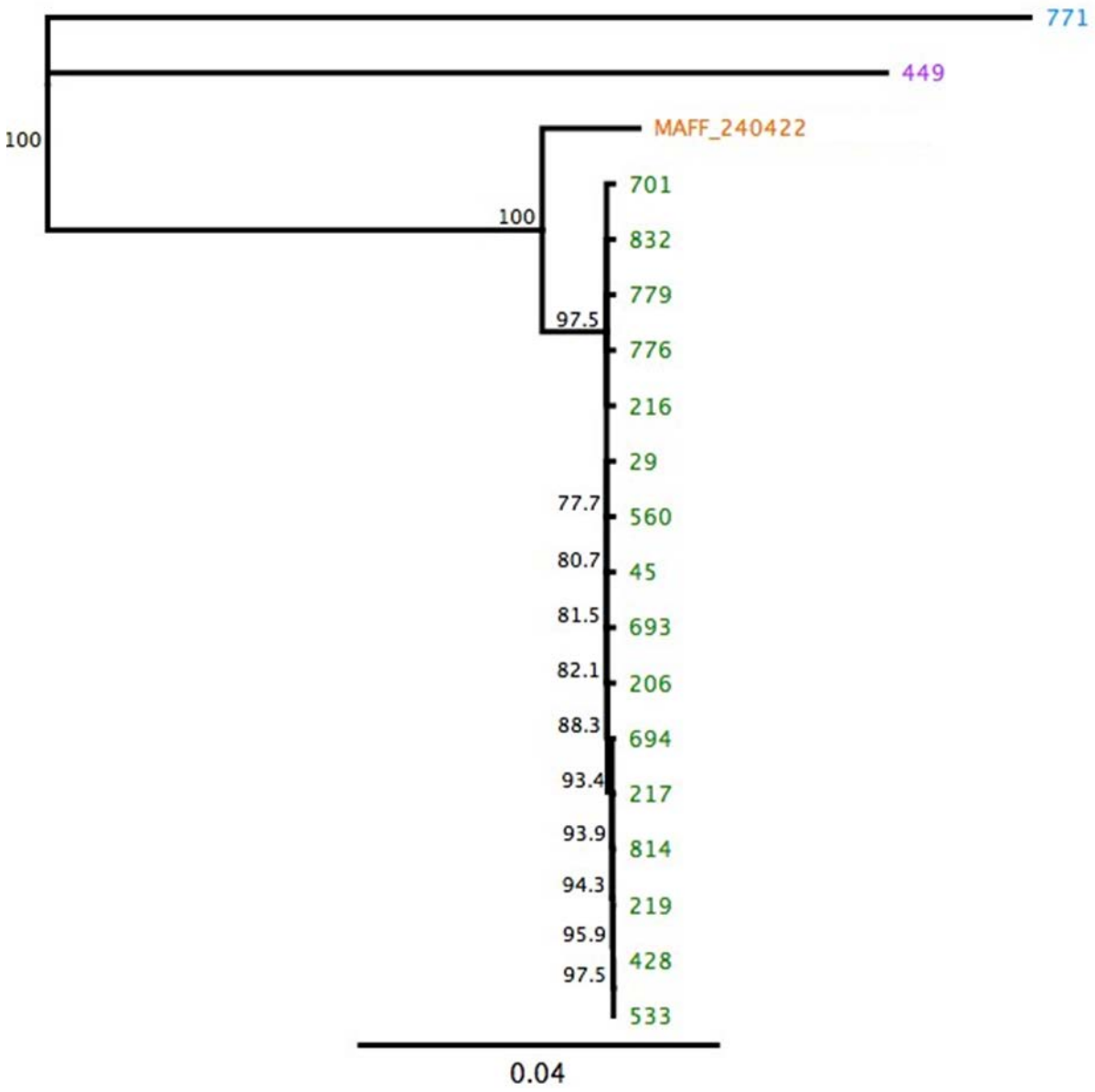


Fig 3.32. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Beta-Tubulin (TUB) Gene.

The TUB locus was the most conserved amongst the genetic markers used in this study with 100 % homology (Appendix VII Table 4) between all *C. lindemuthianum* isolates, which were clustered together to form one genetic group at 97.5 % bootstrap value. However, this locus resolved the four *Colletotrichum* species at 100 % bootstrap value including the relatedness between *C. orbiculare* and *C. lindemuthianum* as representatives of the *orbiculare* clade (Fig 3.32.).

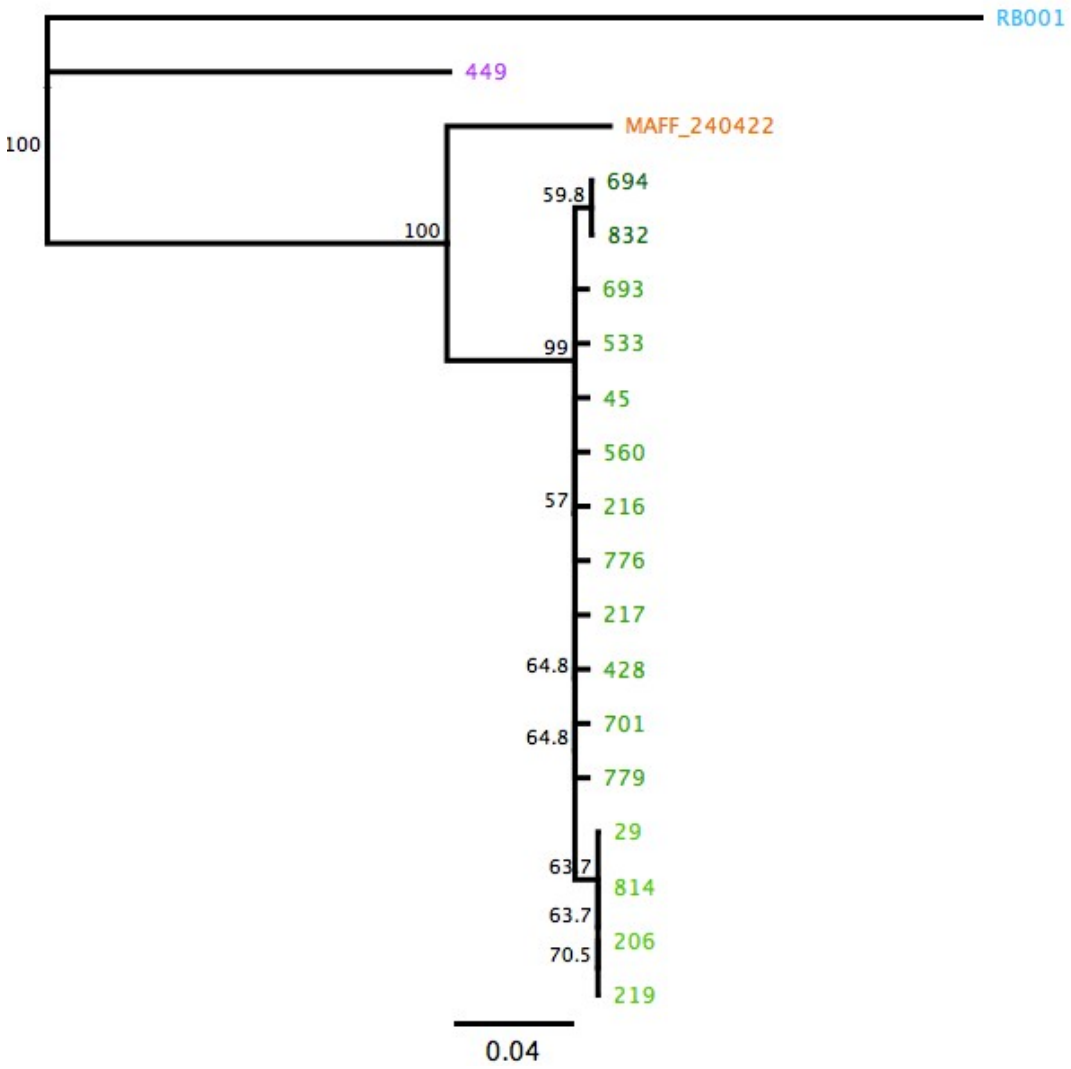


Fig 3.33. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis Based on Multiple Sequence Alignment of the Mating Type Gene/High Mobility Group Domain.

The mating type locus MAT1-2-1 (HMG) differentiated the *C. lindemuthianum* isolates into two main groups each represented by 4 and 12 isolates. Within the larger group a cluster of two isolates 694 and 832 represented by a particular haplotype (HT3, Appendix VII Table 5) was differentiated albeit with a lower bootstrap value 59.8 %. This locus resolved the four *Colletotrichum* species including *C. gloeosporioides* represented by RB001 at 100 % bootstrap value displaying the close relatedness between *C. orbiculare* and *C. lindemuthianum* as representatives of the *orbiculare* clade (Fig 3.33.).

3.4.3. Generation of Consensus Tree Using Concatenated Multiple Sequence Alignment of the five Loci

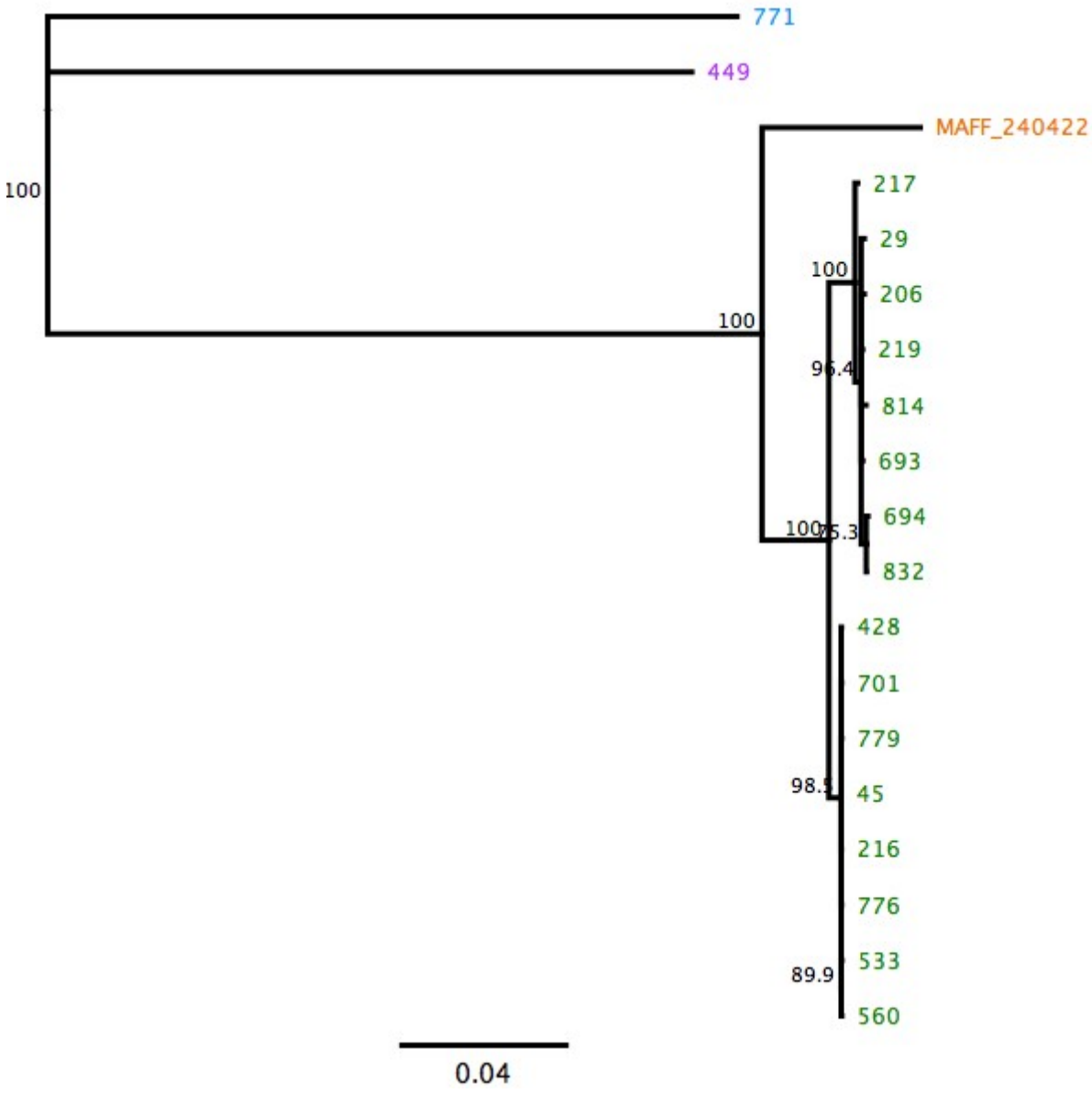


Fig 3.34. Phylogenetic Tree of *Colletotrichum* Isolates Obtained Using Bayesian Analysis using Jukes and Cantor (1969) Model Based on Concatenated Multiple Sequence Alignment (including: ITS, TUB, GS, GAPDH, and HMG)*

The concatenated sequence data produced a phylogenetic tree with an overall topology that was well supported by high bootstrap values of 89.9 to 100 % (Fig 3.34), where *C. gloeosporioides*, *C. truncatum* and the *orbiculare* clade including *C. orbiculare* and the *C. lindemuthianum* isolates were well resolved at 100%

bootstrap values. *C. lindemuthianum* isolates representing seven haplotypes (Appendix VII Tables 6; Table 3.9) were clustered into two main genetic groups of eight isolates each with 98.5 % and 100 % bootstrap support. One of the groups was further sub-divided into three clusters (e.g. 694 and 832 with 75.3% bootstrap value); within the second group isolates 533 and 560 were represented as a cluster with 89.9 % bootstrap support).

3.4.4. Sequence Homology and Divergence among Colletotrichum spp. Isolates

Sequence homology values were calculated based on the pairwise analysis of all *Colletotrichum* spp. isolates used in the study. Homology range data is shown in Table 3.8; data for individual loci and the concatenated sequence are presented in Appendix VII. This provided a detailed view of the levels of genetic diversity identified by various loci as reflected by the number of haplotypes (HT) identified (Table 3.9.), and also the relatedness amongst the four species namely *C. lindemuthianum, C. orbiculare, C. gloeosporioides* and *C. truncatum*. For example, the TUB locus proved to be the most conserved with 100 % homology across all *C. lindemuthianum* isolates, GS differentiated three haplotypes, and the HMG differentiated four haplotypes. Concatenated sequence data analysis of the five loci namely ITS, TUB, GAPDH, GS, and HMG provided a comprehensive synopsis of the genetic diversity amongst the 16 *C. lindemuthianum* isolates with seven haplotypes.

Table 3.8. Homology Ranges within *C. lindemuthianum* Isolates, and between the Four Different *Colletotrihcum* Species Compared*

Locus	*C. lindemuthianum*	*C. lindemuthianum* and *C. orbiculare*	*C. lindemuthianum* and *C. truncatum*	*C. lindemuthianum* and *C. gloeosporioides*
ITS	99.0-100.0	96.3-96.8	87.3-87.8	89.9-90.1
TUB	100.0	96.8	83.6	82.4
GD/GADPH	98.0-100.0	73.5	49.7	57.6-58.6
GS	96.8-100.0	91.1-92.5	59.6-60.0	51.2-51.9
HMG	99.0-100.0	90.0-90.5	72.5-73.5	60.5-61.0
Concatenated	97.8-100.0	88.3-89.1	67.9-68.2	63.0-63.4

*based on the data presented in Appendix VII Tables 1 to 6.

Table 3.9. Summary of Haplotype Allocations for *C. lindemuthianum* Isolates Based on Sequence Data Generated for ITS, TUB, GS, GD and HMG Loci*

Locus	***Haplotypes (HT) Allocations***	***Isolates Representing the Haplotype***
ITS	HT1	216, 701, 776, 779
	HT2	832
	HT3	29, 206, 219, 693, 694, 814
	HT4	45, 428, 533, 560
	HT5	217 *(0.2% difference from HT4)
TUB	HT1	All isolates
GD	HT1	216, 701, 776, 779, 29, 45, 206, 217, 219, 428, 533, 560, 693
	HT2	832, 694, 814
GS	HT1	216, 45, 428, 533, 560, 701, 776, 779
	HT2	217, 814, 832
	HT3	29, 206, 219, 693, 694,
HMG	HT1	45, 216, 533, 560, 693, 776
	HT2	217, 428, 701, 779,
	HT3	694, 832
	HT4	29, 206, 219, 814
Concatenated	HT1	45, 216, 428, 701, 776, 779
	HT2	694, 832
	HT3	29, 206, 219
	HT4	533, 560
	HT5	693
	HT6	217*(0.3% difference from HT3)
	HT7	814*(0.1% difference from HT2 and HT3)

*Based on the data contained in the Appendix VII Tables 1 to 6; The number of haplotypes identified for each locus are presented along with *C. lindemuthianum* isolate codes representing particular HT. Table 2.1. contains biogeographic diversity details of the isolates.

CHAPTER 3

PART III. Arbitrary-Primed PCR (AP-PCR) Analysis of *Colletotrichum spp.* Isolates

3.5. Preliminary Screening of 10 AP-PCR Primers

A selection of 10 AP-PCR primers (Table 2.7.) identified from the literature were tested with *C. lindemuthianum* isolates 701 and 832 and *C. gloeosporioides* isolate 771 (Fig 3.35.). Primers $(CAG)_5$,$(CAC)_5$, $(GAC)_5$ and $(GCA)_5$ generated profiles consistently with the three isolates tested. . Primers $(TCC)_5$, $(GACG)_4$, and $(TGTC)_4$ showed very few or no banding; primers $(ACTG)_4$, $(GACAC)_4$,and, $(GACA)_4$ were inconsistent in *C. lindemuthianum* amplification with no banding in *C. gloeosporioides*. Based on these overall results, primers $(CAG)_5$,$(CAC)_5$, and $(GAC)_5$ were selected for further work.

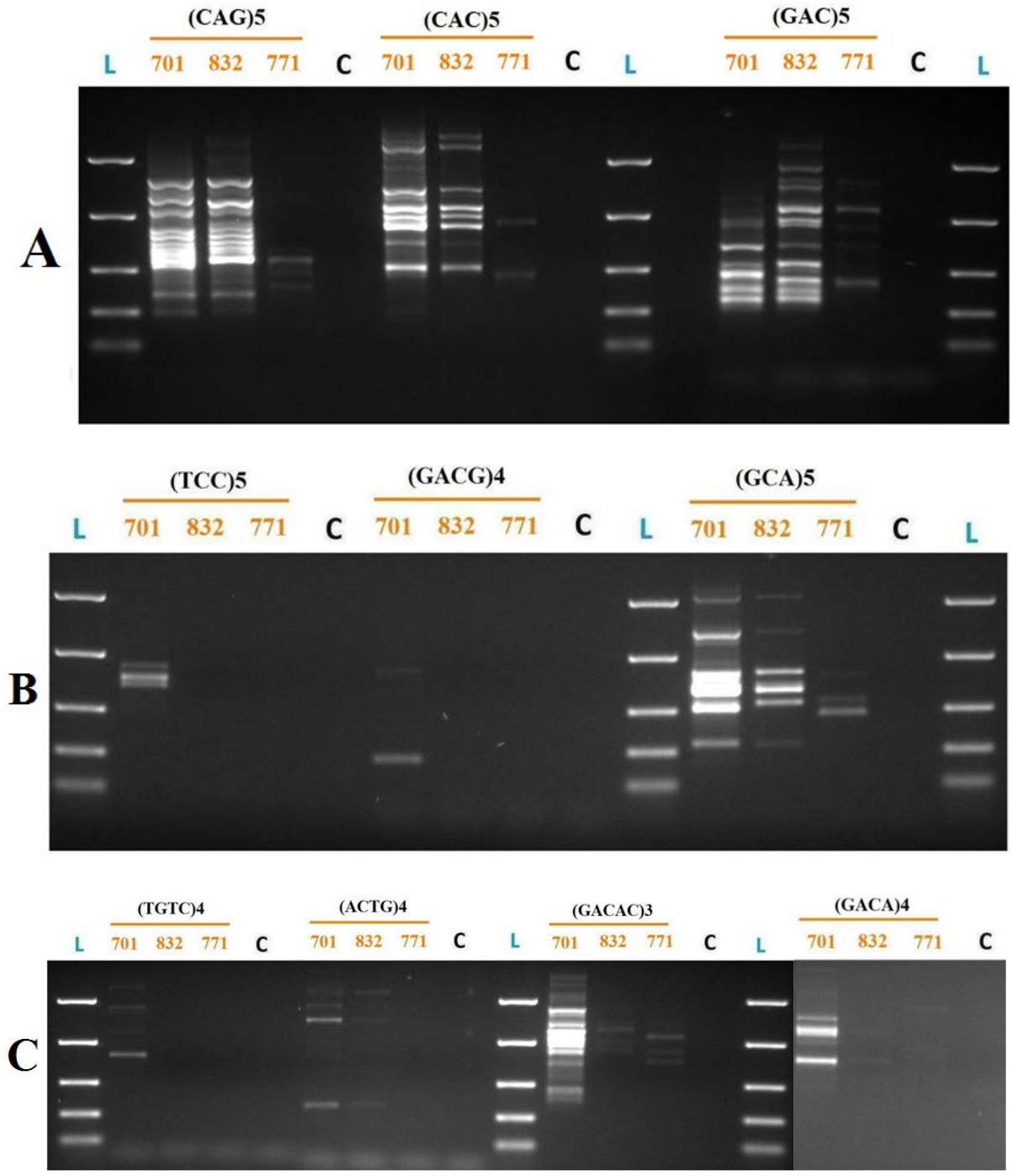


Fig 3.35. Preliminary Screening of AP-PCR Primers with a Set of *Colletotrichum* spp. Isolates (701 and 832, *C. lindemuthianum*; 771, *C. gloeosporioides*; C, Control with no DNA)

3.6. AP-PCR Analysis of 18 *Colletotrichum* Isolates

The banding patterns of the 18 isolates including 16 *C. lindemuthianum* isolates, *C. gloeosporioides* (771) and *C. truncatum* (449) on the gel (Fig 3.36.) were visually compared and isolates with similar profiles were grouped together. This provided the basis for the haplotype allocation of the *C. lindemuthianum* isolates (Table 3.11.).

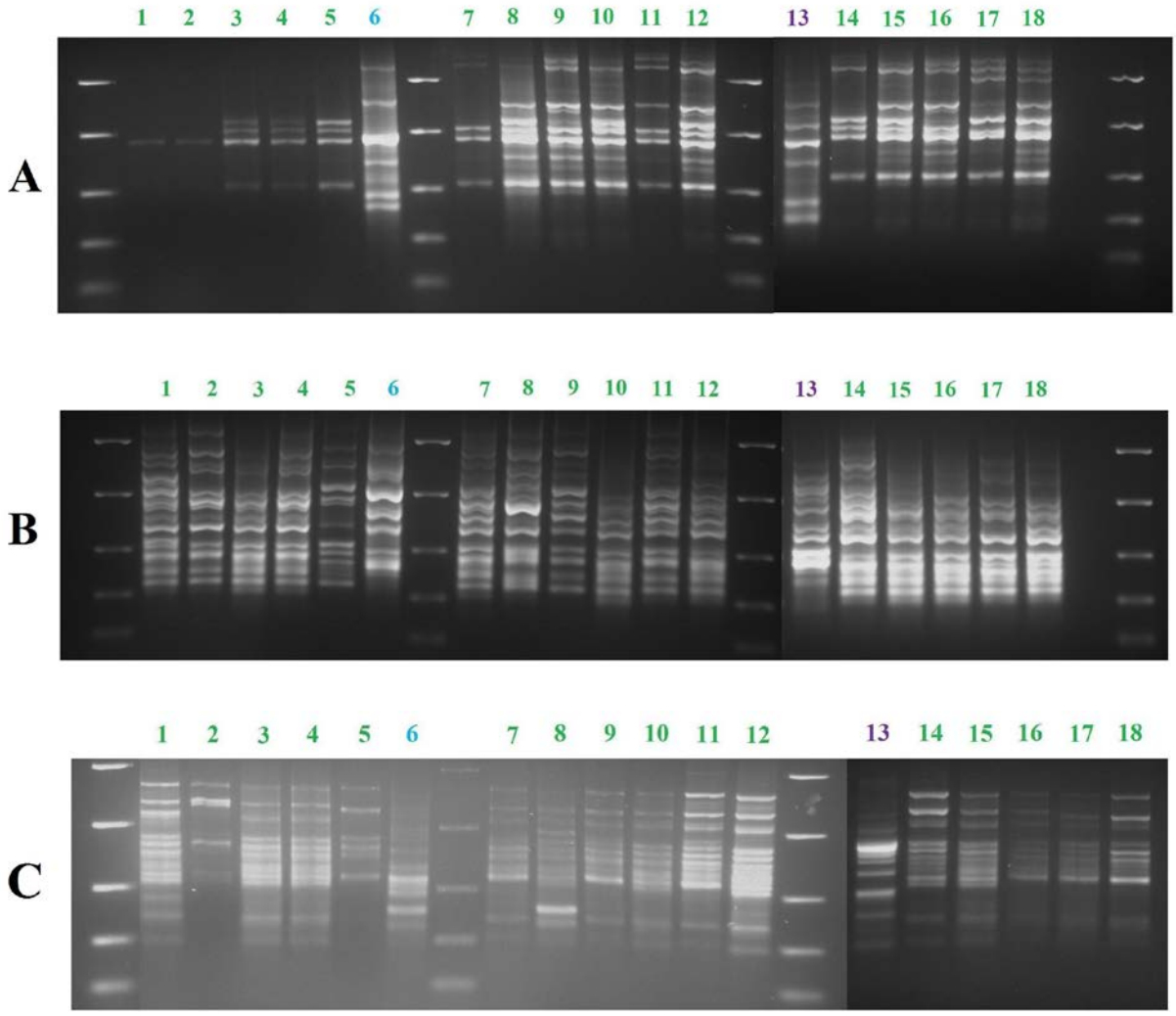


Fig 3.36. Banding Patterns Produced by *Colletotrichum* Isolates After Amplification with AP-PCR Primers $(CAG)_5$,$(CAC)_5$, and $(GAC)_5$*

*Table 3.10. provides details of the labelling.

Table 3.10. Labelling Legend to AP-PCR Results Shown in Fig. 3.45 A, B and C (above)*

Number on Picture	Isolate Code	Species
1	701	*C. lindemuthianum*
2	216	*C. lindemuthianum*
3	776	*C. lindemuthianum*
4	779	*C. lindemuthianum*
5	832	*C. lindemuthianum*
6	771	*C. gloeosporioides*
7	29	*C. lindemuthianum*
8	45	*C. lindemuthianum*
9	206	*C. lindemuthianum*
10	217	*C. lindemuthianum*
11	219	*C. lindemuthianum*
12	428	*C. lindemuthianum*
13	449	*C. truncatum*
14	533	*C. lindemuthianum*
15	560	*C. lindemuthianum*
16	693	*C. lindemuthianum*
17	694	*C. lindemuthianum*
18	814	*C. lindemuthianum*
	A- $(CAC)_5$ **B- $(GAC)_5$** **C- $(CAG)_5$**	

* The labelling legend depicts the numerical representation and colour coding for isolate identification. A, B and C represent the panels with the respective primers in Fig 3.36.

Position and number of bands was taken under consideration while assigning isolates to haplotypes. The brightness of bands was not a factor in allocation process. Primer that had the highest resolution was $(CAC)_5$ that distinguished nine haplotypes, while $(GAC)_5$ resolved eight haplotypes, and $(CAG)_5$ showed the more conserved part of the genome and differentiated only five haplotypes (Table 3.11). The $(CAC)_5$ produced around six-seven bands while $(GAC)_5$ and $(CAG)_5$ on average 11 bands. The number of characters was not specified as more runs of PCR are required in order to clearly resolve the banding pattern.

The AP-PCR proved more useful in illustrating the intraspecific diversity within *C. lindemuthianum* species complex giving a broad overview of their genetic background. On the other hand, multilocus phylogenetic approach proved to be beneficial for identification of the isolates (ITS on its own or supported by other sequence information, which was required for *C. truncatum* species classification) and designating them to appropriate species complexes as well as and gave closer, more specific outlook on genetic biodiversity (Talhinhas *et al*., 2002).

Table 3.11. Haplotype Allocations Based on the AP-PCR Results Generated for Three Primers for *Colletotrichum* Isolates*

Isolate Code*	$(CAC)_5$	$(GAC)_5$	$(CAG)_5$
701	HT1	HT1	HT1
216	HT1	HT2	HT2
776	HT2	HT2	HT1
779	HT2	HT2	HT1
832	HT2	HT3	HT3
29	HT3	HT4	HT4
45	HT4	HT5	HT5
206	HT4	HT6	HT4
217	HT4	HT7	HT4
219	HT5	HT4	HT4
428	HT5	HT4	HT4
533	HT6	HT8	HT4
560	HT7	HT8	HT4
693	HT8	HT8	HT4
694	HT9	HT8	HT4
814	HT7	HT8	HT4
Total Number of Haplotypes	9	8	5

*Isolates number according to the Labelling Legend (Table 3.10.);**HT-haplotype.

CHAPTER 3

PART IV. Genomic DNA Preparation and Quality Assessment for Genome Sequencing

3.7. NanoDrop-based Assessment of DNA Quality and Quantity

Genomic DNA from ***C. lindemuthianum*** **isolates** 216 and 776 extracted using the Qiagen DNeasy Plant Mini Kit (prepared in quadruplicates) was tested for the quality and concentration to fulfil the requirements set by the Illumina MiSeq technology. The MiSeq specification included 50 ng of DNA in max 20µl; 260/280 ratio of ~1.8; and 260/230 ratio of ~2.0. The samples that fit this model were 216(2) and 776(2) as shown below (Table 3.12, Fig 3.37).

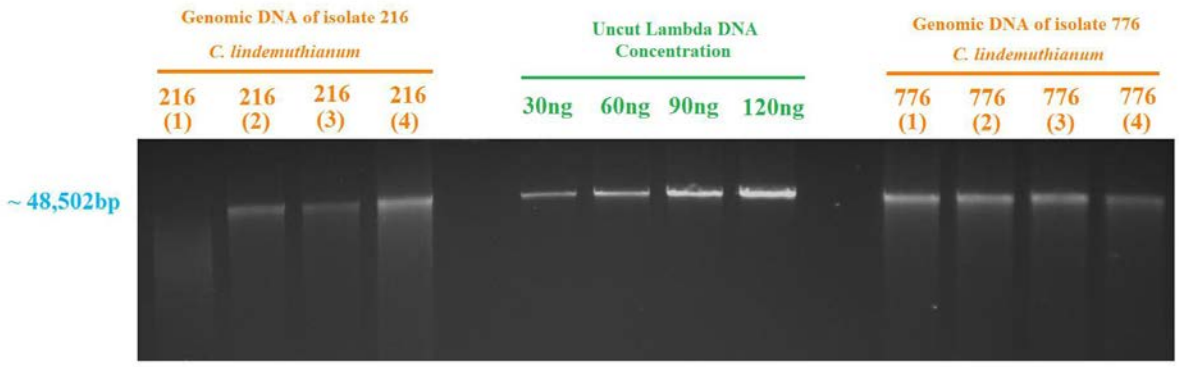
Table 3.12. Summary of NanoDrop Data on DNA Quantity and Quality for *C. lindemuthianum* Isolates 216 and 776 Prepared in Quarduplicate*

Sample	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230
216(1)	33.8	ng/µl	0.676	0.366	1.85	3.07
216(2)	43.9	ng/µl	0.878	0.483	1.82	2.29
216(3)	32.6	ng/µl	0.653	0.365	1.79	2.50
216(4)	117.9	ng/µl	2.358	1.505	1.57	1.85
776(1)	105.3	ng/µl	2.105	1.283	1.64	1.15
776(2)	79.8	ng/µl	1.595	0.905	1.76	2.32
776(3)	62.8	ng/µl	1.256	0.718	1.75	2.20
776(4)	51.5	ng/µl	1.030	0.575	1.79	2.51

*The readings represent the concentration of DNA in ng/µland absorbance measurements at 260, 280, 260/280, and 260/260nm respectively.

3.8. Genomic DNA Integrity and Quantity Compared to Uncut Lambda DNA

All samples were electrophoresed on agarose gel (Fig 3.37) against four different concentrations of lambda DNA in order to cover the range of DNA concentration previously estimated using NanoDrop. Visual inspection, and comparison of the fluorescence levels of the samples and the marker enabled a clear assessment of the integrity and concentration of the genomic DNA samples. For example, 216(1) was partially degraded and unsuitable for genome sequencing work. Other samples, in terms of the size of the fragments, the concentration range as well as the removal of RNA were suitable for further genome sequencing processes.



*Loading volume for all wells was 10ul;
**1ul of DNA sample 216(1-4) and 776 (1-4) was loaded on gel;
***Lambda DNA was loaded on gel in four different concentrations:
30ng (1ul of DNA), 60ng (2ul), 90ng (3ul), and 120ng (4ul).

Fig 3.37 Genomic DNA Integrity and Quality Assessment of *C. lindemuthianum* Isolates Targeted for Genome Sequencing

CHAPTER 3

PART V. Growth Rate, Colony Morphology and Sporulation Patterns of *Colletotrichum* Isolates

The data was generated for 5 *C. lindemuthianum* (216, 701, 776, 779, and 832) and 1 *C. gloeosporioides* isolate (771) (Table 2.1) during incubation at 20 and 25°C over a 15 day period.

3.9. Growth Rate Monitoring of *Colletotrichum* Isolates at 20 and 25ºC

The fastest growing isolate was 771 *C. gloeosporioides* (Fig 3.38. and Fig 3.39.), however its growth rate was higher at 25 ºC with the average values of 4.109 mm, while at 20 º C it was 3.35mm /24hours. The slowest growth rate was recorded for isolate 832 (Fig 3.47. and Fig 3.48.). The rest of the isolates had comparable growth rates. However, isolate 776 was growing slightly faster than 216, 701, and 779 at 25 ºC, while this pattern was not observed at 20 ºC where its growth rate was lower than 216 and 779. Fig 3.38. is based on the data contained in Table 3.13., while Fig 3.39. reflects data from Table 3.14.

Table 3.13. Average Growth Values for Each *Colletotrichum* Isolate Incubated at 20°C and Monitored Periodically*

Average Growth Numbers (mm) for Each Isolate							
Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours	Average mm/24hours
216	3.375	5.575	13.325	17.4	19.925	28.025	1.751
701	4.75	6.9	14.325	17.125	18.3	22.25	1.39
771	8.925	13.4	31.65	36.85	N/A	N/A	3.35
776	4.1	5.775	13.5	17.675	20.075	23.875	1.492
779	4.3	6.625	14.525	18.3	20.2	24.35	1.521
832	1.55	2.7	7.7	10.15	11.2	15.375	0.96

*Table demonstrates the average values calculated based on the raw data (Appendix XI) measurements taken for 5 plates at 8 different positions (Fig 2.1.).

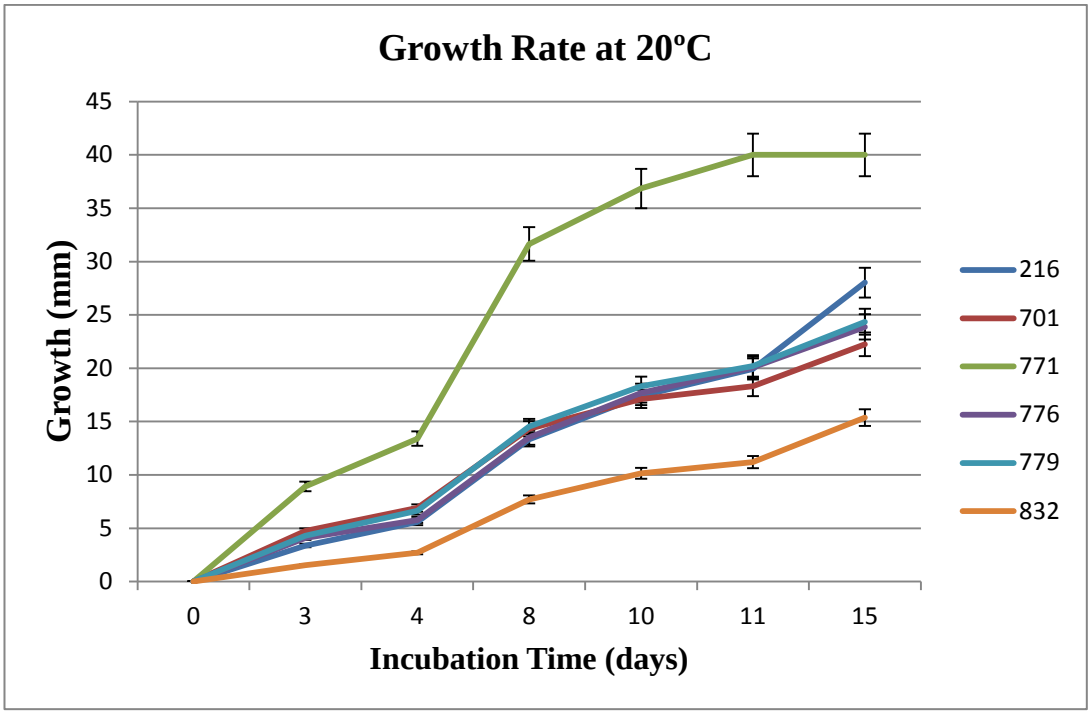


Fig 3.38. Graph Showing the Growth Rate of *Colletotrichum* spp. Isolates Incubated at 20ºC.*

*Graph displays error bars for the selected chart series with 5% value. Chart based on the average measurements contained in Table 3.13. The isolates are colour coded with the legend on the right side from the linear graph.

Table 3.14. Average Growth Values for Each *Colletotrichum* Isolate Incubated at 25°C and Monitored Periodically

Average Growth Numbers (mm) for Each Isolate						
Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours	Average mm/24hours
216	7.5	11.425	13.825	22.275	25.6	1.828
701	10.875	15	17.275	23.375	25.475	1.819
771	17.175	27.425	32.875	N/A	N/A	4.109
776	10.2	15	17.7	26.825	30.35	2.167
779	9.575	13.05	14.825	21.475	25.325	1.8
832	5.625	8.375	10.1	15.425	17.4	1.242

*Table demonstrates the average values calculated based on the raw data (Appendix XII) measurements taken for 5 plates at 8 different positions (Fig 2.1.).

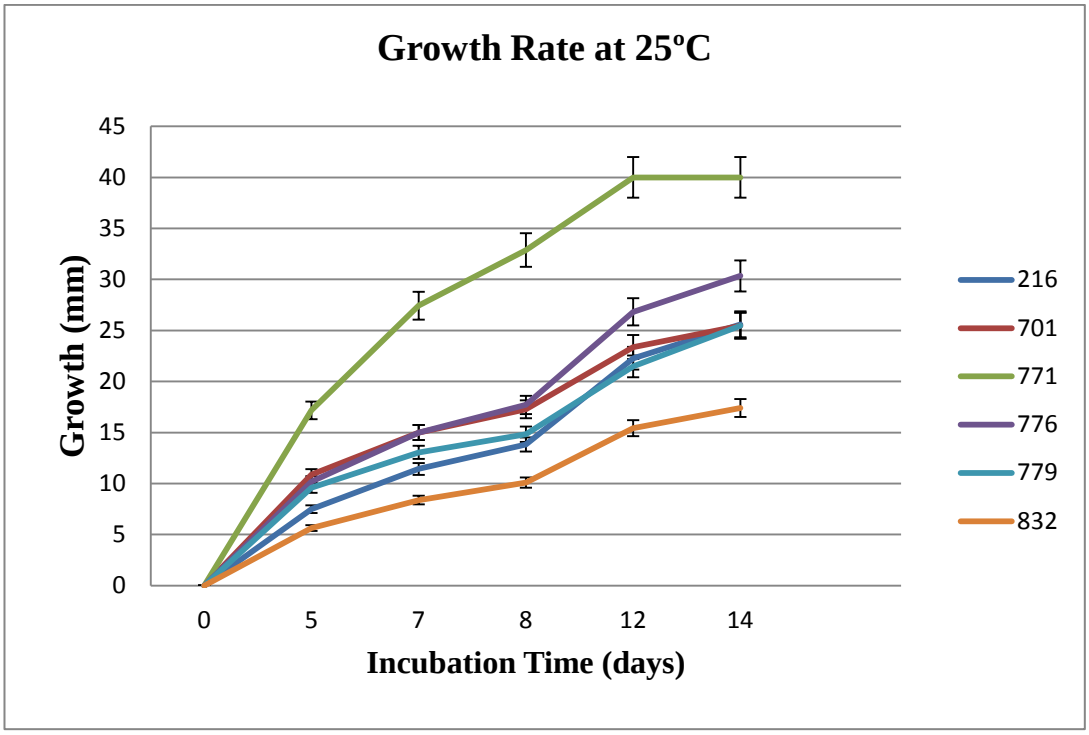


Fig 3.39. Graph Showing the Growth Rate of *Colletotrichum* spp. Isolates Incubated at 25ºC*

*Graph displays error bars for the selected chart series with 5% value. Chart based on the average measurements contained in Table 3.14. The isolates are colour coded with the legend on the right side from the linear graph.

3.10. Level of Sporulation amongst a Set of *Colletotrichum* Isolates

An assessment of the level of sporulation in *Colletotrichum* isolates (216, 701, 776, 779, 832 and 771) was performed. The highest level of sporulation was observed in 779 especially in the middle and outer edges of the culture. Isolate 216 showed good level of sporulation but lower compared to isolate 779. Very low level of sporulation was observed in isolates 701 and 776; isolates 832 and 771 had no sporulation. A semi-quantitative scale was used to record the preliminary observations (Table 3.15.).

Table 3.15. Level of Sporulation Observed amongst a set of *Colletotrichum* Isolates*

Isolates	216	701	776	779	832	771
Sporulation Level*	2	1	1	3	0	0

* Level of sporulation was recorded according to the following scale: 0- no sporulation, 1-very low sporulation, 2-moderate sporulation, 3-highly sporulating.

3.11. Morphological Variability of *Colletotrichum* Isolates Based on PDA Cultures

C. lindemuthianum isolates showed considerable variation in their morphological characteristics like texture and colour (Fig 3.40). Isolates 701 (B), 776 (C) and 832 (E) had similar appearance with white cottony mycelium and creamy/beige surface. Isolate 216 (A) had grey cottony centre with brown and a lighter outer edges of growth. Isolate 779 (D) had flattened mycelia with grey/green centre and light cream outermost edge. Isolate 771 (F) was the *C. gloeosporioides*, mycelium quickly covered the whole plate and had white, grey cottony appearance with darker patches (Fig 3.40).

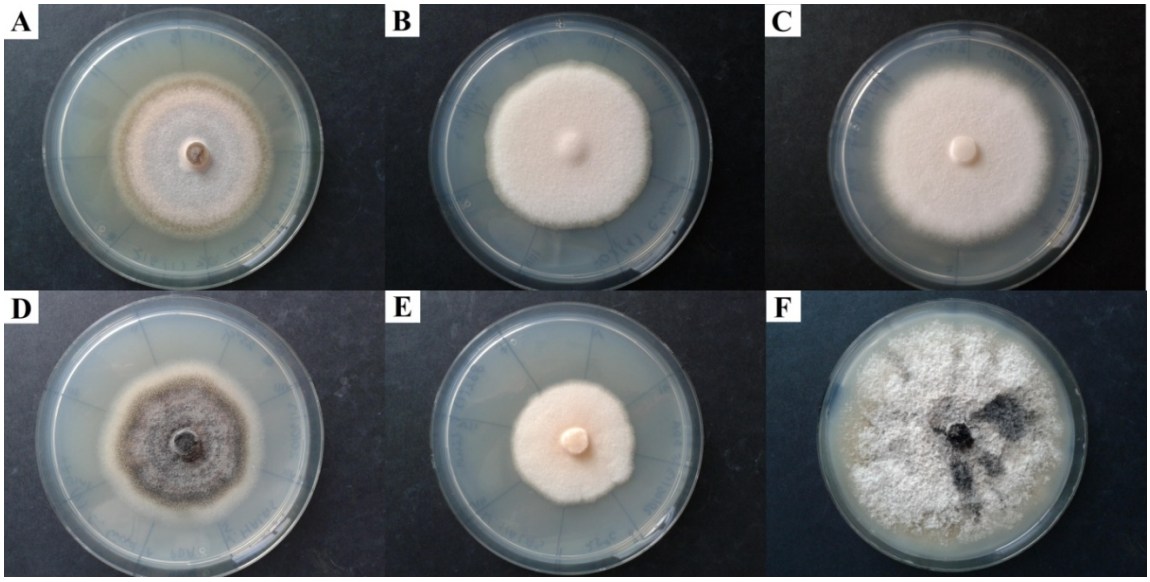


Fig 3.40 Morphological Variation among *Colletotrichum* spp. Isolates in PDA*

*Culture plates incubated at 25ºC for 10 days; Pictures A-E are of *C. lindemuthianum* (A-isolate 216, B-isolate 701, C-isolate 776, D-isolate 779, E-isolate 832), while F is of *C. gloeosporioides* isolate 771.

CHAPTER 4: DISCUSSION

For the present study, 18 isolates representing the biogeographic diversity in the Colletotrichum-bean pathosystem were selected from a historical collection spanning nearly 30 years and more than 200 isolates. This collection, currently maintained by research scientists at the University of Bedfordshire (Professor S Sreenivasaprasad) and the university of Warwick (Professor Eric Holub), mainly originated from the early work by a group of research scientists based at Long Ashton and Rothamsted (previously known as Institute of Arable Crops Research IACR and now known as Rothamsted Research). All 18 isolates were originally deposited in the collection as the anthracnose pathogen *C. lindemuthianum* associated with the common bean *Phaseolus vulgaris*. These 18 isolates represented various countries in Africa, Asia, Europe and the Americas and belonged to diverse races.

Sequence data from the multiple loci analysed in this study confirmed the identity of 16 isolates as *C. lindemuthianum*. However, isolates 771 and 449 were distinct from these, but their identity was not entirely clear based on ITS sequence data alone. Various studies have recently pointed to the insufficiency of the ITS marker for species identification in Colletotrichum (e.g. Talhinhas *et al*., 2011; Cannon *et al*., 2012). Based on the multilocus sequence data isolates 771 and 449 were identified as *C. gloeosporioides* and *C. truncatum*, respectively. These species have not been widely reported as bean anthracnose pathogens in the literature so far and this needs further investigation.

ITS and HGM had the highest resolution differentiating five and four haplotypes respectively. Moderate resolution was expressed by several markers where GADPH, ACT, CHS resolved two haplotypes, while GS and HIS3 distinguished three. Although loci TUB and CAL were useful in species identification, they are highly conserved and were unable to detect genetic diversity within *C. lindemuthianum*. Though ITS resolution was highest, the

concatenated data provided most detailed information, wherein among the two major groups, one of the groups included three sub-groups.

The ITS, GS, concatenated and to some degree GADPH sequence data differentiated two distinct genetic groups of around 7-9 isolates each representing various geographic locations and races suggesting that these genetic groups have separate origins. This phenomenon was most apparent for GS DNA fragment that contained 25 substitutions (Appendix IX) or variables within the sequence that recognized three haplotypes amongst *C. lindemuthianum* isolates and separated *C. lindemuthianum* isolates into two genetic groups. North Andean regions along with Mesoamerica and South of Andes serve as one of the main centres of genetic diversity in common bean (Gepts and Bliss, 1985; Koinange and Gepts, 1992; Bitocchi *et al*., 2012). There is a possibility that haplotypes represent the three main gene pools established for *P. vulgaris* suggesting co-evolution of the host and its pathogen particularly associated with the resistance gene cluster reported by Geffroy *et al*. (1999).

The two genetic groups within *C. lindemuthianum* differentiated by Damm *et al*. (2013) contained isolates from different geographic locations including: USA, Europe, and South America. The Costa Rican isolates were grouped together complying with the results presented in this study. However the Brazilian isolate was separated into another genetic group which is inconclusive as it was placed in the same genetic group along with the Costa Rican isolates. There is a clear distinction of the two genetic groups within *C. lindemuthianum* and further research is required to examine their evolutionary lineages/origins.

This finding may suggest a strong relationship between evolution and the origins of the pathogen and reflects the current knowledge about the origins of *P. vulgaris*. Recent reports point at Mesoamerica as the origin of the *P. vulgaris* (Bitocchi *et al*., 2012), which gave rise to two main gene pools: Mesoamerican and Andean serving as two separate evolutionary lineages (Gepts and Bliss, 1985; Koinange and Gepts, 1992). Evidence based AFLP studies of wild and domesticated *P. vulgaris* indicates the Mesoamerican origin (Rossi *et al*., 2009) and higher diversity in these regions further support the hypothesis (Gepts *et al*.,

1986; Koening and Gepts, 1989). The third gene pool developed around Peru and Ecuador territories that according to certain researchers are the ancestral regions of *P. vulgaris* (Freyre *et al*., 1996, Gepts *et al*., 1999) that spread into two opposite directions forming Mesoamerican (Colombia, Mexico, Central America) and Andean (Bolivia, Argentina, South of Peru) gene pools. That theory arose from the research carried out by Kami *et al*. (1995) that found ancestral protein phaseolin type I exclusive to plants from the Peru and Ecuador.

Pathogen populations and their adaptation processes in a geographic location could be driven by factors such as local climate change, temperature, humidity etc. Colombia and Costa Rica regions are said to be intermediate phase separating two main gene pools of *P.vulgaris* from its ancestral origins (Bitocchi *et al*., 2012), which was partially supported by the results grouping Costa Rican and Colombian isolates in the same genetic groups and occasionally haplotypes for all five loci. It included isolates 832 and 814 -race unknown from Costa Rica and 694 representing 137-epsilon race from Colombia (Table 2.1). High homology between the three organisms may have its roots in similar coevolution of the same cultivars of *P. vulgaris* and adapted *C. lindemuthianum* strains belonging to the same gene pool. Alternatively, pathogen spread via the environment and/or the planting material by means of air currents many generations ago followed by adaptation processes that involved changes in nucleotide sequence is a possibility.

Mesoamerican *C. lindemuthianum* race 137-epsilon has previously been reported by Pastor-Corrales *et al*. (1995) and Mahuku *et al*. (2002) corresponding to isolate CL94 collected in Colombia in 1989. This isolate was exposed to 12 differential cultivars and 3 have been susceptible: Michelite, Cornell 49242, and PI 207262. Amongst other Mesoamerican isolates, CL94 expressed moderate pathogenicity just below median value. The source of resistance in Michelite is *Co-1* gene, in PI207262 it is *Co-4* and *Co-9* (Poletine *et al*., 1999), while in Cornell 49242 the resistance is facilitated by *Are Co-2* locus (Mastenbroek, 1960). While race 137-epsilon is pathogenic to the *P. vulgaris* cultivars above, other epsilon races 69 and 453 were non-pathogenic to PI 207262 and Cornell 49242 differentials (Poletine *et al*., 1999; Poletine *et al*., 2000). Michelite cultivar

susceptible to epsilon races, have proven resistant to other races of *C. lindemuthianum* like alpha, βeta (130), gamma (102), and amongst others: 8, 64, 1088, 1344, MA-1 (Mexican) (Goncalves Vidigal *et al*., 2007). Thus, even in situation of the same race type as in this case of epsilon, variable resistance/susceptibility (R/S) results have been recorded. Moreover, R/S assessment can be very subjective depending on the degree of infection required to designate the cultivar as susceptible leading to mischaracterization of the *C. lindemuthianum* races.

Molecular markers ACT, CAL, HIS3 and TUB were more conserved and did not differentiate any specific genetic groups. However, this pattern could change if a much larger number of isolates are screened.

Although HMG/MAT1 locus did not identify distinct genetic groups, there were two sub-groups where two Costa Rican isolates were separated into HT3 and HT4 indicating that occurrence in the same geographic location does not necessarily signify genetic identity or common origin. This differentiation was also observed for ITS and concatenated data. There was no clearly evident relationship overall between the HMG haplotype allocations of *C. lindemuthianum* isolates and their geographic origins or race..

The opposite is observed based on GADPH analysis where Colombian and two Costa Rican isolates were grouped together suggesting that this locus could potentially reflect the biogeographic origins of the *C. lindemuthianum*; however more isolates from these regions should be screened in order to confirm this potential. Interestingly, isolates belonging to HT1 despite different origins showed 100% homology (Appendix VII Table 2), which could be due to their association with the recent deployment of common bean cultivars.

Grouping of isolates from different geographic locations into the same haplotypes was observed for all molecular markers in this study and although the genetic group allocations vary in some cases, there are some trends observed as discussed above linked to host variety deployment. There is a high possibility that these isolates were sourced from the same host gene pool either of Mesoamerican

or Andean origin. The races may have developed later on as a consequence of interaction with different cultivars of *P. vulgaris* that may have driven the adaptive responses in *C. lindemuthianum* and their pathogenic specificity e.g. Cornell 49-242 containing *Are* resistance gene capable of differentiating race kappa that is virulent to other crop varieties (Alzate-Marin, 1999). Theory on dissemination processes of *P. vulgaris* around Africa and Europe was proposed by Gepts and Bliss (1988) based on the phaseolin type observed in common bean cultivars. Crop exchange began soon after the discovery of the Americas. In first instance it reached Iberian Peninsula (Portugal) followed by spreading to the rest of Europe and other parts of the world (Simmonds, 1976). First record of common bean in Europe was made by Turner in 1538 (Gepts and Bliss, 1988). However, there is no clear information on the source or introduction of *P. vulgaris* in Africa.

There are several types of phaseolin observed amongst *P. vulgaris* cultivars associated with its geographic origins: 'S'-small seeded variety originating from Middle America, 'B'- also small seeded from Colombia, while 'T', 'A','C' and 'H' were large seeded varieties found in South of Andes (Gepts, 1984; Gepts *et al*., 1986). Evidence showed that the most common phaseolin type in Europe and Africa was 'T' found in 72% and 69% of cultivars respectively (Gepts and Bliss, 1988). Abundance of 'T' type phaseolin type cultivars is said to be due to their green pods or better adaptability to the European climate (Brown *et al*., 1982). Crucially, type 'B' phaseolin was not reported for the differentials from Europe or Africa (Gepts and Bliss, 1988), suggesting that cultivars from Colombian regions were not included in large scale deployment processes. Genetic diversity of *C. lindemuthianum* observed in GADPH dataset relates to these findings through distinct separation of isolates from Costa Rica and Colombia on the basis of their unique nucleotide sequence. There were two substitutions observed in all three isolates, where 'A' has been replaced by 'T' in 33rd position, while 'C' compensated for 'T' in 69th base of the 100bp long sequence. Interestingly, the same characters feature in *C. orbiculare* sequence that may further support the belief that these changes have ancestral lineage. Nevertheless, relationship between the 832, 814 and 694 is not so apparent for AP-PCR result analysis, where they were split into separate haplotypes.

Frequent association of isolates of various races into the same haplotypes suggests that the molecular markers used in this study do not differentiate isolates on the basis of their pathogenic specificity, and different markers may have to be investigated, which could be revealed through large-scale genome studies.

With the concatenated data, 693-kappa from Brazil was separated from the rest, which imply their distinct genetic background. Brazil is the main provider of common beans in the world with 39.85% being produced between 2000-2004 (FAO STAT, 2005); however, Brazil is not considered in the main gene pools of *P. vulgaris.* This suggests that the pathogen may have originated from a separate gene pool. Dissemination of the pathogen into new/different geographic locations either through infected seeds and/or environmental factors needs to be further investigated. Multialleic gene cluster linked to pathogen specificity and the corresponding resistance gene cluster in the host could be explored in an attempt to establish the links between race and genetic diversity in the *C. lindemuthianum* (Crute and Pink, 1996).

The available evidence suggests that pathogen has adapted to the cultivars from the same geographic location generally. The pathotypes of Andean origin have narrower virulence range affecting bean cultivars with large seeds. On the other hand, Mesoamerican pathogens are able to infect wide range of hosts particularly the small-seeded varieties (Pastor-Corrales *et al*., 1995). Geffroy *et al*. (1999) identified ancestry resistance specificity gene cluster in common bean commencing from the period before the separation into two pathotype gene pools identified as *Co-9* in Mesoamerican and *Co-y*/*Co-2* in Andean cultivars. The host-pathogen coevolution was revealed when plants expressed resistance to most of the 'non-native' races while remaining susceptible to local races (Geffroy *et al*., 1999). More research needs to be carried out on evolutionary lineage of European and African pathotypes, where *P. vulgaris* is not a conventional crop, which would improve the selection process of cultivars in those areas of the world (Ansari *et al*., 2004).

AP-PCR profiles for the *C. gloeosporioides* isolate 771 and *C. truncatum* isolate 449 were distinctive with each of the 3 primers used, confirming their

distant genetic background. Within *C. lindemuthianum*, AP-PCR methodology was useful in revealing the genetic diversity enabling the identification of various haplotypes. For example, CAC_5 distinguished 9 haplotypes, while CAG_5 provided a more conservative estimate of 5 haplotypes reflecting the sequence differences in different parts of the genome. These haplotype groupings were not fully reflective of the results of the multilocus phylogenetic analysis. For example, CAG_5 separated isolate 216 from 701, 776 and 779 that were all represented by a single haplotype based on the concatenated sequence analysis. Similarly, isolates 45 and 832 each were distinguished as individual haplotypes by GAC_5 and CAG_5 primers. Interestingly, an assessment of biological parameters such as growth rate, level of sporulation and colony morphology also revealed variation amongst the five *C. lindemuthianum* isolates examined. *C. lindemuthianum* isolates were in general considerably slower growing compared to *C. gloeosporioides* which is well recognised as one of the faster growing species within the genus Colletotrichum (e.g. Talhinhas *et al*., 2002).

Thus, the AP-PCR profiling approach is likely to be more suitable for the characterisation and monitoring of local populations of *Colletotrichum* spp. than in the context of phylogenetic analysis of global populations. Consistent AP-PCR profiles from various primers under standardized PCR conditions, can be subjected to binary matrix analysis (Paul, 2001). Band-matching software (e.g. GeneDirectory) in combination with binary data analysis software such as Treeson version 1.3b that employs the UPGMA clustering system are especially useful when population studies are carried out involving a large number of isolates (Van de Peer and De Wachter, 1997).

4.1. *Conclusions and Future Directions*

Results indicate significant genetic diversity within *C. lindemuthianum* associated with *P. vulgaris*. The multilocus analysis indicated some level of correlation between the geographic origin and genetic diversity, by separating the *C. lindemuthianum* isolates into two distinct genetic groups. It reflects the current

information of two main gene pools of Mesoamerican and Andean locations associated with *P. vulgaris* origins (Pastor-Corrales *et al*., 1995).

Multilocus analysis was useful in species delimitation and identification of genetic diversity within *C. lindemuthianum*. Resolution of the markers ranged from high (ITS, HMG), moderate (GD, ACT, CHS-1, GS, HIS3) to low (TUB, CAL). Nonetheless, conserved low resolution markers such as β-tubulin (TUB) were able to establish the right taxonomic order for *Colletotrichum* genus. All molecular markers differentiated between 1 and 5 haplotypes for *C. lindemuthianum* isolates. Results established by GD parsimony analysis positioned the *C. orbiculare* isolate MAFF_240422 further from *C. lindemuthianum* than *C. truncatum*. However, the sequence homology showed higher similarity of *C. lindemuthianum* with *C. orbiculare* at 73.5% than *C. truncatum* calculated at 49.7%. Results generated for AP-PCR were not compatible with the multilocus phylogenetic analysis and provided more general overview of genetic diversity. However, it did identify *C. truncatum* and *C. gloeosporioides* as separate haplotypes outlining their distinct genetic background. Hence, multilocus analysis remained a crucial element in the study giving basic information about genetic diversity and phylogenetic relationships within *C. lindemuthianum* species that serve as a useful platform for further research.

The homology ranges between *C. lindemuthianum*, *C. truncatum* and *C. gloeosporioides* revealed that homology within *C. lindemuthianum* based on all molecular markers and concatenated data was 97.8-100%, *C. lindemuthianum* in relation to *C. truncatum* range was 49.7-87.8%, while for *C. gloeosporioides* it was 51.2-90.1%.

The limited number of molecular markers only provided restricted amount of information about the genetic diversity of the *C. lindemuthianum* isolates. Genome sequencing would provide a much better understanding of the adaptive responses in relation to the biotic and abiotic environmental variables. More specifically, which genomic regions and genes are affected the most and the

extent of change, e.g. based on comparison of the historical isolates with contemporary isolates.

Results of the present study provide an overview of the population biogeographic diversity in *C. lindemuthianum*. Further development of the research would involve genome sequencing of selected *C. lindemuthianum* isolate(s) using NGS technology that would serve as reference genome(s) adopting the methodologies and strategy used with *C. orbiculare* (Gan *et al.*, 2013). Genome sequences constitute a platform for further research using appropriate molecular strategies that would provide the experimental validation of gene function and the genotype- phenotype-environmental interactions, the developmental focus of this project.

A combination of single nucleotide polymorphisms (SNPs) and individual haplotypes (HTs) are an important resource in understanding population level adaptations as demonstrated with human demographic investigations (e.g. Nielsen, 2000). This strategy also requires the development and/or use of stringent statistical models/analysis for the robust identification of SNPs and HTs (Ewing and Greeen, 1998). Identifying highly polymorphic segments of genome whilst avoiding underestimation of SNPs (Li *et al*., 2008) and maintaining the accuracy and prediction of any erros are all critical issues (Schaffner *et al*., 2005). Principle component analysis (PCA), genome-wide association studies and the use of software like STRUCTURE have proved suitable to large-scale population studies (Kaeuffer *et al*., 2007).

Functional genomics to investigate and understand gene function and the evolution of gene networks is another area that is evolving dramatically with the availability of vast quantities of genome data emerging from the application of NGS. There are several different approaches for assessing gene function in filamentous fungi (Weld *et al*., 2006). This includes random and targeted insertional mutagenesis/gene knockout (Alberts *et al*., 2002) based on homologous recombination (Weld *et al*., 2006), RNA interference (RNAi) for gene expression knockdown (Arenz and Schepers, 2003) and the use of Agrobacterium-mediated fungal transformation (Michielse *et al*., 2005). High

throughput gene disruption strategies have been adapted for large scale genomic studies, where large number of genes needs to be assessed e.g. use of overlapping or fusion PCR (e.g. Wendland, 2003).

The present study has contributed to the development of new knowledge and resources that would serve as a platform for further NGS-based investigations to decipher environmental change adaptation in *Colletotrichum* species such as *C. lindemuthianum*. Comparative analysis of historical isolates characterised in this study with contemporary isolates would be a key in this strategy.

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Appendix

Appendix I

GenElute Plant Genomic DNA Miniprep Kit (Sigma) Protocol

Sigma based method for multilocus molecular phylogenetic analysis purposes.

GenElute Plant Genomic DNA Miniprep Kit was used for DNA extractions for multilocus phylogenetic analysis purposes. Sigma protocol was followed as indicated by manufacturer with omission of the first step. Hot block was set for 65ºC and 100µl of molecularly sterile water (Sigma) was heated up for each sample allowing an additional 50µl in case of evaporation.

a) The first step involves disruption of cells, which was achieved by previously described chelex/sand method.

b) In order to lyse the cells 350µl of Lysis solution (Part A) and 50µl of Lysis Solution (Part B) were added to the supernatant and mixed by vortexing and inverting. The tubes were incubated on hot block for 10min. Upon formation of white precipitate, the tubes were inverted few times during incubation process in order to dissolve it.

c) Subsequently, 130µl of Precipitation Solution was added and mixed by inversion. Then tubes were placed on ice for 5min followed by centrifugation at max speed for 5 min to precipitate debris.

d) The supernatant was removed and pipetted onto the GenElute filtration column placed in 2ml collection tube. Tubes were centrifuged at max speed for 1min to ensure debris-free solution.

e) Then the flow-through liquid was topped with 700µl of Binding Solution and mixed by inversion.

f) To prepare the GenElute Miniprep Binding column, 500µl of the Column Preparation Solution was added and centrifuged at max speed for 30s to 1min. The flow-through liquid was discarded. This process ensures optimal adsorption of nucleic acid to the solid phase.

g) 700µl of lysate from step e) was loaded into prepared the GenElute Miniprep Binding column followed by centrifugation at max speed for 1min. Flow-through liquid was discarded and step was repeated with remaining sample.

h) The binding column was placed in fresh 2ml collection tube and 500µl of diluted Wash Solution was loaded. Tubes were span at max speed for 1min. Flow-through liquid was discarded and collection tube was re-used for second wash with 500µl of diluted Wash Solution. Tubes were span for 3min in order to dry the column.

i) DNA was eluted in water previously heated up to 65ºC instead of elution buffer provided in the kit. The 100µl of water was loaded onto the column and after 1min centrifuged at max speed. The flow-through liquid was re-loaded into the column and after 1 min centrifuged one more time. This process ensured high concentration of DNA extract.

Appendix II

DNeasy Plant Mini Kit (Qiagen) Protocol

Qiagen kit method for extraction of genomic DNA.

The DNeasy Plant Mini Kit (Qiagen) was used for DNA extractions prior genome sequencing processes following manufacturer's protocol.

The cultures were grown in 20ml beakers filled with thin layer of PDB (between 3-5mm). Minimal amount of liquid media ensures that the mycelia float on top of PDB instead of drowning which would in turn create anaerobic conditions halting fungal growth. Previously prepared culture plates were cut into squares 3/3mm in diameter and dropped on top of PDB. Minimal amount of agar was removed while inoculating liquid cultures to maximize optimal results during DNA extraction. Beakers were tightly closed and incubated at 25ºC for 3-5days. After incubation period the PDB was removed and mycelial mat was washed twice in autoclaved water. Then mat was placed on filter paper and excess moisture was removed. Subsequently, fungal material was wrapped in 3 layers of aluminium foil and frozen in dry ice to stop the fungi from dying.

a) Dry ice (BOC) (CO_2) and coffee blender (mortar and pestle with dry ice worked equally well) were used for grinding of the fungal material. The fungal material was weighted and ~100mg wet weight was used for grinding (with additional allowance in order to compensate for the loss of mycelia due to grinding process). Ground material was placed in 20ml beakers and 400µl of Buffer AP1 was added straight away to stop biochemical reactions within DNA. The mixture was left till the dry ice evaporated.

b) Subsequently, the mycelia-buffer solution was placed in fresh 2ml eppendorf tube and 4µl of RNase A was added to remove the RNA from the solution. Mix was vortexed and incubated at 65ºC for 10min. Tubes were inverted 2-3 times during incubation.

c) Afterwards, 130µl of Buffer P3 was added and tubes were incubated on ice for 5min in order to halt the process. Lysate was centrifuged at max speed for 5 min.

Then supernatant was pipetted into QIAshreader spin column (placed in 2ml collection tube) in a way to avoid the disturbance of pellet. The tubes were span at max speed for 2min.

d) The flow-through liquid was transferred into the fresh 2ml microcentrifuge tube without disturbing the pellet and 1.5 volumes of Buffer AW1 was added and mixed by pipetting.

e) The 650µl of solution was removed into the DNeasy Mini spin column placed in 2ml collection tube and centrifuge for 1min at ≥6,000 x g. The supernatant was discarded and procedure was repeated with the remaining sample.

f) Column was put into a fresh collection tube and 500µl of Buffer AW2 was added followed by centrifugation for 2min at max speed. Then, spin column was placed in fresh 1.5ml eppendorf tube.

g) The elution buffer was heated to 65ºC and 100µl was pipetted into each column. Tubes were incubating at room temperature for 5min followed by centrifugation at ≥6,000 x g for 1 min. The 100µl of flow-through liquid was re-loaded into the column and procedure was repeated. This process ensures high concentration of DNA extract.

Appendix III

QIAquick PCR Purification Kit Protocol

Add ethanol (96–100%) to Buffer PE before use (see bottle label for volume). All centrifugation steps are carried out at 17,900 x *g* (13,000 rpm) in a conventional table-top microcentrifuge at room temperature. Add 1:250 volume pH indicator I to Buffer PB. The yellow color of Buffer PB with pH indicator I indicates a pH of ≤7.5. If the purified PCR product is to be used in sensitive microarray applications, it may be beneficial to use Buffer PB without the addition of pH indicator I. Do not add pH indicator I to buffer aliquots.

1. Add 5 volumes Buffer PB to 1 volume of the PCR reaction and mix. If the color of the mixture is orange or violet, add 10 µl 3 M sodium acetate, pH 5.0, and mix. The color of the mixture will turn yellow.
2. Place a QIAquick column in a provided 2 ml collection tube or into a vacuum manifold. For details on how to set up a vacuum manifold, refer to the *QIAquick Spin Handbook*.
3. To bind DNA, apply the sample to the QIAquick column and centrifuge for 30–60 s or apply vacuum to the manifold until all the samples have passed through the column. Discard flow-through and place the QIAquick column back in the same tube.
4. To wash, add 0.75 ml Buffer PE to the QIAquick column centrifuge for 30–60 s or apply vacuum. Discard flow-through and place the QIAquick column back in the same tube.
5. Centrifuge the QIAquick column once more in the provided 2 ml collection tube for 1 min to remove residual wash buffer.
6. Place each QIAquick column in a clean 1.5 ml microcentrifuge tube.
7. To elute DNA, add 50 µl Buffer EB (10 mM Tris•Cl, pH 8.5) or water (pH 7.0–8.5) to the center of the QIAquick membrane and centrifuge the column for 1 min. For increased DNA concentration, add 30 µl elution buffer to the center of the QIAquick membrane, let the column stand for 1 min, and then centrifuge.
8. If the purified DNA is to be analyzed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.

Appendix IV

DNA Sample Requirements for Sequencing Provided by Cambridge Sequencing Facility

New! For information on our Next-Gen DNA sequencing service go to our MiSeq Sequencing page or our 454 Sequencing page.

For information on our Cosmid DNA sequencing service go to our Cosmid Sequencing page.

For large sequencing orders we now offer a DNA preparation service. Please contact John Lester for further information.

DNA submitted to the facility needs to be very pure, much purer for instance than for manual sequencing. It is for this reason that we recommend that DNA should be prepared using a commercial kit such as Qiagen, the most commonly used type being the Tip 20. Traditional methods, ie. alkaline lysis, can work if care is taken.

For plasmids we require 10µl of DNA in water at a concentration of 100ng/µl per sequencing reaction.
Cosmids should be submitted at a concentration of 150ng/µl in water.
PCR fragments should be supplied at a concentration of 20ng per 100 base pairs in 10µl water.

Any non-standard primers submitted should be at a concentration of 10pm/µl (10µM) in water. We use 2µl of primer solution per reaction but please give us an excess to allow for evaporation or any other potential loss.

We need the correct amount of DNA and most experienced sequencers will be able to make an accurate assessment of DNA quantity but some may have difficulty. One method is to use a Pharmacia Gene Quant. This can give consistent accurate results and automatically provides abs. 260/280 ratio (which for best results should be around 1.8) however one must ensure careful use and that the cuvette used is very clean if spurious results are to be avoided.

N.B. DNA for sequencing should always be supplied in water only and not TE or Tris buffer. Also please submit samples in tubes no smaller than 0.5ml to avoid handling problems.

Samples and completed DNA sequencing and Cosmid sequencing request forms can be dropped off in the basket provided in the Biochemistry reception (Sanger Building) or posted to the facility at the following address:

Older Macs use Editview
MacOSX use 4Peaks
Windows, Linux and MacOSX use FinchTV

Appendix V

Assessment of DNA Concentration and Purity Using NanoDrop Technology

NanoDrop technology entails microvolume UV-Vis spectrophotometers and fluorospectrophotometers enabling to load 0.5-2.0 µl of sample, which greatly reduce the wastage of resources. It provides fast information about quality and quantity of the nucleic acid. The surface tension between the two optical fibers eliminates the need for cuvettes. The concentration of nucleic acid is measured at 260 nm, while purity is assessed using 260/230 and 260/280 ratios. The 260/280 ratio of ~1.8 for DNA and 2.0 for RNA suggest high purity of the sample. Lower values could indicate contamination with protein (especially aromatic amino acids), phenols and other impurities that strongly absorb light at 280 nm. The 260/230 ratio expected values range from 2.0-2.2, lower results indicate contamination with carbohydrates, phenolic solutions and buffers used for DNA/RNA isolation/purification e.g. EDTA, TRIzol reagent. Carbohydrates and phenols absorb light at 230 nm, while phenolic solutions like TRIzol reagent will absorb light both at 230 and 270nm (Thermo Fisher Scientific). DNA samples were analysed using a Thermo Scientific NanoDrop 2000 Spectrophotometer.

Appendix VI

Multiple Sequence Alignments Generated for *Colletotrichum* Isolates and the Corresponding *C. orbiculare* MAFF 240442

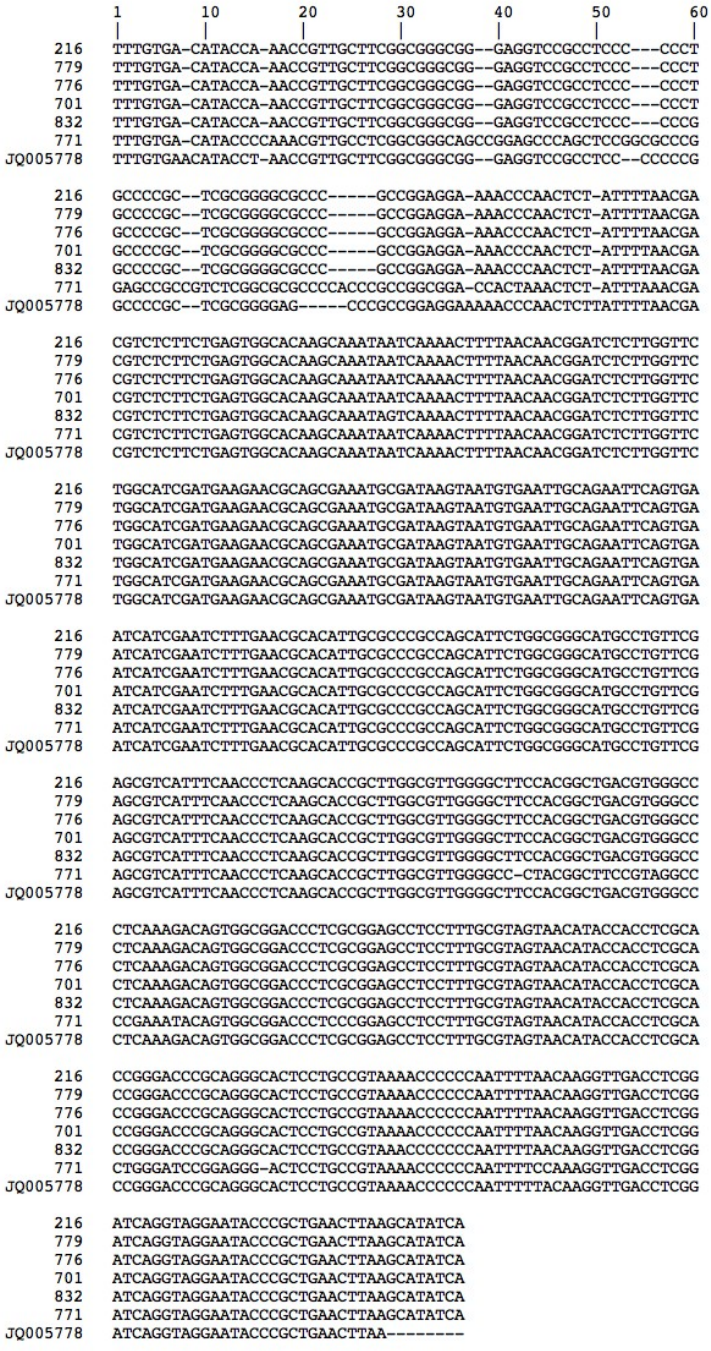

Fig **1.** Multiple Sequence Alignment of Ribosomal RNA Gene Block Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

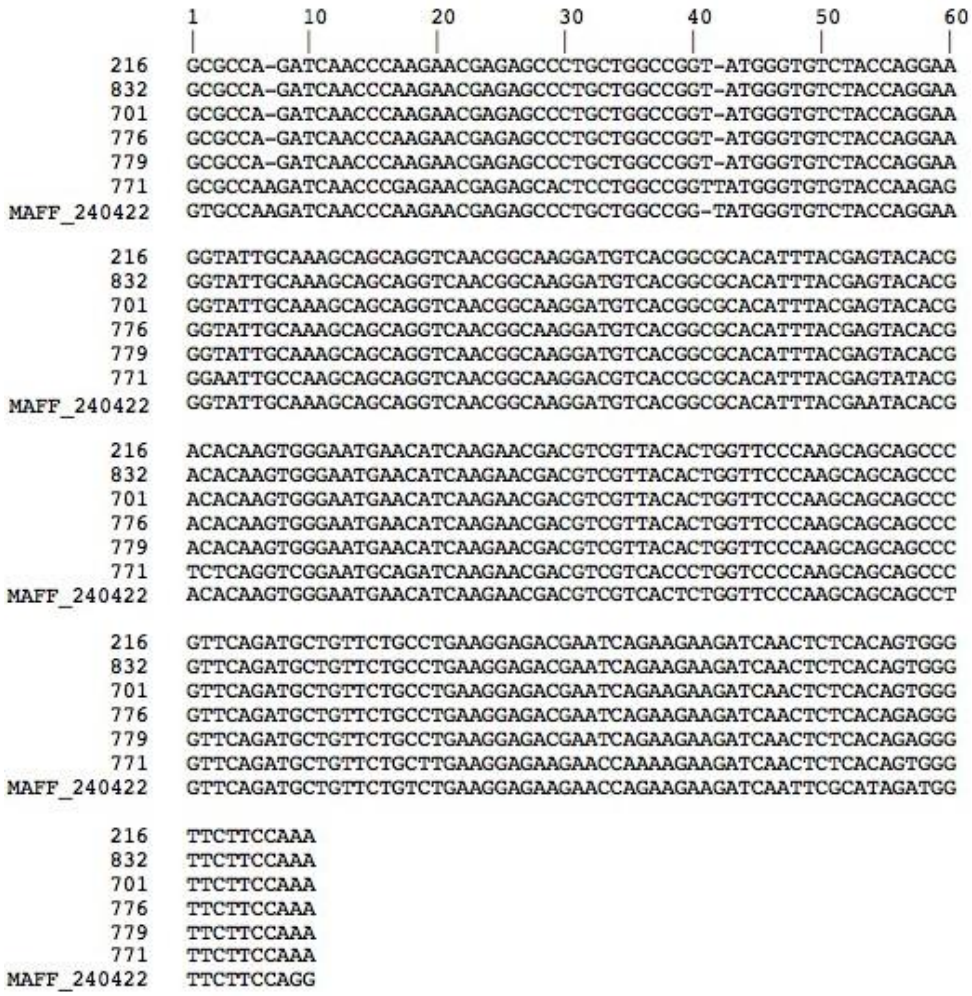

Fig 2. Multiple Sequence Alignment of Chitin Synthase (CHS) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

```
                1         10         20         30         40         50         60
                |         |          |          |          |          |          |
        216     AGGGTATGTTGATCCCGATAAGCCCCCCATTCCTTCCTTTTTG-CTTCAGTTCGGGTGGC
        701     AGGGTATGTTGATCCCGATAAGCCCCCCATTCCTTCCTTTTTG-CTTCAGTTCGGGTGGC
        776     AGGGTATGTTGATCCCGATAAGCCCCCCATTCCTTCCTTTTTG-CTTCAGTTCGGGTGGC
        779     AGGGTATGTTGATCCCGATAAGCCCCCCATTCCTTCCTTTTTG-CTTCAGTTCGGGTGGC
        832     AGGGTATGTTGATCCCGATAAGCCCCCCATTCCTTCCTTTTTG-CTTCAGTTCGGGTGGC
        771     AGGGTATGTCCACCCCGATAAGCCGCCTTCCCATCCCTTATCATCCTCATTTC-------
MAFF_240422     AGGGTATGTCGATTCCGATAAGCCCCCCATTCCTTCCTTTTTG-CTTCAGTTCGGATGGC

        216     ATCTCTCTTCGTGTCCTGTCAGCCCTATGCGACTGGCGCCCGCACTGCCGCGCACATGAT
        701     ATCTCTCTTCGTGTCCTGTCAGCCCTATGCGACTGGCGCCCGCACTGCCGCGCACATGAT
        776     ATCTCTCTTCGTGTCCTGTCAGCCCTATGCGACTGGCGCCCGCACTGCCGCGCACATGAT
        779     ATCTCTCTTCGTGTCCTGTCAGCCCTATGCGACTGGCGCCCGCACTGCCGCGCACATGAT
        832     ATCTCTCTTCGTGTCCTGTCAGCCCTATGCGACTGGCGCCCGCACTGCCGCGCACATGAT
        771     ----CCTTTTGTCTCTTGCTGG-----GGAAAGTGGC-CCCGCGCCGTTGGGTG------
MAFF_240422     ATCTCTCTTCGTGTCTTGTCAGCTCTATGTAACTGGCGCCCGCACTGCTGCGCACCTGAT

        216     CCTCTTC-AACCAGAAGATTTTGTGCTGCCCTCCAGCTTCGTTACTGTTGTTGCACCTGC
        701     CCTCTTC-AACCAGAAGATTTTGTGCTGCCCTCCAGCTTCGTTACTGTTGTTGCACCTGC
        776     CCTCTTC-AACCAGAAGATTTTGTGCTGCCCTCCAGCTTCGTTACTGTTGTTGCACCTGC
        779     CCTCTTC-AACCAGAAGATTTTGTGCTGCCCTCCAGCTTCGTTACTGTTGTTGCACCTGC
        832     CTTCTTC-AACCAGAAGATTTTGTGCTGCCCTCCAGCTTCGTTACTGTTGTTGCACCTGT
        771     -------------GAGGGTTC------GCTCTCCTCCTTCGG----GGTGTCGC------
MAFF_240422     CCTCTTCAAACCAGAAGATTTTGTGCTGCCCTCCAGCTTCGTTACTGTTGTTGCACCTGC

        216     ATTGTGAGCTGGCACTTGCCTCCTCTCAGCACAGCACGACAAAGCCAAGCTGGGGAAGCG
        701     ATTGTGAGCTGGCACTTGCCTCCTCTCAGCACAGCACGACAAAGCCAAGCTGGGGAAGCG
        776     ATTGTGAGCTGGCACTTGCCTCCTCTCAGCACAGCACGACAAAGCCAAGCTGGGGAAGCG
        779     ATTGTGAGCTGGCACTTGCCTCCTCTCAGCACAGCACGACAAAGCCAAGCTGGGGAAGCG
        832     ATTGTGAGCTGGCACTTGCCTCCTCCCAGCACAGCATAACAAAGCCAAGCTGGGGAAGCG
        771     ----------------------------------------------------------CG
MAFF_240422     AATGTGAGCTGGCACCTGCCTCCTCCCAGCACAGCACGACAAAGCAAAGCTGGGGAAGCA

        216     AGCGGCCCCGTGCTTCTTTTTTGGCATTGAGCTCCAAACAGGCGGAGGGGCTGCTGCAGC
        701     AGCGGCCCCGTGCTTCTTTTTTGGCATTGAGCTCCAAACAGGCGGAGGGGCTGCTGCAGC
        776     AGCGGCCCCGTGCTTCTTTTTTGGCATTGAGCTCCAAACAGGCGGAGGGGCTGCTGCAGC
        779     AGCGGCCCCGTGCTTCTTTTTTGGCATTGAGCTCCAAACAGGCGGAGGGGCTGCTGCAGC
        832     AGCGGCCCCGTGCTTCTTTTTTGGCATTGAGCTCCAAATAGGCGGAGGGGCTGCTGCTGC
        771     AGCGCTCCC------------CGGGGCTGA-----AAATAGGC----GGGTTGCTGCAGC
MAFF_240422     AGCGGCCCCGTGTTTCCTTTTTGGCATTGAGGTCCAAATAGGTGGAGGGGCTGCTGCTGC

        216     AG-ATACCGCAATG----GAAAAGCACTGGGGCTTGGCGGGG--CCAAAACATCGT--TT
        701     AG-ATACCGCAATG----GAAAAGCACTGGGGCTTGGCGGGG--CCAAAACATCGT--TT
        776     AG-ATACCGCAATG----GAAAAGCACTGGGGCTTGGCGGGG--CCAAAACATCGT--TT
        779     AG-ATACCGCAATG----GAAAAGCACTGGGGCTTGGCGGGG--CCAAAACATCGT--TT
        832     AG-ATACCGCAATG----GAAAAGCACTGGGGCTTGGCGGGG--CCAAAACATCGT--TT
        771     CGTGTGCGGCAACGCATCGGTAAGCACTGGGGCTTGGCGGGGGGTCAAACCACCGTCTTT
MAFF_240422     AG-AAACCGCATTG----GAAAAGCACTGGGGCTTGGC--GGGGCCAAAACATCGTCT--

        216     GC----CCCTCTGG---CCA----AGT-----TTTGTGGATGGATCGGTGGTATCAGCAT
        701     GC----CCCTCTGG---CCA----AGT-----TTTGTGGATGGATCGGTGGTATCAGCAT
        776     GC----CCCTCTGG---CCA----AGT-----TTTGTGGATGGATCGGTGGTATCAGCAT
        779     GC----CCCTCTGG---CCA----AGT-----TTTGTGGATGGATCGGTGGTATCAGCAT
        832     GCTCGCCCCTCTGG---CCA----AGT-----TTTGTGGGTGGATGGGTGGTATCAGCAT
        771     GC----CCCTCCAGAGCCCAGAACAGTACGGGTGTGCGGGTG--CGGATGGTTTGTGGAC
MAFF_240422     ----GCCCCTCTGG---CCA----AGT-----TTTGTGGATGGAT-GGTGGTATCAGCAT

        216     CAGCGTCGCTTGCCAGACAG-TACGTACCCGGTCAGCTCAGCTCGGTCTGGCTGGACCGG
        701     CAGCGTCGCTTGCCAGACAG-TACGTACCCGGTCAGCTCAGCTCGGTCTGGCTGGACCGG
        776     CAGCGTCGCTTGCCAGACAG-TACGTACCCGGTCAGCTCAGCTCGGTCTGGCTGGACCGG
        779     CAGCGTCGCTTGCCAGACAG-TACGTACCCGGTCAGCTCAGCTCGGTCTGGCTGGACCGG
        832     CAGCGTCGCTTGCCAGACAG-TACGTACCCGGTCAGCTCAGCTCGGTCTGGCTGGACCGG
        771     TTGTGCC-CGTGTCGGACGGGTACGTACCTGG----CTCAGGTCGGCT---CTGAGCCGG
MAFF_240422     CAGCGTCGCT-GCCAGACA-GTACGTACCCGGTCAGCTCAGCTCGGTCTGGCTGGACCGG

        216     CTCTTAGCCGG--TCGGTCGT-TGGCTCTGCTGGCTGACGGGATTC-GTTCTCGAACACG
        701     CTCTTAGCCGG--TCGGTCGT-TGGCTCTGCTGGCTGACGGGATTC-GTTCTCGAACACG
        776     CTCTTAGCCGG--TCGGTCGT-TGGCTCTGCTGGCTGACGGGATTC-GTTCTCGAACACG
        779     CTCTTAGCCGG--TCGGTCGT-TGGCTCTGCTGGCTGACGGGATTC-GTTCTCGAACACG
        832     CTCTTAGCCGG--TCGGTCGT-TGGCTCTGTTGGCTGACGGGATTC-GTTCTCGAACACG
        771     CTCTGA-CTGGATTCGCTCCCACGACTCGGGTGATGCAAGGAACCCTGCCCCCCCACCACC
MAFF_240422     CTCTTAGCCGG--TCGGTCGT-TGGATCTTCTGGCTGACAGGATTC-GTTCTCGAACACG
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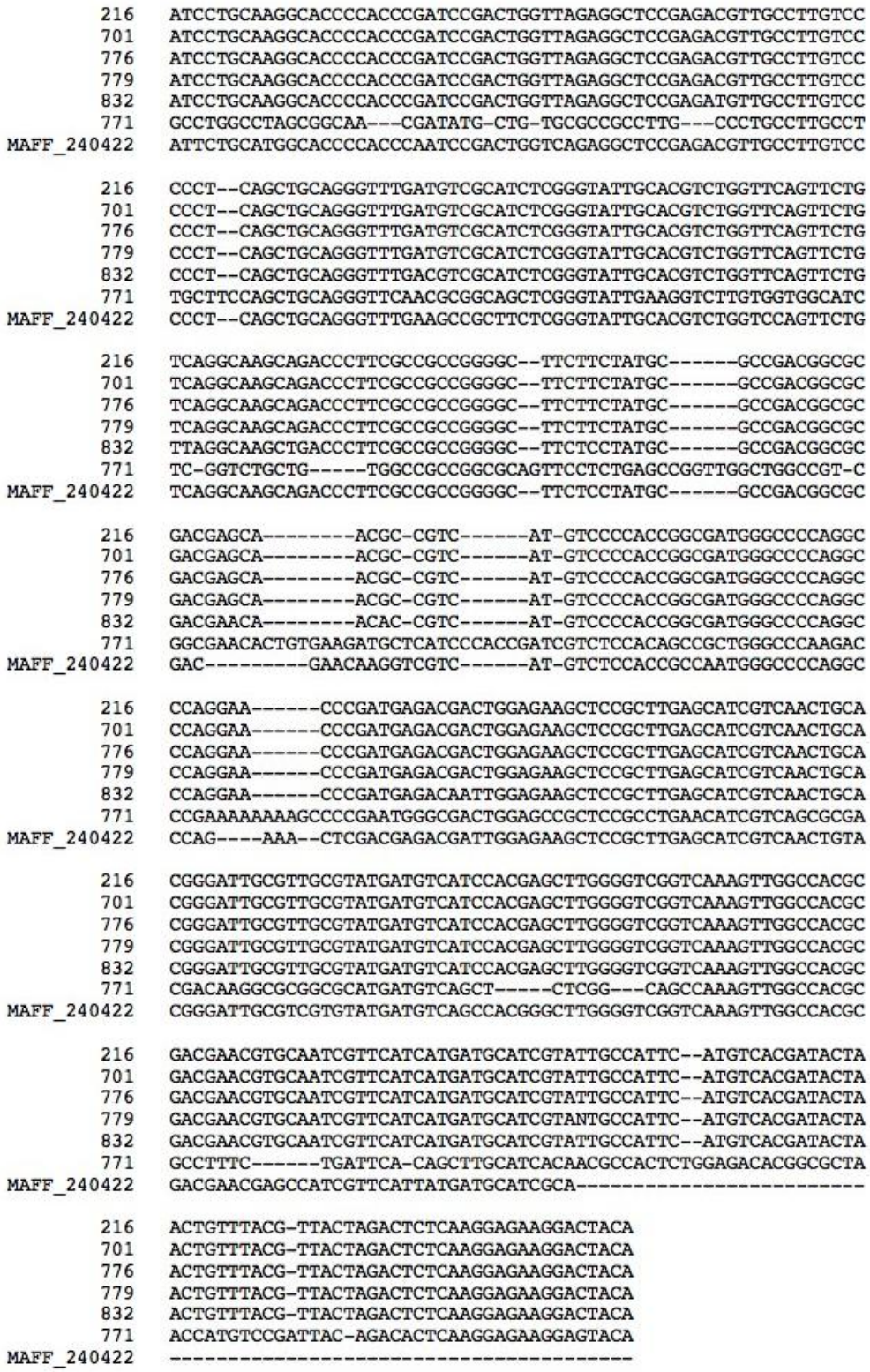


Fig 3. Multiple Sequence Alignment of Glutamine Synthetase (GS) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence

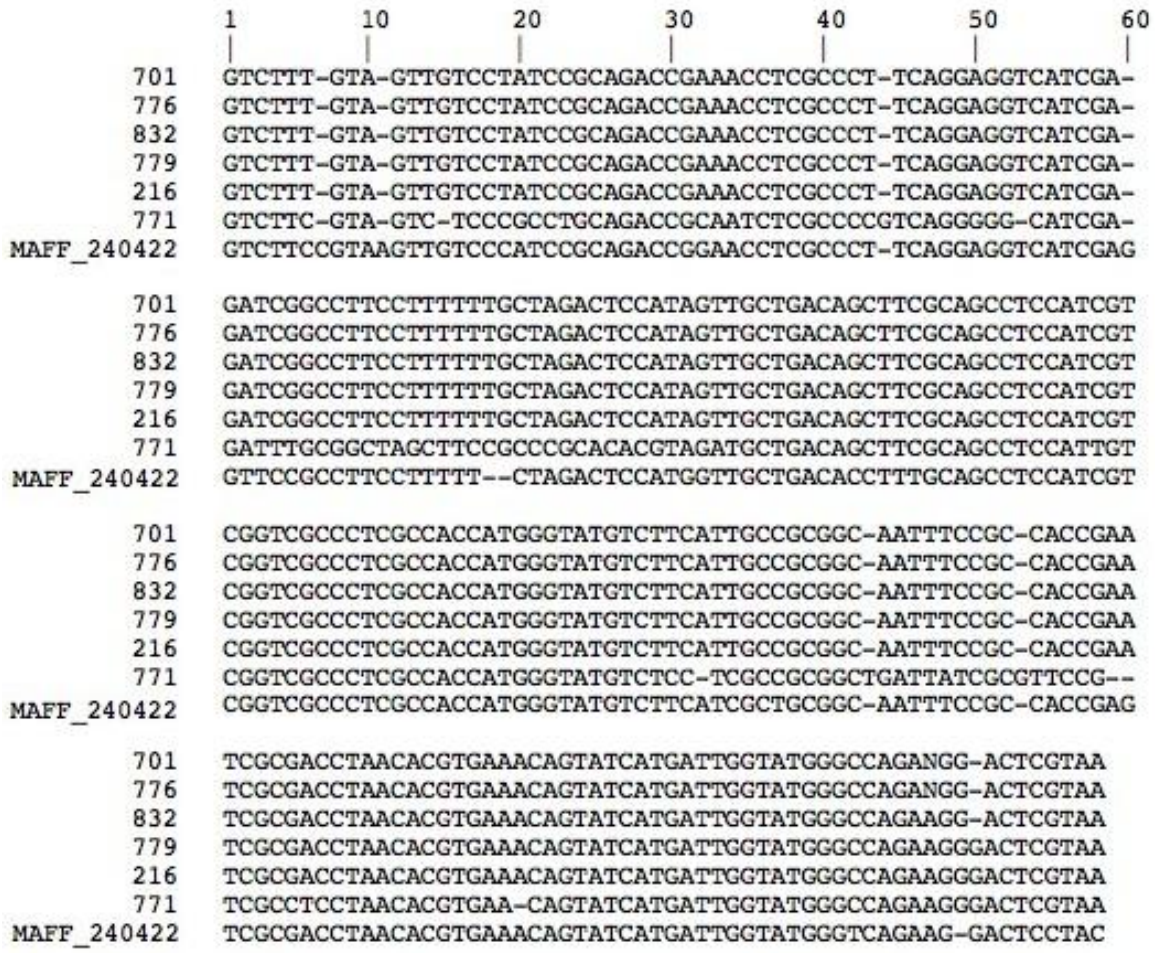
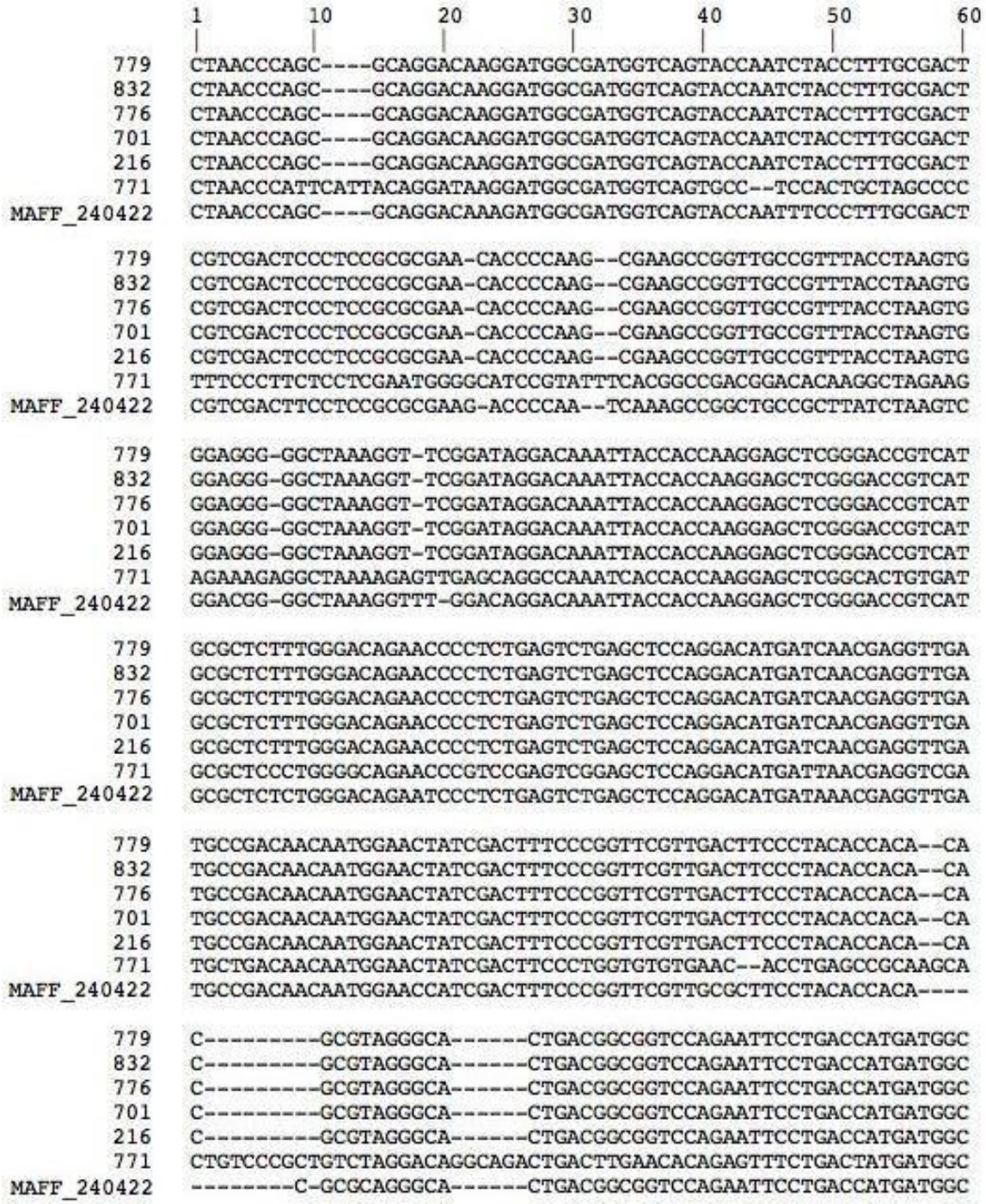


Fig 4. Multiple Sequence Alignment of Actin (ACT) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.


1 10 20 30 40 50 60
779 CTAACCCAGC----GCAGGACAAGGATGGCGATGGTCAGTACCAATCTACCTTTGCGACT
832 CTAACCCAGC----GCAGGACAAGGATGGCGATGGTCAGTACCAATCTACCTTTGCGACT
776 CTAACCCAGC----GCAGGACAAGGATGGCGATGGTCAGTACCAATCTACCTTTGCGACT
701 CTAACCCAGC----GCAGGACAAGGATGGCGATGGTCAGTACCAATCTACCTTTGCGACT
216 CTAACCCAGC----GCAGGACAAGGATGGCGATGGTCAGTACCAATCTACCTTTGCGACT
771 CTAACCCATTCATTACAGGATAAGGATGGCGATGGTCAGTGCC--TCCACTGCTAGCCCC
MAFF_240422 CTAACCCAGC----GCAGGACAAAGATGGCGATGGTCAGTACCAATTTCCCTTTGCGACT
779 CGTCGACTCCCTCCGCGCGAA-CACCCCAAG--CGAAGCCGGTTGCCGTTTACCTAAGTG
832 CGTCGACTCCCTCCGCGCGAA-CACCCCAAG--CGAAGCCGGTTGCCGTTTACCTAAGTG
776 CGTCGACTCCCTCCGCGCGAA-CACCCCAAG--CGAAGCCGGTTGCCGTTTACCTAAGTG
701 CGTCGACTCCCTCCGCGCGAA-CACCCCAAG--CGAAGCCGGTTGCCGTTTACCTAAGTG
216 CGTCGACTCCCTCCGCGCGAA-CACCCCAAG--CGAAGCCGGTTGCCGTTTACCTAAGTG
771 TTTCCCTTCTCCTCGAATGGGGCATCCGTATTTCACGGCCGACGGACACAAGGCTAGAAG
MAFF_240422 CGTCGACTTCCTCCGCGCGAAG-ACCCCAA--TCAAAGCCGGCTGCCGCTTATCTAAGTC
779 GGAGGG-GGCTAAAGGT-TCGGATAGGACAAATTACCACCAAGGAGCTCGGGACCGTCAT
832 GGAGGG-GGCTAAAGGT-TCGGATAGGACAAATTACCACCAAGGAGCTCGGGACCGTCAT
776 GGAGGG-GGCTAAAGGT-TCGGATAGGACAAATTACCACCAAGGAGCTCGGGACCGTCAT
701 GGAGGG-GGCTAAAGGT-TCGGATAGGACAAATTACCACCAAGGAGCTCGGGACCGTCAT
216 GGAGGG-GGCTAAAGGT-TCGGATAGGACAAATTACCACCAAGGAGCTCGGGACCGTCAT
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779 GCGCTCTTTGGGACAGAACCCCTCTGAGTCTGAGCTCCAGGACATGATCAACGAGGTTGA
832 GCGCTCTTTGGGACAGAACCCCTCTGAGTCTGAGCTCCAGGACATGATCAACGAGGTTGA
776 GCGCTCTTTGGGACAGAACCCCTCTGAGTCTGAGCTCCAGGACATGATCAACGAGGTTGA
701 GCGCTCTTTGGGACAGAACCCCTCTGAGTCTGAGCTCCAGGACATGATCAACGAGGTTGA
216 GCGCTCTTTGGGACAGAACCCCTCTGAGTCTGAGCTCCAGGACATGATCAACGAGGTTGA
771 GCGCTCCCTGGGGCAGAACCCGTCCGAGTCGGAGCTCCAGGACATGATTAACGAGGTCGA
MAFF_240422 GCGCTCTCTGGGACAGAATCCCTCTGAGTCTGAGCTCCAGGACATGATAAACGAGGTTGA
779 TGCCGACAACAATGGAACTATCGACTTTCCCGGTTCGTTGACTTCCCTACACCACA--CA
832 TGCCGACAACAATGGAACTATCGACTTTCCCGGTTCGTTGACTTCCCTACACCACA--CA
776 TGCCGACAACAATGGAACTATCGACTTTCCCGGTTCGTTGACTTCCCTACACCACA--CA
701 TGCCGACAACAATGGAACTATCGACTTTCCCGGTTCGTTGACTTCCCTACACCACA--CA
216 TGCCGACAACAATGGAACTATCGACTTTCCCGGTTCGTTGACTTCCCTACACCACA--CA
771 TGCTGACAACAATGGAACTATCGACTTCCCTGGTGTGTGAAC--ACCTGAGCCGCAAGCA
MAFF_240422 TGCCGACAACAATGGAACCATCGACTTTCCCGGTTCGTTGCGCTTCCTACACCACA----
779 C---------GCGTAGGGCA------CTGACGGCGGTCCAGAATTCCTGACCATGATGGC
832 C---------GCGTAGGGCA------CTGACGGCGGTCCAGAATTCCTGACCATGATGGC
776 C---------GCGTAGGGCA------CTGACGGCGGTCCAGAATTCCTGACCATGATGGC
701 C---------GCGTAGGGCA------CTGACGGCGGTCCAGAATTCCTGACCATGATGGC
216 C---------GCGTAGGGCA------CTGACGGCGGTCCAGAATTCCTGACCATGATGGC
771 CTGTCCCGCTGTCTAGGACAGGCAGACTGACTTGAACACAGAGTTTCTGACTATGATGGC
MAFF_240422 --------C-GCGCAGGGCA------CTGACGGCGGTCCAGAATTCCTGACCATGATGGC

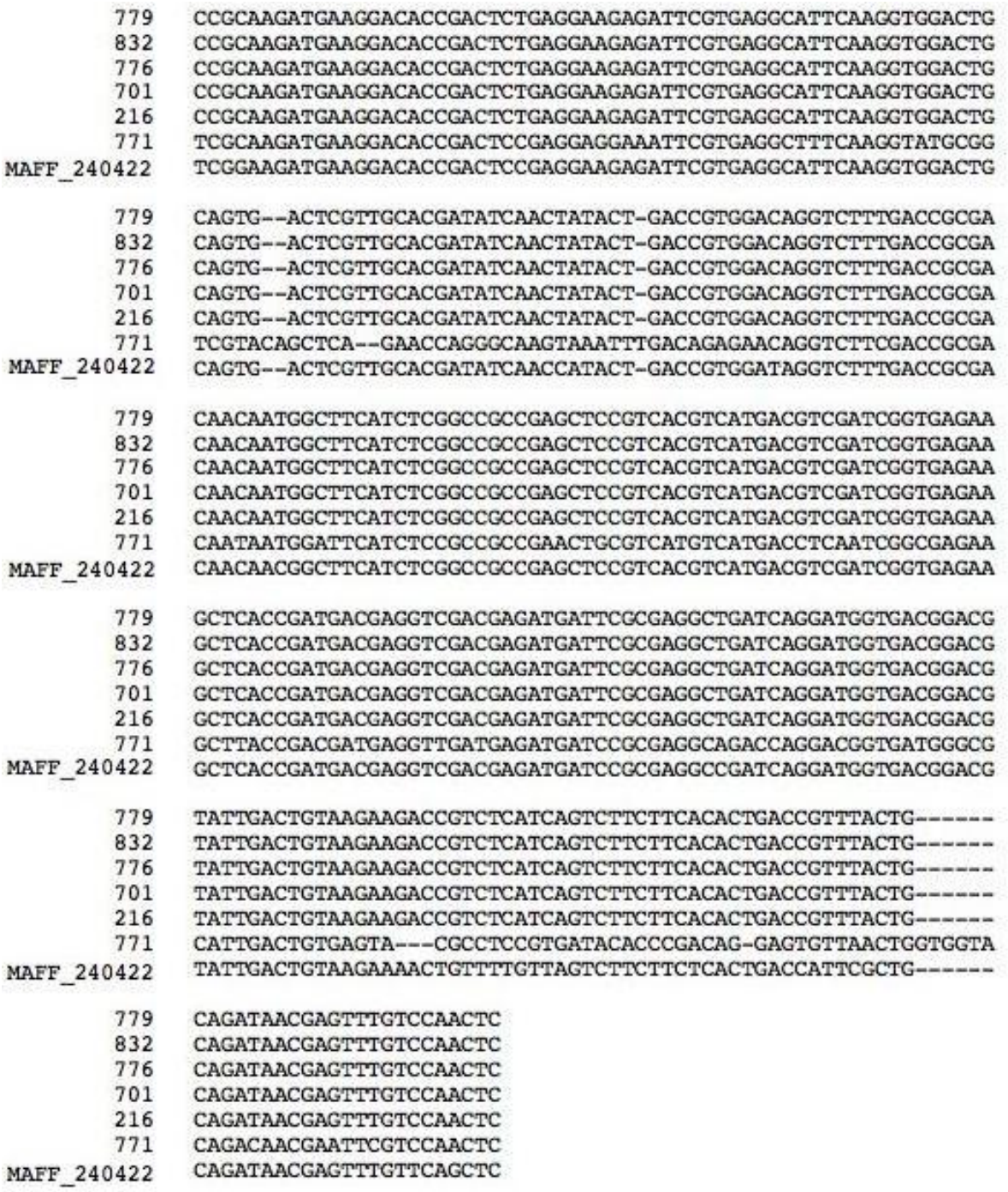


Fig 5. Multiple Sequence Alignment of Calmodulin (CAL) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

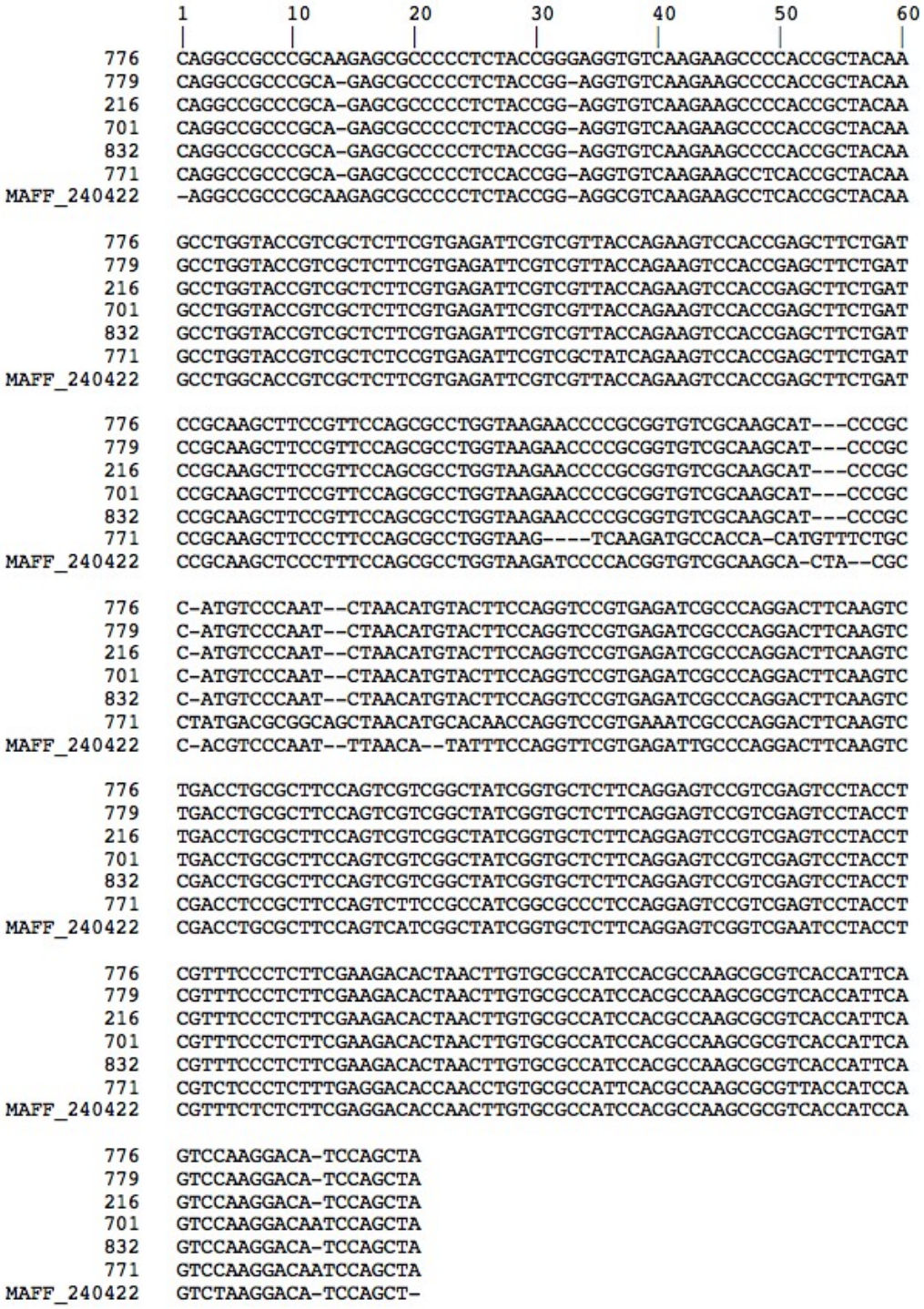


Fig 6. Histone (His3) Multiple Sequence Alignment of Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

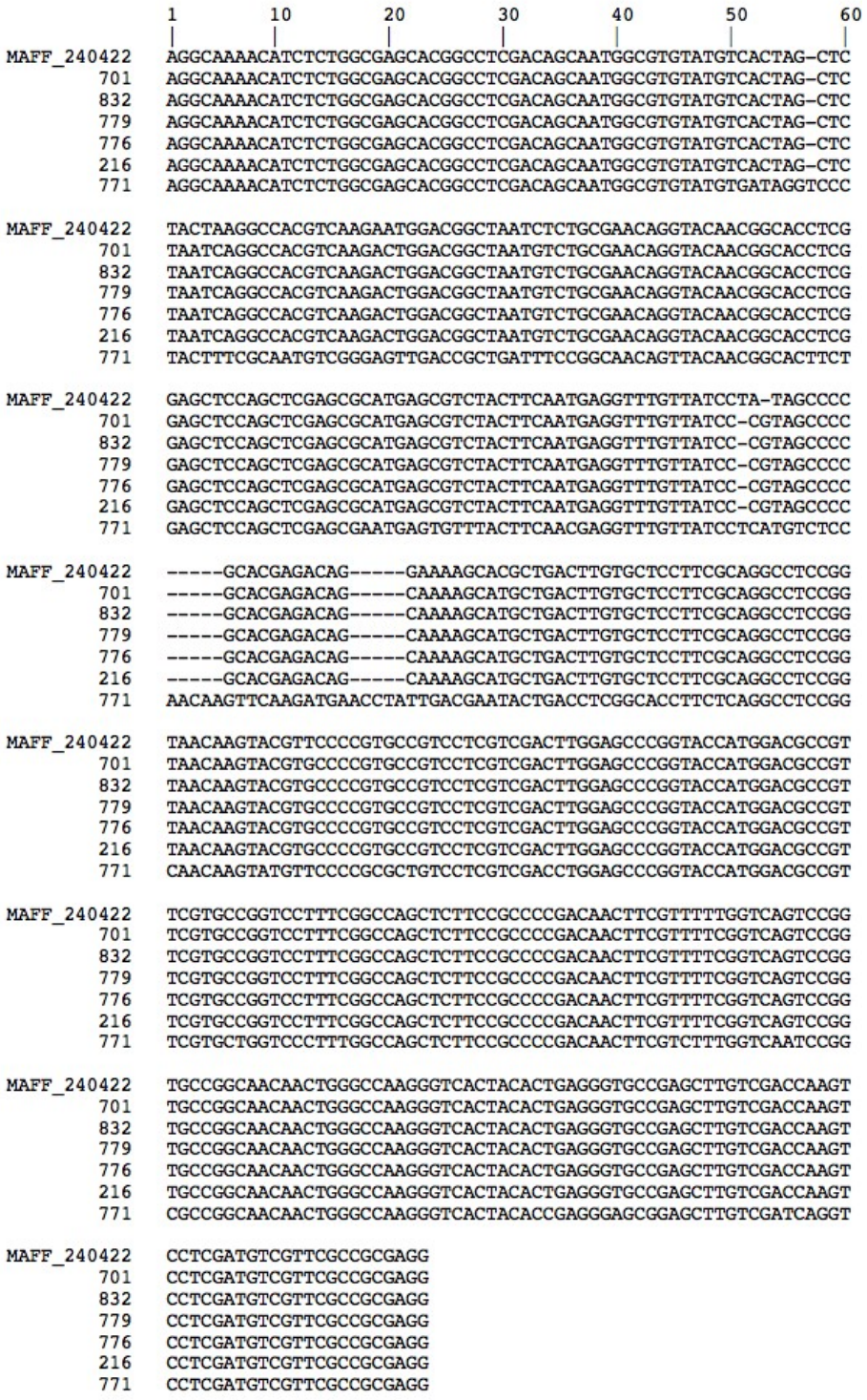


Fig 7. Multiple Sequence Alignment of Beta-Tubulin (TUB) Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

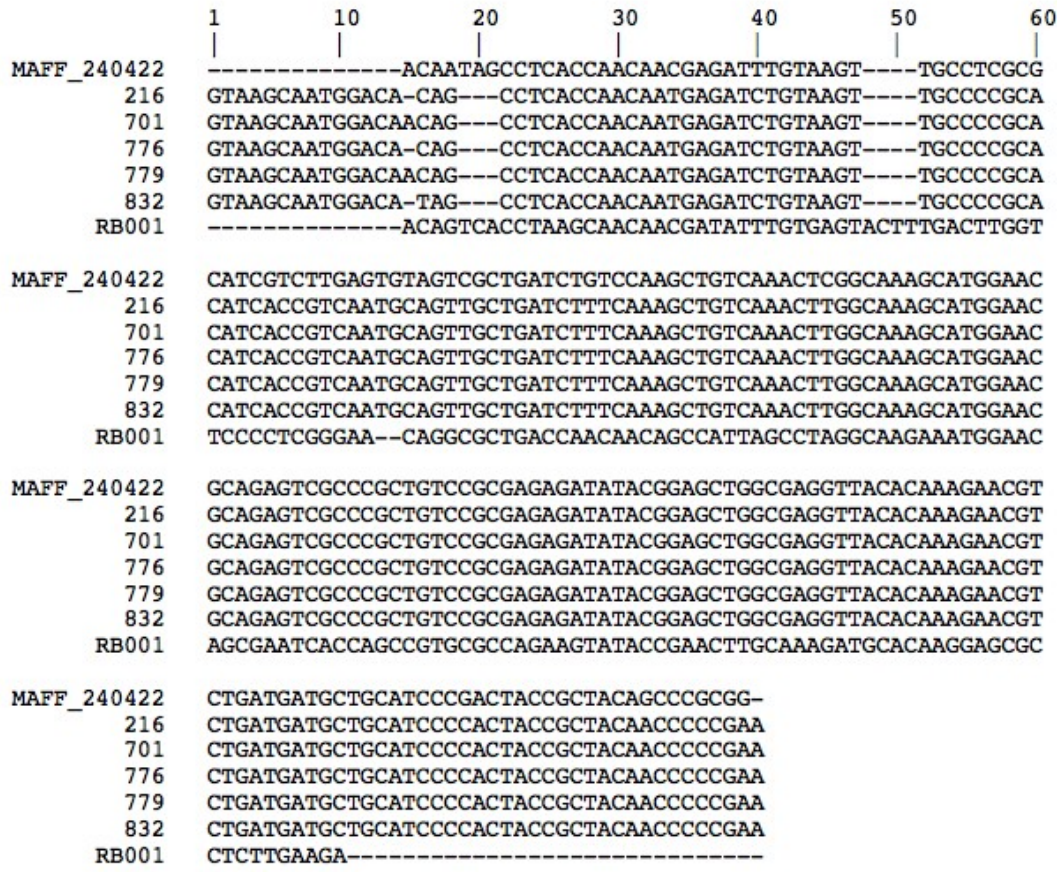


Fig 8. Mating Type Gene/High Mobility Group Domain (HMG) Multiple Sequence Alignment of Sequence Data Generated for *Colletotrichum* Isolates along with the MAFF_240422 Reference Sequence.

Appendix VII

Sequence Homology and Divergence among *Colletotrichum* spp. Isolates

Isolate	216	701	776	779	832	29	45	206	217	219	428	533	560	693	694	814	MAFF	771	449
216	-	0	0	0	0.6	1.0	0.4	1.0	0.6	1.0	0.4	0.4	0.4	1.0	1.0	1.0	3.7	9.9	12.5
701	100.0	-	0	0	0.6	1.0	0.4	1.0	0.6	1.0	0.4	0.4	0.4	1.0	1.0	1.0	3.7	9.9	12.5
776	100.0	100.0	-	0	0.6	1.0	0.4	1.0	0.6	1.0	0.4	0.4	0.4	1.0	1.0	1.0	3.7	9.9	12.5
779	100.0	100.0	100.0	-	0.6	1.0	0.4	1.0	0.6	1.0	0.4	0.4	0.4	1.0	1.0	1.0	3.7	9.9	12.5
832	99.4	99.4	99.4	99.4	-	0.4	1.0	0.4	1.2	0.4	1.0	1.0	1.0	0.4	0.4	0.4	3.7	10.1	13.1
29	99.0	99.0	99.0	99.0	99.6	-	0.6	0	0.8	0	0.6	0.6	0.6	0	0	0	3.2	10.1	12.7
45	99.6	99.6	99.6	99.6	99.0	99.4	-	0.6	0.2	0.6	0	0	0	0.6	0.6	0.6	3.2	9.9	12.2
206	99.0	99.0	99.0	99.0	99.6	100.0	99.4	-	0.8	0	0.6	0.6	0.6	0	0	0	3.2	10.1	12.7
217	99.4	99.4	99.4	99.4	98.8	99.2	99.8	99.2	-	0.8	0.2	0.2	0.2	0.8	0.8	0.8	3.5	10.1	12.4
219	99.0	99.0	99.0	99.0	99.6	100.0	99.4	100.0	99.2	-	0.6	0.6	0.6	0	0	0	3.2	10.1	12.7
428	99.6	99.6	99.6	99.6	99.0	99.4	100.0	99.4	99.8	99.4	-	0	0	0.6	0.6	0.6	3.2	9.9	12.2
533	99.6	99.6	99.6	99.6	99.0	99.4	100.0	99.4	99.8	99.4	100.0	-	0	0.6	0.6	0.6	3.2	9.9	12.2
560	99.6	99.6	99.6	99.6	99.0	99.4	100.0	99.4	99.8	100.0	100.0	100.0	-	0.6	0.6	0.6	3.2	9.9	12.2
693	99.0	99.0	99.0	99.0	99.6	100.0	99.4	100.0	99.2	100.0	99.4	99.4	99.4	-	0	0	3.2	10.1	12.7
694	99.0	99.0	99.0	99.0	99.6	100.0	99.4	100.0	99.2	100.0	99.4	99.4	99.4	100.0	-	0	3.2	10.1	12.7
814	99.0	99.0	99.0	99.0	99.6	100.0	99.4	100.0	99.2	100.0	99.4	99.4	99.4	100.0	100.0	-	3.2	10.1	12.7
MAFF	96.3	96.3	96.3	96.3	96.3	96.8	96.8	96.8	96.5	96.8	96.8	96.8	96.8	96.8	96.8	96.8	-	11.6	13.8
771	90.1	90.1	90.1	90.1	89.9	89.9	90.1	89.9	89.9	89.9	90.1	90.1	90.1	89.9	89.9	89.9	88.4	-	14.1
449	87.5	87.5	87.5	87.5	86.9	87.3	87.8	87.3	87.6	87.3	87.8	87.8	87.8	87.3	87.3	87.3	86.2	85.9	-

Table 1 RNA Gene Block Internal Transcribed Spacer (ITS) Region Sequence Homology and Divergence (%) amongst *Colletotrichum* spp. Isolates

Isolate	216	701	776	779	832	29	45	206	217	219	428	533	560	693	694	814	MAFF	771	449
216	-	0	0	0	2.0	0	0	0	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
701	100.0	-	0	0	2.0	0	0	0	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
776	100.0	100.0	-	0	2.0	0	0	0	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
779	100.0	100.0	100.0	-	2.0	0	0	0	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
832	98.0	98.0	98.0	98.0	-	0	0	0	0	0	0	0	0	0	0	0	24.5	41.4	50.3
29	100.0	100.0	100.0	100.0	98.0	-	0	0	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
45	100.0	100.0	100.0	100.0	98.0	100.0	-	0	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
206	100.0	100.0	100.0	100.0	98.0	100.0	100.0	-	0	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
217	100.0	100.0	100.0	100.0	98.0	100.0	100.0	100.0	-	0	0	0	0	0	2.0	2.0	26.5	42.4	50.3
219	100.0	100.0	100.0	100.0	98.0	100.0	100.0	100.0	100.0	-	0	0	0	0	2.0	2.0	26.5	42.4	50.3
428	100.0	100.0	100.0	100.0	98.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	2.0	2.0	26.5	42.4	50.3
533	100.0	100.0	100.0	100.0	98.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	2.0	2.0	26.5	42.4	50.3
560	100.0	100.0	100.0	100.0	98.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	2.0	2.0	26.5	42.4	50.3
693	100.0	100.0	100.0	100.0	98.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	2.0	2.0	26.5	42.4	50.3
694	98.0	98.0	98.0	98.0	100.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	-	0	24.5	41.4	50.3
814	98.0	98.0	98.0	98.0	100.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	98.0	100.0	-	24.5	41.4	50.3
MAFF	73.5	73.5	73.5	73.5	75.5	73.5	73.5	73.5	73.5	73.5	73.5	73.5	73.5	73.5	75.5	75.5	-	44.4	61.4
771	57.6	57.6	57.6	57.6	58.6	57.6	57.6	57.6	57.6	57.6	57.6	57.6	57.6	57.6	58.6	58.6	55.6	-	61.7
449	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	49.7	38.6	38.3	-

Table 2 Glyceraldehyde-3-Phosphate Dehydrogenase (GD/GAPDH)Sequence Homology and Divergence between *Colletotrichum* Isolates (%)

Isolate	216	701	776	779	832	29	45	206	217	219	428	533	560	693	694	814	MAFF	771	449
216	-	0	0	0	2.6	2.7	0	2.9	2.6	2.7	0	0	0	3.1	2.7	2.7	8.0	48.2	40.3
701	100.0	-	0	0	2.6	2.7	0	2.9	2.6	2.7	0	0	0	3.1	2.7	2.7	8.0	48.2	40.3
776	100.0	100.0	-	0	2.6	2.7	0	2.9	2.6	2.7	0	0	0	3.1	2.7	2.7	8.0	48.2	40.3
779	100.0	100.0	100.0	-	2.6	2.7	0	2.9	2.6	2.7	0	0	0	3.1	2.7	2.7	8.0	48.2	40.3
832	97.4	97.4	97.4	97.4	-	0.1	2.7	0.1	0	0.1	2.7	2.7	2.7	0.5	0.1	0	8.9	48.3	40.4
29	97.3	97.3	97.3	97.3	99.9	-	2.9	0	0.1	0	2.9	2.9	2.9	0	0	0.2	8.6	48.2	40.3
45	100.0	100.0	100.0	100.0	97.3	97.1	-	3.0	2.7	2.9	0	0	0	3.2	2.9	2.9	7.5	48.8	40.0
206	97.1	97.1	97.1	97.1	99.9	100.0	97.0	-	0.1	0	3.0	3.0	3.0	0	0	0.1	8.5	48.2	40.4
217	97.4	97.4	97.4	97.4	100.0	99.9	97.3	99.9	-	0.1	2.7	2.7	2.7	0.5	0.1	0	8.3	48.3	40.4
219	97.3	97.3	97.3	97.3	99.9	100.0	97.1	100.0	99.9	-	2.9	2.9	2.9	0	0	0.2	8.6	48.1	40.3
428	100.0	100.0	100.0	100.0	97.3	97.1	100.0	97.0	97.3	97.1	-	0	0	3.2	2.9	2.9	7.5	48.8	40.0
533	100.0	100.0	100.0	100.0	97.3	97.1	100.0	97.0	97.3	97.1	100.0	-	0	3.2	2.9	2.9	7.5	48.8	40.0
560	100.0	100.0	100.0	100.0	97.3	97.1	100.0	97.0	97.3	97.1	100.0	100.0	-	3.2	2.9	2.9	7.5	48.8	40.0
693	96.9	96.9	96.9	96.9	99.5	100.0	96.8	100.0	99.5	100.0	96.8	96.8	96.8	-	0.3	0.6	8.7	48.4	40.3
694	97.3	97.3	97.3	97.3	99.9	100.0	97.1	100.0	99.9	100.0	97.1	97.1	97.1	100.0	-	0.2	8.4	48.2	40.3
814	97.3	97.3	97.3	97.3	100.0	99.8	97.1	99.9	100.0	99.8	97.1	97.1	97.1	99.4	99.8	-	8.4	48.3	40.4
MAFF	92.0	92.0	92.0	92.0	91.1	91.4	92.5	91.5	91.7	91.4	92.5	92.5	92.5	91.3	91.6	91.6	-	49.2	41.4
771	51.8	51.8	51.8	51.8	51.7	51.8	51.2	51.8	51.7	51.9	51.2	51.2	51.2	51.6	51.8	51.7	50.8	-	57.2
449	59.7	59.7	59.7	59.7	59.6	59.7	60.0	59.6	59.6	59.7	60.0	60.0	60.0	59.7	59.7	59.6	58.6	42.8	-

Table 3 Glutamine Synthetase (GS) Sequence Homology and Divergence between *Colletotrichum* Isolates (%)

Isolate	216	701	776	779	832	29	45	206	217	219	428	533	560	693	694	814	MAFF	771	449
216	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
701	100.0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
776	100.0	100.0	-	0	0	0	0	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
779	100.0	100.0	100.0	-	0	0	0	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
832	100.0	100.0	100.0	100.0	-	0	0	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
29	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
45	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	0	0	0	0	0	3.2	17.6	16.4
206	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	0	0	0	0	3.2	17.6	16.4
217	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	0	0	0	3.2	17.6	16.4
219	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	0	0	3.2	17.6	16.4
428	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	0	3.2	17.6	16.4
533	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	0	3.2	17.6	16.4
560	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	0	3.2	17.6	16.4
693	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	0	3.2	17.6	16.4
694	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	0	3.2	17.6	16.4
814	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	-	3.2	17.6	16.4
MAFF	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	96.8	-	17.4	17.5
771	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.4	82.6	-	20.0
449	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	83.6	82.5	80.0	-

Table 4 Beta-Tubulin (TUB) Sequence Homology and Divergence between *Colletotrichum* Isolates (%)

Isolate	216	701	776	779	832	29	45	206	217	219	428	533	560	693	694	814	MAFF	RB001	449
216	-	0.5	0	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0	0	0	0.5	0.5	10.0	39.0	27.0
701	99.5	-	0.5	0	1.0	1.0	0.5	1.0	0	1.0	0	0.5	0.5	0.5	1.0	1.0	9.5	39.0	27.0
776	100.0	99.5	-	0.5	0.5	0.5	0	0.5	0.5	0.5	0.5	0	0	0	0.5	0.5	10.0	39.0	27.0
779	99.5	100.0	99.5	-	1.0	1.0	0.5	1.0	0	1.0	0	0.5	0.5	0.5	1.0	1.0	9.5	39.0	27.0
832	99.5	99.0	99.5	99.0	-	1.0	0.5	1.0	1.0	1.0	1.0	0.5	0.5	0.5	0	1.0	9.5	39.5	27.5
29	99.5	99.0	99.5	99.0	99.0	-	0.5	0	1.0	0	1.0	0.5	0.5	0.5	1.0	0	10.4	39.5	26.5
45	100.0	99.5	100.0	99.5	99.5	99.5	-	0.5	0.5	0.5	0.5	0	0	0	0.5	0.5	10.0	39.0	27.0
206	99.5	99.0	99.5	99.0	99.0	100.0	99.5	-	1.0	0	1.0	0.5	0.5	0.5	1.0	0	10.4	39.5	26.5
217	99.5	100.0	99.5	100.0	99.0	99.0	99.5	99.0	-	1.0	0	0.5	0.5	0.5	1.0	1.0	9.5	39.0	27.0
219	99.5	99.0	99.5	99.0	99.0	100.0	99.5	100.0	99.0	-	1.0	0.5	0.5	0.5	1.0	0	10.4	39.5	26.5
428	99.5	100.0	99.5	100.0	99.0	99.0	99.5	99.0	100.0	99.0	-	0.5	0.5	0.5	1.0	1.0	9.5	39.0	27.0
533	100.0	99.5	100.0	99.5	99.5	99.5	100.0	99.5	99.5	99.5	99.5	-	0	0	0.5	0.5	10.0	39.0	27.0
560	100.0	99.5	100.0	99.5	99.5	99.5	100.0	99.5	99.5	99.5	99.5	100.0	-	0	0.5	0.5	10.0	39.0	27.0
693	100.0	99.5	100.0	99.5	99.5	99.5	100.0	99.5	99.5	99.5	99.5	100.0	100.0	-	0.5	0.5	10.0	39.0	27.0
694	99.5	99.0	99.5	99.0	100.0	99.0	99.5	99.0	99.0	99.0	99.0	99.5	99.5	99.5	-	1.0	9.5	39.5	27.5
814	99.5	99.0	99.5	99.0	99.0	100.0	99.5	100.0	99.0	100.0	99.0	99.5	99.5	99.5	99.0	-	10.4	39.5	26.5
MAFF	90.0	90.5	90.0	90.5	90.5	89.6	90.0	89.6	90.5	89.6	90.5	90.0	90.0	90.0	90.5	89.6	-	38.4	27.9
RB001	61.0	61.0	61.0	61.0	60.5	60.5	61.0	60.5	61.0	60.5	61.0	61.0	61.0	61.0	60.5	60.5	61.6	-	35
449	73.0	73.0	73.0	73.0	72.5	73.5	73.0	73.5	73.0	73.5	73.0	73.0	73.0	73.0	72.5	73.5	72.1	65.0	-

Table 5 Mating Type Gene/High Mobility Group Domain (HMG) Sequence Homology and Divergence between *Colletotrichum* Isolates (%).

Isolate	216	701	776	779	832	29	45	206	217	219	428	533	560	693	694	814	MAFF	771	449
216	-	0	0	0	1.8	1.8	0	1.8	1.7	1.7	0	0.7	0.7	2.0	1.9	1.8	10.9	36.7	31.8
701	100.0	-	0	0	1.9	1.8	0	1.8	1.6	1.8	0	0.8	0.8	2.0	1.9	1.9	10.9	36.7	31.8
776	100.0	100.0	-	0	1.8	1.8	0	1.8	1.7	1.7	0	0.7	0.7	2.0	1.9	1.8	10.9	36.7	31.8
779	100.0	100.0	100.0	-	1.9	1.8	0	1.8	1.6	1.8	0	0.8	0.8	2.0	1.9	1.9	10.9	36.7	31.8
832	98.2	98.1	98.2	98.1	-	0.2	1.8	0.2	0.4	0.2	1.9	2.1	2.1	0.9	0	0.1	10.9	36.8	32.2
29	98.2	98.2	98.2	98.2	99.8	-	1.8	0	0.3	0	1.8	2.0	2.1	0.8	0.2	0.1	11.1	36.6	32.0
45	100.0	100.0	100.0	100.0	98.2	98.2	-	1.8	1.7	1.7	0	0.7	0.7	2.0	1.9	1.8	10.9	36.7	31.8
206	98.2	98.2	98.2	98.2	99.8	100.0	98.2	-	0.3	0	1.8	2.0	2.1	0.8	0.2	0.1	11.1	36.6	32.0
217	98.3	98.4	98.3	98.4	99.6	99.7	98.3	99.7	-	0.3	1.6	1.9	1.9	1.0	0.4	0.4	11.1	36.7	32.1
219	98.3	98.2	98.3	98.2	99.8	100.0	98.3	100.0	99.7	-	1.8	2.0	2.0	0.8	0.2	0.1	11.1	36.7	32.1
428	100.0	100.0	100.0	100.0	98.1	98.2	100.0	98.2	98.4	98.2	-	0.8	0.8	2.0	1.9	1.9	10.9	36.7	31.8
533	99.3	99.2	99.3	99.2	97.9	98.0	99.3	98.0	98.1	98.0	99.2	-	0	1.3	2.1	2.1	11.3	37.0	31.9
560	99.3	99.2	99.3	99.2	97.9	97.9	99.3	97.9	98.1	98.0	99.2	100.0	-	1.3	2.2	2.1	11.3	37.0	31.9
693	98.0	98.0	98.0	98.0	99.1	99.2	98.0	99.2	99.0	99.2	98.0	98.7	98.7	-	0.8	0.9	11.7	37.0	32.4
694	98.1	98.1	98.1	98.1	100.0	99.8	98.1	99.8	99.6	99.8	98.1	97.9	97.8	99.2	-	0.1	11.0	36.7	32.1
814	98.2	98.1	98.2	98.1	99.9	99.9	98.2	99.9	99.6	99.9	98.1	97.9	97.9	99.1	99.9	-	11.0	36.7	32.1
MAFF	89.1	89.1	89.1	89.1	89.1	88.9	89.1	88.9	88.9	88.9	89.1	88.7	88.7	88.3	89.0	89.0	-	41.2	36.1
771	63.3	63.3	63.3	63.3	63.2	63.4	63.3	63.4	63.3	63.3	63.3	63.0	63.0	63.0	63.3	63.3	58.8	-	33.2
449	68.2	68.2	68.2	68.2	67.8	68.0	68.2	68.0	67.9	67.9	68.2	68.1	68.1	67.6	67.9	67.9	63.9	66.8	-

Table 6 Concatenated Sequence Homology and Divergence (%) amongst *Colletotrichum* Isolates including: ITS, TUB, GS, GAPDH, and HMG.

Appendix VIII

Examples of Sequencing Results for Each Locus of *C. lindemuthianum* Isolate 216 opened Using Geospiza Software.

Fig 1 FinchTV Sequencing Results of ITS- Raw Data for Isolate 216 (Sample).

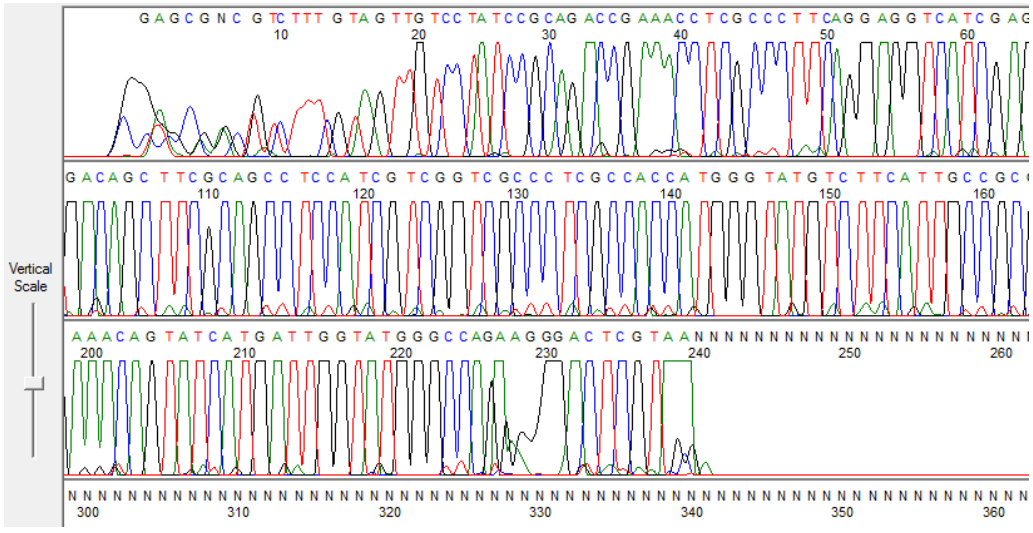


Fig 2 FinchTV Sequencing Results of ACT- Raw Data for Isolate 216 (Sample).

Appendix IX

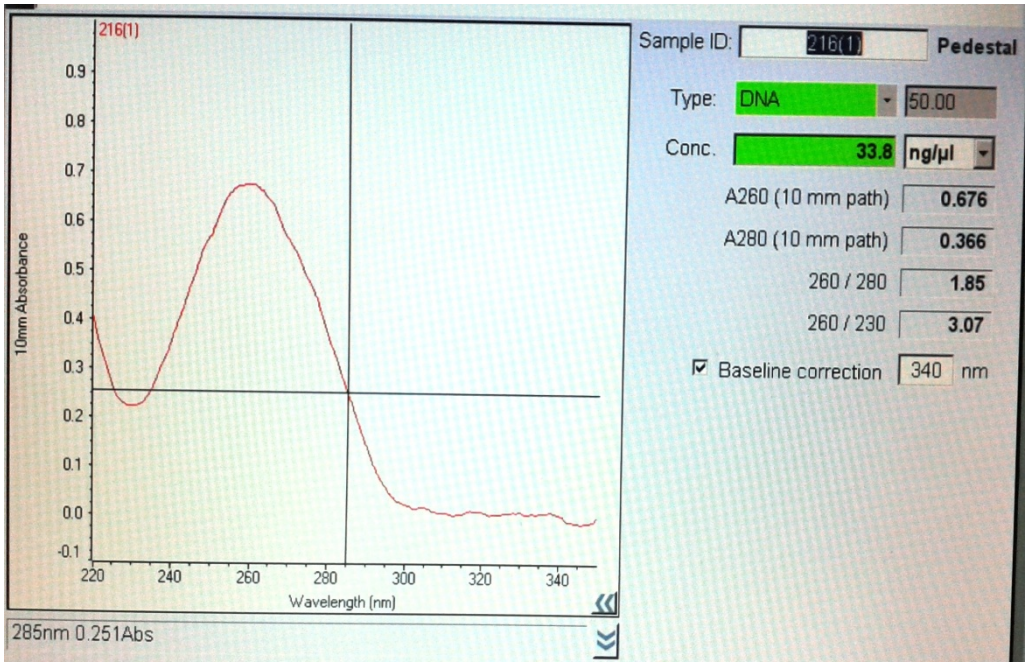
Table 1 Nucleotide Variables among *C. lindemuthianum* Isolates clustered into Two Genetic Groups on the Basis of GS Multiple Sequence Alignment (Fig 3.36)*

Position within the sequence	Genetic group 1	Genetic group 2
140bp	C	T
199bp	C	T
229bp	T	C
240bp	C	T
241bp	G	A
317bp	C	T
352bp	A	T
413bp	-	T
414bp	-	T
415bp	-	G
416bp	-	C
418bp	T	C
454bp	A	G
460bp	C	G
569bp	C	T
648bp	C	T
683bp	T	C
718bp	C	A (apart from 217,814, 832 that had 'C' in this position)
722bp	C	T
731bp	A	T
757bp	T	C
786bp	G	A
797bp	G	A
868bp	G	A
870bp	C	T

**C. lindemuthianum* isolates were separated into two groups in the phylogenetic tree (Fig 3.40).

Appendix X

Examples of DNA Quality and Quantity Assessment using NanoDrop for two *C. lindemuthianum* Isolates 216 and 776



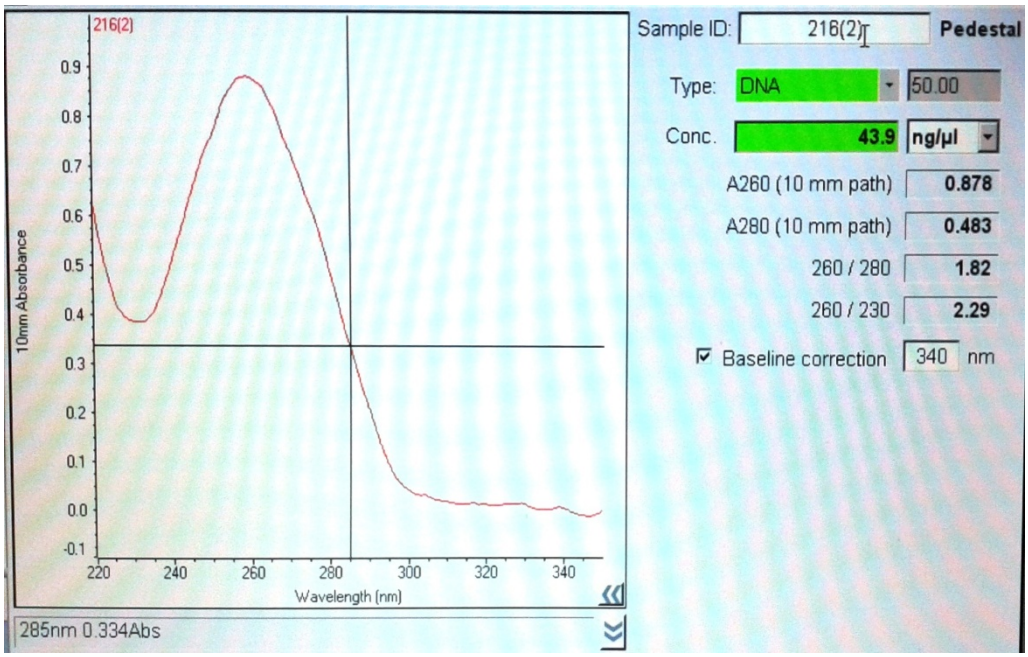


Fig 1 Examples of NanoDrop Read-out.for Isolate 216 *C. lindemuthianum* Sample 1 and 2.

Appendix XI

Tables Containing Raw Data Growth Rate Monitoring at 20ºC

Table 1 Growth Measurements Taken from culture plates of isolate 216 incubated at 20°C

Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours
216 (Plate:1)						
I	2	5	13	18	20	28
II	3	5	13	17	20	28
III	4	5	14	18	21	29
IV	3	6	14	18	21	28
V	3	5	13	17	20	28
VI	3	5	13	18	20	28
VII	4	5	14	18	20	29
VIII	3	5	14	17	20	29
3216 (Plate:2)						
I	3	5	13	17	20	28
II	3	6	13	17	20	28
III	4	6	13	18	20	29
IV	4	6	14	18	20	29
V	3	6	13	17	20	27
VI	4	6	13	18	20	28
VII	3	6	13	17	20	28
VIII	3	5	13	17	19	27
216 (Plate:3)						
I	3	5	13	17	20	28
II	3	6	13	17	20	28
III	5	7	14	18	21	29
IV	4	7	14	18	21	29
V	4	7	14	18	20	28
VI	4	6	13	17	20	28
VII	3	5	13	17	20	28
VIII	3	5	12	17	19	28
216 (Plate:4)						
I	4	6	14	19	20	29
II	4	6	14	18	20	28
III	5	6	14	17	20	28
IV	4	6	14	18	20	28
V	3	5	13	17	20	28
VI	3	5	13	17	20	30
VII	3	5	13	17	19	29
VIII	4	5	13	16	19	28
216 (Plate:5)						
I	3	5	13	17	19	26
II	4	6	14	18	20	27
III	4	7	14	18	20	28
IV	4	6	14	18	21	28
V	3	6	13	18	20	27
VI	2	4	13	17	19	27
VII	2	5	12	16	19	26
VIII	2	5	13	16	19	27

Table 2 Growth Measurements Taken from culture plates of isolate 701 incubated at 20°C

Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours
701 (Plate:1)						
I	5	7	14	17	18	21
II	6	8	15	17	19	22
III	5	7	14	17	18	22
IV	5	7	14	17	18	21
V	5	7	14	17	19	23
VI	5	7	14	16	18	22
VII	3	6	13	15	17	21
VIII	4	7	14	16	18	22
701 (Plate:2)						
I	5	7	14	17	19	23
II	5	7	15	17	18	22
III	5	7	14	17	18	22
IV	5	7	15	18	19	23
V	5	7	14	17	18	22
VI	4	6	14	17	18	22
VII	4	6	14	16	18	22
VIII	5	7	15	17	19	22
701 (Plate:3)						
I	3	6	13	16	17	21
II	5	7	15	20	20	25
III	6	8	16	19	20	24
IV	2	4	12	14	15	18
V	4	6	14	17	18	21
VI	5	7	15	18	19	24
VII	6	8	15	19	19	23
VIII	5	8	15	18	19	23
701 (Plate:4)						
I	5	8	15	18	19	23
II	5	8	15	18	20	24
III	5	7	15	17	19	23
IV	4	6	14	17	17	22
V	4	7	14	17	18	23
VI	4	6	14	17	18	23
VII	5	7	15	18	19	24
VIII	5	7	15	18	19	23
701 (Plate:5)						
I	5	7	14	17	18	20
II	6	8	15	19	19	23
III	6	8	15	18	19	23
IV	6	7	15	18	19	23
V	5	7	14	16	18	22
VI	4	6	13	16	17	21
VII	4	6	14	16	17	21
VIII	5	7	14	16	17	21

Table 3 Growth Measurements Taken from culture plates of isolate 776 incubated at 20°C

Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours
776 (Plate:1)						
I	3	6	12	16	19	22
II	5	8	15	19	21	25
III	5	7	14	19	21	25
IV	5	7	14	19	20	25
V	4	7	14	18	20	25
VI	3	6	13	18	19	24
VII	3	5	12	16	18	23
VIII	3	6	13	17	18	23
776 (Plate:2)						
I	4	6	14	18	20	25
II	3	5	14	17	19	23
III	4	4	13	17	19	23
IV	3	5	13	17	19	22
V	4	5	13	17	18	23
VI	4	5	13	17	19	23
VII	5	6	14	18	20	24
VIII	5	6	14	19	20	25
776 (Plate:3)						
I	4	5	13	18	20	23
II	3	5	13	16	19	23
III	4	5	14	18	20	24
IV	5	6	14	18	20	24
V	5	7	15	20	22	26
VI	5	7	15	20	22	27
VII	5	7	15	19	21	26
VIII	5	7	14	17	20	24
776 (Plate:4)						
I	3	5	12	18	19	23
II	4	5	13	17	19	23
III	4	6	13	18	20	24
IV	4	6	14	18	20	25
V	4	5	14	18	20	24
VI	3	5	13	18	19	23
VII	3	5	13	17	19	23
VIII	2	5	12	16	18	24
776 (Plate:5)						
I	5	7	14	18	20	24
II	5	6	13	17	20	24
III	5	6	14	17	19	23
IV	5	5	14	17	19	23
V	4	5	13	17	19	23
VI	4	5	13	17	20	24
VII	5	6	14	18	20	24
VIII	5	6	13	18	20	24

Table 4 Growth Measurements Taken from culture plates of isolate 779 incubated at 20°C

Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours
779 (Plate:1)						
I	3	6	15	19	21	25
II	3	6	15	19	21	25
III	4	7	16	20	22	27
IV	5	7	15	20	22	27
V	5	8	16	21	23	28
VI	4	6	15	20	22	27
VII	4	6	15	19	21	24
VIII	4	5	15	19	21	25
779 (Plate:2)						
I	3	6	14	18	20	24
II	4	6	15	18	20	24
III	5	9	17	20	22	26
IV	9	11	19	22	24	27
V	5	7	15	19	20	24
VI	4	12	15	19	20	24
VII	4	6	15	18	20	24
VIII	4	6	14	18	20	25
779 (Plate:3)						
I	3	6	13	16	18	22
II	4	5	12	16	18	22
III	4	6	14	17	19	23
IV	4	5	12	16	18	22
V	7	9	16	20	21	25
VI	4	6	13	16	19	23
VII	4	6	13	16	18	22
VIII	4	6	14	17	19	23
779 (Plate:4)						
I	4	6	13	16	18	23
II	4	5	12	16	18	22
III	4	6	14	17	19	23
IV	4	5	12	16	18	22
V	4	9	16	20	21	25
VI	4	6	13	16	19	22
VII	5	6	13	16	18	23
VIII	4	6	14	17	19	23
779 (Plate:5)						
I	4	5	14	18	20	24
II	4	7	16	20	22	26
III	5	9	17	21	22	27
IV	5	7	15	20	21	26
V	5	7	15	19	21	25
VI	4	6	15	19	21	25
VII	4	6	14	19	21	25
VIII	4	6	15	19	21	25

Table 5 Growth Measurements Taken from culture plates of isolate 771 incubated at 20°C

Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours
771 (Plate:1)						
I	8	13	30	33	N/A	N/A
II	9	13	31	31		
III	8	13	31	39		
IV	8	12	31	40		
V	8	12	31	41		
VI	8	13	30	38		
VII	8	12	31	35		
VIII	8	12	30	33		
771 (Plate:2)						
I	9	14	32	36	N/A	N/A
II	9	14	32	38		
III	9	13	32	39		
IV	9	14	32	40		
V	9	13	31	38		
VI	9	14	32	36		
VII	9	14	32	36		
VIII	10	13	33	36		
771 (Plate:3)						
I	10	14	32	35	N/A	N/A
II	9	14	32	37		
III	9	14	33	39		
IV	10	14	33	40		
V	10	14	33	40		
VI	9	14	32	36		
VII	9	13	31	35		
VIII	9	13	32	34		
771 (Plate:4)						
I	9	13	32	36	N/A	N/A
II	8	13	31	38		
III	9	14	32	40		
IV	9	14	32	39		
V	9	14	32	38		
VI	8	13	31	35		
VII	9	14	31	34		
VIII	9	14	31	35		
771 (Plate:5)						
I	9	14	32	32	N/A	N/A
II	8	12	31	40		
III	9	13	32	41		
IV	10	14	33	41		
V	10	14	33	38		
VI	10	14	32	35		
VII	9	13	30	33		
VIII	9	14	32	34		

Table 6 Growth Measurements Taken from culture plates of isolate 832 incubated at 20°C

Date	20/06 72 hours	21/06 96 hours	25/06 192 hours	27/06 240 hours	28/06 264 hours	02/07 360 hours
832 (Plate:1)						
I	1	2	7	9	10	14
II	1	2	6	9	10	15
III	1	2	7	9	10	15
IV	1	2	6	10	11	15
V	1	3	8	11	12	16
VI	3	4	9	12	13	17
VII	2	3	7	10	12	16
VIII	1	3	7	10	11	15
832 (Plate:2)						
I	1	2	8	12	13	17
II	0.5	2	8	10	12	16
III	0.5	2	6	8	10	14
IV	0.5	2	7	9	10	14
V	1	2	7	11	11	15
VI	2	3	8	11	12	17
VII	1	2	8	10	12	16
VIII	1	2	7	11	11	16
832 (Plate:3)						
I	2	3	8	10	12	16
II	2	3	8	10	11	15
III	2	2	7	9	10	14
IV	1	2	7	9	10	14
V	2	3	7	9	10	15
VI	2	3	7	9	11	15
VII	2	3	8	10	11	15
VIII	2	3	8	10	11	16
832 (Plate:4)						
I	2	4	8	11	13	17
II	2	3	8	8	8	12
III	1	2	7	7	8	12
IV	0.5	2	7	9	9	13
V	1	2	8	10	11	15
VI	2	3	7	11	11	16
VII	2	4	9	11	12	16
VIII	3	4	9	11	13	17
832 (Plate:5)						
I	2	3	8	10	11	15
II	2	3	9	11	11	15
III	1	2	10	13	14	18
IV	2	3	11	13	15	19
V	2	3	8	11	12	16
VI	2	3	7	10	10	14
VII	2	3	8	11	12	16
VIII	2	4	8	11	12	16

Appendix XII

Tables Containing Raw Data Growth Rate Monitoring at 25° C

Table 1 Growth Measurements Taken from culture plates of isolate 216 incubated at 25°C

Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours
			216 (Plate:1)		
I	12	11	14	22	25
II	7	11	14	22	25
III	8	12	14	23	26
IV	8	11	14	22	26
V	7	11	14	22	25
VI	6	10	13	20	24
VII	6	10	13	21	24
VIII	7	11	13	21	25
			216 (Plate:2)		
I	8	12	14	22	25
II	7	12	14	22	25
III	8	12	14	22	25
IV	7	11	14	22	26
V	7	11	13	22	24
VI	7	11	13	21	25
VII	7	11	13	22	25
VIII	7	11	14	22	25
			216 (Plate:3)		
I	7	12	13	22	26
II	8	12	14	23	26
III	8	12	14	23	26
IV	8	12	15	23	27
V	8	12	14	23	26
VI	7	11	14	22	26
VII	8	12	14	23	26
VIII	7	11	14	22	26
			216 (Plate:4)		
I	8	12	14	22	26
II	8	12	14	23	27
III	8	12	15	24	27
IV	7	12	14	23	26
V	7	12	14	23	25
VI	7	11	14	23	26
VII	7	12	14	23	26
VIII	8	12	14	23	26
			216 (Plate:5)		
I	8	13	15	23	26
II	9	12	15	23	27
III	8	12	14	22	26
IV	6	10	13	22	25
V	6	10	13	22	25
VI	7	11	13	22	26
VII	8	11	13	22	25
VIII	8	11	14	22	26

Table 2 Growth Measurements Taken from culture plates of isolate 701 incubated at 25°C

Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours
701 (Plate:1)					
I	11	15	17	23	25
II	11	15	18	23	26
III	11	15	17	23	25
IV	10	14	17	23	25
V	10	14	17	22	25
VI	11	15	17	23	25
VII	11	15	18	24	26
VIII	12	16	18	24	26
701 (Plate:2)					
I	9	14	16	22	25
II	11	15	18	24	26
III	11	15	17	24	26
IV	11	15	18	24	26
V	11	15	17	23	26
VI	12	16	18	25	27
VII	10	14	16	23	25
VIII	10	15	17	23	25
701 (Plate:3)					
I	11	15	17	24	26
II	11	16	18	25	27
III	11	15	17	23	26
IV	12	16	19	25	27
V	13	17	19	25	27
VI	12	15	18	23	25
VII	11	15	17	23	25
VIII	11	15	18	24	26
701 (Plate:4)					
I	11	15	17	23	25
II	11	15	17	23	25
III	11	15	17	23	25
IV	11	15	18	23	25
V	11	15	17	23	25
VI	10	14	16	22	24
VII	10	14	16	23	25
VIII	11	15	17	23	25
701 (Plate:5)					
I	11	15	17	23	26
II	11	16	18	25	27
III	11	15	17	24	25
IV	11	15	17	24	25
V	10	14	17	23	25
VI	10	15	17	22	24
VII	10	15	17	23	24
VIII	11	15	17	23	26

Table 3 Growth Measurements Taken from culture plates of isolate 776 incubated at 25°C

Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours
776 (Plate:1)					
I	11	13	19	28	32
II	9	15	17	26	29
III	10	14	17	26	30
IV	9	14	17	26	30
V	10	15	17	27	30
VI	11	15	19	28	31
VII	10	14	17	26	30
VIII	10	14	17	27	30
776 (Plate:2)					
I	11	15	19	28	31
II	11	17	19	28	32
III	11	16	18	26	31
IV	10	16	17	27	30
V	10	15	17	26	30
VI	11	16	18	27	30
VII	10	15	18	27	31
VIII	11	15	18	28	30
776 (Plate:3)					
I	10	15	17	26	30
II	11	16	18	28	32
III	12	17	20	29	33
IV	12	15	18	27	31
V	11	15	18	27	31
VI	10	14	17	25	29
VII	10	14	16	26	29
VIII	10	14	17	26	29
776 (Plate:4)					
I	10	15	18	27	30
II	11	17	19	28	32
III	10	15	18	28	31
IV	10	16	18	27	31
V	10	16	18	27	31
VI	11	16	18	28	30
VII	10	15	18	27	30
VIII	10	16	18	27	31
776 (Plate:5)					
I	9	14	17	26	29
II	9	15	17	26	30
III	9	14	17	26	30
IV	10	14	17	27	30
V	10	15	18	27	30
VI	10	15	18	26	30
VII	9	14	17	26	29
VIII	9	14	17	25	29

Table 4 Growth Measurements Taken from culture plates of isolate 779 incubated at 25°C

Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours
779 (Plate:1)					
I	9	13	14	20	25
II	10	12	14	20	25
III	9	13	13	21	25
IV	9	12	14	21	25
V	9	13	14	20	24
VI	9	12	14	21	25
VII	9	12	14	20	24
VIII	9	12	14	20	23
779 (Plate:2)					
I	9	13	14	21	25
II	11	14	15	23	26
III	10	14	14	21	25
IV	10	14	15	22	25
V	9	13	14	21	25
VI	9	13	14	21	25
VII	9	13	13	20	24
VIII	9	12	13	20	25
779 (Plate:3)					
I	10	14	15	22	25
II	10	13	16	22	26
III	10	14	15	23	26
IV	11	13	15	21	26
V	11	13	16	21	25
VI	10	13	15	22	26
VII	10	13	16	21	25
VIII	10	13	15	21	25
779 (Plate:4)					
I	10	16	20	29	32
II	10	15	16	25	30
III	10	13	16	22	26
IV	10	14	16	22	25
V	9	12	14	21	25
VI	9	13	15	21	25
VII	10	13	15	21	25
VIII	9	12	16	25	28
779 (Plate:5)					
I	10	14	15	22	25
II	11	14	16	22	26
III	11	14	16	23	26
IV	9	12	15	21	24
V	8	12	14	20	24
VI	8	12	14	19	23
VII	9	12	14	20	24
VIII	9	13	15	21	25

Table 5 Growth Measurements Taken from culture plates of isolate 771 incubated at 25°C

Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours
		771 (Plate:1)			
I	18	27	35	N/A	N/A
II	14	25	32		
III	20	31	36		
IV	19	30	36		
V	19	29	34		
VI	21	31	36		
VII	20	31	35		
VIII	19	30	35		
		771 (Plate:2)			
I	20	30	34	N/A	N/A
II	20	31	37		
III	20	32	37		
IV	20	30	36		
V	18	28	34		
VI	16	23	26		
VII	17	28	33		
VIII	18	27	33		
		771 (Plate:3)			
I	13	24	30	N/A	N/A
II	20	30	36		
III	20	28	31		
IV	20	30	36		
V	18	28	33		
VI	15	25	32		
VII	11	22	27		
VIII	8	16	21		
		771 (Plate:4)			
I	10	20	27	N/A	N/A
II	13	24	28		
III	12	21	27		
IV	13	24	30		
V	14	24	29		
VI	14	24	30		
VII	14	25	31		
VIII	13	22	29		
		771 (Plate:5)			
I	21	32	38	N/A	N/A
II	13	26	33		
III	20	30	36		
IV	21	33	38		
V	21	31	37		
VI	20	29	33		
VII	21	32	36		
VIII	23	34	38		

Table 6 Growth Measurements Taken from culture plates of isolate 832 incubated at 25°C

Date	25/06 120 hours	27/06 168 hours	28/06 192 hours	02/07 288 hours	04/07 336 hours
832 (Plate:1)					
I	6	9	10	16	19
II	6	9	11	16	18
III	6	9	10	16	18
IV	6	9	10	16	18
V	6	10	11	17	20
VI	7	9	11	17	19
VII	7	9	10	16	19
VIII	6	8	10	16	18
832 (Plate:2)					
I	5	7	8	13	13
II	7	10	12	16	18
III	6	9	11	17	19
IV	6	9	11	16	19
V	6	10	12	17	20
VI	6	10	11	17	19
VII	7	10	11	17	19
VIII	5	8	10	15	17
832 (Plate:3)					
I	5	8	10	14	16
II	6	8	10	15	16
III	5	9	10	15	17
IV	7	10	12	17	19
V	4	7	10	16	18
VI	4	8	10	14	16
VII	5	7	9	14	16
VIII	5	8	10	15	16
832 (Plate:4)					
I	5	8	9	14	16
II	4	7	8	13	15
III	5	7	8	13	15
IV	5	7	10	15	16
V	6	7	10	16	17
VI	5	8	9	15	16
VII	6	9	10	15	17
VIII	5	8	9	15	16
832 (Plate:5)					
I	6	10	12	17	20
II	6	8	11	16	19
III	6	8	10	16	18
IV	5	7	9	14	15
V	5	8	10	14	16
VI	6	8	10	16	17
VII	5	7	9	14	17
VIII	6	8	10	16	19