Species of *Phytophthora* associated with a native ecosystem in Gauteng

by

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Declaration

I, Jan Hendrik Nagel, declare that the thesis/dissertation, which I hereby submit for the degree *Magister Scientiae* at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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PREFACE

Species of *Phytophthora* are globally known as destructive pathogens of hardwood trees and agricultural crop plants, causing severe root disease, wilt, cankering and in most cases mortality. Rivers play an integral role in the dissemination of *Phytophthora* and often contain the zoospores of many different *Phytophthora* spp. Identification of *Phytophthora* spp. from rivers provides an effective approach to determine the *Phytophthora* species diversity within an area. Unfortunately, few such studies have been conducted in South Africa and almost nothing is known regarding the diversity of *Phytophthora* spp. within rivers and native environments outside the Western Cape Province. The aim of the studies making up this thesis was to survey one such system in the Gauteng Province of South Africa.

Oomycetes (including *Phytophthora*) superficially resemble Fungi, but belong to a different evolutionary lineage. Chapter One of this thesis reviews the taxonomy, diversity, economic and environmental impact of Oomycetes, with special reference to *Phytophthora*. In order to effectively distinguish between species, well defined criteria are necessary. Many species recognition concepts, methods and techniques exist and their application with regards to *Phytophthora* are discussed. The last section of this chapter considers the strengths and weaknesses of the available techniques used to identify *Phytophthora* spp. from native ecosystems.

Among the *Phytophthora* isolates recovered during the sampling phase of this thesis (treated in Chapter 2), there were a large number of isolates that could not be identified using ITS sequence comparisons and phylogenetic inference. These isolates consistently yielded ITS sequences with multiple polymorphic sites and sequence truncation after a few hundred bases. Similarly, isolates independently recovered from Australia were found to resemble the South African isolates phylogenetically. This led to the supposition that these isolates had a hybrid origin. Chapter two of this thesis considered the proposed hybrid nature of these isolates.

In the third chapter of this thesis, the diversity of *Phytophthora* spp. present in the Crocodile River in the Gauteng Province was investigated. This was achieved by a year-long, biweekly sampling of the Crocodile River for *Phytophthora* spp. using on-site baiting. The identities of the recovered *Phytophthora* isolates were determined through phylogenetic analysis of nuclear and mitochondrial gene regions. The resultant species diversity was contrasted to what is known from South Africa and to that found by other similar studies done abroad.

Diseases caused by *Phytophthora* spp. are responsible for severe crop losses and damage to native ecosystems globally. This is also the case in Africa. However, there is a lack of available literature summarizing the impact of *Phytophthora* on this continent. In the last chapter of this thesis, which also appears in a condensed form as a book chapter, this impact is reviewed. Most research in this area has been done on *Phytophthora* spp. causing diseases of important crops, such as potato late blight, cacao black pod and avocado root rot. Only a small number of *Phytophthora* spp. are known to cause tree diseases in Africa. However, the increasing number of *Phytophthora* tree diseases being reported internationally suggests a substantial threat to African forestry. It is hoped that the results of the studies presented in this thesis will contribute meaningfully to understanding these threats.

Chapter 1

Diversity, species recognition and environmental detection of *Phytophthora* spp.

Abstract

More than a hundred *Phytophthora* species are known at present and more species are continually being described. *Phytophthora* and other Oomycetes superficially resemble Fungi, but they belong to two distinct Eukaryotic lineages. There has been much debate surrounding the higher order taxonomy of Oomycetes, however the two dominant opposing classifications mostly agree on the taxonomy below Class level. Oomycete species differ vastly in their lifestyles, ranging from saprobes to parasites of animals and plants. *Phytophthora* spp. are exclusive plant pathogens and cause many important plant diseases around the world. The morphological and phylogenetic species concepts are widely applied in *Phytophthora*. The biological species concept, however, is not useful for identifying species in this genus. Several techniques are available to study *Phytophthora* spp. from native ecosystems. Culture based techniques are most commonly used, but suffer from several biases. Molecular techniques are able to circumvent these biases in some cases. However, many are not amendable with multiplexing. Techniques that have a high multiplex capability are generally much more expensive.

1. Introduction

Phytophthora is a large genus and it is one that continues to expand as new species are increasingly being described. The genus was first described in 1876, when the type species *Phytophthora infestans* was implicated as the cause for Late Blight of potatoes (De Bary 1876). Since then many more species have been described. In the late 20th Century, approximately sixty *Phytophthora* species were known (Erwin and Ribeiro 1996). Thereafter, with the emergence of molecular techniques that enhanced recognition of species and through the exploration of novel niches, the number of species known almost doubled to approximately 100 (Brasier 2009; Kroon *et al.* 2012). The number of *Phytophthora* species will most likely continue to rise as more unexplored areas are being investigated and as modern techniques are refined to define cryptic species.

Phytophthora spp. are Oomycetes (Peronosporomycetes), a diverse fungus-like class of organisms (Dick 2001a). *Phytophthora* is closely related to the Oomycete genera *Pythium* and *Peronospora* that both include important plant pathogens, but reside in two different orders. The taxonomic placement of *Phytophthora* with regard to the other, closely related genera, is at present unresolved. Currently, *Phytophthora* is classified in the Pythiales but it has been suggested that it should be moved to Peronosporales based on phylogenetic evidence (Riethmuller *et al.* 2002).

Plant diseases due to infection by *Phytophthora* spp. have had a profound effect on the world agriculture and human well-being. Examples of such diseases include late blight of potatoes (Fry *et al.* 1993), black pod of *Theobromea cacao* (Cacao) (Appiah *et al.* 2004b) and sudden oak death (Rizzo *et al.* 2002). The impact of *Phytophthora* spp. has been most noticeable when they occur in agricultural systems, especially on food crops. The resulting diseases are, however, not limited to agricultural systems, but also cause substantial damage and losses to commercial forestry plantations, nurseries (Donald and von Broembsen 1977; Barnard *et al.* 1985; Linde *et al.* 1994; Reglinski *et al.* 2009), and ornamental plant industries (Slawson *et al.* 2009). *Phytophthora* spp. are also well known due to the damage to natural ecosystems caused by species in this genus that have become invasive aliens in the environment (Von Broembsen and Kruger 1985; Shearer *et al.* 2004).

This review explores the diversity of the Peronosporomycetes and its importance. Because *Phytophthora* spp. cause some of the most important plant diseases their impact and importance will also be considered. Furthermore, the species concepts currently applied in *Phytophthora* will be discussed. Studying *Phytophthora* in native ecosystems poses some important challenges. Several different techniques and approaches are available, each with

there own set of advantages and disadvantages. The last section of this review will thus focus on these approaches and their application.

2. The Oomycetes

2.1. Oomycetes versus Fungi

Historically, Oomycetes were regarded as fungi and were included in the Phycomycetes, which is an archaic term that is no longer used (Erwin and Ribeiro 1996). Oomycetes were thought to be affiliated with the fungal Kingdom as they shared features such as filamentous hyphae and a heterotrophic nature (Table 1). Even though the Oomycetes are not affiliated with or closely related to the Kingdom Fungi, they are still regarded as fungi in the loose sense by mycologists because of their shared characteristics including their role as pathogens and saprobes (Money 1998).

Oomycetes are affiliated with the heterokont (possessing two different flagella) algae and are included within the Kingdom Chromista (Cavalier-Smith 1986). This Kingdom is far removed from that of the Kingdom Mycota and many differences between these two Kingdoms are apparent (Table 1). Most noticeably differences in flagellation exist between Oomycetes and Fungi, as Oomycetes produce heterokont zoospores whereas Fungi rarely have flagellate spores and where they do occur, these spores are mono-flagellate. Differences in hyphal structure and architecture can also be applied to differentiate between these two groups of organisms. The hyphae of Oomycetes are generally aseptate (coenocytic) with cell walls composed mainly of cellulose, whereas fungi have hyphae that are commonly septate and with cell walls of chitin.

2.2. Taxonomy of Oomycetes

Oomycetes belong to the Kingdom Chromista. The taxonomic relevance and validity of the Chromista, as defined by Cavalier-Smith (1986), has been opposed by other definitions, most notable is that of Dick (2001b) with the Kingdom Straminipila. Due to the large diversity of organisms included in these two competing classifications of the Kingdoms, only issues relevant to the Oomycetes will be discussed below.

Diagnostic characters for Chromista include tubular mitochondrial cristae, chloroplast endoplasmic reticulum (CER) if chloroplasts are present and/or TTH's present on flagella (Cavalier-Smith 1986). The Heterokonta represents one of three phyla originally included in this Kingdom and they are simply characterized as organisms with an anterior tinsel flagellum and with a posterior whiplash flagellum, which is either present or absent. This phylum is morphologically heterogeneous and includes photosynthetic chromophyte algae

(Ochrista), unicellular, free living and phagotrophic bicosecids and the heterotrophic Pseudofungi.

Flagellar characteristics are phylogenetically important (Dick 1997) and have been used for defining super-ordinal taxa in the Chromista (Cavalier-Smith 1986; Dick 2001b). Characteristics important for classification include the number of flagella, flagellar ornamentation and length differences between flagella. In Chromista, the flagellation can either be absent, uniflagelate or biflagellate (Cavalier-Smith 1986). Flagellar ornamentation refers to the presence or absence of tubular tripartite hairs (TTH) (mastigonemes) on flagella. These TTH's are rigid hair like protrusions situated perpendicular to the flagellar shaft and their presence results in a flagellar thrust reversal, i.e changes the flagella's locomotion from pushing to pulling (Cahill *et al.* 1996). Flagella ornamented with TTH's are referred to as straminipilous flagella or tinsel flagella and non ornamented flagella are referred to as whiplash flagella. Some taxa in the Chromista possess two flagella that differ either in terms of ornamentation, length or both. In the case where two flagella are of different length, the taxa are referred to as being anisokont and where both tinsel and whiplash flagellae are present, they are referred to as heterokont. In heterokont zoospores, the tinsel flagellum generates the thrust and the whiplash flagella controls the zoospore's rotation (Erwin and Ribeiro 1996).

The opposing classification of Dick (2001b) argues against Chromista as a Kingdom and defines the Straminipila as a new Kingdom in its place. The latter Kingdom's etymology stems from the Latin term *stramen* meaning "straw", which refers to the TTH's found in this group. The Straminipila is described for organisms possessing anisokont and heterokont flagellation, with the anterior flagella being straminipilous, or derived from organisms that have possessed these characters (Dick 2001b). This circumscription makes this Kingdom equivalent to the Phylum Heterokonta of Kingdom Chromista sensu Cavalier-Smith (1986), which is also used as the sole Phylum within Straminipila.

It remains unclear which of the two classification systems should be used. The circumscription by Dick (2001b) tends to be much more complete than that of Cavalier-Smith (1986). Straminipila is defined more clearly and appears to be more visibly monophyletic although less inclusive than Chromista (Blackwell 2009). If Straminipila is to be used as a Kingdom, the placement of the excluded taxa (Cryptophyta and Haptophyta) becomes an additional problem. The other option is to make use of the super-group Chromalveolata (Adl *et al.* 2005). At present the best option is to use Chromista as Kingdom as it is more inclusive and visibly more holophyletic than Straminipila (Blackwell 2009).

The lower level classification of the two Kingdoms discussed above initially differed significantly. The Pseudofungi *sensu* Cavalier-Smith (1986) includes Labyrinthista, Oomycetes and Hypochytrids, whereas its synonym *sensu* Dick (2001b), Peronosporomycotina, excludes Labyrinthista. Later Pseudofungi was amended by raising it to phylum level (and Heterokonta to infrakingdom) and by excluding Labyrinthista (Cavalier-Smith 2004; Cavalier-Smith and Chao 2006). Dick (2001b) includes several synonyms in his classification, such as Peronosporomycetes for Oomycetes, and although his descriptions are more extensive than those of Cavalier-Smith (1986) they correspond fairly well with one another.

2.3. Diversity and Impact of Oomycetes

Within the Oomycetes there are three subclasses namely the Saprolegniomycetidae, Rhipidiomycetidae, and Peronosporomycetidae (Dick 2001a). The Saprolegniomycetidae produce a thallus that is either mycelial, corraloid or blastic. Multiple oospores with granular ooplasts are often formed centrifugally within a single oogonium. Saprolegniomycetidae are not known to utilize sulphates as a sulphur source (Dick 2001a). The Rhipidiomycetidae is not a well known subclass and species in this group are not known to cause disease of plants. The Rhipidiomycetidae is distinct among the Peronosporomycetes as its members produce monocentric thalli consisting of a single cell and they also possess rhizoids. The Peronosporomycetidae is characterized by having predominantly aseptate mycelial thalli, which are mostly eucarpic (i.e. only a portion of the thalus is converted into fruiting structures), they produce oospores centripetally and in most cases only a single oospore (with transparent ooplast) is formed within the oogonia. The Peronosporomycetidae can also utilize sulphates as a sulphur source (Dick 2001a).

The Saprolegniomycetidae contains the orders Saprolegniales, Sclerosporales, Salilagenidiales and Leptomitales (Dick 2001b). Genera in the Saprolegniales include *Achlya*, *Aphanomyces* and *Saprolegnia*. These genera cause diseases in organisms such as fish and aquatic arthropods (Vennerstrom *et al.* 1998; Czeczuga *et al.* 2005). Recently it was also shown that these genera can infect plant material (Kiziewicz 2005). The Leptomitales are phytosaprophytes of aquatic systems and although their status as pathogens is as yet unknown, many species have been found to infect and colonise fruits of plants (Czeczuga *et al.* 2007). Within Sclerosporales, the best known species is *Sclerospora graminicola*, which is a serious pathogen of pearl millet (*Pennisetum glaucum*) (Gilijamse *et al.* 1997).

The Rhipidiomycetidae has not been studied extensively. This subclass contains genera

such as *Rhipidium*, *Sapromyces* and *Araiospora*, which are thought to be aquatic saprobes (Thaxter 1896). Although Czeczuga *et al.* (2007) treated these genera as Leptomitales, they were found to occur in aquatic habitats and were isolated by fruit baiting. Phylogenetic studies of the Peronosporomycetes have also afforded a disproportionately small amount of focus to this subclass, mainly because it is usually only represented by the single taxon of *Sapromyces* (Hudspeth *et al.* 2000; Petersen and Rosendahl 2001; Riethmuller *et al.* 2002). The Rhipidiomycetidae still remains largely unstudied and only when more of the constituent taxa are used for phylogenetic studies will its taxonomic placement be resolved.

The Peronosporomycetidae includes two orders, namely the Pythiales and Peronosporales. The main differences between these two orders are that the Peronosporales has intersporangial zoosporogenesis with plasma membrane vesicles, while Pythiales may undergo intersporangial zoosporogenesis, with or without plasma membrane vesicles, or extrasporangial zoosporogenesis in a homohylic vesicle (Dick 2001a). The Peronosporales can also additionally produce conidiosporangia, while Pythiales do not have this feature. Another difference between Pythiales and Peronosporales is that species in the Peronosporales have a persistent periplasm surrounding the oospore while Pythiales do not (Dick 2001a).

Although the Pythiales includes important plant pathogens, these are not restricted to plants. Genera such as *Lagenidium* and *Pythium*, among others, are included in this order. *Lagenidium* has an aquatic lifestyle and species are pathogens of dipteran larvae (Couch 1935; Frances *et al.* 1989) and crustaceans such as planktonic copepods (Couch 1935) and cirripedes (Johnson Jr 1958). *Pythium* species are well known plant pathogens (Martin and Loper 1999), but are also known to infect aquatic algae (Fuller *et al.* 1966), humans and other mammals (Gaastra *et al.* 2010).

The Peronosporales contains two families, namely the Albuginaceae and the Peronosporaceae. The Albuginaceae is characterized by its members having small spherical haustoria and unbranched conidiosporangiophores, which produce deciduous conidiosporangia (Dick 2001a). The Peronosporaceae is characterized as having lobate haustoria, branched and persistent conidiosporangiophores, which produce deciduous conidiosporangia (Dick 2001a).

The Peronosporales occur only on plants. The Albuginaceae are commonly known as white rusts and are pathogenic on various plant species (Hartmann and Watson 1980). Their effect has been most noticeable as pathogens of *Brassica* species (Rimmer *et al.* 2000). The

family Peronosporaceae contains several important plant pathogenic genera, most of which cause downy mildews. This family is the largest in the Peronosporomycetes and genera include *Bremia, Peronospora, Plasmopara* and *Pseudoperonospora* (Dick 2001a). *Bremia* is best known for the species *Bremia lactucae,* which is a serious pathogen of lettuce (*Lactuca sativa*) (Norwood and Crute 1983). Many species of *Peronospora* have been described, even though these obligate biotrophs cannot be cultured *in vitro*. Two of the most important species are *Peronospora tabacini* the cause of blue mould of tobacco (Aylor and Taylor 1983) and *Peronospora parasitica,* which causes downy mildew of economically important *Brassica* species (Sherriff and Lucas 1990). *Plasmopara viticola* is an important pathogen of grapevines, which causes substantial losses where grapes are cultivated in temperate regions (Williams 2005). *Pseudoperonospora humuli* is the causal agent of downy mildew of hops (*Humulus lupulus*) and can cause significant crop losses (Chee *et al.* 2006).

The ordinal and familial classification of *Phytophthora* is currently uncertain. *Phytophthora* is classified in the Pythiales, but recently molecular phylogenies have shown that *Phytophthora* should be reclassified in the Peronosporales (Cooke *et al.* 2000a; Hudspeth *et al.* 2000; Petersen and Rosendahl 2001; Riethmuller *et al.* 2002). Phylogenetic studies that focussed on *Phytophthora* (Cooke *et al.* 2000a) have included only a few species of *Peronospora* and those that focussed on *Peronospora* (Goker *et al.* 2003) only include a few *Phytophthora* species.

2.4. Impact of Phytophthora

Phytophthora spp. are hemibiotrophic, implying that they infect living tissue but also have limited saprotrophic abilities (Perfect and Green 2001). This is in contrast to the lifestyle of *Pythium* species, which are much more typically saprotrophic than *Phytophthora* spp. (Erwin and Ribeiro 1996), and to the obligate biotrophic lifestyle of the downy mildews, e.g. species of *Peronospora* (Thines 2009). *Phytophthora* spp. only infect plants unlike some *Pythium* spp. eg. *Pythium insidiosum,* which can infect animals (Mendoza *et al.* 1993).

Phytophthora species have been, and continue to be, among the world's most destructive pathogens. The resultant plant diseases have had different levels of impact on the world and on society. Among these, economic losses to agriculture and/or famine are the most prominent (Erwin and Ribeiro 1996). The best known example of a destructive species is *Phytophthora infestans,* the causal agent of potato late blight which caused the Great Irish Famine of the 1840's (De Bary 1876; Fry and Goodwin 1997). This epidemic caused widespread famine in Ireland due to the people's dependence on potato as the staple food. Late blight remains a problem today and the associated crop losses can be as high as 90%

(Sengooba and Hakiza 1999).The economic impact of late blight is hard to quantify, but it is clear that it causes severe losses. In one study, it was estimated that for an American potato grower, the cost of fungicide application and loss of revenue due to crop loss amounts to $500 per hectare (Guenthner *et al.* 2001).

Phytophthora pod rot of cacao (Cacao black pod) is the most important disease of cacao in the world (Appiah *et al.* 2004a) and is caused by several species of *Phytophthora* (Guest 2007). In this regard, four are considered important i.e. *P. capsici*, *P. citrophthora*, *P. megakarya* and *P. palmivora*. *Phytophthora capsici and P. citrophthora* occur mostly in South America (Kellam and Zentmyer 1986) and *P. megakarya* occurs in Central and West Africa (Opoku *et al.* 2007b; Pokou *et al.* 2008), whereas *P. palmivora* has a global distribution (Zentmyer *et al.* 1973). Of these species, *P. megakarya* is the most virulent as it can cause losses of up to 100% (Dakwa 1987; Appiah *et al.* 2004b; Ndoumbe-Nkeng *et al.* 2004; Opoku *et al.* 2007a) while the other three species cause roughly 20% crop losses (Wood 1975; Appiah *et al.* 2004b).

The impact of *Phytophthora* diseases is not always in the form of direct economic losses. Many species impact negatively on native ecosystems. Apart from causing diseases of several important crops and trees, *P. cinnamomi* has also been observed to be pathogenic on a wide variety of native plants. In Australia, it was found that *P. cinnamomi* could infect more than 3000 native plant hosts (Shearer *et al.* 2004). This allows *P. cinnamomi* to cause widespread damage to natural plant populations upon its introduction into new habitats, as was the case in Australia (Cahill *et al.* 2008). In South Africa, it was first identified causing crown and root rot of Silver trees (*Leucadendron argenteum*) (Van Wyk 1973) in the Western Cape. It was subsequently found on other native plants in the Fynbos vegetation of the Western Cape area such as *Erica* spp., *Leucadendron* spp., *Leucaspermum* spp., *Widdringtonia cederbergensis* and *Protea* spp. (Von Broembsen and Brits 1985; Von Broembsen and Kruger 1985). Most of these studies on *P. cinnamomi* in South Africa focussed on the Western Cape and few studies on native ecosystems outside of the Western Cape have been done to determine the spread of *P. cinnamomi* (Von Broembsen 1984).

In the past decade, several *Phytophthora* spp. causing severe forest diseases have been discovered. Sudden Oak Death is a serious disease of natural North American oak forests caused by *Phytophthora ramorum* (Rizzo *et al.* 2002). It most noticeably affects tanoak (*Notholithocarpus densiflorus*) and three native *Quercus* (oak) species (*Quercus agrifolia, Q. kelloggii* and *Q. parvula*) (McPherson *et al.* 2010) and causes among other symptoms wilting

of shoots, bleeding stem/branch cankers and eventually tree mortality (Garbelotto *et al.* 2001). *P. ramorum* has a wide host range and infects several other trees and understory plants. Apart from the lethal stem/branch infection observed in tanoak and oak species, *P. ramorum* also causes non-lethal foliar and twig infections in some plants (*Arbutus menziesii, Rhododendron macrophyllum* and *Umbellularia californica*) (Garbelotto *et al.* 2001). Non-lethal foliar and twig infection plays an important role in the epidemiology of *P. ramorum* as the hosts act as reservoirs for it (Garbelotto *et al.* 2003; DiLeo *et al.* 2009).

Phytophthora pinifolia is a newly discovered species that causes a severe disease of *Pinus radiata* (Monterey Pine) in Chile known as 'Daño Foliar del Pino' (DFP) (Durán *et al.* 2008). Thus far only *P. radiata* stands were infected whereas other conifer species such as *P. pinaster* and *Pseudotsuga menziessii* remain healthy. *P. pinifolia* is a foliar pathogen and is thought to have been introduced into Chile (Durán *et al.* 2010). Chilean commercial forestry relies heavily on Monterey Pine (Guerrero and Bustamante 2007) and thus *P. pinifolia* is a serious threat to this country's forestry industry and economy (Durán *et al.* 2008). *P. pinifolia* represents a considerable risk to any country growing large areas of *P. radiata.*

3. Species recognition in *Phytophthora*

To identify species effectively, there should be well defined criteria of the definition and recognition of a species. The definition of a species has been debated in the last century and this has come to be known as the "species problem" (Ghiselin 1974; De Queiroz and Donoghue 1988). Species level taxa are distinct categories that represent, often non-distinct, evolutionary groups. This results in disparity between species taxa and the evolutionary groups they represent (Hey 2001).

The evolutionary species concept, which defines a species as "a single lineage of ancestor descendent populations which maintains its identity from other such lineages and which has its own evolutionary tendencies and historical fate" (Wiley 1978), is the preferred species concept for fungi and fungi-like organisms (Taylor *et al.* 2000). This species concept implies actual reproductive isolation between species units, as this will result in separated lineages forming even if two populations can potentially interbreed. The evolutionary species concept does not have any implied recognition criteria associate with it, which limits its practical usefulness (Taylor *et al.* 2000). It has been noted by Taylor *et al*. (2000) that three other species concepts are in fact merely species recognition criteria that attempt to identify evolutionary species. These three species recognition concepts are the morphological, biological and phylogenetic species recognition concepts. Mayden (1999) considered twenty-four species concepts to be secondary recognition concepts for the evolutionary

species concept. With so many species concepts available, it is imperative to know which is most appropriate. For this review, I only focus on the morphological, biological and phylogenetic species recognition concepts as these are the three most widely applied.

3.1. Morphological species concept

The morphological species recognition (MSR) makes use of phenotypic differences to distinguish between evolutionary distinct lineages. Characters considered for use in MSR are assessed for their informativeness in recognizing and differentiating between species (Simpson 1951). The advantages of using MSR is that it is relatively easy to apply and because large data sets are usually available, making comparisons easy (Taylor *et al.* 2000).

The MSR has a few major shortcomings. Not all phenotypic characters are indicative of evolutionary history as selective pressure on such characters are generally low in fungi (Harrington and Rizzo 1999). Morphological differences are also not always present between distinct lineages, causing the MSR to underestimate the true species diversity (Taylor *et al.* 2000). Species described using the MSR often contains multiple species when reevaluated using other criteria such as the phylogenetic species recognition (see below). This is exemplified by the case of *Phytophthora* spp. associated with cacao, where multiple species were regarded as *P. palmivora*. However, it was later shown that at least two species, *P. megakarya* and *P. tropicalis* could be distinguished from *P. palmivora* (Brasier and Griffin 1979; Zhang *et al.* 2004).

In *Phytophthora,* the morphological species concept has been applied extensively and ample data exist for morphological comparisons. Prior to the prevalence of molecular techniques, it was widely applied (Tucker and Milbrath 1942; Chee 1969) and it continues to be used in conjunction with these molecular techniques to identify and delineate species units in this genus e.g. Reeser *et al* (2008), Abad *et al.* (2008) and Nelson and Abad (2009). Characters such as the presence of sexual structures, antheridial placement, zoosporangial features, chlamydospores and hyphal swellings have been considered informative for species delineation (Tucker 1931). Waterhouse (1963) placed *Phytophthora* species into six groups based on morphology and physiology (Table 2). These groupings have been revised by (Newhook *et al.* 1978) and extended to include a seventh group by Stamps *et al*. (1990). This seventh group represents the separate marine genus *Hyalophytophthora*.

3.2. Biological species concept

Biological species recognition (BSR) relies on the mating incompatibility between distinct lineages to identify species boundaries. In other words, if two specimens cannot interbreed

they are considered to be distinct lineages and thus two separate species. The limitation of the BSR is that the reverse is not true, for instance when two specimens can interbreed, it does not necessarily imply that they are from the same lineage or that actual gene flow is occurring between the populations. This is because the BSR determines potential interbreeding and not the actual observed interbreeding occurring in nature. Thus the BSR will underestimate the true species diversity (Taylor *et al.* 2000).

In *Phytophthora,* two mating compatibility types exist, namely A1 and A2 mating types (Erwin and Ribeiro 1996). Heterothallic *Phytophthora* spp. can only produce sexual structures in the presence of a strain with the opposite mating type. The mating hormones of *Phytophthora* spp. are not species specific and the mating type hormone of one species can be used to induce the formation of sexual structures in a isolate of opposite mating type of another species (Qi *et al.* 2005; Harutyunyan *et al.* 2008). Thus mating studies in *Phytophthora* cannot be used to recognize biological species units.

The ability of *Phytophthora* spp. to hybridize further complicates the application of biological species recognition in this genus. Despite the fact that interspecific matings can induce sexual structures in *Phytophthora,* actual hybridization is rare and the resulting oospores are usually due to matings between hyphae of a single species (Boccas and Zentmyer 1976; Boccas 1981; Erselius and Shaw 1982). In contrast to the above observations, it has been shown that some *Phytophthora* spp. can form hybrids, i.e. *P. infestans* with *P. mirabilis* (Goodwin and Fry 1994). Several natural hybrids of *Phytophthora* spp. have also been detected e.g. *P. alni* (Brasier *et al.* 1999; Brasier *et al.* 2004), *P.* X*pelgrandis* and *P.* X*serendipita* (Nirenberg *et al.* 2009; Man in't Veld *et al.* 2012).

3.3. Phylogenetic species concept

The Phylogenetic species recognition (PSR) concept attempts to identify evolutionary lineages by using DNA nucleotide differences to distinguish species units. Originally, the phylogenetic species concept defined species as being the smallest monophyletic clade of organisms sharing a derived character state (Taylor *et al.* 1999). Phylogenetics can detect intraspecific as well as interspecific nucleotide diversity. If intraspecific nucleotide diversity of any one locus is used, then what appears as two distinct species lineages will be only two distinct gene lineages (Taylor *et al.* 1999). This problem is solved by comparing multiple gene genealogies, a process known as Genealogical Concordance Phylogenetic Species Recognition (GCPSR) (Taylor *et al.* 2000). GCPSR relies on the assumption that chromosomal recombination occurs within a species but not between species. Thus, within a species, different loci will assort independently and be independently inherited. In other

words these loci will have different genealogies. When genealogies of two or more genes are concordant, i.e. have similar tree topographies, it supports the absence of recombination, whereas incongruous genealogies will support the presence of recombination (Avise and Ball 1990; Taylor *et al.* 2000). The species boundary can thus be defined as existing between the incongruous and concordant portions of the genealogies. Unfortunately, this strategy is not compatible for exclusively asexual/clonal populations as no recombination occurs within these populations.

Phylogenetic species concepts have been widely applied to *Phytophthora* during the course of the last decade. New species descriptions supported by the PSR were mainly based on only a single gene region, usually the ribosomal DNA Internal transcribed spacer (ITS) region (Mirabolfathy *et al.* 2001; Flier *et al.* 2002; Jung *et al.* 2002; Man in't Veld *et al.* 2002; Polashock *et al.* 2005; Dick *et al.* 2006; Greslebin *et al.* 2007; Abad *et al.* 2008; Jung and Nechwatal 2008; Reeser *et al.* 2008; Hansen *et al.* 2009). Recent studies used data from multiple loci to construct phylogenies (Belbahri *et al.* 2006; Blair *et al.* 2008; Gomez-Alpizar *et al.* 2008; Jung and Burgess 2009; Bezuidenhout *et al.* 2010). Some studies only considered loci from the nuclear genome (Blair *et al.* 2008), whereas others used loci from both the nuclear and mitochondrial genomes (Kroon *et al.* 2004; Villa *et al.* 2006; Gomez-Alpizar *et al.* 2008). No species delineation in *Phytophthora* has been based solely on phylogenetic evidence, instead most of these species descriptions makes use of both morphology and phylogeny for support. Multigene phylogenies are especially important in *Phytophthora*, not only because they allow for the application of GCPSR but also because inconsistencies between individual phylogenies can be used to identify hybrid taxa (Nirenberg *et al.* 2009).

Phylogenetic analyses in *Phytophthora* spp. have led to the identification of ten consistent clades within the larger phylogeny (Figure 1) (Cooke *et al.* 2000a; Kroon *et al.* 2004; Blair *et al.* 2008). These clades only became apparent when many species, representative of the diversity within the genus, were included in phylogenies. Initially clades 1 to 8 were regarded as separate from clades 9 and 10 (Cooke *et al.* 2000a), although later studies did not support this view (Kroon *et al.* 2004; Blair *et al.* 2008). The ten clades within *Phytophthora* do not correlate with the morphological classification of the Waterhouse groupings (Cooke and Duncan 1997; Cooke *et al.* 2000a; Förster *et al.* 2000). Sporangial papillation does seem to correlate with phylogenetic structure, as there is a definite separation between papillate/semi-papillate and non-papillate sporangia in phylogenies (Crawford *et al.* 1996; Cooke and Duncan 1997; Kroon *et al.* 2004). Although thallism and antheridial attachment do not show a distinct pattern in the phylogeny, it has been suggested that homothalism and

amphigyny are ancestral conditions of the genus (Kroon *et al.* 2004).

The pace at which genomic data is becoming available is increasing at a staggering rate. Advances in sequencing technology as well as the decreasing cost of these technologies allow for the rapid increase in sequenced genomes that we see today (Liolios *et al.* 2006; Liolios *et al.* 2010). Several *Phytophthora* species' genomes have been sequenced. *P. sojae* and *P. ramorum* were the first (Tyler *et al.* 2006), followed by *P. infestans* (Haas *et al.* 2009), *P. ipomoeae, P. mirabilis* and *P. phaseoli* (Raffaele *et al.* 2010). Additionally, the genome projects of *P. capsici* and *P. cinnamomi* are underway (http://genomesonline.org). The available genomic data of *Phytophthora* spp. is a powerful resource, with application in genome evolution (Raffaele *et al.* 2010), horizontal gene transfer (Richards *et al.* 2006) and mechanisms of plant pathogenesis (Lamour *et al.* 2007; Soanes *et al.* 2007)

Genomic data can be applied towards phylogenetic and taxonomic issues such as resolving species relationships (e.g. Blair *et al.* (2008)) as well as deeper Kingdom level relationships (Burki *et al.* 2007; Burki *et al.* 2008). Phylogenomic inferences can be made by three methods, namely sequence based, whole genome and rare genomic change methods (Delsuc *et al.* 2005). Sequence based methods entail mining the available genomes for a large number of orthologous genes and if necessary the design of PCR primers in order to acquire sequence data for taxa without genomic data. This data is then used in subsequent alignment and phylogenetic analises. This approach was used by Blair *et al.* (2008) to construct a genus-wide phylogenetic tree of *Phytophthora* using a concatenation of seven nuclear genes. Whole genome methods rely on the comparison of the amount of shared orthologs (Snel *et al.* 1999), presence/absence of orthologs (Fitz-Gibbon and House 1999; Wolf *et al.* 2001) and gene orders (Blanchette *et al.* 1999; Sankoff *et al.* 2000; Belda *et al.* 2005). Several genomic features such as indels, positions of introns, retroposons and gene fusion/fission are sufficiently rare to exclude the probability of convergence (Delsuc *et al.* 2005). Such features are known as rare genetic changes (Rokas and Holland 2000; Roy and Irimia 2008; Rogozin *et al.* 2009). Whole genome and rare genetic change approaches have not been applied to *Phytophthora* yet.

4. Studying *Phytophthora* in native ecosystems

4.1. Isolation and culture of Phytophthora

Conventional approaches to collect *Phytophthora* spp. are based on the specific isolation and culturing of *Phytophthora* spp. from a certain source of inoculum. These methods involve either isolation directly from diseased plant material, or initial baiting of soil or water with leaves and subsequent isolation from lesions on leaf baits (Erwin and Ribeiro 1996).

Isolations are generally done on *Phytophthora*-specific media containing various antimicrobial chemicals to suppress growth of bacteria, fungi and some *Pythium* spp. (Masago *et al.* 1977). Colonies growing on these media are then subsequently sub-cultured.

Baiting has several biases occurring at different stages of the isolation procedure. Firstly baiting involves *Phytophthora* zoospores infecting living plant material, but not all *Phytophthora* spp. can infect all baiting material equally well (Ferguson and Jeffers 1999; Cooke *et al.* 2007; O'Brien *et al.* 2009). When studying a single or relatively low number of known *Phytophthora* spp., the choice of bait can be made with relative ease in order to optimize detection. In natural ecosystems, one would expect to encounter numerous *Phytophthora* spp., and these could be either native or introduced species (Balci and Halmschlager 2003; Brasier *et al.* 2003; Hüberli *et al.* 2010). In such cases, the choice of which baits to use is more difficult as one is potentially dealing with multiple known and unknown species and the possibility exists that the choice of bait may prevent the detection of one or more *Phytophthora* spp. An additional problem is that some *Phytophthora* spp. might grow asymptomatically on certain baits. These species will then be missed if only lesions or visibly infected parts of baits are used in isolations (Hüberli *et al.* 2000).

Baiting exploits the ability of *Phytophthora* zoospores to actively seek and infect living plant material. Baiting will thus not be able to detect other forms of inoculum such as oospores, chlamydospores and hyphae directly (Cooke *et al.* 2007). When soil is baited by first adding water to create a soil suspension, survival structures such as oospores and chlamydospores can germinate and produce sporangia that can then form zoospores capable of infecting the baiting material. In these cases, the isolation will be biased against those *Phytophthora* species that do not readily produce sporangia (Cooke *et al.* 2007).

Finally, isolation of *Phytophthora* can be difficult due to competitive exclusion by other microorganisms. Both inside plant baits and on selective media, *Phytophthora* spp. might not be detected as antagonistic species such as *Pythium* can parasitize *Phytophthora* spp. (Picard *et al.* 2000). *Pythium* spp. with a faster growth rate than *Phytophthora* can often mask the growth of *Phytophthora* spp. in culture (Ghimire *et al.* 2009).

An alternative technique to baiting is filtration, which is especially useful when isolating from water sources. Filtering entails vacuum pumping a water sample through a membrane filter with pore size smaller than that of a zoospore (~10µm). The filter membrane can then be plated out on selective media to isolate *Phytophthora* spp. As filtering does not suffer from the biases of baiting, it is more effective in its ability to detect *Phytophthora* spp. (Hwang *et*

al. 2008). Filtering will retrieve the inoculum of all organisms present in the water sample larger than the pore size and thus will lose some of the specificity provided by baiting. Because some species of *Pythium* are insensitive to Hymexazol (Tsao and Guy 1977; Kato *et al.* 1990), isolation from filter membranes can result in a high level of *Pythium* contamination.

4.2. Molecular detection and identification techniques for Phytophthora

Molecular detection techniques can detect species by exploiting unique, detectable characteristics in biological molecules occurring in the cells of a species. Molecular detection methods are not used to define species units, but mostly to recognize already defined species units. Several biological molecules can be used for targets of molecular detection, including proteins (Amouzou-Alladaye *et al.* 1988; Gabor *et al.* 1993), genomic material (Duncan and Cooke 2002) and metabolites (Karlshoj and Larsen 2005). The difficulty in finding a molecule that provides the needed specificity limits the molecules available as targets for molecular detection. Most detection techniques are also dependent on having a pure culture containing only a single species. These techniques are not amendable to environmental detection. Some techniques can only be applied to detect known or specific species and unknown species may then go undetected or be misidentified. Several techniques, however, are capable of detecting single or multiple species from heterogeneous samples, which makes them ideal for environmental detection and independent from culture-based biases. A brief overview of the most prominent molecular techniques and their efficacy for detecting *Phytophthora* from environmental samples is presented in the following sections.

4.3. Restriction fragment length polymorphism (RFLP)

Restriction fragment length polymorphism (RFLP) assay is a technique where DNA is digested with one or more type II restriction endonucleases and then resolved electrophoretically to reveal polymorphisms between samples. Type II restriction endonucleases are those that cleave their DNA substrate within their DNA recognition motif (restriction site) (Pingoud and Jeltsch 2001). The presence of restriction sites in the DNA will determine the positions where the DNA will be digested and thus the sizes of the DNA fragments formed. This technique can detect DNA polymorphisms based on presence/absence of the relevant restriction sites, as well as nucleotide length polymorphisms between the restriction sites (Botstein *et al.* 1980). These DNA polymorphisms will result in polymorphisms in the restriction fragment lengths, which can be used to differentiate between samples.

RFLP analysis can be carried out on genomic DNA to produce complex gel electrophoresis profiles containing many restriction fragment bands. This approach was used to develop species-specific profiles for 12 *Phytophthora* spp. (Panabieres *et al.* 1989). However, the complexity of the RFLP profiles generated from genomic DNA makes differentiation between profiles difficult. When RFLP is coupled to polymerase chain reaction (PCR) by performing the restriction reactions on amplified DNA instead of genomic DNA, the complexity of the resultant profiles is significantly reduced, thus making the development and application of the PCR-RFLP assay easier. Most of the PCR-RFLP assays for *Phytophthora* are based on the ribosomal DNA Internal transcribed spacer (ITS) region (Ristaino *et al.* 1998; Cooke *et al.* 2000b; Camele *et al.* 2005; Drenth *et al.* 2006; Bowman *et al.* 2007; Durán *et al.* 2009). The mitochondrial *cox*I and *cox*II genes have also been used as the target for PCR-RFLP (Martin and Tooley 2004).

RFLP analysis is a powerful tool for *Phytophthora* spp. identification. It can be performed faster than conventional morphological identification and is relatively easily interpreted. The most important shortcoming of RFLP is that it is dependent on a homogeneous DNA sample. Thus it is not amendable with multiplexing or environmental detection and is usually performed on DNA retrieved from cultured mycelia. Furthermore, RFLP identification profiles are designed for the detection of specific species and if these methods are applied to novel species or those not considered during the analysis design, spurious identifications might result.

4.4. Single-strand Conformation Polymorphism (SSCP)

Single-strand Conformation Polymorphism (SSCP) analysis is an electrophoretic technique whereby DNA molecules can be resolved based on their nucleotide sequence composition (Sunnucks *et al.* 2000). This technique requires DNA to be single stranded, which will allow the single stranded molecules to form into complex structures. The tertiary structure of the single-stranded (ss) DNA determines its mobility during electrophoresis (Liu *et al.* 1999). Thus, two DNA fragments of equal length but of different nucleotide sequence will be distinguishable with this technique. SSCP analyses are able to detect much more sequence variation than RFLP and can be combined with PCR to increase its specificity.

PCR-SSCP has been developed for the identification of about thirty *Phytophthora* spp. (Kong *et al.* 2003b; Kong *et al.* 2004). These markers are based on ribosomal DNA internal transcribed spacer (ITS) amplicons and were species-specific for all of the species tested. The above studies by Kong *et al.* (2003b; 2004) also developed and applied a SSCP molecular marker ladder for SSCP electrophoresis, which facilitates comparison of

electrophoretic profiles between gels.

Although this technique is less time consuming than morphological identification, it utilizes toxic acrylamide (LoPachin *et al.* 2002) to perform the polyacrylamide gel electrophoresis. Additionally, it requires either radio-isotope labelling (Sunnucks *et al.* 2000) or silver staining (Kong *et al.* 2003b) to visualize the DNA fragments as intercalating dyes do not bind well to ssDNA. SSCP analysis is dependent on homogeneous DNA samples, thus its application is restricted to identification of culturable isolates.

4.5. PCR detection with species-specific primers

PCR is a commonly used diagnostic tool to identify various plant pathogens when species-specific primer pairs are utilized (Henson and French 1993; Vincelli and Tisserat 2008). Species specific primer sets will only allow the amplification of the species for which they were designed. These make it possible to identify cultured isolates, but also to specifically detect a single species from a heterogeneous sample such as soil, water and plant samples (for review see O'Brien *et al.* (2009)). PCR reactions are analyzed electrophoretically and the presence/absence of relevant species is scored based on the presence or absence of an amplified fragment band on the gel. Most species-specific primers are designed by using pre-existing sequencing data generated by phylogenetic studies. The most popular locus is the ITS region (Tooley *et al.* 1997; Liew *et al.* 1998; Grote *et al.* 2002; Ippolito *et al.* 2002; Kong *et al.* 2003a; Silvar *et al.* 2005), but some have been designed from the *Ypt*1 gene (Schena *et al.* 2008; Meng and Wang 2009).

An alternative approach is to identify species-specific fragments generated by other techniques such as random amplified DNA polymorphism (RAPD) or RFLP and then to isolate and sequence these fragments. These fragments are then known as sequence-characterized amplified regions (SCAR) and they can be used to develop specific primers. SCAR markers have been used to produce specific primer sets for the identification of *Phytophthora* spp. (Schubert *et al.* 1999; Causin *et al.* 2005; De Merlier *et al.* 2005; Ioos *et al.* 2005)

The most prominent advantage of PCR is that it represents a relatively easy, sensitive and specific technique that can be performed in a short amount of time. The sensitivity of PCR detection can be further improved by performing nested PCR, which entails a preceding amplification set using general primers flanking the species-specific primers. This results in an amplicon containing the primer binding sites of the species-specific primers. This is followed by the normal PCR reaction containing the species-specific primers. Nested PCR

has been widely applied in conjunction with species-specific primers for the detection of *Phytophthora* spp. (Grote *et al.* 2002; Ippolito *et al.* 2002; Silvar *et al.* 2005; Schena *et al.* 2008; Meng and Wang 2009). A disadvantage of the technique is that prior knowledge of the DNA sequence is necessary, and it is usually quite specific to a species or phylogenitically closely related species.

4.6. Quantative PCR

Quantative PCR (qPCR) is a modification of the conventional PCR technique that makes it possible to quantify the amount of DNA template sample present (Heid *et al.* 1996). The basic qPCR technique is similar to conventional PCR in that it requires a primer pair and *Taq* polymerase among others, to function. In addition, qPCR also requires a fluorescent reporter system, which is used to measure the amplification of DNA. The reporter system can either be intercalating dyes or fluorescently labelled probes. Intercalating dyes bind to double stranded DNA and thus will detect all amplified DNA, whereas fluorescent probes will detect only those amplicons to which they can hybridize. The use of probes gives additional specificity to the qPCR compared to intercalating dyes, but the design of probes can be problematic. The qPCR reaction is also accomplished by thermocycling as with conventional PCR and as the amount of amplified DNA (and fluorescence) increases with every cycle, it is detected by CCD camera. The change in detected fluorescence over a cycle is then plotted as a graph. The point (C_T), where the detected fluorescence rises above a certain point (threshold) is used to quantify the sample (Heid *et al.* 1996).

qPCR is a powerful technique that has the specificity and sensitivity needed to be used as a detection tool with the added advantage of quantification. Thus it has been applied extensively to detect and measure the level of *Phytophthora* spp. in a sample. There have been various studies where qPCR was used to quantify and detect *Phytophthora* spp. from environmental sources, such as *P. medigacinis* (Vandemark and Barker 2003), *P. ramorum* (Hayden *et al.* 2004; Tomlinson *et al.* 2005; Hughes *et al.* 2006; Belbahri *et al.* 2007) and *P. cryptogea* (Minerdi *et al.* 2008). Most of the above-mentioned studies based their detection on the ITS region.

Multiplex qPCR is possible when using species-specific primer-probe combinations, each probe labelled with a different fluorescent dye. The degree of multiplexing that is possible in qPCR is dependent on the available fluorescent dyes with non-overlapping light emission ranges. Multiplex qPCR can simultaneously detect and quantify up to four different moieties. A multiplex qPCR for *P. ramorum, P. kernoviae, P. citricola* and *P. quercina* was designed based on the Ypt1 gene region (Schena and Cooke 2006). The Ypt1 gene region is more

variable than ITS, thus better suited for design of 4-fold multiplex qPCR species specific primer-probe sets. qPCR is an excellent technique for use on a heterogeneous DNA sample as it has the specificity to exclusively detect the desired DNA moiety. The disadvantages of multiplex qPCR are that the fluorescent labelled probes are expensive and that it cannot be used to detect unknown species from a heterogeneous/environmental DNA sample.

An additional technique that can be used in conjunction with standard qPCR on certain platforms, is high resolution melting (HRM) analysis. This technique subjects amplicons to a gradual increase in temperature in order to denature the double stranded (ds)DNA. The melting of dsDNA is detected by a decrease of fluorescence of intercalating dyes such as SYBR Green and LCGreen (Ririe *et al.* 1997; Wittwer *et al.* 2003). This decrease in signal is used to construct a melting curve for each sample and differences in melting curves are used to distinguish samples (Ririe *et al.* 1997). This technique has been extensively applied in single nucleotide polymorphism (SNP) genotyping and discovery (Liew *et al.* 2004; Reed and Wittwer 2004). HRM-qPCR has also been applied to identify bacterial species of medical importance (Odell *et al.* 2005; Skow *et al.* 2005; Cheng *et al.* 2006). HRM analysis has the advantage of being a cheap and rapid diagnostic tool (Vossen *et al.* 2009) but cannot be applied for the detection of multiple targets in a single reaction as one would find in environmentally derived samples.

4.7. DNA Hybridization based techniques

DNA hybridization techniques detect whether a specific DNA fragment is present in a sample by hybridization to a complementary, labelled oligonucleotide probe. Probes can be labelled either with radioactive isotopes or with fluorescent dyes. The basic technique where DNA is detected by a probe is called Southern blotting and consists of electrophoretic separation of the DNA samples, transfer and fixation of the DNA samples to nylon or nitrocellulose membranes and hybridization with labelled probes (Primrose and Twyman 2006). Detection by hybridization was a widely used technique prior to the advantages of automated PCR.

Hybridization techniques have been used in the past to detect and identify *Phytophthora* spp. These include *P. citrophthora* (Goodwin *et al.* 1990), *P. parasitica* (Goodwin *et al.* 1989), *P. capsici, P. cinnamomi, P. megakarya* and *P. palmivora* (Lee *et al.* 1993). Hybridization techniques can detect specific *Phytophthora* species from heterogeneous samples but due to their use of radioactive isotope labelling, the long and complicated procedure and low sensitivity, they have not been used widely.

DNA microarrays (Schena *et al.* 1995) are the modern equivalent for conventional

hybridization techniques. Microarrays use specialized glass slides on which hundreds of probes can be "printed". The fluorescently labelled sample DNA is then allowed to hybridize to the probes and the resultant signal is detected. Microarrays are used for high throughput detection of specific DNA fragments as well as their relative quantification. Microarrays have been developed to detect *Phytophthora* species (Anderson *et al.* 2006). Although microarrays represent a powerful tool able to detect a very large number of *Phytophthora* species simultaneously, their application is hampered by high cost and the inability to detect unknown species. Application of microarrays for the detection of known *Phytophthora* spp. from environmental samples is a viable and effective option, except for its inability to detect novel species.

4.8. Serological techniques

Serological techniques refer to the use of mammalian antibodies to detect specific proteins. Specific antibodies must first be made, which is a difficult and lengthy procedure involving the use of live animals. Once the antibodies are produced, they can be used to recognize and bind the protein of interest in a sample. Detection of this antibody-antigen complex is achieved by staining with specific dyes, which change colour when bound to antibodies (Hahn and Werres 1997). For example, a novel assay was developed that uses an antibody coated membrane to detect *Phytophthora cinnamomi* directly from environmental samples (Cahill and Hardham 1994).

An alternative detection method is to make use of antibody-enzyme conjugations in a technique known as enzyme-linked immunosorbent assay (ELISA) (Engvall and Perlmann 1972). Here the antigen (protein) is fixed to the wall of the reaction tube and the antibody is allowed to recognize and bind to the antigen. The unbound antibody is removed by washing so that only antibodies bound to the antigen remains. Reagents are then added and the conjugated enzyme catalyzes a reaction resulting in a colour change to the reaction solution. This change in colour is subsequently used to score the presence and concentration of the antigen present in the assay. Many studies have used ELISA to detect various *Phytophthora* spp. (Amouzou-Alladaye *et al.* 1988; Ali-Shtayeh and MacDonald 1991; Benson 1991; Gabor *et al.* 1993).

Serological techniques such as ELISA described above can detect *Phytophthora* spp. directly from environmental samples with a high sensitivity (Amouzou-Alladaye *et al.* 1988; Ali-Shtayeh and MacDonald 1991) as well as specificity (Gabor *et al.* 1993; Cahill and Hardham 1994). This makes these techniques very desirable, but it requires antibody production, which is a lengthy and complicated procedure. Serological techniques are also

unable to identify unknown *Phytophthora* spp. and these techniques cannot be conducted in multiplex.

4.9. PCR amplification with genus specific primers

The process of developing primer sets specific only to members of a single genus, can be difficult but if it is achieved, it can be an invaluable tool. This has been done for *Phytophthora* based on two gene regions, namely ITS (Drenth *et al.* 2006) and Ypt1 (Schena *et al.* 2008). These primers should be able to selectively amplify all *Phytophthora* spp. from environmental DNA samples, without amplifying from the DNA of other organisms. Used on their own, these primer sets provide the ability to assess the presence or absence of *Phytophthora* spp., but they lack the ability to give an identification to species level.

When using these primers in conjunction with a heterogeneous/environmental DNA sample, it is impossible to use direct sequencing, RFLP or SSCP and thus a technique able to detect the separate amplicon moieties is needed. qPCR can detect multiple species from such an sample but can only detect known species. Microarrays are able to detect a larger number of species but still suffer from inability to detect unknown species.

Cloning of the heterogeneous PCR reaction into a plasmid vector and transforming a suitable bacterial host with this will separate the different amplicon moieties. This will then allow conventional Sanger sequencing to be used to identify the different species. This has been done for *Phytophthora* based on DNA isolated from rhizosphere soil (Arcate *et al.* 2006) and has shown its ability to detect *Phytophthora* spp. that had not been detected by baiting. By combining genus specific primers with cloning and sequencing a high number of *Phytophthora* spp. including the unknown species can be detected from a heterogeneous DNA sample. The drawback of this approach is that it is relatively expensive and time consuming.

An alternative to cloning and sequencing is 454 pyrosequencing. Pyrosequencing is a second generation sequencing technology that can sequence millions of amplicons in parallel (Mardis 2008). Unlike the other second generation sequencing technology platforms the 454 platform can sequence much longer amplicons, which is desirable for species identification or phylogenetics. Pyrosequencing of heterogeneous amplicon samples has been used to study microbial diversity from various niches (Buee *et al.* 2009; Jumpponen and Jones 2009). Pyrosequencing is an effective and relatively easy, but expensive method for studying *Phytophthora* diversity from environmental samples.

5. Conclusions

Phytophthora is an important genus residing in the class Peronosporomycetes. The organisms represented by this class bear superficial similarity to fungi. Various morphological and physiological characters, as well as molecular data can be used to differentiate between fungi and Peronosporomycetes. The Peronosporomycetes form part of the Kingdom Chromista, which is also referred to as the Straminipila. However, there remain disagreements on which of the two names for this Kingdom should be used. Within the Peronosporomycetes, *Phytophthora* has an uncertain position. Previously *Phytophthora* was contained within Pythiales, but phylogenetic evidence supports its reclassification into the Peronosporales. Furthermore, there remains some confusion on the exact placement of *Phytophthora* in this order.

Phytophthora is a genus of hemibiotrophic and important plant pathogens that have had a serious impact on humans in many parts of the world. It has a very rapid lifecycle and makes use of sporangia for quick and effective infection of large numbers of plants. Additionally *Phytophthora* spp. produce resting spores, which allows them to survive extended periods in soil. *Phytophthora* spp. are the cause of many diseases of plants of agricultural and silvicultural importance. They have also been the cause of serious disease problems in natural ecosystems. All indications are that they are growing in importance and that they will cause greater numbers of disease problems in the future.

The preferred species concept applied to *Phytophthora* is the evolutionary species concept. The morphological and phylogenetic species recognition criteria are used to identify species units in *Phytophthora*. Morphological species recognition in *Phytophthora* is based on certain characteristics of the hyphae, sporangia, chlamydospores and sexual structures. Ample morphological data exists in the form of morphological descriptions and keys, which makes morphological comparisons relatively easy. The disadvantage of MSR is that the characters often cannot resolve closely related species. Phylogenetic species recognition in *Phytophthora* allows for more robust species delineation when based on two or more gene regions. By using both these species recognition criteria in conjunction with one another more robust species delineations will result.

Phylogenetics has also allowed the evolutionary relationships between the species of *Phytophthora* to be determined. Based on this approach, the species of *Phytophthora* have been grouped into ten clades. The phylogenetic structure of *Phytophthora* does not correspond to the morphological grouping system of Waterhouse (1963), but can be correlated to sporangial papillation, reproductive strategy and antheridial placement. These

phylogenies serve as a scaffold for use in species identification and delineation as well as aid in our understanding the evolutionary relevance of various characters.

Early detection of *Phytophthora* spp. are critical for disease prevention and mitigation. New *Phytophthora* spp. represent a considerable biosecurity risk as their potential impact on both cultivated crops and trees and native vegetation are undeterminable. Various methods are available for the detection of *Phytophthora* spp. Each of these techniques has specific strengths and shortcomings. The choice of which technique to employ for *Phytophthora* detection is affected by several factors including number of *Phytophthora* species, the presence of unknown *Phytophthora* spp., whether the *Phytophthora* sp. can be isolated in culture, the number of samples to screen as well as the financial resources available. As advances in molecular technologies are made the power, throughput and cost of techniques for molecular detection will decrease.

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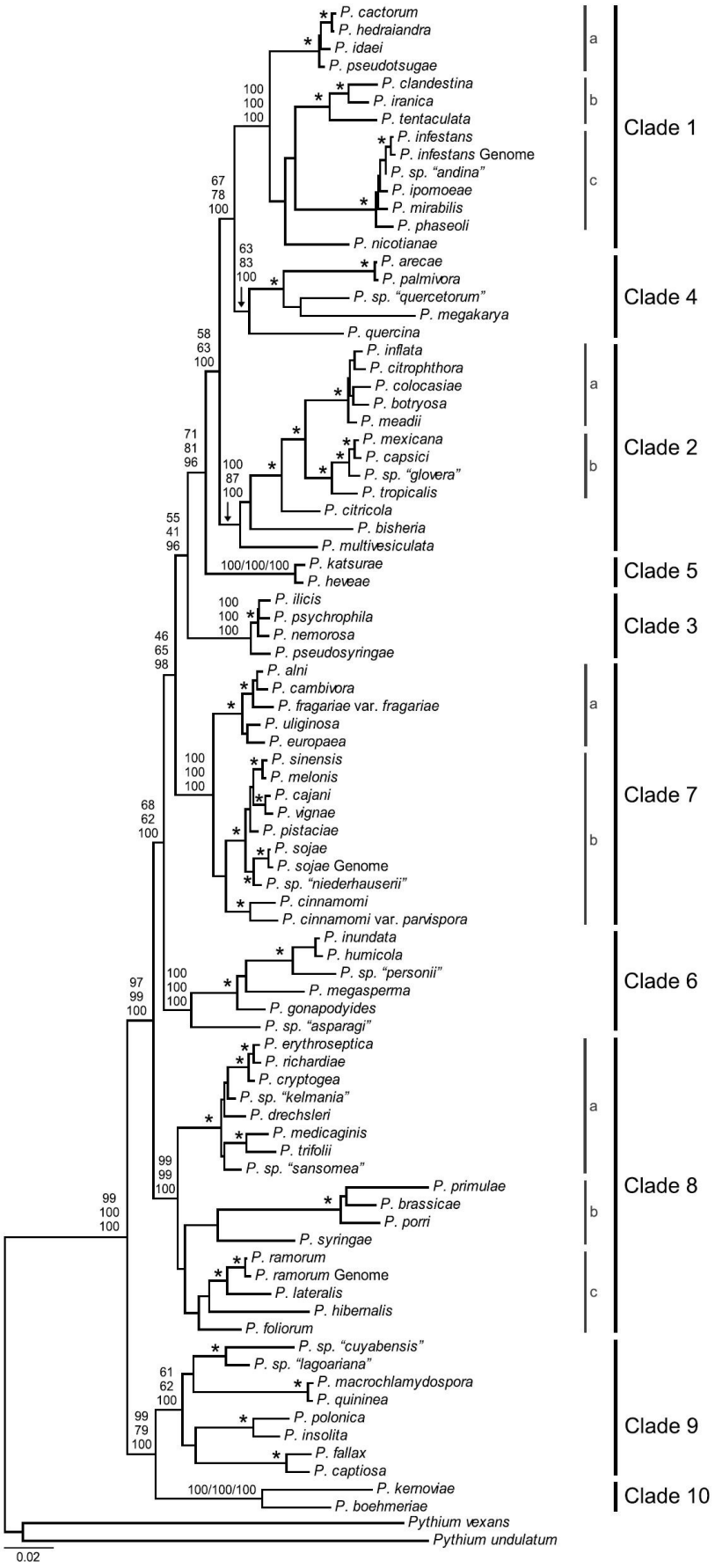
Table 1: Major differences between oomycete 'fungi' and true fungi (Judelson and Blanco 2005)

Feature	Oomycete	True fungi
Neighbouring taxonomic groups	Diatoms and golden-brown algae	Animals
Hyphal architecture	Aseptate and coenocytic tubular hyphae	Either single cell or septated hyphae, with one or more nuclei per compartment
Ploidy of vegetative hyphae	Diploid, except for transient haploid nuclei in gametangia	Typically haploid or dikaryotic; often with a stable or semi-stable diploid stage following mating
Typical size of genome	50–250 Mb	10–40 Mb
Major glucans in cell walls	Cellulose (β-1,4-linked glucose) and β-1,3, and β-1,6-linked glucose polymers	Usually chitin (β-1,4-linked N-acetylglucosamine) and/or chitosan (β-1,4-linked glucosamine), often with other β-1,3, and β-1,6 glucans
Pigmentation	Usually unpigmented	Very common in hyphae or spores, or secreted (for example, melanin, carotenoids and others)
Toxic secondary metabolites	None described	Common (typically aromatic, heterocyclic compounds)
Mating hormones	Non-peptide, probably lipid-like	Usually small peptides or lipopeptides
Predominant asexual spore	Undesiccated, unicellular sporangia (multinucleate cells)	Desiccated single or multicellular conidia (one nucleus per cell)
Motile asexual spores	Nearly universal, biflagellated zoospore	Uncommon, only in chytrids, which are monoflagellate
Sexual spores	Oospores, formed on the termini of specialized hyphae, each containing one viable zygotic nucleus	Various types, often formed in large numbers within complex enclosures (for example, perithecia, mushroom caps and others)
Mitochondria	With tubular cristae	With flattened cristae
Major energy reserves used by spores	Mycolaminarin and lipid, possibly polyphosphate	Glycogen and trehalose, also sugar alcohols and lipid

Table 2: Waterhouse's morphological grouping of *Phytophthora* spp.

	Waterhouse group					
Characters	**I**	**II**	**III**	**IV**	**V**	**VI**
Sporangial papilation	Papillate	Papillate	Semi-papillate	Semi-papillate	Non-papillate	Non-papillate
Sporangial exit pore size	Narrow	Narrow	Narrow	Narrow	Wide	Wide
Antheridial attachement	Paragynous	Amphigynous	Paragynous	Amphigynous	Paragynous	Amphigynous
Sporangial caducity	Caducous	Caducous	Caducous or non-caducous	Caducous	Non-caducous	Non-caducous
Internal sporangial proliferation	Absent	Absent	Absent	Absent	Present	Present

Figure 1. A genus-wide phylogeny for *Phytophthora* (Blair *et al.* 2008), indicating the ten clades and the species within each.


P. cactorum
P. hedraiandra
P. idaei
P. pseudotsugae
P. clandestina
P. iranica
P. tentaculata
P. infestans
P. infestans Genome
P. sp. "andina"
P. ipomoeae
P. mirabilis
P. phaseoli
P. nicotianae
P. arecae
P. palmivora
P. sp. "quercetorum"
P. megakarya
P. quercina
P. inflata
P. citrophthora
P. colocasiae
P. botryosa
P. meadii
P. mexicana
P. capsici
P. sp. "glovera"
P. tropicalis
P. citricola
P. bisheria
P. multivesiculata
P. katsurae
P. heveae
P. ilicis
P. psychrophila
P. nemorosa
P. pseudosyringae
P. alni
P. cambivora
P. fragariae var. fragariae
P. uliginosa
P. europaea
P. sinensis
P. melonis
P. cajani
P. vignae
P. pistaciae
P. sojae
P. sojae Genome
P. sp. "niederhauserii"
P. cinnamomi
P. cinnamomi var. parvispora
P. inundata
P. humicola
P. sp. "personii"
P. megasperma
P. gonapodyides
P. sp. "asparagi"
P. erythroseptica
P. richardiae
P. cryptogea
P. sp. "kelmania"
P. drechsleri
P. medicaginis
P. trifolii
P. sp. "sansomea"
P. primulae
P. brassicae
P. porri
P. syringae
P. ramorum
P. ramorum Genome
P. lateralis
P. hibernalis
P. foliorum
P. sp. "cuyabensis"
P. sp. "lagoariana"
P. macrochlamydospora
P. quininea
P. polonica
P. insolita
P. fallax
P. captiosa
P. kernoviae
P. boehmeriae
Pythium vexans
Pythium undulatum
0.02
a
b
c
Clade 1
Clade 4
Clade 2
Clade 5
Clade 3
Clade 7
Clade 6
Clade 8
Clade 9
Clade 10

Chapter 2

Characterization of *Phytophthora* hybrids from ITS clade 6 associated with riparian ecosystems in South Africa and Australia

This chapter represents a collaborative study with Dr TI Burgess and has been accepted Fungal Biology.

Abstract

Surveys of Australian and South African rivers revealed numerous isolates of *Phytophthora* spp. residing in Clade 6 of the genus, which had ITS gene regions that were either highly polymorphic or unsequenceable. These isolates were suspected to be hybrids. Three nuclear loci, the ITS region, two single copy loci (ASF and GPA) and one mitochondrial locus (*cox*I) were amplified and sequenced to validate this hypothesis. Abundant recombination within the ITS region was observed. This, combined with phylogenetic comparisons of the other three loci, confirmed the presence of four distinct hybrids involving the three known parental species *P. amnicola*, *P. thermophila* and *P.* taxon PgChlamydo. Two of these hybrids were found in South Africa and all four were found in Australia. In all cases, only a single *cox*I allele was obtained suggesting that hybrids arose from sexual recombination. The hybrid species were sterile in culture and had physiological traits similar to those of the maternal parents. Nothing is known regarding the host range or pathogenicity of these hybrids, but several isolates from Western Australia were obtained from the rhizosphere soil of dying plants. Regardless of the unknown pathogenicity of these hybrids, they should be regarded as potential threats. Their serendipitous and simultaneous discovery on two continents is intriguing yet their broad geographic distribution, frequent occurrence and their presence together with their parental strains in Australia strongly suggests an origin in this country.

1. Introduction

Riparian ecosystems are transitional zones between rivers and the surrounding landscape. These areas have been referred to as 'critical transitional zones' as they perform several important ecological functions, e.g. alleviation of flooding, sediment trapping and mediating nutrient and energy transfer between the aquatic and terrestrial zones (Ewel *et al.* 2001). Species of the Oomycetes genus *Phytophthora*, which represents a large group of plant pathogens, are uniquely adapted to exist in rivers as they produce motile zoospores (Judelson and Blanco 2005). Often, multiple *Phytophthora* spp. are isolated in surveys of waterways (Hwang *et al.* 2008; Hüberli *et al.* 2010; Reeser *et al.* 2011). Not surprisingly, several *Phytophthora* spp. are involved with riparian tree diseases, such as *P. lateralis* (clade 8) that causes Port-Orford-cedar (*Chamaecyparis lawsoniana*) decline (Hansen *et al.* 2000), *P. alni* (clade 7) causing alder (*Alnus* spp.) decline (Brasier *et al.* 2004) and *P. ramorum* (clade 8) causing sudden oak death on oak (*Quercus* spp.) and tanoak (*Lithocarpus densiflorus*) (Rizzo *et al.* 2002).

Phytophthora residing in ITS Clade 6 includes 24 taxa in three sub-clades, with several species not yet formally described (Crous *et al.* 2012; Kroon *et al.* 2012). These species occur abundantly in rivers and riparian ecosystems. The Clade 6 Phytophthoras are thought to be adapted to survival in rivers because they are able to rapidly colonize leaves and other plant debris (Brasier *et al.* 2003a; Jung *et al.* 2011). Additionally, these species typically have high temperature optima for growth and survival, which is hypothesized to be an adaptation to their aquatic lifestyle where the littoral zones of rivers and lakes can reach high temperatures (Jung *et al.* 2011). Initially, there were few taxa in Clade 6 but this has increased rapidly as *Phytophthora* spp. in riparian systems have received growing attention.

Many Clade 6 *Phytophthora* spp. cause serious diseases. In sub-clade 1, *P. inundata* is associated with disease on *Aesculus hippocastanum* and *Salix matsudana* in the United Kingdom and *Olea europaea* in Spain in riparian zones. (Brasier *et al.* 2003b). *P. asparagi*, the only species in sub-clade III, causes disease on *Asparagus officinalis* in Australia, Europe, New Zealand and USA (Förster and Coffey 1993; Cunnington *et al.* 2005; Saude *et al.* 2008), as well as basal root rot of plants in the family Agavaceae in Australia (Cunnington *et al.* 2005). However, it is the species residing in sub-clade II, with the exception of *P. pinifolia* (Durán *et al.* 2008) that have a very strong association with rivers and riparian ecosystems (Brasier *et al.* 2003a; Jung *et al.* 2011). Most taxa in this sub-clade are only weakly pathogenic, opportunistic pathogens or are of unknown pathogenicity (Brasier *et al.* 2003a; Jung *et al.* 2011). Others cause diseases on several hosts such as *P. gonapodyides* that commonly infects feeder roots of various woody plants in the UK, Europe and USA

(Brasier *et al.* 1993). *P. megasperma* frequently causes root and collar rots of of various agricultural and horticultural crops in temperate and subtropical regions of the world (Hansen *et al.* 1986; Brasier *et al.* 2003a). *P. pinifolia* is the causal agent of the serious 'Daño Foliar del Pino' disease on *Pinus radiata* in Chile (Durán *et al.* 2008), but it has not been found in aquatic ecosystems.

Clade 6 *Phytophthora* species include roughly equal numbers of homothallic and sterile taxa and only a single heterothallic species, *P. inundata* (Jung *et al.* 2011). This is in contrast to the *Phytophthora* spp. in other clades, where the majority are homothallic, about a quarter are heterothallic and the remaining species are sterile. However, it is hypothesized that the tendency towards homothallism and sterility seen in the Clade 6 *Phytophthora* spp. is an adaptation to their aquatic lifestyle (Brasier *et al.* 2003a; Jung *et al.* 2011). As probable saprotrophs, these *Phytophthora* spp. depend on their ability to rapidly colonize fresh plant material (such as fallen leaves) in order to outcompete other saprotrophic organisms (Jung *et al.* 2011). In this situation, the formation of oospores is not advantageous as these are resting structures that do not assist in the rapid and opportunistic colonization of plant material. The *Phytophthora* spp. in Clade 6 thus appear to have abandoned sexual reproduction in order to thrive in their aquatic niche.

Several important natural *Phytophthora* species hybrids have previously been reported. The best known example is *P. alni* and its variants (Brasier *et al.* 1995; Streito *et al.* 2002; Nagy *et al.* 2003). The parental species of this hybrid were initially thought to be *P. cambivora* and a *P. fragariae*-like species (Brasier *et al.* 1999), but it was later shown that three novel lineages are involved (Ioos *et al.* 2006). These 'alder Phytophthoras' are not the product of a single hybridization event because three distinct sub-species i.e. *P. alni* subsp. *alni (Paa), P. alni* subsp. *uniformis (Pau),* and *P. alni* subsp. *multiformis (Pam)* are found (Brasier *et al.* 2004). These three variants differ genetically in their chromosome number and the number of different alleles for selected single copy genes (Ioos *et al.* 2006). Other examples of hybrids include those commonly forming between *P. cactorum* and *P. nicotianae* and known as *P.* x *pelgrandis* in the Netherlands (Man in't Veld *et al.* 1998; Bonants *et al.* 2000), Germany (Nirenberg *et al.* 2009), Peru and Taiwan (Hurtado-Gonzales *et al.* 2009). Additionally, hybrids between *P. cactorum* and *P. hedraiandra*, described as *P.* x *serendipita,* were found in the Netherlands (Man in't Veld *et al.* 2007; Man in't Veld *et al.* 2012). Experimental hybridization between *P. capsici* and *P. nicotianae* produced offspring that had a wider host range than either parental species (Ersek *et al.* 1995), re-inforcing the view that hybridization can lead to novel or altered pathogenic capabilities.

Although the ITS region is the most frequently used locus for phylogenetic inference, it is not particularly well suited for studying interspecific hybrids. The rDNA, of which the ITS region is a part, exists in the genome as a tandem repeat array and hence it is impossible to distinguish between allelic variants (ITS variants occurring at the same locus but on different homologous chromosomes) and copy variants (ITS variants within the rDNA repeat array on a single chromosome) of the ITS region. It is for this reason that the ITS region cannot be used to differentiate between homoploid and allopolypoid hybrids. Single copy nuclear genes are much better suited to study the origins of hybrid species such as those occurring in *Phytophthora* as they are, like rDNA, also biparentally inherited, however unlike rDNA they are not under concerted evolution and can be used to identify hybrids (Ioos *et al.* 2006). Mitochondrial genes are also useful in studies on hybrids because their uniparental inheritance through the maternal line (Whittaker *et al.* 1994) can be used to determine which species acted as the maternal parent.

Numerous isolates from *Phytophthora* Clade 6 have been recovered from riparian ecosystems in South Africa and Australia. Due to the presence of multiple polymorphisms in the ITS sequence or, in many cases, the inability to obtain readable sequences for the ITS region, many of these isolates have been suspected to be hybrids. The aim of this study was to characterize these hybrid isolates using nuclear and mitochondrial molecular markers, as well as physiological and morphological traits. Furthermore, the reasons why the ITS sequences have been difficult to read and the feasibility to use these sequences to differentiate between hybrids was considered.

2. Materials and methods

2.1. Sampling and isolations

Phytophthora isolates were collected from a river in a single region of South Africa and from river systems and soil from several locations in Australia (Table 1). In rivers, samples were collected using mesh bags containing baits of (a) *Rhododendron indicum* leaves (South Africa) or (b) *Banksia attenuata*, *Pittosporum undulatum*, *Hakea* sp. and *Quercus robur* leaves and germinated seedlings of *Lupinus angustifolius* (Western Australia). Baits were collected after 10-14 days. Leaves were rinsed with distilled water, after which sections of the leaves and lupin seedlings containing lesions were excised. These sections were surface disinfested using 70% ethanol for ten seconds, rinsed in distilled water and plated onto NARPH agar (Hüberli *et al.* 2000). Hyphal tips where excised from colonies, after the NARPH plates had been incubated for three to five days in the dark at room temperature, and transferred to 10% V8 agar (V8A) (100mL Campbell's V8 juice, 3 g $CaCO_3$, 16 g agar, 900 mL distilled water) in Petri dishes.

Isolates from Tasmania and Victoria were obtained by filtration of 1 litre stream water through a 5 µm mixed cellulose filter (A500A047A, Advantec, Toyo Roshi Kaisha LtD, Japan). Filters were placed on NARPH plates and after 24 hours individual colonies were transferred onto new NARPH plates. Additionally two isolates from Western Australia, VHS5185 and VHS22715, were recovered from the rhizosphere soil of dying plants within natural vegetation by baiting with *Eucalyptus sieberi* cotyledons (Marks and Kassaby 1974). Regardless of the isolation technique, isolates were further sub-cultured to 2% water agar (WA), after which single hyphal tips were transferred to corn meal agar (CMA). South African isolates have been maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa. Australian isolates are maintained in the Murdoch University Culture Collection and the Vegetation Health Service Collection, Department of Environment and Conservation, Western Australia.

The isolates used in this study (Table 1) were collected together with other *Phytophthora* spp. During the course of the identification process using ITS sequencing, multiple isolates exhibited additivity (i.e. double chromatogram peaks) at several positions, as well as unusable sequence data after approximately 200 bases. This result suggested that these isolates could be hybrids and further experiments were conducted on them, as described below.

2.2. Analysis of polymorphisms in ITS sequence data

Phytophthora isolates were grown for two weeks on 10% V8A at room temperature. Mycelium was harvested by scraping the surface of cultures with a sterile scalpel blade and transferring it to 1.5 ml Eppendorf tubes. DNA was extracted using the protocol described by Möller *et al.* (1992) with slight modification: Samples were not lyophilized before DNA extraction but rather were frozen using liquid nitrogen after the addition of TES buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS) and Proteinase K. Furthermore, the samples were not treated with NH_4Ac but were directly precipitated with 450 µl isopropanol.

The polymerase chain reaction (PCR) was used to amplify the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA). PCR mixtures were set up so as to contain 1 x PCR reaction buffer (Roche Diagnostics, Mannhein, Germany), 2 mM $MgCl_2$ (Roche Diagnostics, Mannhein, Germany), 2.5 units of FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannhein, Germany), 200 µM of each dNTP, 0.45 µM of each primer, 2 µl template DNA (20-50 ng) and sterile water to a final volume of 25 µl. PCR amplification reactions were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City,

California, USA), using the following programme. The samples were subjected to an initial denaturation step at 95 ºC for 4 minutes followed by 35 cycles of denaturation at 95 ºC for 30 seconds, primer annealing at 55 ºC for 45 seconds and extension at 72 ºC for 60 seconds. This was followed by an additional extension step of 72 ºC for 4 minutes after which samples were held at 4 ºC. All DNA and PCR samples were electrophoretically analyzed on a 1.5 % agarose gel using Gel Red (Biotium, Hayward, California, USA) as fluorescent dye and were visualized under UV illumination.

Amplification products of the ITS region were cloned into a bacterial plasmid vector, pGEM®-T Easy Vector System (Promega, Madison, Wisconson, USA). Competent JM109 *E. coli* cells were transformed with recombinant plasmids and plated on LB/ampicillin/IPTG/X-Gal plates. Plates were incubated overnight at 37 ºC and recombinant transformant colonies were identified using blue/white screening. Colony PCR's were done on the white colonies, which were lifted from plates and transferred to PCR reaction mixtures. These were set up with the same reagent concentrations as mentioned previously, but without the addition of DNA and to a final volume of 50 µl. The plasmid T7 and SP6 primers (Table 2) were used for the amplification of the inserted DNA fragment using the same conditions as for the ITS loci.

PCR and sequencing reactions were purified either by sodium acetate and ethanol precipitation (Zeugin and Hartley 1985) or using sephadex, as described previously (Sakalidis *et al.* 2011). PCR amplicons were sequenced in both directions using the T7 and SP6 primers. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used and 1/16th reactions were set up to a final volume of 10 µl. Sequencing reactions were run on an ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, California, USA). For the ITS region, 10 amplicons were sequenced for each of the putative hybrids.

Preliminary data, from the sequenced ITS amplicons of the putative hybrids isolated, suggested that three parental species were involved in producing the hybrids. These included *P. amnicola*, *P. thermophila* and *P.* taxon PgChlamydo. The level of sequence variation within the ITS region for each of these putative parental species was established to validate the ITS consensus sequence for each of the three parental species. This was also accomplished in order to be able to accurately identify which ITS sequences from the hybrid isolates were recombinant. To this end, 50 amplicons of the type isolate of *P. thermophila* (CBS127954), 20 amplicons of the type isolate of *P. amnicola* (CBS131652), 10 amplicons of *P. amnicola* isolate VHS19503 and 20 amplicons of *P.* taxon PgChlamydo isolate

VHS6595 were cloned and sequenced. The number of cloned amplicons sequenced for each species was influenced by the level of variation seen e.g. *P. thermophila* had variation across more sites than that observed for *P.* taxon PgChlamydo. Sequences from each species were then separately aligned and intraspecific single nucleotide polymorphisms (SNPs) identified. These intraspecific polymorphisms were quantified by expressing their frequency of occurrence as a percentage of the total number of amplicons sequenced. A SNP was regarded as rare when it was present in less than 10 % of amplicons. The total number of rare SNPs was further quantified by expressing their frequency as a percentage of the total sequence length. The sites, in the consensus sequence of each species, where high frequency intraspecific SNPs occurred were noted and excluded from the interspecific SNP comparisons because they were not useful to distinguish between the parental species.

Interspecific SNPs from the consensus sequences of the parental species were used to assess the origin of the ITS sequences from the hybrid isolates. These were also used to identify whether any recombination took place within ITS copies. This was achieved by aligning the consensus ITS sequence data for *P. amnicola*, *P. thermophila* and *P.* taxon PgChlamydo and manually comparing the variable sites between these three species. Unique sites were identified for each of the three species and were then used as a template to compare the ITS sequences from the hybrid isolates. Sequences were considered recombinant when they contained SNPs unique to one of the known species at some sites and SNPs unique to another species at other sites.

2.3. Phylogenetic relationships of nuclear and mitochondrial genes

To elucidate the parentage of the hybrid isolates, two single copy nuclear and a mitochondrial gene regions were sequenced and were subjected to phylogenetic analyses. The anti-silencing factor (ASF)-like and G protein alpha subunit (GPA1) genes were chosen as the single copy nuclear genes and the cytochrome oxidase c subunit I (*cox*I) as the mitochondrial gene. These loci were amplified by PCR using the same DNA as that used for the ITS amplification. Primers used to amplify these loci are given in Table 2. The reaction mixtures were the same as those used for the ITS amplification. The GPA1 locus was amplified using the same thermocycling programme as that for the ITS region, whereas for amplification of the *cox*I locus the annealing temperature was changed to 65 ºC and the length of time for the cycled primer annealing and extension steps was increased to 60 seconds and 2 minutes, respectively. The programme used to amplify the ASF-like locus needed to be modified to incorporate a touchdown PCR (Don *et al.* 1991) cycle. This cycle followed directly after the initial denaturation step and consisted of 95 ºC for 30 seconds, the annealing temperature for 45 seconds and 72 ºC for 60 seconds. The annealing temperature

in the above cycle was initially 65 ºC, but was lowered by 0.2 ºC per cycle for 25 cycles, followed by 15 cycles of 60 ºC.

The ASF-like and GPA1 amplicons were cloned into a bacterial plasmid and used to transform competent bacterial cells. This was followed by the retrieval of the amplicons by colony PCR. The colony PCR products were sequenced in both directions using the T7 and SP6 primers. For ASF and GPA1, between six and ten amplicons were sequenced for each of the putative hybrid isolates and for the known taxa (Table 1). The *cox*I amplicons were sequenced using the same primers as those used in the PCR, as well as the FM50 and FM85 internal primers (Table 2) when needed. The cloning, transformation, colony PCR and sequencing were done as described above for the ITS sequencing. For ASF-like and GPA1, sequences of closely related *Phytophthora* species were obtained following the protocol described above, including the cloning step.

Sequence data were analyzed in CLC Main Workbench 6.0 (CLC Bio, Aarhus, Denmark) by combining forward and reverse sequences into contigs and manually verifying dubious sequence calls. Identities of the derived sequences were verified against data in GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST), prior to phylogenetic analyses. Additional sequences were retrieved from GenBank and aligned with the sequences generated in this study using MAFFT (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh *et al.* 2005).

Maximum parsimony (MP) analyses were performed using Phylogenetic Analysis Using Parsimony (PAUP*) ver 4.0b10 (Swofford 2002). The most parsimonious phylogenetic trees were generated through a heuristic search whereby the initial tree was generated randomly by 100 stepwise additions of taxa and subsequent trees were generated using the tree bisection reconnection branch swapping algorithm. All characters were unordered and of equal weight and gaps in the alignments were regarded as a fifth character. A thousand bootstrap replicates were performed to calculate branch and branch node support values (Felsenstein 1985).

Bayesian statistical inferences were used to generate phylogenetic trees and node support values through the Metropolis-coupled Monte Carlo Marcov Chain (MC^3) algorithm. In order to determine the optimal evolutionary model, each locus was subjected to hierarchical Likelihood ratio tests (hLRT) using MrModeltest2.2 (Nylander 2004). Bayesian analyses were done using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) and each analysis was run

for 5 000 000 generations. Tracer 1.4 (Rambaut and Drummond 2003) was used to determine burn-in values prior to parameter and tree summarization.

2.4. Colony morphology and growth rates

In order to compare the hybrid groups with the three reference species, as well as with each other, their colony morphology and temperature-growth relationships were determined. Colony growth pattern and growth rates were determined for all hybrid isolates, as well as for *P.* taxon PgChlamydo (VHS6595, VHS3753, MUCC766), *P. amnicola* (VHS19503 and CBS131652) and *P. thermophila* (VHS7474, CBS127954, VHS3655 and VHS16164) (Table 1). Colony growth patterns were described from 7-day-old cultures grown at 20 ºC in the dark on V8A, half-strength potato dextrose agar (PDA) (19.5 g PDA, 7.5 g agar and 1 L distilled water) and carrot agar (CA) (0.1 L filtered carrot juice, 17 g agar and 1 L distilled water).

Growth rates were determined on V8A. Mycelial plugs (5 mm in diameter) cut from actively growing cultures were transferred to the centers of 90 mm V8A plates and incubated at 20 ºC for 24 hours in the dark. The growth that occurred during the 24 hour incubation was noted on each plate following to the method described by (Hall 1993). Plates were then transferred to incubators set at 15 ºC, 20 ºC, 25 ºC, 30 ºC, 32.5 ºC, 35 ºC, 37.5 ºC. Three replicate plates were used for each isolate at each temperature. After 5-7 days, the radial growth of each culture was measured along two perpendicular axes and the mean radial growth rates (mm per day) were calculated. The mean radial growth rates per day were plotted against each temperature and standard errors for each group were calculated and included in the graph as error bars. Plates, incubated at a high temperature and where no growth was observed, were moved to a 20 ºC incubator in order to establish their viability.

2.5. Morphology of sporangia and gametangia

Besides colony characteristics, dimensions of selected morphological characters were measured to further compare the hybrid groups with the three reference species. Isolates used to determine morphological characters (Table 1) were also compared to the characters determined in previous studies for *P. thermophila* (Jung *et al.* 2011) and *P. amnicola* (Crous *et al.* 2012). Sporangia and hyphal swellings produced on V8A were measured using the methods described in Jung *et al.* (1999). Sporangia were produced by flooding 15 x 15 mm V8A agar pieces, taken from the growing margins of 7-day-old colonies, so that their surfaces were covered with distilled water in 90 mm Petri dishes. These were incubated at room temperature around 22 ºC in natural daylight. The water was decanted and replaced after 2 and 8 hours. Two millilitres of diluted non-sterile soil extract was added to the

replaced water at 8 hours. The soil extract was made from 20 g of rhizosphere soil from beneath a planted *Quercus* sp. suspended in 200 mL distilled water, incubated for 24 hours at 20 ℃, filtered through cheesecloth and refiltered through Whatman no. 1 paper.

After 15-24 hours, dimensions and characteristic features of 50 mature sporangia and 25 exit pores and zoospore cysts were chosen at random per isolate, and measured. Likewise, after 3-7 days, 25 hyphal swellings were also measured. All measurements were made at x400 magnification (BX51, Olympus). In order to stimulate the formation of gametangia, isolates were paired with *P. cinnamomi* tester strains of the A1 (CMW 29606, CMW 29607) and A2 (CMW 29597, CMW 29598) mating type. Paired cultures were incubated at 20 ºC in the dark for 2-4 weeks. Cultures were monitored throughout this period for the presence of sexual structures.

3. Results

3.1. Analysis of polymorphisms in ITS sequence data

The alignment of the consensus sequences of the three reference *Phytophthora* species was 823 bp in length and for consistency, position numbers given in all species and hybrids are based on this alignment (Table 3).

3.1.1. Intraspecific polymorphisms of P. amnicola, P. thermophila *and* P. *taxon PgChlamydo*

Phytophthora amnicola isolates (VHS19503 and CBS131652) had two prominent ITS copy types, one which was 820 bp and another which was 823 bp. This size difference was due to a 3 bp insertion/deletion (indel) at sites 750-752 in the alignment. This indel occurred in a ratio of approximately 50:50 among 30 cloned fragments (Table S1). There were three high frequency SNPs within these two isolates of *P. amnicola*; a T-C transition at site 756, a T-G transversion at site 757 and a G-T/C transversion at site 788 (Table S1). The transition and transversion at sites 756 and 757 were linked to the indel at sites 750-752 and thus occurred at the same frequency, whereas the transversion at site 788 occurred in 60% of ITS copies. Additionally, rare SNPs occurred at a frequency of approximately 0.08% (Table S1).

Phytophthora thermophila produced ITS sequences that were 819 bp in length. Among 50 cloned amplicons of the *P. thermophila* type isolate (CBS127954) there were 3 high frequency SNPs (Table S2); a T-A transversion at position 464 and C-T transitions at positions 513 and 573. Each of these variable sites appeared in approximately 30 % of ITS copies and were linked. Additionally, rare SNPs occurred at a frequency of approximately 0.09 % (Table S2).

Phytophthora taxon PgChlamydo also produced ITS sequences of 819 bp. Two high frequency SNPs were present within the 20 cloned amplicons. The ITS sequence of the *P.* taxon PgChlamydo isolate VHS6595 contained C-T transitions at positions 172 and 668 and occurring with a frequency of 45 % and 25 %, respectively (Table S3). These two high frequency SNPs were not linked. There were also very few rare SNPs (frequency <0.02 %).

3.1.2. Interspecific polymorphisms and comparisons of hybrid isolates

Interspecific variation in the form of SNPs and indels were seen in the alignment between the consensus sequences of the three reference *Phytophthora* species. However, sites containing intraspecific variation, as identified above, were excluded (Table 3). Interestingly, the interspecific SNPs were not at the same positions as the intraspecific SNPs. The intraspecific 3 bp indel within sequences of *P. amnicola* was included in Table 3 to demonstrate its occurrence within hybrid isolates, even though it was not informative for distinguishing between species. All other sites with interspecific variation were fixed within each species. In total this alignment included 19 SNPs and one indel (Table 3). This indel occurred at position 171 and consisted of a single thymine insertion within all sequences from *P. amnicola* that was not present in *P. thermophila* or *P.* taxon PgChlamydo. The consensus sequences for *P. amnicola* differed from that of *P.* taxon PgChlamydo by 15 SNPs and the one indel. There were 10 SNPs and one indel differentiating *P. amnicola* and *P. thermophila* and 13 SNPs differentiating *P. thermophila* and *P.* taxon PgChlamydo.

The ITS sequences obtained from hybrid isolates were added to the above alignment between the three reference species (Table 3). The identified interspecific SNPs and indels were then used to establish the similarity of sequences from the hybrid isolates with the consensus sequences of the reference species. They were also used to identify any sequences from hybrid isolates of ITS copies where recombination took place between parental ITS types. Some sequences from hybrid isolates were identical to the consensus sequences of the reference species. Other sequences appeared to be composites between the consensus sequences of the three reference species. Such sequences appeared to be the result of recombination, because portions of a single sequence matched to two different reference sequences. Each hybrid isolate had sequences either identical to or derived from the consensus sequences of two of the three reference species. No hybrid isolate possessed sequences originating from all three reference species simultaneously. From the above comparison, three hybrid groups could be identified, namely those with ITS sequences originating from *P. amnicola* and *P.* taxon PgChlamydo (CMW37727, CMW37728, CMW37729, CMW37730, MUCC774, MUCC777, MUCC778 and MUCC779),

P. amnicola and *P. thermophila* (CMW37731, CMW37732, CMW37733, CMW37734, VHS22715, VHS5185, MUCC780, MUCC781 and MUCC782) and *P.* taxon PgChlamydo and *P. thermophila* (MUCC783 and MUCC784).

Different proportions of recombinant sequences were observed between the three groups of hybrids. Those isolates with *P.* taxon PgChlamydo and *P. thermophila* ITS types (MUCC783 and MUCC784) did not yield any recombinant sequences, compared to the isolates of the other two hybrid groups that did so abundantly. Additionally, considerable variation was observed in the proportion of recombinant sequences obtained between isolates of the same hybrid group. Within the group of hybrids with *P. amnicola* and *P.* taxon PgChlamydo ITS types, no recombinant sequences were obtained from CMW37728, but many were obtained from MUCC774 (Table 3). Likewise, in the hybrid group with *P. amnicola* and *P. thermophila* ITS types, no recombinant sequences were obtained for CMW37731, but many were obtained for CMW37733 and MUCC782.

3.2. Phylogenetic relationships of nuclear and mitochondrial genes

The ASF-like sequence alignment was 328 characters in length and of these 27 were parsimony informative. Five most parsimonious trees of 41 steps were obtained (CI = 0.85, RI = 0.97, RC = 0.83) and there were only small differences in the terminal branches. All species formed clades well supported by bootstrap values (Figure 1, TreeBASE S12996). The generalized time reversible (Tavaré 1986) nucleotide substitution model with gamma distributed among-site variation (GTR+G) was applied during Bayesian inference. Posterior probabilities supported the same nodes as those observed with the MP analyses.

The GPA1 sequence alignment was 306 characters in length with 71 parsimony informative characters. Four most parsimonious trees were generated of 141 steps (CI = 0.79, RI = 0.91, RC = 0.71) and there were only small differences in the terminal branches. All species resided in well supported clades (Figure 2, TreeBASE S12996). Bayesian inference was done using a GTR+G nucleotide substitution model and the resulting posterior probabilities supported the same node as the bootstrap values.

The *cox*I sequence alignment had a length of 1149 characters of which 141 were parsimony informative and resulted in 162 most parsimonious trees of 297 steps (CI = 0.63, RI =0 89, RC = 0.56.) All species resided in clades with high bootstrap support (Figure 3, TreeBASE S12996). Bayesian inference was run using the generalized time reversible nucleotide substitution model with gamma distributed among-site variation and a proportion of

invariable sites (GTR+I+G). The resulting posterior probabilities supported the bootstrap values.

In the ASF-like phylogeny (Figure 1) the three reference species, *Phytophthora amnicola*, *P. thermophila* and *P.* taxon PgChlamydo each resided in a well supported clade. Each isolate of these three species had a single ASF-like allele. The hybrid isolates, however, each possessed two different alleles for the ASF-like locus, each corresponding to the allele of one of the three reference species. Based on this analysis, the hybrid isolates could be divided into three groups: those with ASF-like alleles grouping with both *P. amnicola* and *P.* taxon PgChlamydo (CMW37727, CMW37728, CMW37729, CMW37730, MUCC774, MUCC777, MUCC778 and MUCC779), with *P. amnicola* and *P. thermophila* (CMW37731, CMW37732, CMW37733, CMW37734, VHS22715, VHS5185, MUCC780, MUCC781 and MUCC782) and those with *P.* taxon PgChlamydo and *P. thermophila* (MUCC783 and MUCC784). The ASF-like phylogeny thus confirmed the hybrid nature of the isolates in question, because each hybrid isolate had two ASF-like alleles originating from two different parental species. Furthermore, the hybrid groups identified using the ASF-like phylogeny supported the same hybrid groups comprised of the same isolates, as those identified using analyses of the ITS region. However, unlike in the ITS sequences, no recombination was observed between alleles of the ASF-like locus of the hybrid isolates.

Phytophthora thermophila and *P.* taxon PgChlamydo formed well supported clades in the GPA1 phylogeny (Figure 2). However, the GPA1 locus failed to be amplified for *P. amnicola* and this species is, therefore, not included in the phylogeny. Each isolate of *P. thermophila* and *P.* taxon PgChlamydo had a single GPA1 allele. The isolates that were identified by the ITS polymorphism analysis and ASF-like phylogeny to be hybrids of *P. thermophila* and *P.* taxon PgChlamydo (MUCC783 and MUCC784), both had two GPA1 alleles. Of these two alleles, one grouped with the GPA1 alleles from *P. thermophila* and the other with that of *P.* taxon PgChlamydo. The isolates previously identified as hybrids between *P. amnicola* and *P. thermophila* (CMW37731, CMW37732, CMW37733, CMW37734, VHS22715, VHS5185, MUCC780, MUCC781 and MUCC782) had only a single GPA1 allele grouping with that of *P. thermophila*. The isolates previously identified as hybrids between *P. amnicola* and *P.* taxon PgChlamydo (CMW37727, CMW37729, CMW37730, MUCC774, MUCC777, MUCC778 and MUCC779) also had a single GPA1 allele grouping with that of *P.* taxon PgChlamydo. One exception was isolate MUCC777, which had one GPA1 allele grouping with *P.* taxon PgChlamydo and another unidentified allele residing in the phylogeny as a sister clade to *P. fluvialis*. This unidentified allele might represent the *P. amnicola* lineage, but without sequence data from the type or other isolates of *P. amnicola* this cannot be verified. The

GPA1 locus could not be amplified for isolate CMW37728. Similar to the ASF-like sequence data, no recombination was observed within the GPA1 sequences obtained from hybrid isolates.

In the *cox*I phylogeny (Figure 3), the three reference species, *Phytophthora amnicola*, *P. thermophila* and *P.* taxon PgChlamydo, each resided in a well supported clade and isolates of each species had a single *cox*I allele. The hybrid isolates all had a single *cox*I allele corresponding to one of the three reference species. Isolates identified by the ITS polymorphism analysis and ASF phylogeny as hybrids between *P. amnicola* and *P. thermophila* (CMW37731, CMW37732, CMW37733, CMW37734, VHS22715, VHS5185, MUCC780, MUCC781 and MUCC782) and *P. thermophila* and *P.* taxon PgChlamydo (MUCC783 and MUCC784), all had a *cox*I allele grouping with those from *P. thermophila*. One subset of the isolates (CMW37727, CMW37728, CMW37729, CMW37730 and MUCC774) identified by the ITS polymorphism analysis and ASF phylogeny as hybrids between *P. amnicola* and *P.* taxon PgChlamydo had a *cox*I allele grouping with those from *P. amnicola*, while another subset (MUCC777, MUCC778 and MUCC779) had a *cox*I allele that grouped with that from *P. taxon* PgChlamydo. The *cox*I phylogeny could not identify any isolates as hybrids, but it did indicate that the *cox*I locus and by extension the mitochondrial genome was inherited uniparentally. Furthermore, the maternal parent for each hybrid isolate could be established using the coxI phylogeny.

Four hybrid groups were identified from the phylogenetic analyses of ASF-like, GPA1 and *cox*I and from the polymorphism comparison of the ITS region. These hybrid taxa were represented by hybrid formulae and since the identity of the maternal parent could be established, the recommendation of the International Code of Botanical Nomenclature (ICBN) article H.2A.1 can be followed, where the name of the maternal parent precedes that of the male. The names of these hybrids are as follows: *P. amnicola* × *P.* taxon PgChlamydo (A-PG, represented by CMW37727, CMW37728, CMW37729, CMW37730, and MUCC774), *P.* taxon PgChlamydo × *P. amnicola* (PG-A, represented by MUCC777, MUCC778 and MUCC779), *P. thermophila* × *P. amnicola* (T-A, represented by CMW37731, CMW37732, CMW37733, CMW37734, VHS22715, VHS5185, MUCC780, MUCC781 and MUCC782) and *P. thermophila* × *P.* taxon PgChlamydo (T-PG, represented by MUCC783 and MUCC784).

3.3. Colony morphology and growth rates

On V8A, all isolates and their parental species produced little to no aerial mycelia but differences in growth patterns could be observed between the reference species and hybrid groups (Figure 4). Isolates of *P.* taxon PgChlamydo grew uniformly without any distinct

pattern, those of *P. amnicola* were densely petaloid to stellate and *P. thermophila* produced faintly petaloid colonies. Overall, the hybrids produced colonies similar to those of their maternal parents. Thus, PG-A hybrids produced uniform colonies with no pattern, A-PG colonies were petaloid to stellate, T-PG colonies were faintly petaloid and T-A colonies were petaloid to stellate.

On PDA, isolates of all reference species and hybrids were slow growing and produced dense, cottony colonies. No discernible differences between the reference species and the different hybrid groups were noted. However, on CA, all isolates produced aerial mycelium with distinct patterns (Figure 5). Isolates of *P.* taxon PgChlamydo produced colonies with a rosaceous growth forms, *P. amnicola* isolates were fast growing and produced a dense "chrysanthemum" pattern and *P. thermophila* isolates were slow growing and produced stellate colonies. As with V8A, hybrid isolates had similar patterns to the maternal parents. However, T-PG hybrids were faster growing than *P. thermophila*, but had similar growth patterns. Most isolates of T-A had identical growth patterns to *P. thermophila*, but with the one exception that isolate MUCC780 produced fast growing colonies with a chrysanthemum pattern similar to that seen in *P. amnicola.*

The average daily growth rates of reference and hybrid isolates were plotted against temperature (Figure 6). *P. amnicola* was represented by two isolates (VHS19503 and CBS131652), *P. thermophila* by four isolates (VHS7474, CBS127954, VHS3655 and VHS16164), *P.* taxon PgChlamydo by one isolate (MUCC766), the PG-A hybrid by three isolates (MUCC777, MUCC778 and MUCC779), the A-PG hybrid by five isolates (CMW37727, CMW37728 CMW37729, CMW37730 and MUCC774), the T-A hybrid by eight isolates (CMW37731, CMW37732, CMW37733, CMW37734, MUCC780, MUCC782, VHS22715 and VHS5185) and the T-PG hybrid by two isolates (MUCC783 and MUCC784). Overall, standard errors were low, except for isolates representing PG-A, indicating high variability amongst isolates. Both isolates of A-PG and PG-A had a temperature-growth relationship similar to that of the isolate representing *P.* taxon PgChlamydo with a broad optimum from 20-32.5 ºC. Isolates of PG-A were able to maintain viability up to 35 ºC, whereas A-PG isolates were more variable and the maximum temperature at which they could survive was 35 ºC for some isolates and 37.5 ºC for others. Isolates of T-A had a profile similar to that of *P. thermophila* with a clear optimal temperature. However, while the optimum for *P. thermophila* was 32.5 ºC, the optimum for T-A isolates was 30 ºC. Isolates of T-PG had a faster growth rate than either of its parental species and a temperature-growth response profile intermediate between the two parental species.

3.4. Morphology of sporangia and gametangia

Isolates of all four hybrid species produced non-papillate sporangia similar to those of *P. amnicola*, *P. thermophila* and *P.* taxon PgChlamydo (Table 4). The sporangial sizes of these hybrids were intermediate between those of the reference species, except for isolates of A-PG that produced smaller sporangia than those of either of its parent species. Ovoid sporangia were most common across sporangia produced for all the reference species and hybrids. This was with the exception of isolates of T-PG that produced ovoid, limoniform and obpyriform sporangia in roughly equal proportions. Isolates of PG-A produced hyphal swellings intermediate in size between those of its two parental species, although they sometimes formed branched hyphal swellings, which have not been observed in either of its parent species, *P. amnicola* or *P.* taxon PgChlamydo. None of the four hybrid species produced oospores or sexual structures in pure culture or when paired with *P. cinnamomi* tester isolates of either mating type.

4. Discussion

Phytophthora isolates collected from water and rhizosphere soil in Australia and South Africa, with highly polymorphic or unsequenceable ITS regions, were shown to represent four distinct interspecific hybrids between *P. amnicola*, *P. thermophila* and *P.* taxon PgChlamydo. Analysis of interspecific polymorphic sites within the ITS region demonstrated the hybrid nature of these isolates and also showed that recombination has occurred within the ITS region. Phylogenetic analysis of the ASF-like single copy nuclear loci (ASF-like and GPA1) demonstrated the biparental inheritance of alleles and phylogenetic analysis of a mitochondrial locus (*cox*I) enabled further differentiation of the hybrid isolates based on maternal species. In general, the colony morphology of the hybrids was consistent with that of the maternal parent, although substantial variation was observed in the growth patterns of some hybrid groups. For temperature-growth relationships and morphology, hybrids exhibited intermediate characteristics between those of their two respective parental species. All the hybrids and parental species were sexually sterile in mating tests conducted in culture.

Of the four loci considered in this study, the combination of the ASF-like and *cox*I loci were the most effective for distinguishing between the four hybrid groups. The ASF-like locus could be used effectively to identify both parental lineages without complication and the *cox*I locus was useful to identify the maternal species. Similar to the ASF-like locus, it was possible to identify both parental lineages using the ITS region, but the large proportion of recombinant sequences reduced the efficacy of using the ITS region for this purpose. Additionally, the presence of indels between the *P. amnicola* ITS type and the *P. thermophila*

and *P.* taxon PgChlamydo ITS types negated the possibility of using conventional sequencing of hybrids containing the *P. amnicola* ITS type. The GPA1 locus had limited application in identifying A-PG, GP-A and T-A due to the fact that the *P. amnicola* allele could not be amplified. However, it could successfully identify both parental lineages from T-PG. The ASF-like and GPA1 loci have both been used previously without complication to characterize the *P. alni* hybrids (Ioos *et al.* 2006).

The absence of the GPA1 allele for *P. amnicola* in these hybrids could indicate backcrossing with an A-PG or a PG-A hybrid with *P.* taxon PgChlamydo or of a T-A hybrid with *P. thermophila* to form hybrids homozygous for GPA1. This is, however, improbable because it is unlikely that the same allele would be eliminated in three separate events. The one allele amplified from isolate MUCC777 could represent *P. amnicola*, but it is not possible to verify this fact without reference sequences for *P. amnicola*. The absence of a *P. amnicola* allele for the GPA1 locus could also be due to a mutation in one or both primer binding sites, which would prevent primer annealing during the PCR amplification procedure. The fact that no GPA1 allele could be amplified from the A-PG hybrid isolate CMW37728, suggests that no *P.* taxon PgChlamydo allele was present. This might represent a single instance of a backcross with *P. amnicola* or a cross of a hybrid with such an allele with the effect that GPA locus becomes homozygous for the *P. amnicola* allele in the resultant progeny. Non-inheritance of alleles, due to meiotic nondisjunction, have been reported for *P. cinnamomi* (Dobrowolski *et al.* 2002) and *P. nicotianae* (Forster and Coffey 1990) and this phenomenon may explain the above observations. Alternatively this may be explained by gene conversion disparity where one allele is always lost (Chamnanpunt *et al.* 2001; Vercauteren *et al.* 2011).

The presence of two parental ITS types and ASF-like alleles per hybrid isolate suggests that each of these hybrids were formed by separate single hybridization events. The GPA1 locus also supports this view in the case of T-PG, where two different alleles were obtained. The mitochondrial genome was inherited uniparentally as each hybrid isolate possessed a single *cox*I allele. This pattern of biparental nuclear inheritance and uniparental mitochondrial inheritance suggests that these hybrids are the result of sexual hybridization. An alternative hypothesis is that these hybrids have a somatic origin with subsequent segregation of mitochondria to a homoplastic state. Interspecific somatic hybridization would combine two diploid genomes resulting in an allopolyploid hybrid that is also heteroplasmic for the mitochondrial genome. Heteroplasmy is quickly reduced to homoplasmy through the random segregation of mitochondrial genomes (Chen and Butow 2005). Little is known, however, about the occurrence and mechanism of parasexual processes like somatic hybridization in *Phytophthora*. It has been shown previously in *P.* x *pelgrandis* and *P.* x *serendipita* that

arose from sexual hybridization because both these hybrids exhibited biparental inheritance of nuclear genes and uniparental inheritance of mitochondrial genes (Man in't Veld *et al.* 1998; Bonants *et al.* 2000; Man in't Veld *et al.* 2007; Hurtado-Gonzales *et al.* 2009). In contrast with this relatively simple situation, the *Paa* hybrid does not exhibit an obvious biparental inheritance pattern of nuclear genes as it possesses three alleles for nuclear loci. However, when *Pam* and *Pau* are regarded, which have two and one allele per nuclear locus respectively, it is evident that these two subspecies hybridized to form *Paa* (Ioos *et al.* 2006).

As with many of the other clade 6 taxa (Brasier *et al.* 2003a; Jung *et al.* 2011), the four hybrids found in this study are sexually sterile in culture and reproduce asexually via sporangia and the release of zoospores. All three parental species are known to be self-sterile (Jung *et al.* 2011; Crous *et al.* 2012). However, it has been shown that a single isolate of *P. thermophila* produced oospores when stimulated with non-sterile soil filtrate (Jung *et al.* 2011). It is, therefore, possible that the conditions used during general laboratory mating tests are not conducive to mating and oospore formation in this clade, but that the ideal conditions for sexual recombination could exist in nature. This could account for the sexual formation of these hybrid species.

Interspecific hybrids are often sterile due to chromosomal, genic or epistatic effects (Rieseberg 2001; Michalak 2008). This was observed in *Paa* as frequent chromosome pairing failures prevented the completion of meiosis (Brasier *et al.* 1999). However, if this is not the case with the hybrids found in the present study, they may only require the correct environmental stimuli to reproduce sexually with the potential for backcrossing with other hybrids (i.e. a hybrid swarm) or introgression with parental species.

In the situation reported here, three separate cases (i.e. in A-PG, PG-A and T-A, but not T-PG) were found where two divergent ITS lineages have recombined. The observed sterility of these hybrids under laboratory conditions precludes the occurrence of meiotic recombination. It can then be assumed that the observed recombination was a result of mitotic events, most notably gene conversion (Andersen and Sekelsky 2010). If, however, these hybrids are capable of sexual reproduction in nature, both meiotic and mitotic recombination will occur. Although recombination created significant variation between the rDNA subunits, the non-recombined parental-type subunits remained. This was also shown with *Paa* (Brasier *et al.* 1999), which also possesses considerable variation in the combinations of polymorphic bases of the ITS region, indicative of chromosomal crossover. Conversely, no evidence for recombination in the ITS region is present in *P.* x *pelgrandis* (Hurtado-Gonzales *et al.* 2009) or *P.* x *serendipita* (Man in't Veld *et al.* 2007).

Both intraspecific and interspecific variation contributed to the heterogeneity of the ITS region of the hybrid isolates, while only intraspecific SNPs contributed to the heterogeneity of the ITS regions of *P. thermophila*, *P. amnicola* and *P.* taxon PgChlamydo. The interspecific SNPs are indicative of the evolutionary divergence between these three species. All three parental species possessed intraspecific SNPs within the ITS region, although the type strains of *P. thermophila* and *P. amnicola* had a higher proportion of SNPs than *P.* taxon PgChlamydo. Intraspecific SNPs in the ITS region are usually generated through point mutations within a single rDNA subunit, that are either lost or fixed due to the homogenizing effect of concerted evolution of the ITS region. It has been noted that in cases where sexual reproduction (and by extension meiotic recombination) is absent, high levels of intra-individual rDNA sequence heterogeneity exists (Sang *et al.* 1995; Campbell *et al.* 1997). This high level of sequence heterogeneity indicates slower rates of concerted evolution. Given the higher levels of ITS heterogeneity caused by the interspecific hybridization and the hypothesized reduced rate of homogenization due to sterility, it can be expected that the hybrids found in this study may never attain a level of homogeneity comparable to that of non-hybrid species.

The level of recombinant sequences observed in the hybrid isolates reported in this study was not identical. For example, T-PG had undergone no recombination, while the other three hybrids did. Furthermore, within A-PG and T-A the absence of recombination in some isolates (CMW37728 and CMW37731) indicates that even within a hybrid group, all isolates are not identical. This suggests that the hybrids encountered in this study are a result of multiple hybridization events and that these events, although rare, are part of an ongoing process. The very high level of similarity of the ASF-like, GPA1 and *cox*I alleles of the hybrid isolates with those of the parent species suggests that little time for divergence has passed, and we can thus assume that the hybrids are relatively new.

The colony growth pattern and rate, as well as sporangial morphology of the hybrid isolates are indicative of their relation to their respective parental species. The resemblance between colony growth patterns of hybrid isolates and their maternal parent species suggests that this trait may be governed, at least in part, by a mitochondrial locus. Colony growth rate and sporangial morphology might be more affected by nuclear loci as hybrids exhibited intermediate growth rates and sporangial measurements compared to that of their parental species. The faster growth rate observed for T-PG isolates, when compared to either parent species, might be due to synergistic interaction between alleles and loci from the two parental genomes.

All four of the hybrids considered in this study were found in Australia, whereas only two (A-PG and T-A) were detected from South Africa. The geographic origin of these four Clade 6 hybrids is unknown. Two of the parental species (*P. amnicola* and *P. thermophila*) are known only from Australia (Jung *et al.* 2011; Crous *et al.* 2012). *Phytophthora* taxon PgChlamydo occurs in Australia (Stukely 2012), Argentina, Europe, USA (Brasier *et al.* 2003a; Hansen *et al.* 2007) and South Africa (Chapter 3 of this thesis) and probably has a global distribution (Hansen *et al.* 2007). Given the shared distribution of the hybrids and parental species in Australia, it is most likely that they originated in that country and that some subsequently spread to South Africa. The alternative hypothesis would be that all the parental species are also present in South Africa, but that they have yet to be detected, and that hybridization has occurred separately on both continents.

Our observations that multiple hybridization events occurred and continue to occur in nature have important implications for plant pathology and ecosystem management. It reinforces the fact that land managers should work to minimise opportunities for *Phytophthora* spp. to spread to new sites where they may come into contact with compatible species and potentially form new hybrids. This possible outcome represents a new level of threat to biodiversity, forestry and agriculture in addition to the well-known threat that is posed directly by the introduction of any pathogenic *Phytophthora* species to non-infested sites. The hybrids reported in this study were retrieved from stream water or from the rhizosphere soil of diseased plants and their pathogenicity has not been tested. The parental species are also mostly associated with soil and river samples from riparian ecosystems, although *P. thermophila* and *P.* taxon PgChlamydo opportunistically occur on plant hosts (Brasier *et al.* 2003a; Jung *et al.* 2011). Clearly, further work is required to test the pathogenicity of the *Phytophthora* hybrids found in Australia and South Africa. However, as recommended by Jung *et al.* (2011) precaution should be applied in managing all soil-borne *Phytophthora* taxa in natural ecosystems, regardless of their present known impact on plant health.

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Table 1: Isolates of *Phytophthora* used in this study.

Reference collection number[a]	Other collection numbers	Identity[b]	Substrate	Host	Location	Isolated by	Date	GenBank accession number		
								ASF	GPA	*cox*I
CBS131652	DH228	*Phytophthora amnicola*	Water	Stream baiting	Lake Jualbup, WA, Australia	D Hüberli	2009	JQ936759		JQ029948
VHS19503		*P. amnicola*	Soil	*Patersonia* sp.	Pemberton, WA, Australia	VHS	2008	JQ936760		JQ029950
CBS129424	DH086	*P. fluvialis*	Water	Stream baiting	Moore River, WA, Australia	D Hüberli	2009	JQ936761	JQ936733	JF701442
VHS17350		*P. fluvialis*	Water	Stream baiting	Badgingarra, WA, Australia	VHS	2007	JQ936762		JF701440
MUCC775	DH213	*P. fluvialis*	Water	Stream baiting	Moore River, WA, Australia	D Hüberli	2009		JQ936734	JF701441
CBS309.62	PFF309	*P. fragariae*	Plant	*Fragaria × ananassa*	Scotland, United Kingdom	CJ Hickman	1962	DQ092832	DQ092858	
MUCC776	TAS35	*P. gonapodyides*	Water	Stream baiting	TAS, Australia		2009	JQ936763	JQ936735	JN547642
MUCC761	SLPA72	*P. gonapodyides*	Water	*Eucalyptus obliqua* forest	Toolangi North State Forest, VIC, Australia	WA Dunstan	2008	JQ936764	JQ936736	HQ012850
VHS17085		*P. litoralis*	Soil	*Banksia* sp.	Hopetoun, WA, Australia	VHS	2007	JQ936766	JQ936738	HQ012864
CBS127953	VHS20763	*P. litoralis*	Soil	*Banksia* sp.	Ravensthorpe, WA, Australia	VHS	2008	JQ936765	JQ936737	HQ012866
DDS3432		*P. megasperma*	Soil	*Banksia* sp.	North Dinninup, WA, Australia	VHS	1992	JQ936768	JQ936740	HQ012867
VHS17183		*P. megasperma*	Soil	*Xanthorrhoea platyphylla*	Esperance, WA, Australia	VHS	2007	JQ936767	JQ936739	HQ012868
VHS17175		*P. asparagi*	Soil	*Banksia media*	Esperance, WA, Australia	VHS	2007			HQ012844
MUCC766*	SLPA121	*P.* taxon PgChlamydo	Water	Stream baiting	Yea Wetlands, VIC, Australia	WA Dunstan	2008	JQ936771	JQ936743	JN547652
VHS6595*		*P.* taxon PgChlamydo	Soil	Native forest	Manjimup, WA, Australia	VHS	1999	JQ936770	JQ936742	HQ012879
DDS3753*		*P.* taxon PgChlamydo	Soil	Native forest	Manjimup, WA, Australia	VHS	1995	JQ936769	JQ936741	HQ012878
IMI 389731	P510	*P.* taxon PgChlamydo	Roots	*Pseudotsuga* sp.	Walley, British Columbia, Canada	PB Hamm & EM Hansen	1984	JQ936772		
VHS7474		*P. thermophila*	Soil	Native forest	Manjimup, WA, Australia	VHS	2000	JQ936773	JQ936752	HQ012871
VHS13530	CBS127954	*P. thermophila*	Soil	*Eucalyptus marginata*	Dwellingup, WA, Australia	VHS	2004	JQ936774	JQ936753	HQ012872

Reference collection number[a]	Other collection numbers	Identity[b]	Substrate	Host	Location	Isolated by	Date	GenBank accession number		
								ASF	GPA	*cox*I
VHS3655		*P. thermophila*	Soil	Native forest	Quinninup, WA, Australia	VHS	1998			HQ012870
VHS16164		*P. thermophila*	Soil	*Banksia grandis*	Pemberton, WA, Australia	VHS	2006			HQ012875
VHS13567		*P. thermophila*	Roots	*E. marginata*	Dwellingup, WA, Australia	VHS	2004			HQ012873
VHS13761		*P. thermophila*	Soil	*E. marginata*	Dwellingup, WA, Australia	VHS	2004			HQ012874
CMW37727	J 2.2 C	A-PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2009	JQ890332 JQ890333	JQ890356	JQ890348
CMW37728	J 2.4 A	A-PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2009	JQ890334 JQ890335		JQ890349
CMW37729	J 2.23 A	A-PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2010	JQ890336 JQ890337	JQ890357	JQ890350
CMW37730	J 2. 24 A	A-PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2010	JQ890338 JQ890339	JQ890358	JQ890351
MUCC774*	TAS21	A-PG	Water	Stream baiting	Carlton River, TAS, Australia	Y Ziqing	2009	JQ936775 JQ936784	JQ936744	JQ936797
MUCC777*	SLPA48	PG-A	Soil	Track drain, native forest	Toolangi North State Forest, VIC, Australia	WD Dunstan	2008	JQ936776 JQ936785	JQ936732 JQ936745	JQ936798
MUCC778*	SLPA56	PG-A	Soil	Track drain, native forest	Toolangi North State Forest, VIC, Australia	WD Dunstan	2008	JQ936777 JQ936786	JQ936746	JQ936799
MUCC779*	SLPA57	PG-A	Soil	Native forest	Toolangi North State Forest, VIC, Australia	WD Dunstan	2008	JQ936778 JQ936787	JQ936747	JQ936800
CMW37731	J 1. 3 A	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2009	JQ890340 JQ890341	JQ890359	JQ890352
CMW37732	J 4.2 D	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2009	JQ890342 JQ890343	JQ890360	JQ890353
CMW37733	J 4.9 A	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2010	JQ890344 JQ890345	JQ890361	JQ890354
CMW37734	J 5.11 C	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	JH Nagel	2010	JQ890346 JQ890347	JQ890362	JQ890355
MUCC780*	DH150	T-A	Water	Stream baiting	Lake Jualbup, WA, Australia	D Hüberli	2009	JQ936779 JQ936792	JQ936754	JQ936803
MUCC781	TAS25	T-A	Water	Stream baiting	TAS, Australia	Y Ziqing	2009	JQ936780 JQ936793	JQ936755	JQ936804
MUCC782*	TAS28	T-A	Water	Stream baiting	TAS, Australia	Y Ziqing	2009	JQ936781 JQ936794	JQ936756	JQ936805
VHS22715*		T-A	Soil	Urban parkland	Mosman Park, Perth, WA, Australia	VHS	2009	JQ936783 JQ936796	JQ936758	JQ936807

Reference collection number[a]	Other collection numbers	Identity[b]	Substrate	Host	Location	Isolated by	Date	GenBank accession number		
								ASF	GPA	*cox*I
VHS5185*		T-A	Soil	Native vegetation	Pemberton, WA, Australia	VHS	1998	JQ936782 JQ936795	JQ936757	JQ936806
MUCC783*	TAS30	T-PG	Water	Stream baiting	TAS, Australia	Y Ziqing	2009	JQ936788 JQ936790	JQ936748 JQ936750	JQ936801
MUCC784*	TAS33	T-PG	Water	Stream baiting	TAS, Australia	Y Ziqing	2009	JQ936789 JQ936791	JQ936749 JQ936751	JQ936802

[a] Abbreviations for culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI); IMI = CABI Bioscience (International Mycological Institute), UK; VHS = Vegetation Health Service Collection, Department of Environment and Conservation, Perth, Australia; DDS = earlier prefix of VHS Collection; MUCC = Murdoch University Culture Collection. Isolates used in the morphological study indicated with an asterisk.
[b] Hybrid identity (maternal parent first): A-PG = *P. amnicola* × *P.* taxon PgChlamydo , T-A = *P. thermophila* × *P. amnicola*, T-PG = *P. thermophila* × *P.* taxon PgChlamydo, PG-A = *P.* taxon PgChlamydo × *P. amnicola*

Table 2. List of primers used in this study

Locus	Primer	Sequence (5'-3')	Reference
Anti-silencing factor (ASF1)	ASF-E1-1F	ACCAACATCACCGTGCTGGAC	Ioos *et al.* (2006)
	ASF-E2-2R	CGTTGTTGACGTAGTAGCCCAC	Ioos *et al.*(2006)
Cytochrome oxidase c subunit I (*cox*I)	FM84	TTTAATTTTTAGTGCTTTTGC	Martin and Tooley (2003)
	FM83	CTCCAATAAAAAATAACCAAAAATG	Martin and Tooley (2003)
	FM50	GTTTACTGTTGGTTTAGATG	Martin and Tooley (2003)
	FM85	AACTTGACTAATAATACCAAA	Martin and Tooley (2003)
G protein alpha subunit (GPA1)	GPA-E1-1F	GGACTCTGTGCGTCCCAGATG	Ioos *et al.*(2006)
	GPA-E2-1R	ATAATTGGTGTGCAGTGCCGC	Ioos *et al.*(2006)
Internal transcribed spacer (ITS)	ITS6	GAAGGTGAAGTCGTAACAAGG	Cooke *et al.* (2000)
	ITS4	TCCTCCGCTTATTGATATGC	White *et al.* (1990)
pGEM®-T Easy plasmid	T7	TAATACGACTCACTATAGGG	
	SP6	ATTTAGGTGACACTATAGAA	

Table 3. Comparison of variable sites between consensus sequences of *Phytophthora amnicola*, *P.* taxon PgChlamydo, *P. thermophila* and sequences from isolates of their hybrid taxa

	n[a]	41	44	59	71	110	148	162	171	180	184	457	476	477	518	519	555	699	746	748	750	751	752	753	CODE[b]
Isolates with *P. amnicola* and *P.* taxon PgChlamydo sequences																									
P. amnicola consensus-1		C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	C	T	T	T	A
P. amnicola consensus-2		C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
PgChlamydo consensus		C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
CMW37727-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
CMW37727-2	2	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	A	A	A	-	-	-	T	A/PG
CMW37727-3	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	-	-	-	T	A/PG
CMW37727-4	5	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37728-1	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
CMW37728-2	9	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37729-1	4	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37729-2	2	C	T	T	C	G	G	A	-	T	T	G	G	T	T	C	C	A	A	A	-	-	-	T	A/PG
CMW37729-3	2	C	A	C	C	A	A	A	-	T	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A/PG
CMW37730-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
CMW37730-2	1	C	A	C	C	A	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A/PG
CMW37730-3	1	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	C	A/PG
CMW37730-4	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC777-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
MUCC777-2	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	-	-	-	T	A/PG
MUCC777-3	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	G	G	-	-	-	C	A/PG
MUCC777-4	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC778-1	6	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
MUCC778-2	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	-	-	-	T	A/PG
MUCC778-3	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC779-1	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
MUCC779-2	1	C	A	C	C	A	A	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	C	A/PG
MUCC779-3	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	G	G	G	-	-	-	C	A/PG
MUCC779-4	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC774-1	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
MUCC774-2	1	C	A	C	C	A	A	A	-	T	T	G	C	G	T	C	C	A	A	A	C	T	T	T	A/PG
MUCC774-3	1	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	C	A/PG
MUCC774-4	1	C	A	C	C	A	A	A	-	T	T	A	G	T	T	C	C	G	G	G	-	-	-	C	A/PG
MUCC774-5	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	G	G	G	-	-	-	C	A/PG
MUCC774-6	1	C	A	C	C	A	A	A	-	T	T	G	C	G	T	C	C	A	A	A	C	T	T	T	A/PG
MUCC774-7	1	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	C	A/PG
MUCC774-8	1	C	T	T	C	G	G	C	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	A/PG
MUCC774-9	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A

	n[a]	41	44	59	71	110	148	162	171	180	184	457	476	477	518	519	555	699	746	748	750	751	752	753	CODE[b]
Isolates with *P. thermophila* and *P.* taxon PgChlamydo sequences																									
PgChlamydo consensus		C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
P. thermophila consensus		T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
MUCC783-1	2	C	A	C	C	A	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
MUCC783-2	8	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
MUCC784-1	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
MUCC784-2	8	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	C	PG
Isolates with *P. amnicola* and *P. thermophila* sequences																									
P. amnicola consensus-1		C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	C	T	T	T	A
P. amnicola consensus-2		C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
P. thermophila consensus		T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
CMW37731-1	4	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	C	T	T	T	A
CMW37731-2	4	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37731-3	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
CMW37732-1	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	C	T	T	T	A
CMW37732-2	8	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37732-3	1	C	T	C	C	G	G	T	T	A	T	A	G	T	T	C	C	A	G	A	-	-	-	C	A/T
MUCC780-1	9	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC780-2	1	T	A	C	T	G	G	C	-	A	A	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
CMW37733-1	5	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37733-2	1	T	A	C	T	G	G	C	-	A	A	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
CMW37733-3	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	G	A	-	-	-	C	A/T
CMW37733-4	1	T	A	C	T	G	G	C	-	A	A	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
CMW37733-5	1	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	A	A	-	-	-	T	A/T
CMW37733-6	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	C	A	A	A	-	-	-	T	A/T
CMW37734-1	4	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
CMW37734-2	2	T	A	C	T	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	C	T	T	T	A/T
CMW37734-3	1	T	A	C	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
CMW37734-4	3	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
MUCC781-1	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	C	T	T	T	A
MUCC781-2	3	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC781-3	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	G	A	-	-	-	T	A/T
MUCC781-4	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	T	A	G	A	-	-	-	C	A/T
MUCC781-5	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
MUCC782-1	3	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
MUCC782-2	1	T	A	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
MUCC782-3	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	C	A	A	A	-	-	-	T	A/T
MUCC782-4	1	C	T	C	T	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
MUCC782-5	1	T	A	C	T	G	G	C	-	A	A	A	G	T	T	C	C	A	A	A	-	-	-	T	A/T
MUCC782-6	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	T
VHS22715-1	7	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A

	n[a]	41	44	59	71	110	148	162	171	180	184	457	476	477	518	519	555	699	746	748	750	751	752	753	CODE[b]
VHS22715-2	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	T	A	A	A	-	-	-	T	A/T
VHS22715-3	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	T	A	G	A	-	-	-	C	A/T
VHS22715-4	1	C	T	T	C	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	C	A/T
VHS5185-1	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	T	A
VHS5185-2	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	T	A	G	A	-	-	-	C	A/T
VHS5185-3	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	C	A	A	A	-	-	-	T	A/T
VHS5185-4	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	T	A	A	A	-	-	-	T	A/T
VHS5185-5	1	C	T	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	A	A	-	-	-	T	A/T

[a] Number of amplicons with the same allele. For each hybrid isolate there were 8-10 amplicons sequenced
[b] Code for the allele: A = *P. amnicola* (orange), T = *P. thermophila* (green), PG = *P.* taxon PgChalmydo (blue); alleles showing recombination are given two codes and a combination of colours

Table 4. Comparison of morphological features of *Phytophthora thermophila*, *P. amnicola*, *P.* taxon PgChlamydo, PG-A, A-PG, T-A and T-PG

	P. thermophila	***P. amnicola***	***P.* taxon PgChlamydo**	**PG-A**	**A-PG**	**T-A**	**T-PG**
No. of isolates	5	2	3	3	1	4	2
Sporangia	Ovoid (65%) to elongated ovoid (15%), ellipsoid (13%), limoniform (7%), nonpapillate, often with tapering base	Ovoid (50%) to limoniform (32%), and ellipsoid (12%), rarely obpyriform (2%) or pyriform (3%), nonpapillate, often with a long tapering base	Ovoid (73%) to obpyriform (16%), occasionally limoniform (7%) or ellipsoid (4%), nonpapillate	Ovoid (67%), often obpyriform (17%) or limoniform (16%), nonpapillate	Ovoid (48%) to broad ovoid (12%), obpyriform (27%), rarely limoniform (8%) or peanut shaped (5%), nonpapillate	Ovoid(57%), limoniform (24%) obpyriform (12%) or ellipsoid (6%), nonpapillate	limoniform (37%), obpyriform (34%), ovoid (29%), nonpapillate
lxb mean (µm)	44.8±6.3x25.7±3.9	62 ± 9.0x35.3 ± 5.6	57.7±7.4x35.5±4.1	56.2±9.6x34.2±6.6	39.1±5.5x27.1±4.5	48.2±8.3x30.3±4.7	48.5±7.7x31.5±3.5
Total range (µm)	29.0–64.8x15.6–39.3	39–78x17–43	34.9-79.3x23.5-49.9	31-93.4x18-50.4	26.6-56.4x17.5-41.2	30.4-74.8x8.8-45.7	31.8-69.7x23.7x39
Isolate means (µm)	44.2–46.8x24.1–26.6		55.7-60.5x32.5-38.3	52.2-63x30.1-39.1		39.8-55.1x28.2x33.2	
l/b ratio	1.78±0.26	1.79±0.17	1.63±0.16	1.63±0.19	1.47±0.24	1.60±0.19	1.54±0.21
Isolate means	1.67–1.86		1.58-1.71	1.59-1.75		1.42-1.75	
Sporangia Exit pores							
Width (µm)	13.9±2.9	12.7±3.5	13.8±4.2	11.9±2.7	11.2±1.5	11.6±1.6	12.5±2.0
Isolate means (µm)	9.7–16.4	10.0–14.6	8.4–14.1	10.7–17.1		9.7-12.5	
Proliferation	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested
Hyphal swellings	Globose or elongated, partly catenulate	Ellipsoid to irregular catenulate hyphal swellings in clusters	Globose	Globose or catenulate and branched	no swellings	no swellings	no swellings
Mean diam (µm)	12.6 ± 2.3	14.2 ± 4.0	22.5 ± 4.4	18.8 ± 4.7			
Chlamydospores	Globose, radiating hyphae	Not observed	Globose, radiating hyphae	Chlamydospore-like globose swellings but no true chlamydospores	Not observed	Not observed	Not observed
Mean diam (µm)	41.5 ± 14.7		41.0 ± 11.7				
Sexual system	Sterile or silent homothallic	sterile	Sterile	sterile	sterile	sterile	sterile
Maximum temperature (ºC)	35	37.5	35	>35<37.5	>35<37.5	>35<37.5	35
Optimum temperature (ºC)	33	25–32.5	20-32.5	20-32.5	20-30	30	25-30

Figure 1. Phylogenetic tree based on the ASF locus of *Phytophthora* spp. generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. *Phytophthora fragariae* is used as an outgroup taxon. Hybrid taxa are indicated in colour: orange = A-PG, blue = PG-A, green = T-A and purple = T-PG.

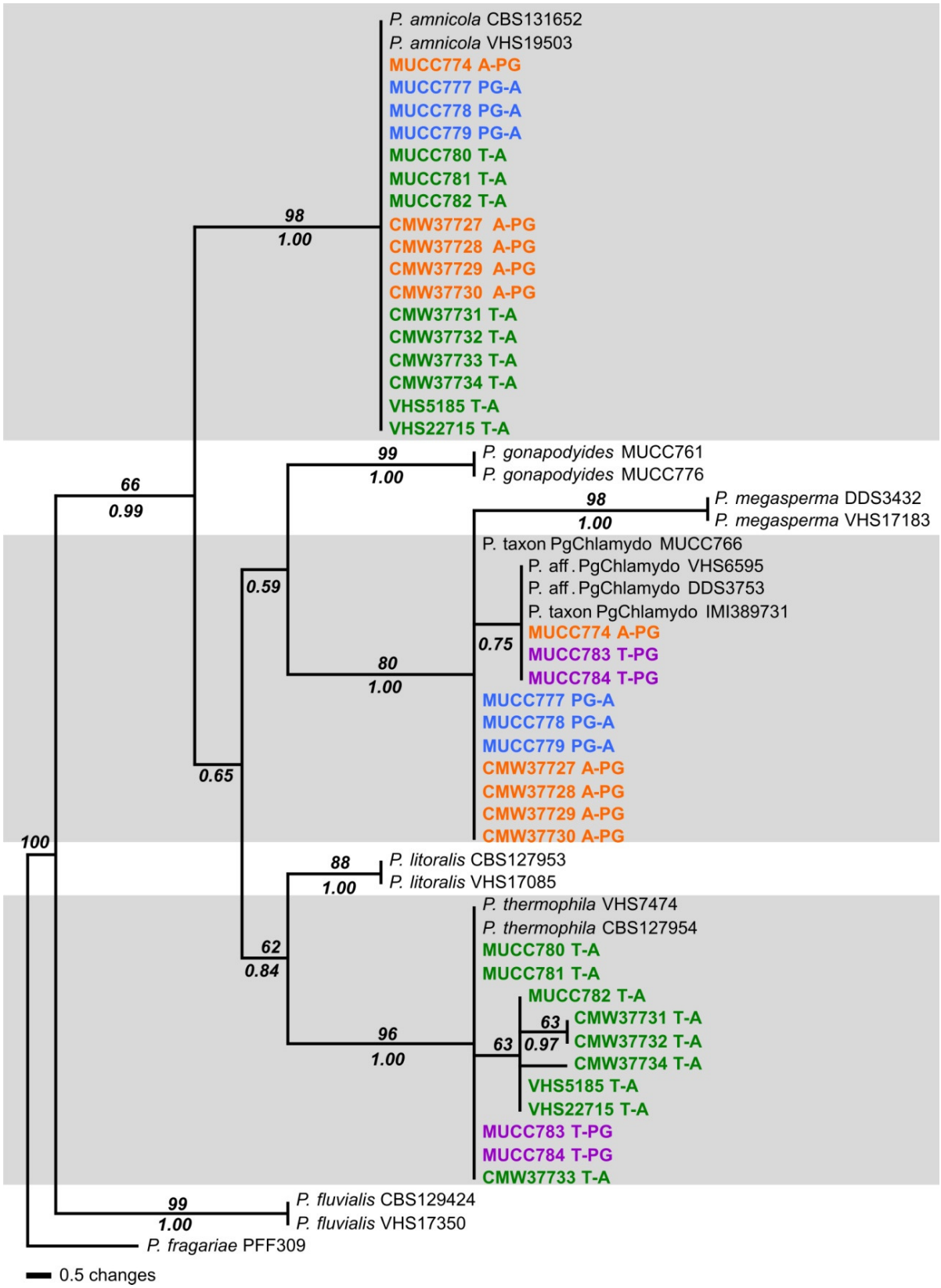

P. amnicola CBS131652
P. amnicola VHS19503
MUCC774 A-PG
MUCC777 PG-A
MUCC778 PG-A
MUCC779 PG-A
MUCC780 T-A
MUCC781 T-A
MUCC782 T-A
CMW37727 A-PG
CMW37728 A-PG
CMW37729 A-PG
CMW37730 A-PG
CMW37731 T-A
CMW37732 T-A
CMW37733 T-A
CMW37734 T-A
VHS5185 T-A
VHS22715 T-A
98
1.00
99
1.00
P. gonapodyides MUCC761
P. gonapodyides MUCC776
66
0.99
98
1.00
P. megasperma DDS3432
P. megasperma VHS17183
P. taxon PgChlamydo MUCC766
P. aff. PgChlamydo VHS6595
P. aff. PgChlamydo DDS3753
P. taxon PgChlamydo IMI389731
0.59
0.75
MUCC774 A-PG
MUCC783 T-PG
MUCC784 T-PG
80
1.00
MUCC777 PG-A
MUCC778 PG-A
MUCC779 PG-A
CMW37727 A-PG
CMW37728 A-PG
CMW37729 A-PG
CMW37730 A-PG
0.65
100
88
1.00
P. litoralis CBS127953
P. litoralis VHS17085
P. thermophila VHS7474
P. thermophila CBS127954
MUCC780 T-A
MUCC781 T-A
62
0.84
MUCC782 T-A
63
0.97
CMW37731 T-A
CMW37732 T-A
CMW37734 T-A
63
96
1.00
VHS5185 T-A
VHS22715 T-A
MUCC783 T-PG
MUCC784 T-PG
CMW37733 T-A
99
1.00
P. fluvialis CBS129424
P. fluvialis VHS17350
P. fragariae PFF309
0.5 changes

Figure 2. Phylogenetic tree based on the GPA locus of *Phytophthora* spp. generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. *Phytophthora fragariae* is used as an outgroup taxon. Hybrid taxa are indicated in colour: orange = A-PG, blue = PG-A, green = T-A and purple = T-PG.

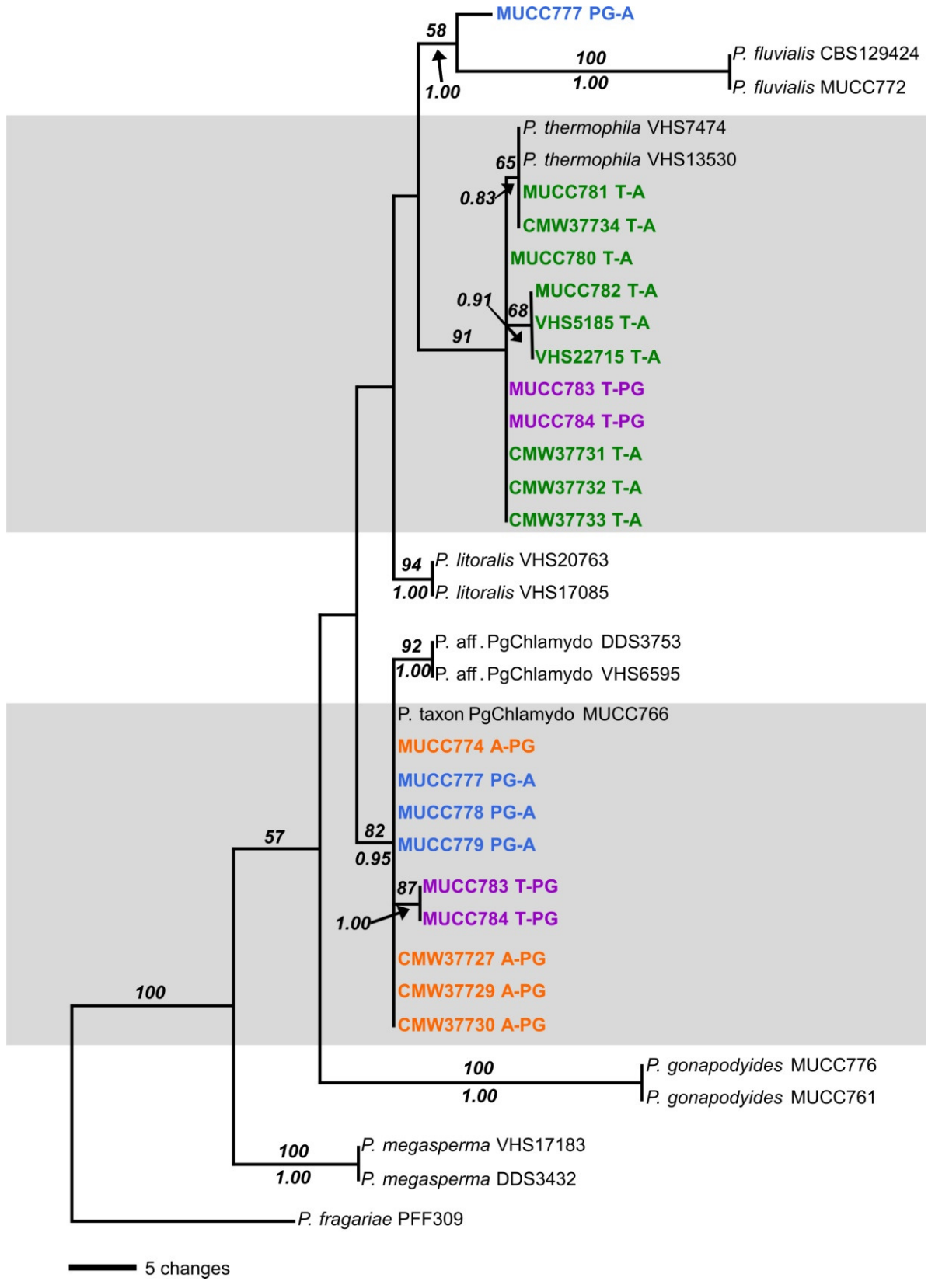

MUCC777 PG-A
58
1.00
100
1.00
P. fluvialis CBS129424
P. fluvialis MUCC772
P. thermophila VHS7474
65
P. thermophila VHS13530
0.83
MUCC781 T-A
CMW37734 T-A
MUCC780 T-A
MUCC782 T-A
0.91
68
VHS5185 T-A
91
VHS22715 T-A
MUCC783 T-PG
MUCC784 T-PG
CMW37731 T-A
CMW37732 T-A
CMW37733 T-A
94
P. litoralis VHS20763
1.00
P. litoralis VHS17085
92
P. aff. PgChlamydo DDS3753
1.00
P. aff. PgChlamydo VHS6595
P. taxon PgChlamydo MUCC766
MUCC774 A-PG
MUCC777 PG-A
MUCC778 PG-A
57
82
MUCC779 PG-A
0.95
87
MUCC783 T-PG
1.00
MUCC784 T-PG
CMW37727 A-PG
CMW37729 A-PG
100
CMW37730 A-PG
100
1.00
P. gonapodyides MUCC776
P. gonapodyides MUCC761
100
1.00
P. megasperma VHS17183
P. megasperma DDS3432
P. fragariae PFF309
5 changes

Figure 3. Phylogenetic tree based on the *cox*I locus of *Phytophthora* spp. generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. *Phytophthora asparagi* is used as an outgroup taxon. Hybrid taxa are indicated in colour: orange = A-PG, blue = PG-A, green = T-A and purple = T-PG.

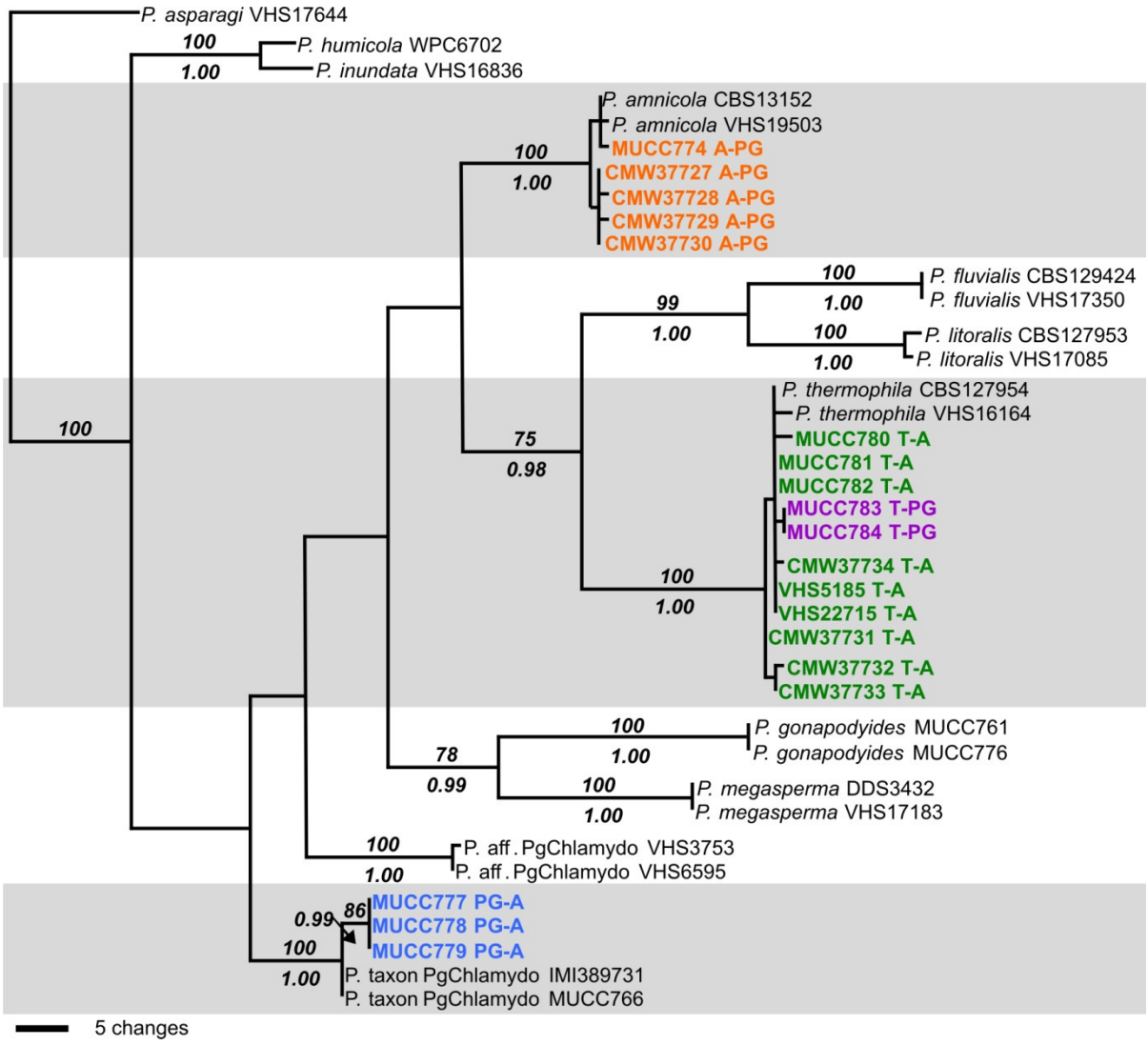

P. asparagi VHS17644
100
1.00
P. humicola WPC6702
P. inundata VHS16836
P. amnicola CBS13152
P. amnicola VHS19503
100
1.00
MUCC774 A-PG
CMW37727 A-PG
CMW37728 A-PG
CMW37729 A-PG
CMW37730 A-PG
100
1.00
P. fluvialis CBS129424
P. fluvialis VHS17350
99
1.00
100
1.00
P. litoralis CBS127953
P. litoralis VHS17085
P. thermophila CBS127954
P. thermophila VHS16164
100
75
0.98
MUCC780 T-A
MUCC781 T-A
MUCC782 T-A
MUCC783 T-PG
MUCC784 T-PG
100
1.00
CMW37734 T-A
VHS5185 T-A
VHS22715 T-A
CMW37731 T-A
CMW37732 T-A
CMW37733 T-A
100
1.00
P. gonapodyides MUCC761
P. gonapodyides MUCC776
78
0.99
100
1.00
P. megasperma DDS3432
P. megasperma VHS17183
100
1.00
P. aff. PgChlamydo VHS3753
P. aff. PgChlamydo VHS6595
0.99
86
MUCC777 PG-A
MUCC778 PG-A
100
1.00
MUCC779 PG-A
P. taxon PgChlamydo IMI389731
P. taxon PgChlamydo MUCC766
5 changes

Figure 4. Colony morphology of four hybrid taxa on V8 agar compared with their known parental *Phytophthora* species. (a) *P*. taxon PgChlamydo (VHS6595), (b-c) PG-A (MUCC778 and MUCC779), (d) *P. amnicola* (CBS131652), (e) A-PG (MUCC774), (f) *P.* taxon PgChlamydo (MUCC766), (g-h) T-PG (MUCC783 and MUCC784), (i) *P. thermophila* (CBS127954), (j-l) T-A (MUCC780, MUCC782 and VHS22715).

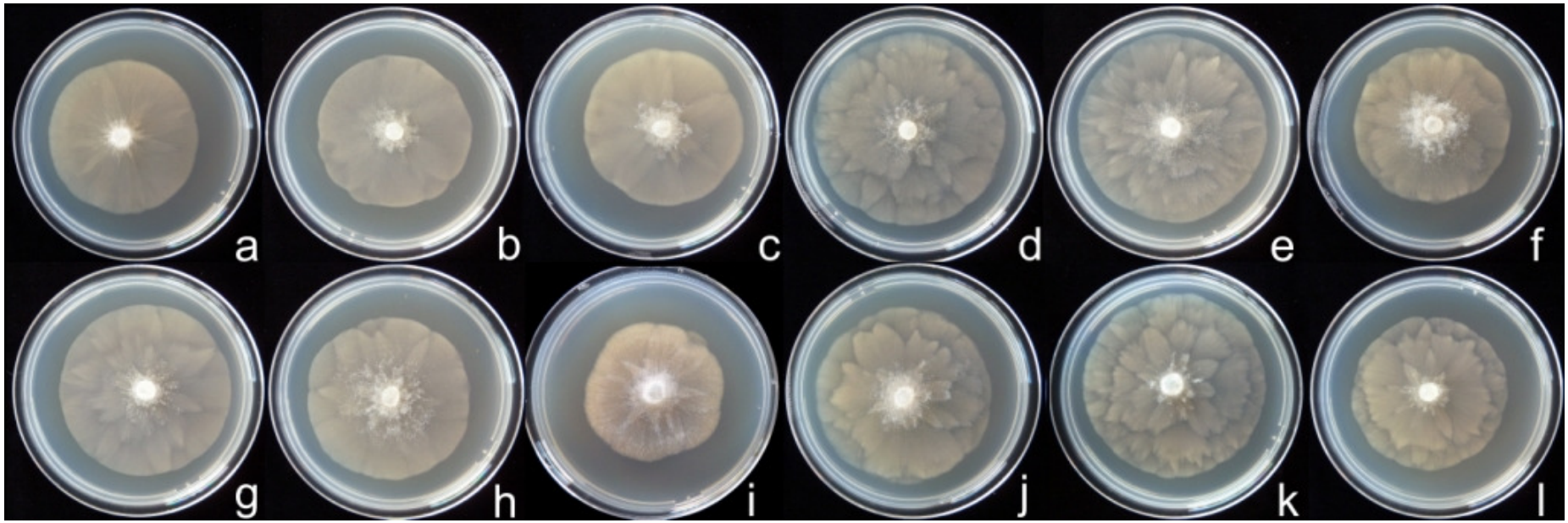

a
b
c
d
e
f
g
h
i
j
k
l

Figure 5. Colony morphology of four hybrid taxa on carrot agar compared with known parental *Phytophthora* species. (a) *P.* taxon PgChlamydo (VHS6595), (b-c) PG-A (MUCC778 and MUCC779), (d) *P. amnicola* (CBS131652), (e) A-PG (MUCC774), (f) *P.* taxon PgChlamydo (MUCC766), (g-h) T-PG (MUCC783 and MUCC784), (i) *P. thermophila* (CBS127954), (j-l) T-A (MUCC780, MUCC782 and VHS22715).

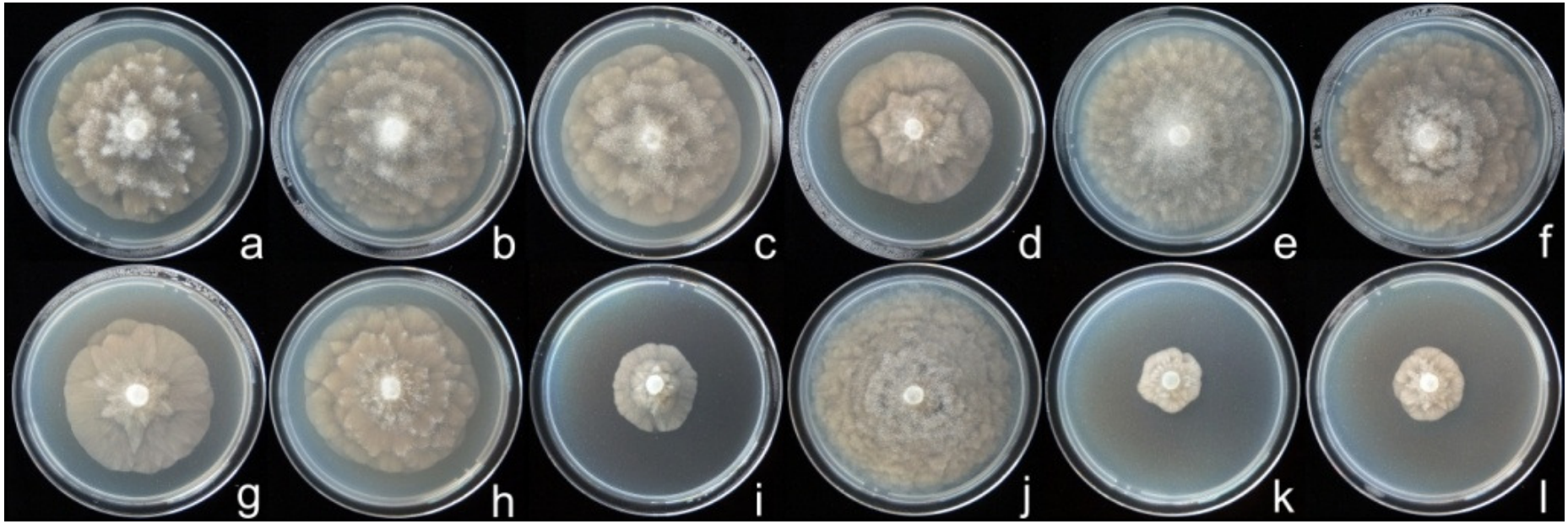
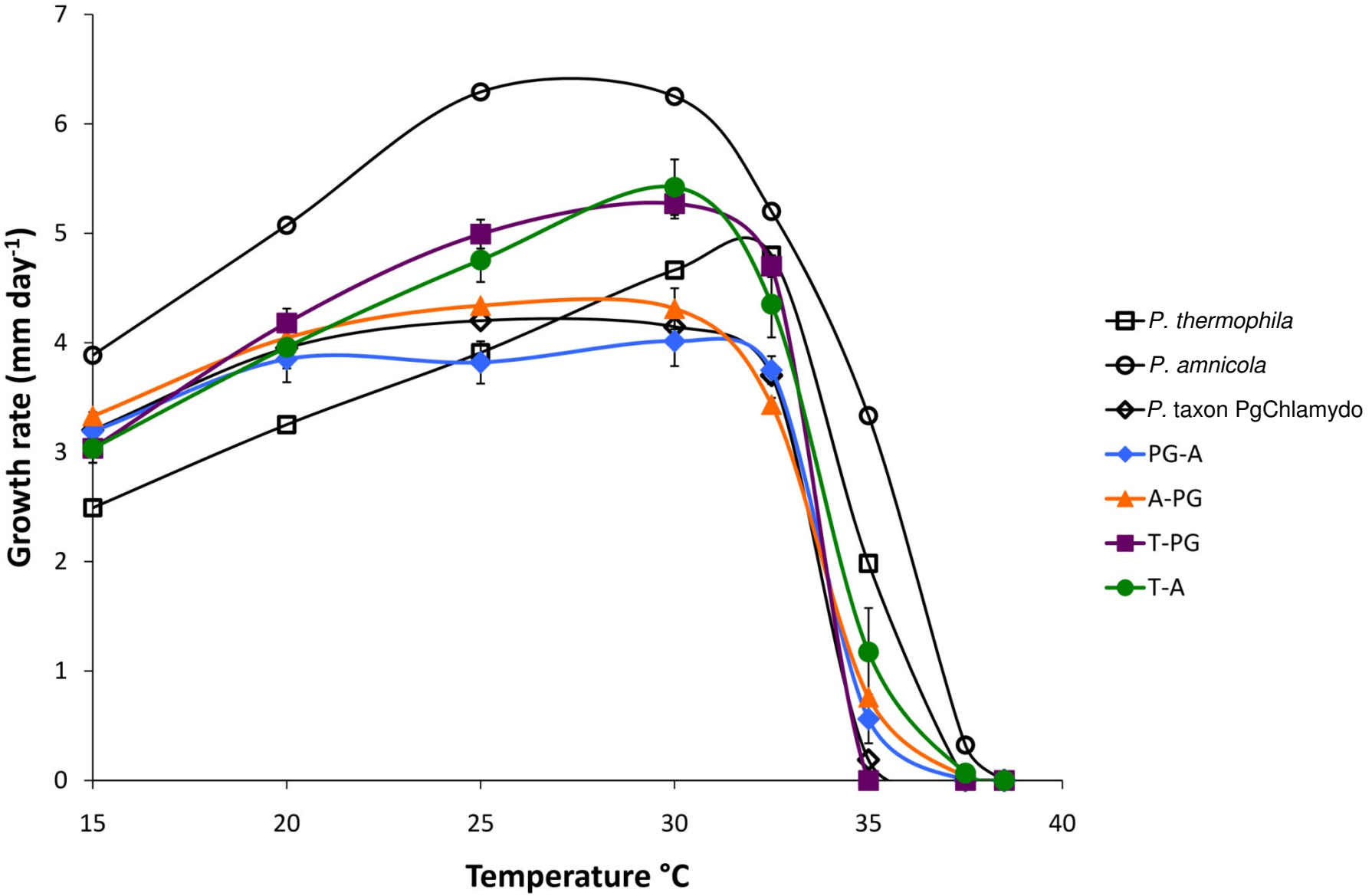

a
b
c
d
e
f
g
h
i
j
k
l

Figure 6. Average radial growth rate (in mm. day^{-1}) at seven temperatures (ºC) of isolates represening the parental species *Phytophthora amnicola, P.* taxon PgChlamydo, and *P. thermophila*, and the four hybrid taxa.


Growth rate (mm day-1)
Temperature °C
0
1
2
3
4
5
6
7
15
20
25
30
35
40
P. thermophila
P. amnicola
P. taxon PgChlamydo
PG-A
A-PG
T-PG
T-A

Appendix A: Supplementary data

Table A.1. Positions of frequent and rare single nucleotide polymorphisms of the ITS region of *Phytophthora amnicola* among 20 cloned amplicons of the type isolate (CBS131652) and 10 cloned amplicons of isolate VHS19503.

clone	67	156	208	375	387	592	596	678	707	719	742	750	751	752	756	757	788	791	801	803	Rare[a]
CBS131652-1	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	C	T	G	T	
CBS131652-2	C	C	A	T	C	G	A	T	C	A	A	-	-	-	C	G	T	T	G	T	1
CBS131652-3	T	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	G	T	G	T	1
CBS131652-4	T	T	A	T	C	G	A	T	C	A	G	-	-	-	C	G	T	T	G	T	2
CBS131652-5	C	C	A	T	C	G	G	T	T	A	G	-	-	-	C	G	C	T	G	T	2
CBS131652-6	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	G	T	G	T	
CBS131652-7	C	C	G	T	C	G	A	T	C	A	G	C	T	T	T	T	G	T	G	T	1
CBS131652-8	C	T	A	T	C	G	A	C	C	A	G	C	T	T	T	T	C	T	G	T	2
CBS131652-9	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	G	A	G	T	1
CBS131652-10	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	C	T	G	T	
CBS131652-11	C	C	A	T	T	G	A	T	C	A	G	-	-	-	C	G	C	T	G	T	1
CBS131652-12	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	T	T	G	T	
CBS131652-13	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	G	T	G	T	
CBS131652-14	C	T	A	T	C	G	A	T	C	A	G	-	-	-	C	G	T	T	A	T	2
CBS131652-15	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	C	T	G	T	1
CBS131652-16	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	C	T	G	T	
CBS131652-17	C	C	A	T	C	G	A	T	C	G	G	C	T	T	T	T	T	T	G	T	1
CBS131652-18	C	C	A	C	C	G	A	T	C	A	G	C	T	T	T	T	C	T	G	A	2
CBS131652-19	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	G	T	G	T	
CBS131652-20	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	T	T	G	T	
VHS19503-1	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	G	T	G	T	
VHS19503-2	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	C	T	G	T	
VHS19503-3	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	T	T	G	T	
VHS19503-4	T	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	G	T	G	T	1
VHS19503-5	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	G	T	G	T	
VHS19503-6	C	C	A	T	C	G	A	T	C	A	G	C	T	T	T	T	C	T	G	T	
VHS19503-7	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	G	T	G	T	
VHS19503-8	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	G	T	G	T	
VHS19503-9	C	C	A	T	C	G	A	T	C	A	G	-	-	-	C	G	C	T	G	T	
VHS19503-10	C	C	A	T	C	A	A	T	C	A	G	C	T	T	T	T	G	T	G	T	1
No.	3	3	1	1	1	1	1	1	1	1	1	14	14	14	14	14	18	1	1	1	0.63
%	10	10	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	46.7	46.7	46.7	46.7	46.7	60	3.3	3.3	3.3	0.08

[a] Refers to the no. of rare SNPs in the sequences. A SNP was considered rare if it occurred in <10% of sequences. Cells shaded in grey indicate nucleotide polymorphisms

Table A.2. Positions of frequent and rare SNPs of the ITS region of *Phytophthora thermophila* among 50 cloned amplicons of the type isolate (CBS127954)

	32	49	62	76	81	127	159	164	222	273	303	310	321	343	356	382	399	432	462	464	471	486	498	513	573	589	595	612	651	666	705	725	798	800	Rare[a]
1	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	
2	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	A	1
3	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	
4	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	
5	T	T	A	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	1
6	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	
7	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
8	G	T	G	T	T	A	T	T	A	A	T	T	T	T	C	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	2
9	T	T	G	T	T	A	T	T	A	A	T	T	T	C	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	1
10	T	T	G	T	T	A	T	T	A	G	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	1
11	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	
12	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	
13	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	A	T	C	C	G	1
14	T	T	G	T	T	A	T	C	A	A	T	T	T	T	T	T	T	T	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	1
15	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	C	T	A	T	T	T	C	T	G	T	A	T	G	T	C	C	G	1
16	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	C	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	1
17	T	T	G	T	T	A	C	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	1
18	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	C	T	C	G	T	A	T	G	T	T	C	G	2
19	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	
20	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	T	T	G	T	C	C	G	1
21	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	
22	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	
23	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	
24	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	C	G	T	C	C	G	1
25	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	C	T	T	T	T	C	G	T	A	T	G	G	C	C	G	1
26	T	T	G	T	T	A	T	T	A	A	T	C	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	1
27	T	T	G	T	T	G	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	1
28	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	
29	T	T	G	T	T	A	T	T	A	A	C	T	T	T	T	T	T	T	T	T	T	T	T	T	C	G	T	A	T	G	T	C	C	G	1
30	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	T	T	G	T	A	T	G	T	C	C	G	
31	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
32	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
33	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
34	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
35	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	C	T	C	C	A	T	A	T	G	T	C	C	G	2
36	T	T	A	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	C	T	T	C	C	G	T	A	T	G	T	C	C	G	2

	32	49	62	76	81	127	159	164	222	273	303	310	321	343	356	382	399	432	462	464	471	486	498	513	573	589	595	612	651	666	705	725	798	800	Rare[a]
37	T	T	G	T	T	A	T	T	A	A	T	T	T	T	C	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	1
38	T	T	G	T	T	A	T	T	G	A	T	T	T	T	T	T	C	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	2
39	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	A	G	1
40	T	T	A	A	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	A	3
41	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	A	1
42	T	T	G	T	A	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	C	A	T	G	T	C	C	A	3
43	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
44	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
45	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
46	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
47	T	C	G	T	T	A	T	T	A	A	T	T	C	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	2
48	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
49	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
50	T	T	G	T	T	A	T	T	A	A	T	T	T	T	T	T	T	T	T	T	T	T	T	C	C	G	T	A	T	G	T	C	C	G	
NO.	1	1	3	1	1	1	1	1	1	1	1	1	1	1	2	1	1	1	1	15	1	1	1	14	13	1	1	1	1	1	1	1	1	4	0.72
%	2	2	6	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	30	2	2	2	28	26	2	2	2	2	2	2	2	2	8	0.09

[a] Refers to the no. of rare SNPs in the sequences. A SNP was considered rare if it occurred in <10% of sequences. Cells shaded in grey indicate nucleotide polymorphisms

Table A.3. Positions of frequent and rare SNPs of ITS region of *Phytophthora* taxon PgChlamydo among 20 cloned amplicons of isolate VHS6595.

	114	172	235	470	668	Rare[a]
1	T	T	C	G	T	
2	T	C	T	G	C	1
3	T	C	C	G	T	
4	T	C	C	G	T	
5	T	C	C	G	T	
6	T	T	C	G	C	
7	T	T	C	G	T	
8	T	C	C	G	T	
9	T	C	C	G	T	
10	T	T	C	G	T	
11	T	T	C	G	C	
12	T	T	C	G	T	
13	T	C	C	G	T	
14	T	C	C	G	C	
15	A	C	C	G	T	1
16	T	T	C	G	T	
17	T	T	C	G	T	
18	T	C	C	A	C	1
19	T	C	C	G	T	
20	T	T	C	G	T	
N	1	9	1	1	5	0.15
%	5	45	5	5	25	0.018

[a] Refers to the no. of rare SNPs in the sequences. A SNP was considered rare if it occurred in <10% of sequences. Cells shaded in grey indicate nucleotide polymorphisms

Chapter 3

Multiple *Phytophthora* species associated with a single riparian ecosystem in South Africa

Abstract

The diversity of *Phytophthora* spp. in rivers and riparian ecosystems has received considerable international attention, although little such research has been conducted in South Africa. This study determined the diversity of *Phytophthora* spp. within a single river situated in the Gauteng Province of South Africa. Sample acquisition was performed over a one year period and entailed biweekly river baiting using *Rhododendron indicum* leaves. *Phytophthora* spp. were identified through phylogenetic analyses of the internal transcribed spacer (ITS) region of the ribosomal DNA, as well as the mitochondrial cytochrome oxidase c subunit I (*cox*I) gene. Eight *Phytophthora* spp. were identified, including a new taxon, *P.* taxon Sisulu-river, and two hybrid species from Cooke's ITS clade 6. Of these, species from Clade 6 were the most abundant, including *P.* taxon PgChlamydo and *P. lacustris*. Species residing in Clade 2 were also encountered, including *P. multivora*, *P. plurivora* and *P. citrophthora*. The detection of eight species in this first investigation into *Phytophthora* diversity of rivers and associated riparian ecosystems in northern South Africa, suggests a high level of diversity of these organisms in South African rivers.

1. Introduction

Rivers and streams in riparian ecosystems play an important role in the dissemination of species in the oomycete genus *Phytophthora* and the diseases they cause. For example, decline of alder (*Alnus* spp.) in Europe and the United Kingdom, caused by *P. alni*, is much more common in trees that are within one meter of a river (Gibbs *et al.* 1999). It has also been demonstrated that riparian alder stands are more likely to become diseased if they share catchment areas with diseased stands (Jung and Blaschke 2004). Once *P. lateralis*, which causes a lethal disease of Port-Orford-cedar (*Chamaecyparis lawsoniana*) in Oregon and California (USA) is present in a stream, *C. lawsoniana* trees with roots exposed to the river water die within a few years (Hansen *et al.* 2000). Likewise, *Phytophthora* diseases in agricultural and horticultural nurseries are closely linked to the presence of *Phytophthora* spp. present in irrigation water (Oudemans 1999; Yamak *et al.* 2002; Gevens *et al.* 2007; Werres *et al.* 2007; Ghimire *et al.* 2009; Orlikowski *et al.* 2009). Although, the incidence of sudden oak death caused by *P. ramorum* appears not to be linked to rivers and streams (Davidson and Shaw 2003), riparian systems play an important role in early disease detection because zoospores make their way into any nearby stream when *P. ramorum* is present in an area (Sutton *et al.* 2009).

Multiple *Phytophthora* spp. often occur simultaneously in rivers. In North Carolina, USA stream monitoring for *P. ramorum* revealed the presence of numerous *Phytophthora* species, including *P. cambivora*, *P. cinnamomi, P. citricola, P. citrophthora, P. gonapodyides, P. heveae* and *P. pseudosyringae* (Hwang *et al.* 2008; Hwang *et al.* 2011). In a similar study conducted in Oregon and Alaska (USA), 18 *Phytophthora* spp. were recovered from streams of which *P. gonapodyides* and *P.* taxon PgChlamydo were the most frequently encountered (Reeser *et al.* 2011). Nine *Phytophthora* spp. were retrieved from rivers and streams in Western Australia and included only two species (*P. cinnamomi* var. *parvispora* and *P. inundata)* known at that time (Hüberli *et al.* 2010). Likewise, a survey of rivers within Argentinean *Austrocedrus chilensis* stands revealed the presence of five *Phytophthora* spp., namely *P. syringae*, *P. gonapodyides, P. cambivora*, *P.* taxon PgChlamydo and *P.* taxon raspberry (Greslebin *et al.* 2005).

It appears that *Phytophthora* species residing in ITS Clade 6 are over represented in rivers and riparian ecosystems. Thus, seven of the eighteen identified from streams in Oregon and Alaska (USA) belonged to Clade 6 (Reeser *et al.* 2011). Similarly, three of the five of the *Phytophthora* spp. retrieved from Patagonian streams in *Austrocedrus chilensis* forests, were from Clade 6 (Greslebin *et al.* 2005). Nine distinct *Phytophthora* spp. were discovered from Australian rivers including six species from Clade 6 (Hüberli *et al.* 2010). This is

consistent with the hypothesis that Clade 6 species are adapted to exist as saprotrophs on fallen leaves and other plant debris in rivers (Brasier *et al.* 2003; Jung *et al.* 2011).

Phytophthora spp. are well known plant pathogens in South Africa (Nagel *et al*. 2012, Chapter 4 of this thesis). Numerous diseases of crop plants in the country are caused by *Phytophthora* spp., including root rot of avocado (*Persea americana*) (Zentmyer 1979; Lonsdale *et al.* 1988) and grapevine (*Vitis* spp.) (van der Merwe *et al.* 1972; Marais 1979) caused by *P. cinnamomi*, and rot and wilt of several solanaceous and curcubitaceous crops caused by *P. capsici* (Thompson *et al.* 1994; Labuschagne *et al.* 2000; Labuschagne *et al.* 2003; Meitz *et al.* 2010). Likewise, *Phytophthora* diseases are widespread in the commercial forestry plantations of South Africa, such as black butt disease of black wattle (*Acacia mearnsii*) caused by *P. nicotianae, P. boehmeriae* and *P. meadii* (Roux and Wingfield 1997), root and collar rot of *Pinus* spp. and *Eucalyptus* spp. caused by *P. cinnamomi* (Linde *et al.* 1994), and several other *Phytophthora* spp. such as *P. alticola*, *P. boehmeriae P. fridiga* and *P. nicotianae* associated with disesases of *Eucalyptus* spp. (Linde *et al.* 1994; Maseko *et al.* 2001; Maseko *et al.* 2007).

The current knowledge of *Phytophthora* spp. from native plant species is restricted to the Western Cape Province of South Africa. *P. cinnamomi* infects numerous species of native Bruniaceae (Lamiales), Ericaceae (Ericales) and Proteaceae (Proteales) species making up the Fynbos vegetation (Van Wyk 1973; Von Broembsen 1984b; Von Broembsen and Kruger 1985). Additionally, several *Phytophthora* spp. including *P. cinnamomi*, *P. cryptogea, P. drechsleri, P. multivora, P. nicotianae* and *P.* taxon *emzansi* are associated with diseases of the native medicinal buchu plant (*Agathosma* spp. Rutaceae, Sapindiales) (Bezuidenhout *et al.* 2010).

The *Phytophthora* spp. diversity in rivers and other natural water bodies has been poorly studied in South Africa. Akin to the situation of *Phytophthora* spp. causing diseases on native plant hosts mentioned above, the current knowledge of *Phytophthora* diversity in rivers is restricted to the Western Cape Province of South Africa. In this area, it was for example found during the late 1970's that *P. cinnamomi* was present in all the major rivers of the Western Cape Province (Von Broembsen 1984a). *P. citricola*, *P. cryptogea* and *P. drechsleri* were present in rivers utilized for irrigation purposes (Von Broembsen 1989). *P. capensis* was also retrieved once from stream water, although it was earlier identified as *P. citricola* (Oudemans *et al.* 1994; Bezuidenhout *et al.* 2010). All of these reports date back to the 1980's and the *Phytophthora* spp. diversity in the rivers of the Western Cape Province

deserve reassessment. Furthermore, nothing is known about the *Phytophthora* spp. diversities in rivers from other parts of South Africa.

It is clear that very little is known about *Phytophthora* spp. diversity from South African rivers and riparian ecosystems. Yet, knowledge of this kind is important as it can serve as an early indicator of the presence of potentially harmful *Phytophthora* spp. This is because *Phytophthora* spp. on crop plants, commercially propaged trees and native plants, would most likely also be present in these rivers. Furthermore, such systems represent a niche potentially rich in *Phytophthora* diversity, as well as novel species. No prior studies have attempted to determine the *Phytophthora* diversity in the Gauteng Province of South Africa. The aim of this study was, therefore, to assess the diversity of *Phytophthora* spp. present in a river associated with both a native and disturbed riparian zone, in the Gauteng Province.

2. Materials and methods

2.1. Sampling and isolation

Sampling was done from the Crocodile River (West) in the Gauteng Province of South Africa. The Crocodile River has its source in the Witwatersrand mountain range from where it flows through the Walter Sisulu National Botanical Garden (NBG) situated in Roodepoort, Johannesburg and residential areas and smallholdings of the Muldersdrift area. Further down river it is joined by other tributary rivers and eventually flows into the Hartebeespoort dam. The Walter Sisulu NBG is one of nine NBG's in South Africa and features the Rocky Highveld Grassland biome, which is characterized by a combination of grassland and savannah vegetation (Bredenkamp and van Rooyen 1996). Additionally, the Crocodile River that runs through the garden is surrounded by a densely forested riparian zone. Outside the NBG, urbanization is developing fast, and the river is typically surrounded by several agricultural and commercial enterprises and disturbed vegetation that includes invasive tree species such as *Acacia mearnsii, Eucalyptus* spp. and *Salix* spp.

Samples were collected from three sites within the garden and two downstream sites outside of the garden (Figure 1). Sampling Site 1 had no riparian forest zone, but instead was surrounded by grassland. Site 1 was separated from the other sites by a waterfall. Sites 2 and 3 had dense riparian forests on either side of the river. However, Site 3 did not possess a complete canopy spanning the river as was found at Site 2. The downstream sampling sites occurred approximately 10 km downstream of the garden and the riparian zone consisted mostly of disturbed vegetation, including several introduced species such as *A. mearnsii, Eucalyptus* spp. and *Salix* spp. The river at Site 4 was narrow and fast flowing, whereas at Site 5 it became broader with slower moving water.

Samples were collected every two weeks by means of on-site river baiting, where mesh bags containing *Rhododendron indicum* leaves were anchored in the river, similar to what has been done in other studies (Hwang *et al.* 2008; Hüberli *et al.* 2010). One bag with four leaves were used per sampling site. Leaf baits were collected after two weeks of exposure and sampling was made over the period of one year, from 2009 to 2010, in order to exclude any effect that seasonal variation might have on the presence of *Phytophthora* spp. Leaves were rinsed in distilled water and sections containing lesions were excised. These sections were surface disinfested in 70% ethanol for ten seconds, rinsed in distilled water and plated onto NARPH media (Hüberli *et al.* 2000). NARPH plates were incubated for three to five days at 22°C and any putative *Phytophthora* colonies were transferred to 10% V8 agar (V8A) (100mL Campbell's V8 juice, 3 g $CaCO_3$, 16 g agar, 900 mL distilled water). Cultures were maintained on V8A and corn meal agar (CMA, Sigma-Aldrich, Steinheim, Germany) at 25°C. Isolates included in the phylogenetic analyses are maintained in the culture collection (CMW) of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, South Africa.

2.2. DNA sequencing comparisons

For DNA sequence comparisons, DNA was extracted from all isolates that had been grown for two weeks on 10% V8A at room temperature. Mycelium was harvested by scraping the surface of cultures with a sterile scalpel blade and transferring this to 1.5 ml Eppendorf tubes. DNA was extracted using the protocol described by Myburg *et al*. (1999).

Polymerase chain reaction (PCR) was used to amplify two gene regions. The cytochrome oxidase c subunit I (*cox*I) mitochondrial gene was amplified for all isolates using primers FM84 and FM83 (Martin and Tooley 2003) to screen for various groupings or species. Additionally, the internal transcribed spacer (ITS) region of the ribosomal DNA (rDNA) was amplified for selected representative isolates in each group using the ITS6 and ITS4 primers (White *et al.* 1990; Cooke *et al.* 2000). PCR mixtures contained 1x PCR reaction buffer (Roche Diagnostics, Mannhein, Germany), 2 mM $MgCl_2$ (Roche Diagnostics, Mannhein, Germany), 2.5 units of FastStart *Taq* DNA polymerase (Roche Diagnostics, Mannhein, Germany), 200 µM of each dNTP, 0.45 µM of each primer, 2 µl template DNA (20-50 ng) and sterile water to a final volume of 25 µl, and were performed in a 2720 Thermal Cycler (Applied Biosystems, Foster City, California, USA). PCR conditions were the same as those used in previous studies for ITS (Cooke *et al.* 2000; Martin and Tooley 2003). All DNA and PCR samples were electrophoretically analyzed on a 1.5 % agarose gel using Gel Red (Biotium, Hayward, California, USA) as fluorescent dye and visualized under UV illumination.

PCR amplicons were sequenced in both directions, using the same primers used in PCR amplification. The BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used and 1/16th reactions were set up to a final volume of 10 µl. Sequencing reactions were run on a ABI PRISM® 3100 Genetic Analyser (Applied Biosystems, Foster City, California, USA). PCR and sequencing reactions were purified by sodium acetate and ethanol precipitation (Zeugin and Hartley 1985).

Forward and reverse sequence reads were combined in CLC Main Workbench 6.0 (CLC Bio, Aarhus, Denmark). Prior to phylogenetic analyses, sequences for both the *cox*I and ITS gene regions were used to verify the identities of the isolates as *Phytophthora* against data in GenBank (http://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool (BLAST). Additional sequences of closely related *Phytophthora* species (Table 1) were retrieved from GenBank and from previous studies (Jung and Burgess 2009; Scott *et al.* 2009; Jung *et al.* 2011) and were aligned with the sequences generated in this study using MAFFT (http://mafft.cbrc.jp/alignment/server/index.html) (Katoh *et al.* 2005).

Isolates obtained in this study grouped in Clade 2 and Clade 6 of the classification of Cooke (Cooke *et al.* 2000). The ITS and *cox*I data were not combined due to the difference in the mode of inheritance between nuclear and mitochondrial genes. Furthermore, the sequence data from clades 2 and 6 were compiled into two separate datasets and subjected to phylogenetic analyses. This was done due to the large number of taxa in each dataset. The shorter sequence lengths of many reference taxa in the Clade 2 *cox*I dataset would also have resulted in the truncation of the Clade 6 dataset if the two clades were combined. The outgroup taxon for both the ITS and *cox*I phylogenies was an isolate of *P. multivesiculata*. Although this species also resides in Clade 2, it was chosen as the outgroup taxon because it is a sister taxon to all other known Clade 2 species. The outgroup taxon for the Clade 6 ITS phylogeny was *P. cinnamomi* because of the proximity of Clade 7 to Clade 6 in some phylogenies, i.e. (Cooke *et al.* 2000). *P. nicotianae* was chosen as the outgroup for the Clade 6 *cox*I phylogeny because no sequences of *P. cinnamomi* spanning the whole *cox*I region used in this study, was available.

Maximum parsimony (MP) analysis was performed using Phylogenetic Analysis Using Parsimony (PAUP*) ver 4.0b10 (Swofford 2002). The most parsimonious phylogenetic trees were generated through a heuristic search whereby the initial tree was generated randomly by 100 stepwise additions of taxa and subsequent trees were generated using the tree bisection reconnection branch swapping algorithm. All characters were unordered and of equal weight and gaps in the alignments were regarded as fifth characters. A thousand

bootstrap replicates were performed to calculate branch and branch node support values (Felsenstein 1985).

Bayesian statistical inferences were used to generate phylogenetic trees and node support probability values through the Metropolis-coupled Monte Carlo Marcov Chain (MC^3) algorithm (Geyer 1991) to support results obtained through MP. Each locus was subjected to hierarchical Likelihood Ratio Tests (hLRT) using MrModeltest2.2 (Nylander 2004) to determine the optimal evolutionary model. Bayesian analyses were done using MrBayes 3.1 (Ronquist and Huelsenbeck 2003) and each analysis was run for 3,000,000 generations. Tracer 1.4 (Rambaut and Drummond 2003) was used to determine burn-in values prior to parameter and tree summarization.

3. Results

3.1. Sampling and isolation

A total of 102 *Phytophthora* isolates were retrieved from the five sites across the 12 month sampling period (Figure 2). The majority of the isolates obtained were sampled during the winter and spring months. The largest number of isolates were retrieved from Site 2, followed by both Site 3 and 4 (Table 2). The least isolates were retrieved from Site 1.

3.2. DNA sequencing comparisons

Four separate phylogenies were created in this study representing the analyses for Clade 2 and Clade 6 taxa (Table 3). All datasets had significant phylogenetic signal compared to random trees ($P<0.01$) (Hillis and Huelsenbeck 1992). Maximum parsimony analyses for the Clade 2 ITS dataset yielded 19 most parsimonious trees with a tree length of 133 steps (Figure 3) and that for the *cox*I dataset of Clade 2 resulted in 39 most parsimonious trees with tree length of 197 steps (Figure 4). Maximum parsimony analyses of the ITS dataset for Clade 6 isolates resulted in a single most parsimonious tree with a length of 285 steps (Figure 5) and 20 most parsimonious trees with a length of 469 steps for the *cox*I dataset (Figure 6). The various trees obtained for each analysis differed only in the length of the branches and by differences between the relationship of isolates within a species.

Species could be readily identified in the various analyses. In both the ITS (Figure 3) and *cox*I (Figure 4) phylogenies of the Clade 2 taxa, all currently recognized *Phytophthora* spp. formed clades well supported by bootstrap and posterior probability values. The ITS and *cox*I phylogenies differed in terms of the relationships between species, but both agreed on the identities of the Clade 2 isolates recovered in this study, namely *P. citrophthora* (CMW37890), *P. multivora* (CMW37891) and *P. plurivora* (CMW 37938). Similarly both the

ITS (Figure 5) and *cox*I (Figure 6) phylogenies of the Clade 6 taxa supported the known *Phytophthora* spp. with high bootstrap and posterior probability values, although the relationships between species again differed between the two gene regions.

The ITS phylogeny grouped isolates into three groups, which corresponded to two known *Phytophthora* spp. (*P.* taxon PgChlamydo and *P. lacustris*) and one group that is closely related to *P.* taxon asparagus. The *cox*I phylogeny grouped the isolates into five distinct clades corresponding to *P.* taxon PgChlamydo, *P. lacustris* and *P.* taxon asparagus-like. The additional groups were further identified as *P. amnicola* (CMW37727, CMW37728, CMW37729, CMW37730, CMW37942 and CMW37943) and *P. thermophila* (CMW37731, CMW37732, CMW37733, CMW37734, CMW37947, CMW37946, CMW37948, CMW37944 and CMW37945). These isolates could not be included in the ITS analyses because unusable data were generated for them (discussed below). Although the identities of all isolates were confirmed through phylogenetic analyses of the *cox*I locus, only a subset of isolates for each species are included in Figure 6.

A clade closely related to *P. asparagi* was present in both the ITS and *cox*I phylogenies of clade 6. These isolates consistently grouped closest to *P. asparagi* but always in a separate, well supported clade. This group differed from *P. asparagi* by 52-54 and 22-24 steps respectively in the *cox*I and ITS phylogenies. Given the large difference between this group and *P. asparagi,* it is regarded as a unique and previously unknown species referred to here as *P.* taxon Sisulu-river.

A large subset of isolates were not included in the ITS phylogeny because of an inability to generate usable ITS sequence data for them. Sequencing from either the forward or reverse direction resulted in unusable sequences after only approximately 200 bases. Additionally, abundant polymorphic sites were present in the useable segments of the ITS sequences. These isolates included those grouping together with *P. amnicola* and *P. thermophila* in the *cox*I phylogeny. Although the *cox*I phylogeny identified these two groups as *P. amnicola* and *P. thermophila* they were previously shown to be interspecific hybrids (Chapter 2 of this thesis). The hybrid with the *P. amnicola cox*I profile is a hybrid between *P. amnicola* and *P.* taxon PgChlamydo, refered to as A-PG and the hybrid with *P. thermophila cox*I profile is a hybrid of *P. thermophila* and *P. amnicola*, T-A.

The T-A and A-PG hybrids were the most frequently retrieved during sampling (Figure 2). Isolates of A-PG were recovered from all sampling sites, although most frequently from Site 2, during August to October 2009 and again from June to August 2010 (Figure 2). Isolates of

T-A were also recovered from all five sampling sites, but were most prevalent at Sites 3 and 4. They were encountered throughout the year but not in November 2009 or January and May 2010 (Figure 2). The next most frequently encountered species was *P. lacustris*, followed by *P.* taxon PgChlamydo and *P.* taxon Sisulu-river. Isolates of *P. lacustris* were recovered during September and October 2009 and June and August 2010 from all but Site 1. Isolates of *P.* taxon PgChlamydo were recovered during August 2009 and June to August 2010 from all but Site 5. Isolates of *P.* taxon Sisulu-river were retrieved from Site 2 and 3 during August, September and October of 2009 (Figure 2). *P. citrophthora*, *P. multivora* and *P. plurivora* were each isolated only once. These three isolates were retrieved from Site 2. The isolates of *P. multivora* and *P. plurivora* were retrieved during December 2009 and the isolate of *P. citrophthora* were recovered during March 2010.

4. Discussion

Numerous *Phytophthora* spp. were collected during a year-long baiting in the Crocodile river. Phylogenetic analyses revealed these to be mostly from Clade 6, but some Clade 2 species were also encountered. Eight *Phytophthora* spp. were identified including *P. citrophthora*, *P. multivora* and *P. plurivora* representing Clade 2 and two hybrid speices T-A and A-PG, *P. lacustris*, *P.* taxon PgChlamydo and *P.* taxon Sisulu-river from Clade 6. Other than for *P. citrophthora* and *P. multivora*, none of these species has been reported from South Africa. Additionally, a novel *Phytophthora* species was discovered, i.e. *P.* taxon Sisulu-river, which has a phylogenetic placement close to that of *P. asparagi.*

The five sampling sites differed in the number and identities of *Phytophthora* isolates recovered. The abundance of retrieved isolates at Site 2 may be explained by the presence of a complete foliar canopy at this site which decreases the direct solar radiation. This may also account for the scarcity of isolates retrieved from Site 1 that had no canopy. Additionally, the waterfall separating Site 1 and Site 2 most likely restricts the movement of *Phytophthora* spp. to Site 1. The paucity of isolates collected during summer and autumn (Figure 2) was not necessarily due to the absence of *Phytophthora.* A more plausible explanation is the high rate of lost baits due to the severe torrential downpours during the summer and autumn months.

The three species from Clade 2 were isolated only once in this study. *P. citrophthora* is found on all continents except Antarctica and has a very wide host range (Erwin and Ribeiro 1996). It is best known as the causal agent of gummosis of *Citrus* trees, was first identified in South Africa during the 1920's and has more recently been implicated in a trunk disease of clementines (*Citrus reticulata*) in the Western Cape Province (Schutte and Botha 2008). *P.*

multivora has been implicated in the decline of *Eucalyptus* spp., *Banksia* spp. and *Agonis* spp. in Australia (Scott *et al.* 2009) and has been isolated from diseased *Agathosma* spp. in South Africa (Bezuidenhout *et al.* 2010). *P. plurivora* is known from various European countries as well as from the USA where it occurs on a wide variety of hosts, including *Abies* spp., *Acer* spp. and *Quercus* spp. (Jung and Burgess 2009). *P. plurivora* has not previously been reported from South Africa. Due to the fact that *P. multivora* and *P. plurivora* have previously been identified as *P. citricola*, their global distribution is most likely larger than is currently reported. For the same reason, the distribution of these two species in South Africa might also be under estimated.

The identification of *P.* taxon Sisulu-river is an important discovery as it expands the currently known diversity of Clade 6 and especially of sub-clade III. *P. asparagi* and *P.* taxon sulawesiensis are the only other taxa in sub-clade III (Brasier *et al.* 2003; Jung *et al.* 2011). *P.* taxon Sisulu-river is more closely related to *P. asparagi* than to *P.* taxon sulawesiensis. *P. asparagi* is a well known species that causes spear and root rot of *Asparagus officinalis* in Australia, Europe, New Zealand and USA (Förster and Coffey 1993; Cunnington *et al.* 2005; Saude *et al.* 2008; Crous *et al.* 2012) as well as basal root rot of plants in the family Agavaceae in Australia (Cunnington *et al.* 2005). Little is known regarding *P.* taxon sulawesiensis, except that it was isolated from a declining clove (*Syzygium aromaticum*) tree from Sulawesi, Indonesia. The host range of *P.* taxon Sisulu-river is not known. Sub-clade III is phylogenetically far removed from the other Clade 6 taxa and might represent an undersampled sub-clade. Studies investigating *Phytophthora* spp. diversity from unsampled environments and regions seem likely to result in the discovery of additional sub-clade III species.

P. lacustris and *P.* taxon PgChlamydo are found in the United Kingdom, Europe, North America and Australia (Brasier *et al.* 2003; Reeser *et al.* 2011; Stukely 2012). Both these species appear to be strongly associated with aquatic habitats where they are thought to exist as saprotrophs on plant debris (Brasier *et al.* 2003; Nechwatal and Mendgen 2006). However, they also infect living plants, such as *Salix* spp. and *Fraxinus* sp. in the case of *P. lacustris* (Brasier *et al.* 2003; Orlikowski *et al.* 2011) and *Prunus* sp., *Rhododendron* sp. and *Taxus* sp. in the case of *P.* taxon PgChlamydo (Brasier *et al.* 2003; Schwingle *et al.* 2007). This study is the first to report these species from Africa.

The two hybrid species that were characterized in Chapter 2 were dominant in the sampled river. Isolates of T-A made up approximately 40% and those of A-PG 30% of the total isolates recovered in this study. Apart from the current study location, these hybrids have

been found only in Australia (Chapter 2 of this thesis). Nothing is currently known regarding their hosts or pathogenicity The parental species of these hybrids are thought to be *P. thermophila* and *P. amnicola* for T-A and *P. amnicola* and *P.* taxon PgChlamydo for A-PG (Chapter 2 of this thesis). *P. thermophila* is found only in Australia, where it mostly is associated with environmental samples from rivers and soil and rarely from roots of *Eucalyptus marginata* (Jung *et al.* 2011). Likewise, *P. amnicola* is known only in Australia where it was found in river water and from soil beneath a diseased *Patersonia* spp. plant (Crous *et al.* 2012).

The species identified from the Crocodile river in this study are different to those known from Gauteng Province. Several *Phytophthora* spp. have been reported from that area, but most were associated with diseases of agricultural crops such as *P. nicotianae* on tree lucern (*Chamaecytisus palmensis*) (Botha 1993) and tomato (*Solanum lycopersicum*) (Ferreira *et al.* 1991), *P. capsici* on pumpkins (*Cucurbita* spp.) and paprika (*Capsicum annuum*) (Labuschagne *et al.* 2000; Meitz *et al.* 2010) and *P. medicaginis* and *P. dreschleri* on lucerne (*Medicago sativa*) (Thompson 1987). Additionally, several *Phytophthora* spp. have been associated with diseases of exotic ornamental plants, i.e. *P. cactorum* causing wilt on snapdragon (*Antirrhinum majus*) (Mes 1934) and carnation (*Dianthus caryophyllus*) (Wijers 1937) and *P. nicotianae* causing wilt of baby's breath (*Gypsophila paniculata*) (Thompson and Naudé 1992) in South Africa. None of these *Phytophthora* spp. were detected in the present study, which is not surprising considering the differences in the environment in which they occurred and the many years between the sampling events.

This is the first study to consider *Phytophthora* species diversity in rivers outside the Western Cape Province of South Africa. None of the species reported from the Western Cape previously (Von Broembsen 1989; Bezuidenhout *et al.* 2010) were recovered in the present study. However, it is difficult to contrast the species diversity found here with that reported from the Western Cape Province as those studies were done more than 20 years ago, in a very different ecosystem far removed from the location of the current study and at a time when the taxonomy of *Phythophthora* spp. had not benefited from the power of DNA sequence comparisons.

This study was conducted in a relatively small geographic area, at the headwaters of the Crocodile River. Even so, an unexpectedly large *Phytophthora* diversity was found, including two interspecific hybrids, one new taxon and several *Phytophthora* species previously unknown in South Africa. Additional surveys of rivers in the poorly studied areas and ecosystems of South Africa will surely result in greater numbers of *Phytophthora* spp. being

discovered. This would increase the knowledge regarding the diversity and distribution of *Phytophthora* in South Africa. Furthermore, the plant hosts of these species in this region are not known, neither whether any of the *Phytophthora* spp. are pathogenic to any of the plant species in this area. The species identified here represent a potential threat to cultivated and natural plants in South Africa and further effort and vigilance will be required to ascertain their role as pathogens. Clearly, expanded surveys and studies should be undertaken in order to address these questions.

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5. References

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Table 1: *Phytophthora* spp. information and GenBank accesion numbers for isolates used in the phylogenetic analyses

Reference collection number[1]	Other collection numbers	Identity[2]	Host	Location	Date	Genbank accesion number	
						ITS	*cox*I
CBS131652	DH228	*P. amnicola*	Stream baiting	Lake Jualbup, Australia	2009	JQ029956	JQ029948
VHS19503		*P. amnicola*	*Patersonia* sp.	Pemberton, Australia	2008	JQ029958	JQ029956
VHS17175		*P. asparagi*	*Banksia media*	Esperance, Australia	2007	EU301167	HQ012844
VHS17644		*P. asparagi*	*Lomandra sonderi*	Murdoch, Australia	2007	EU301168	HQ012845
	P10690	*P. asparagi*	*Asparagus officinalis*	Whakatane, New Zealand	1986	FJ801481	
CBS 581.69	IMI136915	*P. botryosa*	*Hevea brasiliensis*	Perlis, Western Malaysia	1966	AF266784	
P6945	WPC6945A252	*P. botryosa*	*Hevea brasiliensis*	Langkawi, Malaysia	1986	FJ801954	
CBS 533.92		*P. botryosa*	*Hevea brasiliensis*	Chantaburi, Thailand	1967		AY564166
P1819		*P. capensis*	*Curtisia dentata*	South Africa		GU191232	GU191275
P1822		*P. capensis*	Stream water	South Africa		GU191219	GU191277
IMI352321		*P. capsici*	*Piper nigrum*	India	1989	AF266787	
CBS 128.23		*P. capsici*	*Capsicum annuum*		1923	DQ464056	
	302	*P. capsici*	*Capsicum annuum*	Florida, USA			AY129166
	P6490	*P. cinnamomi*	*Persea americana*	Florida, USA	1989	AY302181	
CBS295.29		*P. citricola*	*Citrus* sp.	Tankan, Japan	1929	FJ560913	FJ665244
	CIT-US1	*P. citricola*	*Fagus sylvatica*	New York State, USA	2003	FJ665234	FJ665242
IMI332632		*P. citrophthora*	*Actinidia chinensis*	Chile		AF266785	
	3BO	*P. citrophthora*	*Buxus* spp.	Italy		FJ874794	HM752780
P10785	WPC10785A1229	*P. citrophthora*				GU259187	
CBS 274.33		*P. citrophthora*	*Citrus limonium*	Cyprus	1933		AY564171
	PCRh7.1	*P. citrophthora*					HM752781
IMI368918		*P. colocasiae*	*Colocasia esculenta*	Malaysia	1995	AF266786	
P7180	WPC7180B269	*P. colocasiae*	*Colocasia esculenta*	Hawaii	1990	GU259000	
	345	*P. colocasiae*	*Colocasia esculenta*	China			AY129173
CBS129424	DH086	*P. fluvialis*	Stream baiting	Moore River, Australia	2009	JF701436	JF701442
VHS17350		*P. fluvialis*	Stream baiting	Badgingarra, Australia	2007	EU593261	JF701440
VHS21998		*P. gibosa*	*Acacia pycnantha*	Scott River, Australia	2009	HQ012933	HQ12846
VHS21999		*P. gibosa*	*Xanthorrhoea gracilis*	Scott River, Australia	2009	HQ012934	HQ12847
MUCC776	TAS35	*P. gonapodyides*	Stream baiting	Tasmania	2009		JN547642
MUCC761	SLPA72	*P. gonapodyides*	*Eucalyptus obliqua* forest	Toolangi North State Forest, Australia	2008		HQ012850
IMI389723	P1047	*P. gonapodyides*	*Quercus robur*	Bienwald, Rhineland, Germany	1996	AF541889	

Reference collection number[1]	Other collection numbers	Identity[2]	Host	Location	Date	Genbank accesion number	
						ITS	*cox*I
IMI389727	P897	*P. gonapodyides*	Native forest	Pine Lake, Tasmania	1996	AF541888	
DCE68		*P. gregata*	Native vegetation	Byford, Australia	1965	EU301171	HQ12851
MJS235		*P. gregata*	*Pinus radiata*	Nannup, Australia	1982	EU301172	HQ12853
	NP44	*P. himalsilva*	Soil	Nepal	2005	HM752784	HM752796
	NP60	*P. himalsilva*	Soil	Nepal	2005	HM752785	HM752797
CBS200.81	IMI302303	*P. humicola*	*Citrus* sp.	Taiwan	1981	AF266792	
	P6701	*P. humicola*	*Citrus* sp.	Taiwan	1989	AB367496	
VHS19081		*P. inundata*	*Banksia attenuata*	Bold Park Australia	2008	HQ012945	HQ012861
VHS16836		*P. inundata*	*Xanthorrhoea preissii*	Boyup Brook, Australia	2007	HQ012944	HQ012860
VHS17085		*P. litoralis*	*Banksia* sp.	Hopetoun, Australia	2007	EU593262	HQ012864
CBS127953	VHS20763	*P. litoralis*	*Banksia* sp.	Ravensthorpe, Australia	2008	HQ012948	HQ012866
CBS 219.88		*P. meadii*	*Hevea brasiliensis*	Kottayam, India	1987		AY564192
P6262	WPC6262A424	*P. meadii*	*Hevea brasiliensis*	India	1989	FJ802096	
P7493	WPC7493D1329	*P. meadii*	*Hevea brasiliensis*	India	1988	GU259352	
DDS3432		*P. megasperma*	*Banksia* sp.	North Dinninup, Australia	1992	HQ012949	HQ012867
VHS17183		*P. megasperma*	*Xanthorrhoea platyphylla*	Esperance, Australia	2007	EU301166	HQ012868
CBS 545.96	PD 95/8679	*P. multivesiculata*	*Cymbidium* sp.	Mijdrecht, Netherlands	1998	DQ335639	AY564195
WAC13201		*P. multivora*	*Eucalyptus marginata*	Yalgorup, Australia		FJ237521	FJ237508
WAC13200		*P. multivora*	*Eucalyptus gomphocephala*	Yalgorup, Australia		FJ237522	FJ237509
WAC13204		*P. multivora*	*Eucalyptus gomphocephala*	Yalgorup, Australia		FJ237518	FJ237507
	332	*P. nicotianae*	*Nicotiana tabacum*	Australia			AY129169
CMW26667		*P. pinifolia*	*Pinus radiata*	Llico plantation, Arauco, Chile	2007	EU725805	
CMW26668		*P. pinifolia*	*Pinus radiata*	Llico plantation, Arauco, Chile	2007	EU725806	GU799673
CBS 124090		*P. plurivora*	*Quercus petraea*, soil	Ljubljana, Slovenia	1995	FJ237524	FJ237511
CBS 124093		*P. plurivora*	*Fagus sylvatica*, root lesion	Irschenberg, Germany	2004	FJ665225	FJ665236
CBS 124087		*P. plurivora*	*Quercus robur*, soil	Pulling, Germany	1994	FJ237523	FJ237510
	WA5-030403	*P.* siskiyouensis	Stream water	Oregon, USA	2003	EF523386	
VHS14081		*P.* sp. Personii	*Grevillea mccutcheonii*	Busselton, Australia	2005	EU301169	HQ012877
	STE-U6269	*P.* taxon emzansi	*Agathosma betulina*	South Africa		GU191228	GU191270
	STE-U6272	*P.* taxon emzansi	*Agathosma betulina*	South Africa		GU191220	GU191269
IMI 389747	P1054	*P.* taxon forestsoil	Native forest	Illwald forest, Alsace, France	1998	AF541908	
	UASWS0315	*P.* taxon forestsoil	*Alnus glutinosa*	Kolo, Poland	2006	EF522138	

Reference collection number[1]	Other collection numbers	Identity[2]	Host	Location	Date	Genbank accesion number	
						ITS	*cox*I
	UASWS0321	*P.* taxon hungarica	*Alnus glutinosa*	Adamowizna, Poland	2006	EF522144	
	UASWS0318	*P.* taxon hungarica	*Alnus glutinosa*	Kolo, Poland	2006	EF522141	
IMI 389733	P1055	*P.* taxon oaksoil	*Quercus robur*	Illwald forest, Alsace, France	1998	DQ528749	
	SPLA166	*P.* taxon paludosa	Pond baiting, native forest	Sugarloaf Reservoir Reserve, Australia	2008	HQ012953	HQ12876
VHS6595		*P.* taxon PgChlamydo	Native forest	Manjimup, Australia	1999	EU301159	HQ012879
VHS3753	DDS3753	*P.* taxon PgChlamydo	Native forest	Manjimup, Australia	1995	EU301160	HQ012878
IMI 389731	P510	*P.* taxon PgChlamydo	*Pseudotsuga* sp.	Walley, British Columbia, Canada	1984	AF541902	
	RAS1	*P.* taxon raspberry	*Betula pendula*	Germany, Bavaria, Neuburg	2006		HQ12888
	P1044	*P.* taxon riversoil	Riparian vegetation	Riverbank, Worcestershire, UK	1997	DQ648139	
	HSA1959	*P. lacustris*	Road drainage sump baiting	Welshpool, Australia	1994	HQ012956	HQ012880
IMI389725	P245	*P. lacustris*	*Salix matsundana*	Bexley Heath, Kent, UK	1972	AF266793	JQ626633
IMI 389726	P878	*P. lacustris*	*Alnus* sp.	Odense, Denmark, Funen	1995	AF541909	
	P6306	*P.* taxon sulawesiensis	*Syzygium aromaticum*	Sulawesi, Indonesia	1989	FJ801912	
VHS7474		*P. thermophila*	Native forest	Manjimup, Australia	2000	HQ012952	HQ012871
CBS127954	VHS13530	*P. thermophila*	*E. marginata*	Dwellingup, Australia	2004	EU301155	HQ012872
	P4742	*P. tropicalis*	*Allamanda* sp.	Spain	2004	AY946257	
	PD97/11132	*P. tropicalis*	*Rosa* sp.	Netherlands	1997		AY564161
CMW37727	J 2.2 C	A-PG	Stream water	Crocodile river, Roodepoort, South Africa	2009		JQ890348
CMW37728	J 2.4 A1	A-PG	Stream water	Crocodile river, Roodepoort, South Africa	2009		JQ890349
CMW37729	J 2.23 A	A-PG	Stream water	Crocodile river, Roodepoort, South Africa	2010		JQ890350
CMW37730	J 2.24 A	A-PG	Stream water	Crocodile river, Roodepoort, South Africa	2010		JQ890351
CMW37942	J 2.8 D	A-PG	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272329
CMW37943	J 2.9 A	A-PG	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272330
CMW37731	J 1.3 A1	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2009		JQ890352
CMW37732	J 4.2 d	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2009		JQ890353
CMW37733	J 4.9 A	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2010		JQ890354
CMW37734	J 5.11 C	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2010		JQ890355
CMW37947	J 3.15 B	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2010		JX272330
CMW37946	J 2.3 A3	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272330
CMW37948	J 3.8 A	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272330

Reference collection number[1]	Other collection numbers	Identity[2]	Host	Location	Date	Genbank accesion number	
						ITS	*cox*I
CMW37944	J 3.13 D	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2010		JX272330
CMW37945	J 4.2 C	T-A	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272330
CMW37889	J 3.6 B	*P.* taxon Sisulu-river	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272355	JX272336
CMW37937	J 2.1 A	*P.* taxon Sisulu-river	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272337
CMW37995	J 2.10 A	*P.* taxon Sisulu-river	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272356	JX272338
CMW37996	J 2.14 A	*P.* taxon Sisulu-river	Stream water	Crocodile river, Roodepoort, South Africa	2009		JX272339
CMW37939	J 4.2 B	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272357	JX272340
CMW37998	J 4.8 A	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272363	JX272346
CMW37902	J 4.16 A	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272358	JX272341
CMW37896	J 4.16 B	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272359	JX272342
CMW37999	J 5.1 E	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272364	JX272347
CMW37897	J 5.5 A1	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272360	JX272343
CMW37898	J 5.5 A2	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272361	JX272344
CMW37941	J 5.13 B	*P. lacustris*	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272362	JX272345
CMW37892	J 2.5 A	*P.* taxon PgChlamydo	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272365	JX272348
CMW37893	J 2.25 B	*P.* taxon PgChlamydo	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272366	JX272349
CMW37894	J 2.27 B	*P.* taxon PgChlamydo	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272367	JX272350
CMW37997	J 4.13 A	*P.* taxon PgChlamydo	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272368	JX272351
CMW37890	J 2.19 A	*P.* citrophthora	Stream water	Crocodile river, Roodepoort, South Africa	2010	JX272354	JX272327
CMW37891	J 2.16 B	*P.* multivora	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272353	JX272326
CMW37938	J 2.17 C	*P.* plurivora	Stream water	Crocodile river, Roodepoort, South Africa	2009	JX272352	JX272328

[1] Abbreviations for culture collections: CBS = Centraalbureau voor Schimmelcultures Utrecht, Netherlands; CMW = culture collection of the Forestry and Agricultural Biotechnology Institute (FABI); IMI = CABI Bioscience (International Mycological Institute), UK; VHS = Vegetation Health Service Collection, Department of Environment and Conservation, Perth, Australia; DDS = earlier prefix of VHS Collection; MJS = cultures of MJC Stukely, in VHS Collection; DCE = cultures of EM Davison, in VHS Collection; MUCC = Murdoch University Culture Collection; P = World *Phytophthora* collection, University of California, USA

[2] Hybrid identity (maternal parent first) as determined in Chapter 2: A-PG = *P. amnicola* × *P.* taxon PgChlamydo, T-A = *P. thermophila* × *P. amnicola*

Table 2: Sampling of *Phytophthora* spp. from five sites along the Crocodile River.

	T-A[1]	A-PG[1]	*P. lacustris*	*P.* taxon PgChlamydo	*P.* taxon Sisulu-river	*P. citrophthora*	*P. multivora*	*P. plurivora*	Total
site 1	2	2	0	2	0	0	0	0	6
site 2	5	18	1	4	4	1	1	1	35
site 3	13	6	1	1	1	0	0	0	22
site 4	12	3	5	2	0	0	0	0	22
site 5	9	1	7	0	0	0	0	0	17
Total	41	30	14	9	5	1	1	1	102

[1] Hybrid identity (maternal parent first) as determined in Chapter 2: A-PG = *P. amnicola* × *P.* taxon PgChlamydo , T-A = *P. thermophila* × *P. amnicola*

Table 3: Summary of statistics from phylogenetic trees generated in this study

Clade	Locus	Alignment length	Parsimony informative characters	Phylogenetic signal (g1)	Tree length	Consistency Index	Retention Index	Model used for Bayesian analysis
Clade 2	ITS	820	86	-0.44	133	0.79	0.95	GTR+G
	*cox*I	742	82	-0.52	197	0.57	0.78	GTR+I+G
Clade 6	ITS	933	153	-0.95	285	0.69	0.90	GTR+I+G
	*cox*I	1211	170	-0.52	469	0.52	0.88	GTR+I+G

Figure 1. Geographic area where the stream baiting for *Phytophthora* spp. were conducted with the five sampling sites indicated. The insert is an outline of the Gauteng province and indicated in grey are the Johannesburg and Pretoria metropoles, with the yellow dot indicating the location of the sampling area.

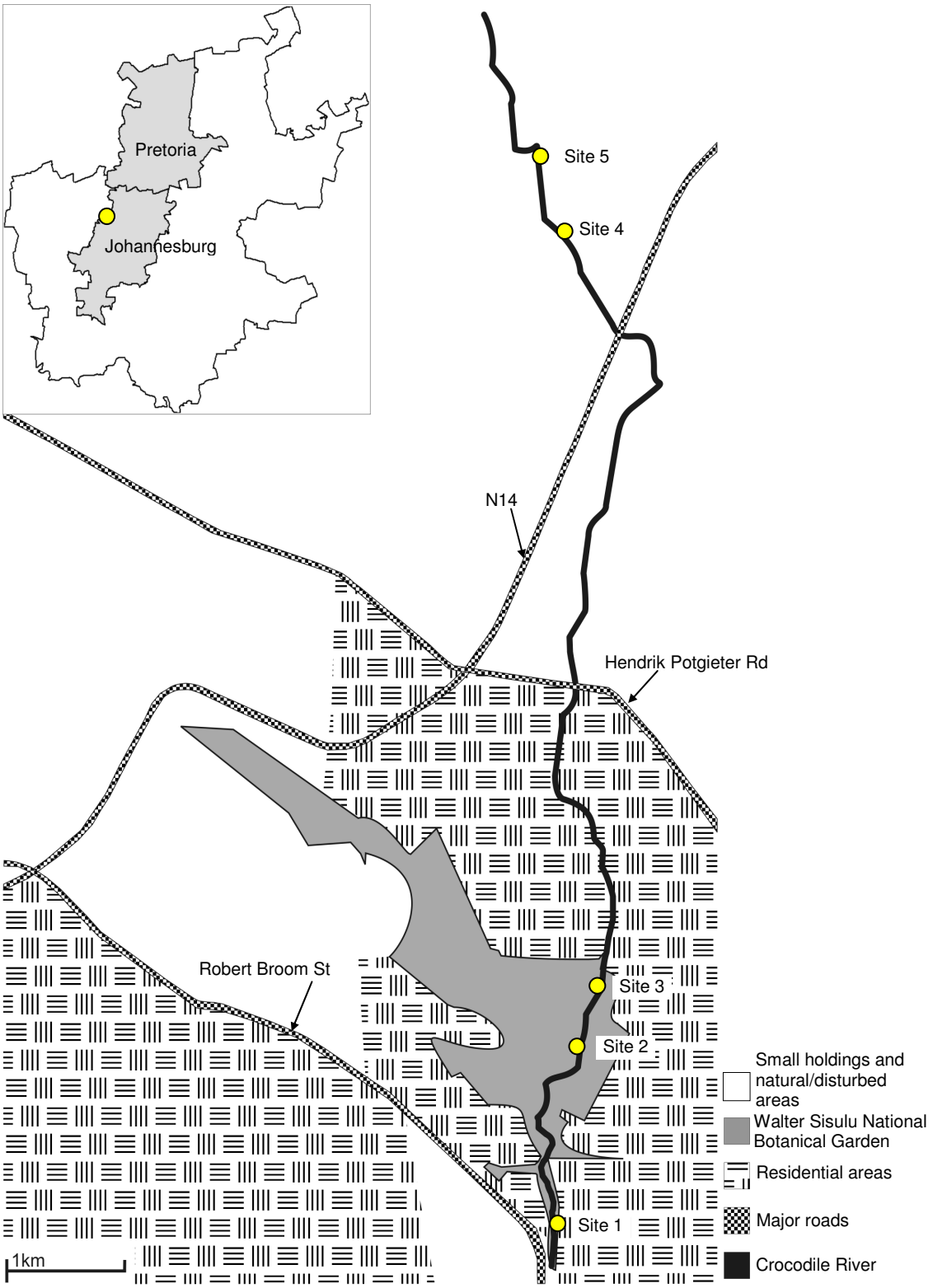

Pretoria
Johannesburg
Site 5
Site 4
N14
Hendrik Potgieter Rd
Robert Broom St
Site 3
Site 2
Site 1
1km
Small holdings and natural/disturbed areas
Walter Sisulu National Botanical Garden
Residential areas
Major roads
Crocodile River

Figure 2. Number of *Phytophthora* isolates retrieved from five sites of the Crocodile river in South Africa over the year long sampling period during 2009 to 2010. The total bar length represents the total number of *Phytophthora* isolated during a specific month and the different colours within a bar relate to the abundance of each species. The total number of isolates per species is indicated within parentheses in the legend. The isolates abbreviated as T-A and A-PG represent interspesific hybrids between *P. thermophila* and *P. amnicola* and between *P. amnicola* and *P.* taxon PgChlamydo, respectively.

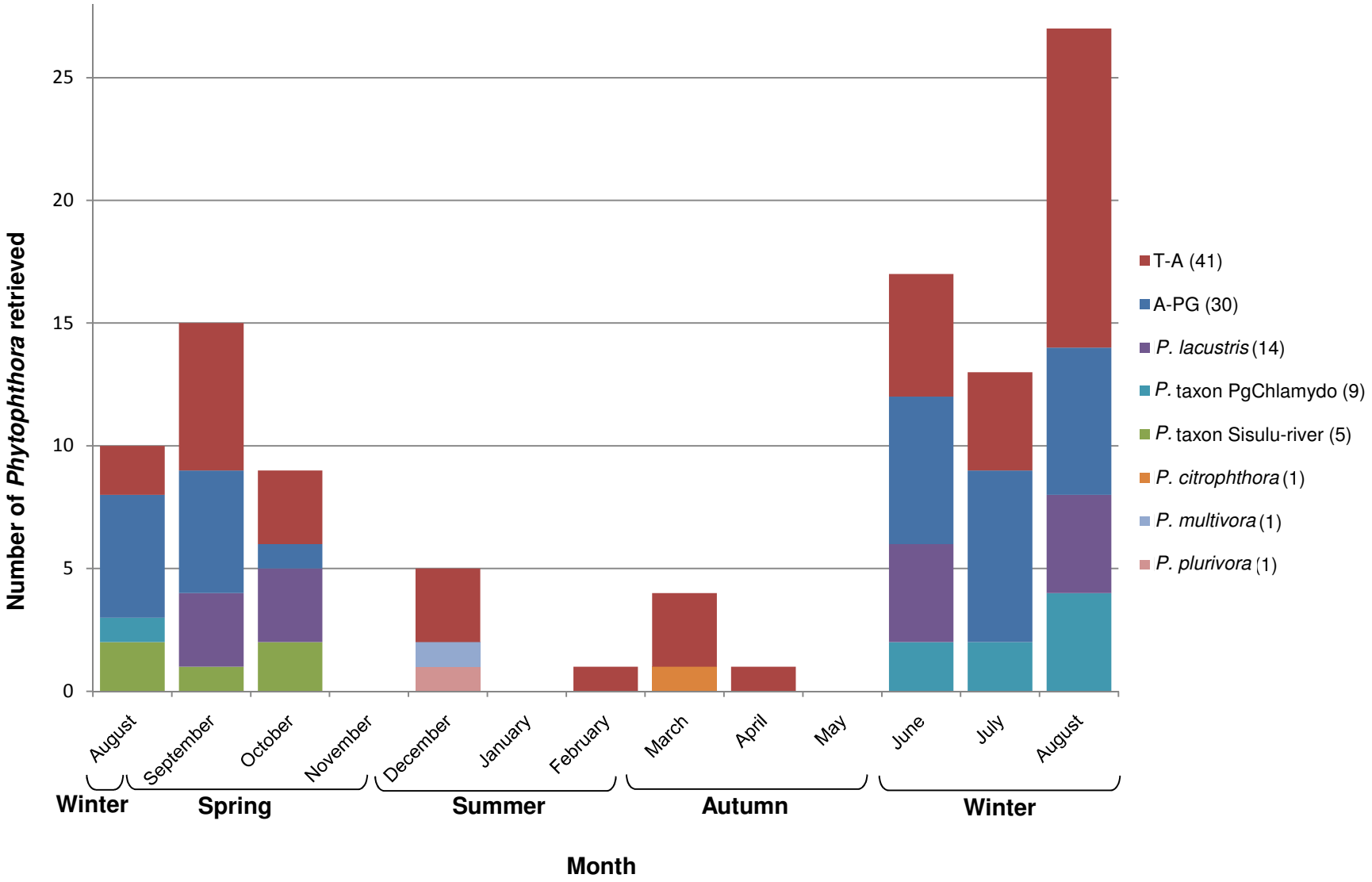

Number of Phytophthora retrieved
25
20
15
10
5
0
August
September
October
November
December
January
February
March
April
May
June
July
August
Winter
Spring
Summer
Autumn
Winter
Month
T-A (41)
A-PG (30)
P. lacustris (14)
P. taxon PgChlamydo (9)
P. taxon Sisulu-river (5)
P. citrophthora (1)
P. multivora (1)
P. plurivora (1)

Figure 3. Phylogenetic tree based on the ITS locus of the *Phytophthora* Clade 2 generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. This tree is rooted using *Phytophthora multivesiculata* as the outgroup. Isolate sequences generated in this study are indicated in bold.

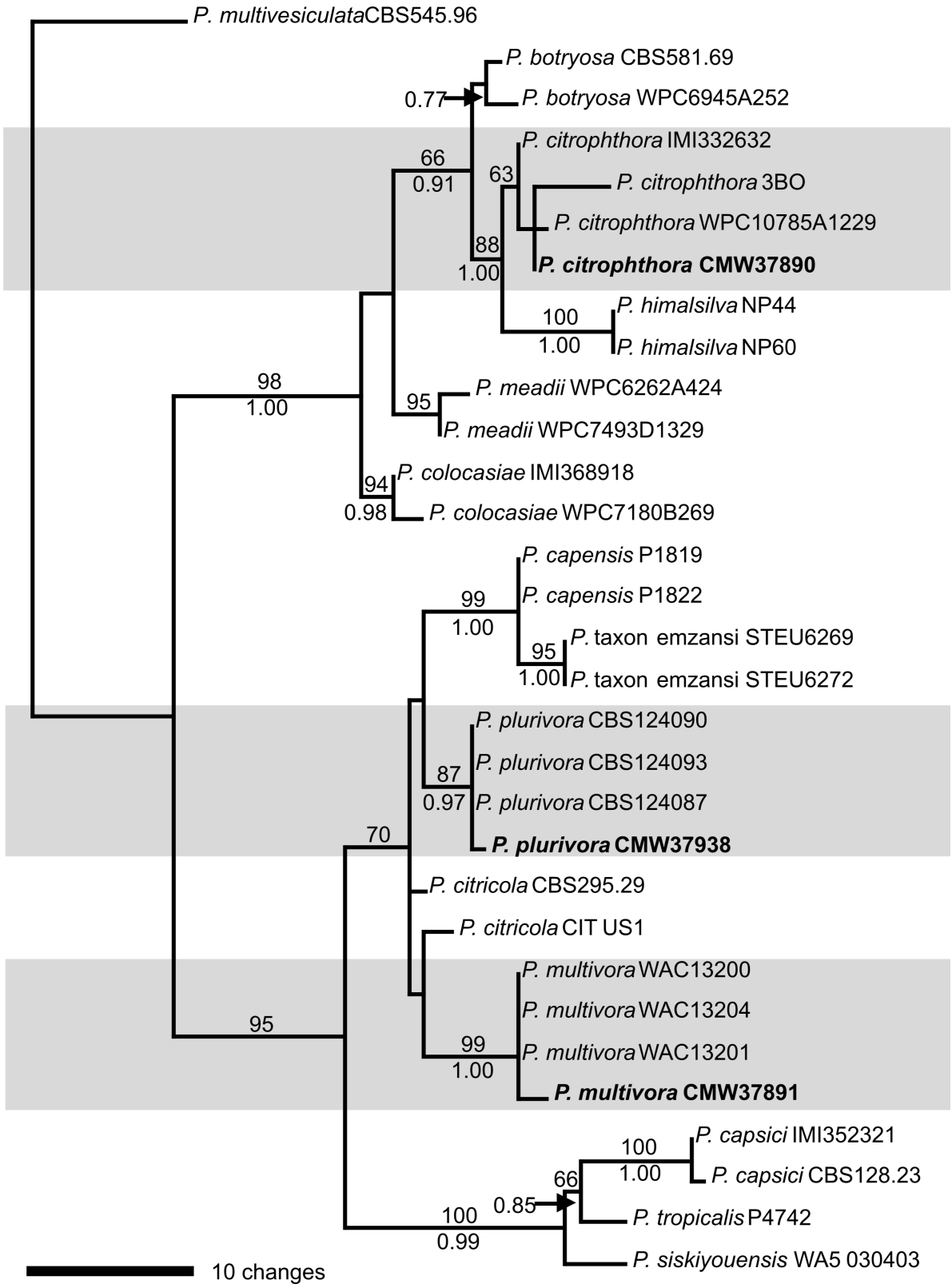


Figure 4. Phylogenetic tree based on the *cox*I locus of the *Phytophthora* Clade 2 generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. This tree is rooted using *Phytophthora multivesiculata* as the outgroup. Isolate sequences generated in this study are indicated in bold.

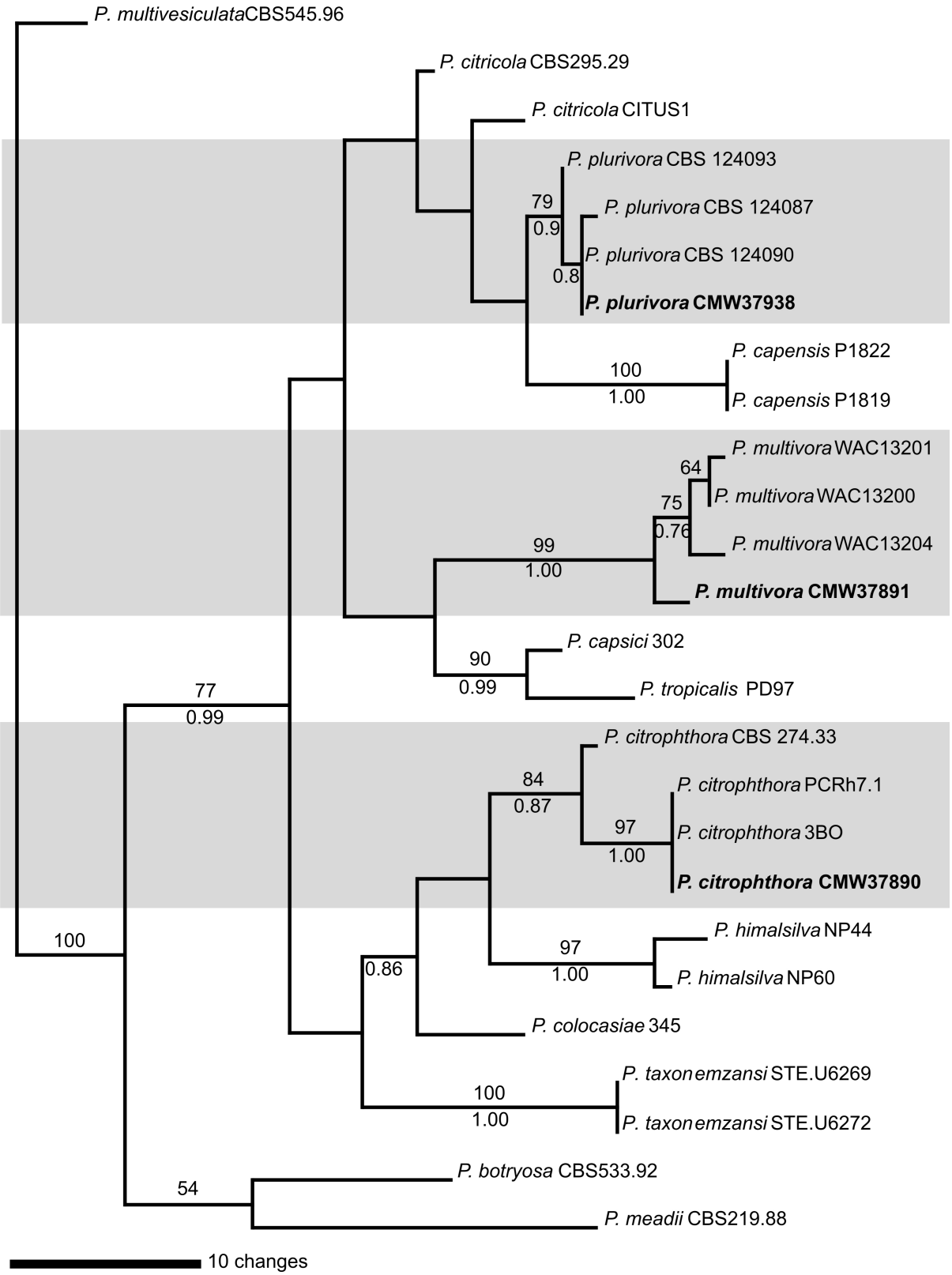

P. multivesiculataCBS545.96
P. citricola CBS295.29
P. citricola CITUS1
P. plurivora CBS 124093
79
0.9
P. plurivora CBS 124087
P. plurivora CBS 124090
0.8
P. plurivora CMW37938
P. capensis P1822
100
1.00
P. capensis P1819
P. multivora WAC13201
64
P. multivora WAC13200
75
0.76
P. multivora WAC13204
99
1.00
P. multivora CMW37891
P. capsici 302
90
0.99
P. tropicalis PD97
77
0.99
P. citrophthora CBS 274.33
84
0.87
P. citrophthora PCRh7.1
97
1.00
P. citrophthora 3BO
P. citrophthora CMW37890
100
P. himalsilva NP44
97
1.00
P. himalsilva NP60
0.86
P. colocasiae 345
P. taxon emzansi STE.U6269
100
1.00
P. taxon emzansi STE.U6272
P. botryosa CBS533.92
54
P. meadii CBS219.88
10 changes

Figure 5. Phylogenetic tree based on the ITS locus of the *Phytophthora* Clade 6 generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. This tree is rooted using *Phytophthora cinnamomi* as the outgroup. Isolate sequences generated in this study are indicated in bold.

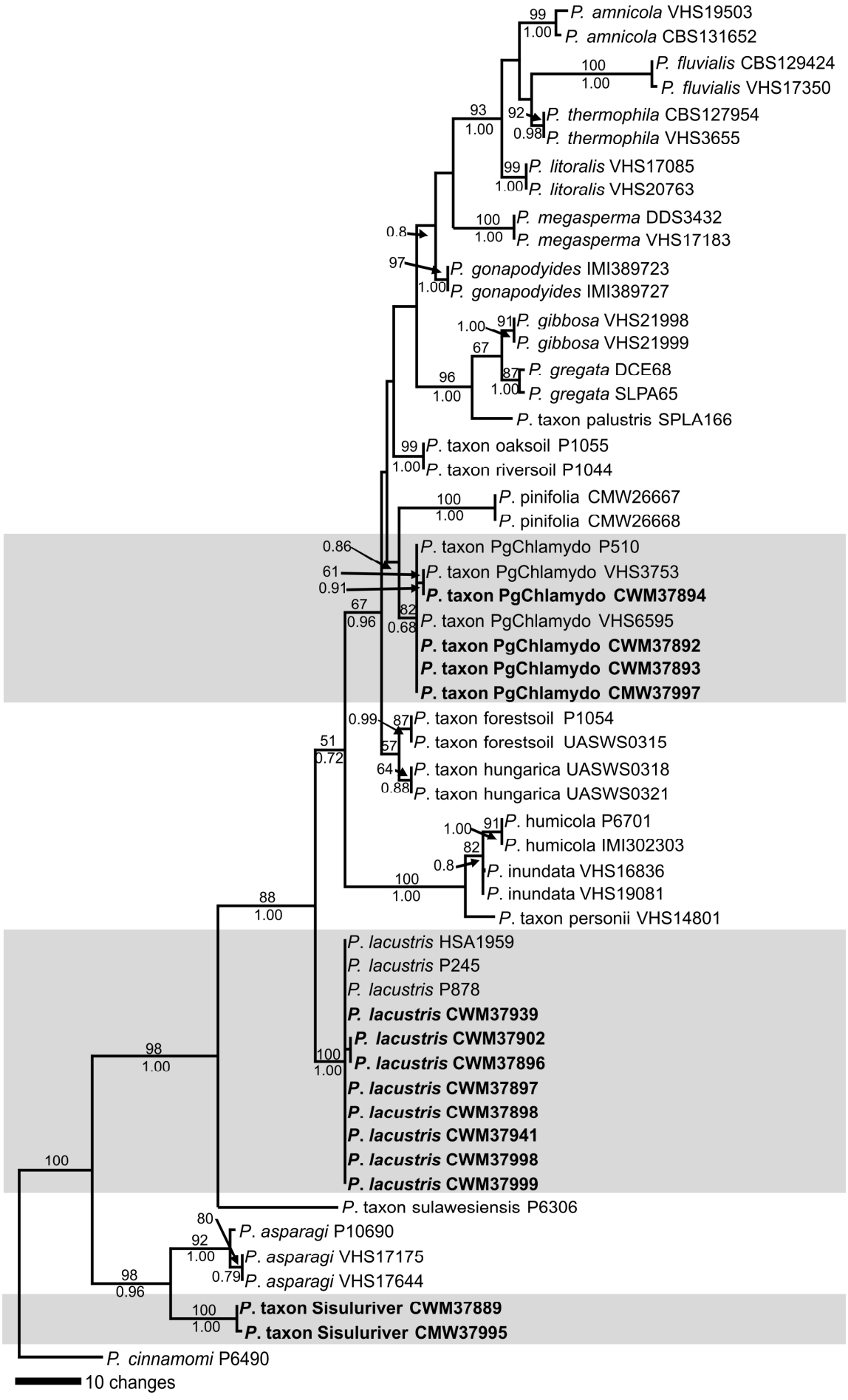
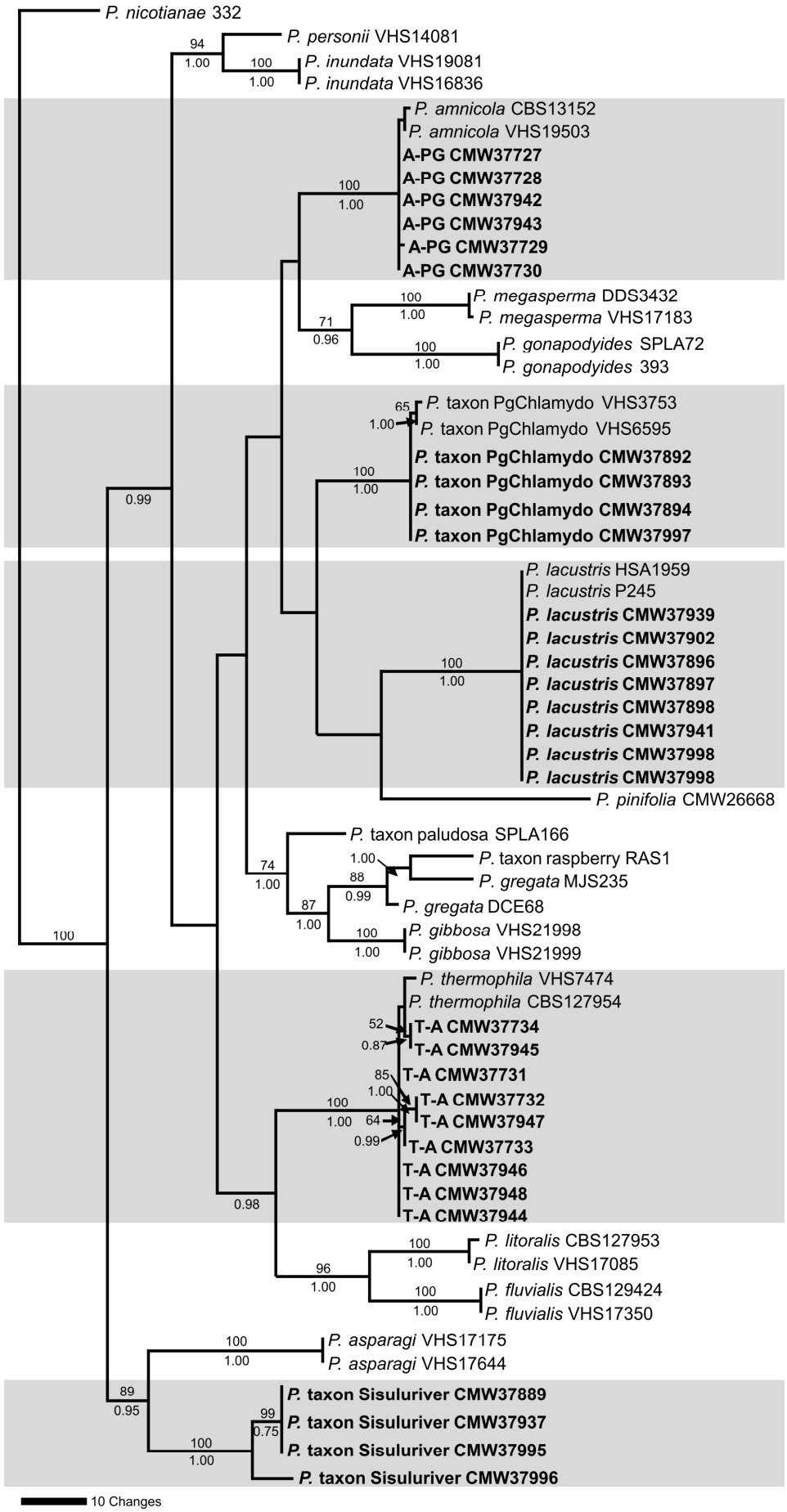


Figure 6. Phylogenetic tree based on the *cox*I locus of the *Phytophthora* Clade 6 generated by a maximum parsimony heuristic search. Bootstrap support values appear above and posterior probabilities below branches. This tree is rooted using *Phytophthora nicotianae* as the outgroup. Isolate sequences generated in this study are indicated in bold.


P. nicotianae 332
P. personii VHS14081
P. inundata VHS19081
P. inundata VHS16836
P. amnicola CBS13152
P. amnicola VHS19503
A-PG CMW37727
A-PG CMW37728
A-PG CMW37942
A-PG CMW37943
A-PG CMW37729
A-PG CMW37730
P. megasperma DDS3432
P. megasperma VHS17183
P. gonapodyides SPLA72
P. gonapodyides 393
P. taxon PgChlamydo VHS3753
P. taxon PgChlamydo VHS6595
P. taxon PgChlamydo CMW37892
P. taxon PgChlamydo CMW37893
P. taxon PgChlamydo CMW37894
P. taxon PgChlamydo CMW37997
P. lacustris HSA1959
P. lacustris P245
P. lacustris CMW37939
P. lacustris CMW37902
P. lacustris CMW37896
P. lacustris CMW37897
P. lacustris CMW37898
P. lacustris CMW37941
P. lacustris CMW37998
P. lacustris CMW37998
P. pinifolia CMW26668
P. taxon paludosa SPLA166
P. taxon raspberry RAS1
P. gregata MJS235
P. gregata DCE68
P. gibbosa VHS21998
P. gibbosa VHS21999
P. thermophila VHS7474
P. thermophila CBS127954
T-A CMW37734
T-A CMW37945
T-A CMW37731
T-A CMW37732
T-A CMW37947
T-A CMW37733
T-A CMW37946
T-A CMW37948
T-A CMW37944
P. litoralis CBS127953
P. litoralis VHS17085
P. fluvialis CBS129424
P. fluvialis VHS17350
P. asparagi VHS17175
P. asparagi VHS17644
P. taxon Sisuluriver CMW37889
P. taxon Sisuluriver CMW37937
P. taxon Sisuluriver CMW37995
P. taxon Sisuluriver CMW37996
10 Changes

Chapter 4

The occurrence and impact of *Phytophthora* on the African continent

A condensed version of this review is in press as: Nagel JH, Gryzenhout M, Slippers B and Wingfield MJ. (2012). The occurrence and impact of *Phytophthora* on the African continent. In *Phytophthora*: A Global Perspective, ed. KH Lamour. CABI, UK.

Abstract

Surprisingly little is known about the diversity of *Phytophthora* on the African continent. *Phytophthora* spp. causing diseases of economically important crops have received considerable attention in Africa. Late blight, caused by *P. infestans*, occurs throughout the African continent and is the most devastating disease of potato and tomato crops. Africa is the world's largest cacao grower, but production is severely affected by black pod disease caused by *P. megakarya* and *P. palmivora*. Many crops in Africa are affected by root rot and damping off caused by *P. cinnamomi*, but avocado trees are the most severely affected. In South Africa, root rot of avocado is especially severe due to climatic conditions. Several *Phytophthora* spp. are important pathogens of plantation forestry tree species in Africa. Black butt disease is a serious disease of black wattle trees caused by several *Phytophthora* spp. Eucalypt and pine trees also often suffer from *Phytophthora* root rot in plantations and in nurseries. The diversity of *Phytophthora* spp. occurring on indigenous plants and in native environments in Africa is poorly studied. Numerous plant species from the Fynbos vegetation type suffers from a devastating die-back caused by *P. cinnamomi* in the Western Cape of South Africa. Several other *Phytophthora* spp. are also known from Fynbos. There is a great need for more investigation into the potentially large undiscovered *Phytophthora* spp. diversity of Africa.

1. Introduction

Phytophthora species are responsible for some of the most important plant diseases in the world. These diseases are most apparent on economically important plants where they result in lost productivity. Consequently, the best known *Phytophthora* spp. have been responsible for severe diseases of a variety of crops (Erwin and Ribeiro 1996) and commercially propagated forest trees (Davison and Bumbieris 1973; Linde *et al.* 1994b; Harrington and Wingfield 1998; Wingfield *et al.* 2008). Substantial attention has also been given to situations where *Phytophthora* spp. exist in and cause damage to native habitats (McDougall *et al.* 2002; Garbelotto *et al.* 2003; Shearer *et al.* 2004). Given the importance of *Phytophthora* diseases elsewhere in the world, it is surprising that very little attention has been paid to these pathogens on the African continent.

It has recently been proposed that the number of extant *Phytophthora* species might be up to ten times greater than the number of species described prior to the year 2000 (Brasier 2009). This view arose from the increasing pace at which new *Phytophthora* spp. are being described as well as fungal diversity estimations (Hawksworth and Rossman 1997; Crous *et al.* 2006) presenting the view that less than 10% of the world's total fungal diversity has been described. These fungal diversity estimates are linked to the biodiversity of vascular plants. In this regard, Africa has exceptionally high numbers of endemic plant species with a remarkable diversity (Kier and Barthlott 2001). Given the host dependant nature of *Phytophthora* species, Africa's extensive plant biodiversity and suitable habitat, it is possible to assume that Africa harbours a large albeit undiscovered *Phytophthora* species diversity. Furthermore, the natural plant diversity of the continent could be damaged by the accidental introduction of non-native *Phytophthora* spp.

Surprisingly little is known regarding the occurrence and diversity of *Phytophthora* species in African countries other than South Africa. Twenty-three species of *Phytophthora* have been reported from Africa (Table 1), of which twenty are known from South Africa and only nine species are known from the rest of Africa. In Africa, *Phytophthora* spp. cause several diseases of cultivated plant species and particularly agronomic crop plants. In addition, some *Phytophthora* spp. are known to occur in native ecosystems. Here we review the occurrence, importance and potential threats of *Phytophthora* spp. to agriculture and natural ecosystems in Africa.

2. *Phytophthora* in agriculture

Africa is a continent that depends heavily on agriculture with many people deriving food and income from small-scale farming (Toenniessen *et al.* 2008). Consequently, plant diseases

and insect pests are crucially important to the livelihood of large numbers of Africans. In this regard, seventeen *Phytophthora* spp. are known to cause diseases of agricultural crops in Africa (Table 1). Little research has been done on all but the most important species, such as *Phytophthora infestans*, *P. megakarya, P. palmivora*, and *P. cinnamomi*. In most cases, only a disease incidence and pathogen reports have been submitted, such as the case with *P. cryptogea* (Marais 1979; 1980; Thompson *et al.* 1995), *P. drechsleri* (Thompson 1987; 1988; Thompson and Phillips 1988), *P. medicaginis* (Thompson 1987; 1988), *P. megasperma* (Marais 1980), *P. porri* (Von Maltitz and Von Broembsen 1984) and *P. syringae* (Wager 1941). Follow-up studies are rare and exceptions include *P. capsici* (Meitz *et al.* 2010), *P. cinnamomi* (Linde *et al.* 1997) and *P. infestans* (McLeod *et al.* 2001) where genetic diversity has been characterized in South African populations.

In most African cases *Phytophthora* spp. have been found associated with the same plant hosts as on other continents. For instance, *P. cactorum* has been found on *Malus domestica* (apple) in the USA, England and Switzerland (Tucker 1933); *P. citrophthora* on *Citrus* spp. in Egypt, Israel, Spain and various Mediterranean islands (Tucker 1933) and *P. porri* on *Allium cepa* (onion) in Japan (Smilde *et al.* 1996) and the Netherlands (Tichelaar and van Kesteren 1967). The known association of several *Phytophthora* spp. with widely cultivated and popular crop hosts prior to their detection in Africa makes it probable that these species were introduced, but no further evidence exist to unequivocally test this hypothesis at this time. The only exception is *P. megakarya*, one of the species involved in cacao black pod disease. *P. megakarya* occurs exclusively in Central to Western Africa (Guest 2007). Exotic crops cultivated in Africa have thus largely been affected by known and probably non-native *Phytophthora* spp.

There are a few cases where new plant associations have been reported. One example is the proposed (but not formally described) *Phytophthora niederhauseri*. This species causes disease on several ornamental plants such as *Banksia* spp. (Proteaceae, Proteales), *Calistemon citrinus* (Myrtacaeae, Myrtales), *Cistus* spp. (Cistaceae, Malvales), *Laurus nobilis* (Lauraceae, Laurales) and *Pistacia lentiscus* (Anacardaceae, Sapindiales) in Europe (Cacciola *et al.* 2009a; Cacciola *et al.* 2009b; Moralejo *et al.* 2009; Scanu *et al.* 2011). *P. niederhauseri* is also involved in almond (*Prunus dulcis*, Rosaceae, Rosales) decline in Spain (Pérez-Sierra *et al.* 2010). Other than the above European incidences, *P. niederhauseri* was reported in Western Australia from a *Banksia* sp. (Burgess *et al.* 2009) as well as from soil associated with nursery plants imported from the Northern Territory of Australia (Davison *et al.* 2006). From South Africa, this species was isolated from the crowns of healthy grapevines (*Vitis* spp., Vitaceae, Vitales) (Spies *et al.* 2011), the first report of *P.*

niederhauseri on this host. Even though most *Phytophthora* spp. present in Africa have only affected previously known hosts, associations with new crop species remains a possibility.

While numerous *Phytophthora* spp. are associated with crop disease in Africa, very few of these are known to have a significant impact. *P. infestans* causes late blight of several solanaceous crops in Africa, most notably of potato and tomato. *P. megakarya* and *P. palmivora* cause a serious disease of cacao (*Theobroma cacao*), called black pod disease. *P. cinnamomi*, with its very wide host range (Hardham 2005) is a serious crop pathogen. Each of these pathogens is discussed individually and an attempt is made to highlight their importance in Africa.

2.1. Late blight of potato and tomato caused by P. infestans

Potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) are globally cultivated for their carbohydrate rich tubers and nutritious fruit, respectively. Global annual potato production is around 300 million metric tons and for tomato 120 million metric tons. By comparison African potato production ranges from 12 to 18 million metric tons and tomato from 13 to 16 million metric tons per year (FAO 2011). Most of Africa's potato production is consumed locally; however a small amount is exported. African countries, on average, import slightly more (470 970 tons) potatoes than they export (386 240 tons), based on 2001-2007 data (FAO 2011). On the other hand African countries export (216 251 tons) almost ten times more tomatoes than they import (24 926 tons). These crops are thus of great economic importance in Africa.

Late blight caused by *P. infestans* (Fry and Goodwin 1997) is one of the most important diseases of solanaceous plants worldwide including Africa. In Africa, late blight can result in total crop losses of potato and tomato if no chemical control measures are taken (Sengooba and Hakiza 1999; Fontem 2003), although disease severity varies considerably. This variation can be attributed to variable weather conditions (Olanya *et al.* 2001; Fontem 2003) and to the degree of resistance in the cultivars being planted (Mukalazi *et al.* 2001b; Tumwine *et al.* 2002b; Mekonen *et al.* 2011).

The impact of late blight in African countries is difficult to quantify. Due to the socio-economic importance of the crops it affects, this disease has a major influence on the people and countries in Africa. Yield losses result in loss of sustenance and/or loss of income for farmers (Gildemacher *et al.* 2009). Due to the severity of late blight on potato and tomato, chemical control agents are a necessity. However, these agents are costly and decrease the net profit generated from the crop. For instance, the lack of chemical control agents and the

high cost associated with it are the major constraints in tomato production in Uganda (Tumwine *et al.* 2002b).

The first record of late blight in Africa was in 1913 when the disease appeared on potato and in 1922 on tomato at various locations in South Africa (Doidge and Bottomley 1931; Wager 1941). The first outbreak of late blight elsewhere in Africa was in Kenya, where it infected potatoes and various solanaceous plants, was recorded in 1941 (Nattrass and Ryan 1951). By the mid 1950's late blight had spread throughout Africa, including Cameroon, Uganda, Tanzania (erstwhile Tanganyika territory) (Russell 1954) and in the 1960's it was found in Egypt (El-Bakry 1972). It is assumed that *P. infestans* spread to all African countries by the 1970's (Fry *et al.* 1993). Late blight is now known in all major potato growing countries of Africa, including Algeria (Beninal *et al.* 2009), Cameroon (Fontem *et al.* 2005), Egypt (Fahim *et al.* 2003; Fahim *et al.* 2007), Ethiopia (Kassa and Beyene 2001; Mekonen *et al.* 2011), Kenya (Erselius *et al.* 1997; Vega-Sanchez *et al.* 2000), Morocco (Sedegui *et al.* 2000; Hammi *et al.* 2002), Rwanda (Muhinyuza *et al.* 2008), South Africa (McLeod *et al.* 2001), Tunisia (Jmour and Hamada 2006) and Uganda (Erselius *et al.* 1997; Vega-Sanchez *et al.* 2000; Mukalazi *et al.* 2001a). Clearly the spread of the pathogen has been closely linked to the development of agriculture on the continent.

The centre of origin of *P. infestans* is believed to be Central Mexico (Goodwin *et al.* 1992). The initial dissemination of *P. infestans* occurred in the 1840's and by the 1950's it had gained a global distribution, including Africa (Fry *et al.* 1993). This original spread of *P. infestans* occurred through a single genotype of the A1 mating type (Goodwin *et al.* 1992). The A2 mating type was first identified outside Mexico, from Switzerland (Hohl and Iselin 1984). By 1990 the A2 mating type had spread through Europe, large parts of Asia, Egypt and South America (Fry et al. 1993). The A2 mating type of *P. infestans* was first reported in Africa from infected potato tubers originating from Egypt (Shaw *et al.* 1985). However a later study failed to detect the A2 mating type amongst Egyptian *P. infestans* isolates (Baka 1997). The A2 mating type has also recently been found in Morocco (Sedegui *et al.* 2000; Hammi *et al.* 2001; Hammi *et al.* 2002), together with the A1 mating type. Other than Egypt and Morocco, there have been no other reports of the A2 mating type in Africa.

The initial spread of *P. infestans* from its centre of origin, Mexico, to the USA included a small number of genotypes and its subsequent spread to Europe and the rest of the world was made by a single clonal lineage (Goodwin *et al.* 1994). Thereafter, on several occasions additional migrations of *P. infestans* introduced new genotypes into USA and the rest of the world (Goodwin 1997). African reports of new genotypes have been very scarce. Most

studies done on population characterization of *P. infestans* have found only the old genotype (Baka 1997; Erselius *et al.* 1997; Vega-Sanchez *et al.* 2000; McLeod *et al.* 2001). Only one case of new genotypes has been reported from Morocco, where both mating types and new *Gpi* and *Pep* profiles were found and where the population underwent a shift from A1 mating type and "old" genotype dominated to that of A2 mating type and "new" genotype dominated (Sedegui *et al.* 2000).

The spread of the A2 mating type and new genotypic diversity is a severe threat to potato and tomato farming in Africa. An increase in genetic diversity of *P. infestans* populations brought on by the introduction of new genotypes could, through selection, result in increased virulence and increased resistance to fungicides (Goodwin 1997) as well as a breakdown of resistance in potato and tomato cultivars. The increase in fungicide resistance, especially to metalaxyl, would be devastating as fungicides are one of the most important measures against late blight (Fontem *et al.* 2005; Jmour and Hamada 2006). The presence of both mating types in a population would, as a result of sexual recombination, augment the speed at which the population can respond to selective pressures. In addition, sexual reproduction would result in the formation of oospores, which can survive greater periods of time outside of its host, compared to asexually reproducing populations (Goodwin 1997). The increased risk of fungicide resistance, increased virulence and long lived, soilborne inoculum that would result from the introduction of the A2 mating type and new genotypic diversity into African *P. infestans* populations could have devastating effects for potato and tomato farmers in Africa.

P. infestans can infect various plant species other than potato and tomato (Erwin and Ribeiro 1996), and this has also been the case in Africa. *P. infestans* infects and causes late blight of huckleberry (*S. scabrum*) in Cameroon (Fontem *et al.* 2003), which is native to Africa, where its leaves and shoots are widely used as a vegetable for subsistence and commercial production (Gockowski *et al.* 2003). The pathogen has also been reported from other solanaceous plants such as gboma eggplant (*S. macrocarpon*) in Cameroon (Fontem *et al.* 2004a), several *Solanum* spp. in Kenya (Nattrass and Ryan 1951), as well as the asteraceous invasive weed Billy goatweed (*Ageratum conyzoides*, Asteraceae, Asterales) and several native plants e.g. Dichrocephala (*Dichrocephala integrifolia,* Asteraceae), haemorrhage plant (*Aspilia africana*, Asteraceae) and Worowo (*Solanecio biafrae*, Asteraceae) from Cameroon (Fontem *et al.* 2004a). The ability of *P. infestans* to infect these alternative hosts complicates control measures, as the alternative hosts can act as an over-wintering cache and a continuous source of inoculum (Flier *et al.* 2003).

In Africa a great deal of research focus has been placed on optimal usage of chemical control methods against late blight. Due to the high cost associated with control chemicals, and important goal has been to maximize the economic benefit and not necessarily to maximize the crop production (Kassa and Beyene 2001; Kankwatsa *et al.* 2003). Commonly used chemicals for the control of *P. infestans* in Africa include the contact fungicide Dithane M-45 (mancozeb), and the systemic fungicides Ridomil (metalaxyl) and Ridomil MZ (metalaxyl and mancozeb) (Olanya *et al.* 2001; Tumwine *et al.* 2002b; Kankwatsa *et al.* 2003). Mancozeb has the advantage of being effective against a broad range of pathogens, but because it is a contact fungicide, its protection is severely reduced after rainfall. Metalaxyl is not influenced by rainfall, but resistance to metalaxyl by *P. infestans* has been reported from several African countries including Uganda (Erselius *et al.* 1997; Vega-Sanchez *et al.* 2000; Mukalazi *et al.* 2001a), Kenya (Erselius *et al.* 1997; Vega-Sanchez *et al.* 2000), Morocco (Sedegui *et al.* 2000; Hammi *et al.* 2002), Cameroon (Fontem *et al.* 2005) and South Africa (McLeod *et al.* 2001).

Chemical control, although the most effective and widely applied control method, is not the only option applied in Africa. Late blight resistant varieties of tomato and potato are important as these can significantly decrease disease incidence and increase yield, but these are only effective when integrated with chemical control (Kankwatsa *et al.* 2002; Namanda *et al.* 2004; Ojiewo *et al.* 2010). Integration of sanitation (the removal of infected material), sheltering, intercropping and planting time is an effective option to control late blight in tomatoes (Tumwine *et al.* 2002a; Fontem *et al.* 2004b) and potatoes (Olanya *et al.* 2001), although not as effective as chemical control. Integrated control strategies, which combine resistant cultivars, chemical agents and cultural practices are advantageous to improve crop yields and reduce associated expenses (Low 1997; Kankwatsa *et al.* 2002; Namanda *et al.* 2004). These various options result in a need to educate farmers on how to best combat late blight and maximize their yields, as most farmers are ignorant of integrated disease management methods (Low 1997; Nyankanga *et al.* 2004). Several farmer field schools have for example been established in Uganda with the aim to educate local farmers on cultivation and disease management of potato (Olanya *et al.* 2010).

2.2. Black pod disease caused by P. palmivora *and* P. megakarya

Cacao (*Theobroma cacao*) native in tropical South America, is commercially cultivated for its cocoa bean seeds in many tropical countries around the world, where it is used to produce cocoa, chocolate and cocoa butter. Africa is the world's largest cacao grower, producing on average 70% of the world's crop, of which 38% is produced in Côte d'Ivoire and 19% in Ghana (ICCO 2007; 2009). When this is compared to the production of the Americas (12%),

Asia and Oceania (17%), it is evident that cacao production is one of Africa's most significant agricultural industries.

Pod rot is the most important symptom of *Phytophthora* infection of cacao because it results in direct loss of crop yield. Infection can occur on mature or immature pods (cherelles) and is readily visible as spreading brown to black lesions (Bowers *et al.* 2001; Guest 2007). Initially the infection does not affect the cacao beans, but as the disease progresses the beans are infected, which renders them unusable (Wood 1975). Diseased pods serve as a source of secondary inoculum of *Phytophthora* spp. and if they are not removed, they mummify and act as reservoirs of inoculum (Bowers *et al.* 2001; Ndoumbe-Nkeng *et al.* 2004).

Phytophthora spp. can infect parts of cacao trees besides pods, such as the main stems (chupons), leaves, flower cushions and roots (Wood 1975; Guest 2007). The importance of these types of infection has been underestimated as they influence cacao tree health and thus indirectly decrease cacao yield (Wood 1975; Evans and Prior 1987; Appiah *et al.* 2004b). Additionally, these infections contribute to the spread, establishment and severity of further infections as they are a source of secondary inoculum (Bowers *et al.* 2001; Guest 2007).

In cacao producing regions of the world, several *Phytophthora* spp., including *P. capsici*, *P. citrophthora*, *P. heavea, P. megasperma* and *P. nicotianae,* cause cacao black pod disease (Ploetz 2007). Black pod is thus a complex disease caused by multiple *Phytophthora* species. However, in Africa only two species are responsible for causing disease of cacao. Initially *P. palmivora* was implicated as the cause for black pod disease (*Phytophthora* pod rot) (Dade 1927), but later it was shown that a second species, *P. megakarya*, was also involved (Brasier and Griffin 1979). *P. palmivora* occurs globally also affecting *Carica papaya*, *Cocos nucifera* and *Hevea braziliensis* (Ashby 1929; Erwin and Ribeiro 1996; Guest 2007), whereas *P. megakarya* is restricted to parts of central and western Africa (Guest 2007). Initially *P. megakarya* was known only to occur in Nigeria and Cameroon on cacao (Brasier and Griffin 1979), but later it was found in Cote d'Ivoire, Equatorial Guinea, Gabon, Ghana and Togo (Nyasse *et al.* 1999; Appiah *et al.* 2004a; Appiah *et al.* 2004b; Pokou *et al.* 2008).

Both mating types of *P. megakarya* and *P. palmivora* are present in Africa. Most isolates of *P. megakarya* encountered on cacao are of the A1 mating type, but both mating types have been found in Equatorial Guinea, Cameroon, Ghana and Nigeria (Nyasse *et al.* 1999; Appiah *et al.* 2003). In contrast, *P. palmivora* is mostly represented by the A2 mating type in

Africa (Brasier and Griffin 1979), with both mating types found only in Ghana and Togo (Appiah *et al.* 2003). The dominance of one mating type and the scarcity of the other in the above two cases, limits the opportunity for sexual reproduction within populations of the pathogen.

Although the two *Phytophthora* spp. occurring on cacao in Africa cause the same disease, they have slightly different disease cycles and attributes. *P. megakarya* is much more virulent than *P. palmivora* on cacao and consequently results in greater yield losses of up to 100% (Appiah *et al.* 2004b; Ndoumbe-Nkeng *et al.* 2004; Opoku *et al.* 2007a) compared to the 20-30% yield loss caused by *P. palmivora* (Wood 1975). *P. megakarya* has a propensity for earlier and more profuse release of zoospores than *P. palmivora*, and this is reflected in the ability of the former species to spread faster than the latter species (Brasier *et al.* 1981). *P. palmivora* is better adapted at surviving in mummified pods in contrast to *P. megakarya,* which is unable to endure the dry season in shrivelled pods (Brasier *et al.* 1981). These two *Phytophthora* spp. also differ in their ability to cause cankers with *P. megakarya* resulting in a higher incidence of tree girdling cankers, whereas *P. palmivora* causes cankers higher up on the stems (Appiah *et al.* 2004b). Although *P. palmivora* is better adapted to surviving within cacao, it is the ability of *P. megakarya* to spread and colonize cacao that allows it to outcompete the former pathogen.

Cacao is a major cash crop in the African countries where it is cultivated (Duguma *et al.* 2001; Oke and Odebiyi 2007). Most of the cacao producers are smallholder farmers (Duguma *et al.* 2001; ICCO 2007) that are reliant on cacao as a source of income. Black pod disease represents a serious constraint of cacao production given high yield loss, especially when caused by *P. megakarya*. *Phytophthora* infested cacao plantations incur additional expenses as chemical control agents are necessary to reduce yield loss (Akrofi *et al.* 2003). The high cost associated with fungicides and their application decreases the farmer's profit per unit of cacao sold. Simply producing more cacao is not a viable option for farmers because of limited land available for production and it may lead to overproduction that decrease cacao prices, and increasing the efficiency of cacao production is the better option (ICCO 2007).

Phytophthora megakarya and *P. palmivora* occur on several tree species other than cacao. In Ghana *P. megakarya* infects four tree species, namely *Funtumia elastica* (Apocynaceae, Gentianales), *Sterculia tragacantha* (Malvaceae, Malvales), *Dracaena mannii* (Asparagaceae, Asparagales) and *Ricinodendron heudelotii* (Euphorbiaceae, Malpighiales) (Opoku *et al.* 2002). These trees are native, naturally occurring trees, which are often

retained in cacao plantations to provide shade. In Cameroon, *P. megakarya* was also reported from the fallen fruit of a native *Irvingia* sp. (Irvingiaceae, Malpighiales) (Holmes *et al.* 2003) occurring in a native forest habitat. Although *P. palmivora* has a very broad host range and occurs globally (Guest 2007), in Africa it has been reported only from a small number of hosts other than cacao. These include *Hevea brasiliensis* (Euphorbiaceae, Malpighiales) and a *Mimusops* sp. (Sapotaceae, Ericales) in Ghana (Ashby 1929; Dade 1940); *Musa* sp. (Musaceae, Zingerberales) in Nigeria (Esenam 1971); and *Cocos nucifera* (Arecaceae, Arecales) in Tanzania (Nsolomo and Venn 1994). All the above mentioned trees, with the exception of the *Mimusops* sp., are commonly intercropped together with cacao trees (Duguma *et al.* 2001). The effect that these alternative hosts have on the disease incidence is not clear, but they could act as reservoirs and sources of inoculum for *P. megakarya* and *P. palmivora* (Opoku *et al.* 2002).

Most of the research done on Cacao Black Pod in Africa has focussed on control methods for this disease. Chemical fungicides have been used widely, including a variety of Copper-based contact fungicides (Opoku *et al.* 2007a), the semi-systemic fungicide “Ridomil 72 plus” (metalaxyl and copper-1-oxide) (Opoku *et al.* 2004; Opoku *et al.* 2007b) and the systemic fungicide potassium phosphonate (Opoku *et al.* 2007a). The disadvantages of using chemical fungicides is that they are costly, labour intensive to apply, multiple applications are required throughout the season and that they are often toxic to humans and other organisms in the environment (Monkiédjé *et al.* 2000).

Recently biological control using mycoparasites of black pod disease have been investigated. Several *Geniculosporium* spp. and *Trichoderma asperellum* are potential biological control agents as the former suppresses growth and sporulation of *Phytophthora megakarya* in artificial inoculations (Tondje *et al.* 2006) and the latter suppresses black pod under field conditions (Tondje *et al.* 2007). Several *Trichoderma* spp. have been shown to provide *in vitro* and *in vivo* suppression of *P. palmivora* growth (Mpika *et al.* 2009). Current biological control agents of Black Pod disease cannot offer complete protection and are used only when they are integrated with chemical fungicides and cultural practises (Deberdt *et al.* 2008).

Cacao resistance to black pod disease is a potential tool to negate or reduce the need for costly chemical fungicides. Several field accessions with high yield and increased resistance to black pod disease have been identified in Cameroon (Efombagn *et al.* 2007) and Côte d’Ivoire (Pokou *et al.* 2008). Detached leaf and pod inoculation is a fast and effective method

developed to facilitate screening for resistance in cacao trees (Nyassé *et al.* 2002; Tahi *et al.* 2006; Efombagn *et al.* 2011).

Control of *Phytophthora* pod rot is best achieved by integration of different control approaches. Phytosanitary cultural practices are aimed at reducing inoculum production by creating unsuitable conditions for *Phytophthora* and by removing infected material, and include weeding, removal of mistletoes, eliminating excess shade and removal of infected and mummified pods (Akrofi *et al.* 2003). Sanitary practices alone are not sufficient to control Black Pod but when combined with fungicide applications, it considerably reduces disease incidence (Opoku *et al.* 2007a). This can also reduce the number of fungicide applications needed per year, although reduction of disease incidence is not as effective as with a standard fungicide regime (Opoku *et al.* 2007b). It has been widely suggested that optimal control can be achieved by combining chemical control, phytosanitary cultural control, biological control and host resistance (Ndoumbe-Nkeng *et al.* 2004; Opoku *et al.* 2007b; Deberdt *et al.* 2008).

2.3. Avocado root root caused by P. cinnamomi

Avocado (*Persea americana*) trees are widely planted in Africa, both for local consumption and export of their fruit. Annually, Africa produces on average 14% of the world's avocados, with Kenya, South Africa, Democratic Republic of the Congo, Ethiopia and Cameroon having the largest production (FAO 2011). The South African avocado industry is mostly export driven with approximately 61 kilotons (65%) of avocados exported in 2008/9 (Directorate Agricultural Statistics 2010). South Africa's largest competitor is Kenya, exporting about 18 kilotons of avocado in 2009 (Anonymous 2009).

The most important disease of avocado in Africa is root rot and die-back caused by *P. cinnamomi*. The first African report of this pathogen was as *P. cambivora* in South Africa (Doidge and Bottomley 1931; Wager 1931), but re-examination showed that these isolates were *P. cinnamomi* (Wager 1941). Currently, root rot is widespread in South African avocado orchards and it is the most destructive disease of this crop (Kremer-Köhne and Mukhumo 2003). Root rot is particularly severe in South Africa, probably because of the high soil temperatures and moisture in the main growing regions in this country that receives summer rainfall (Zentmyer 1979). Other African countries where root rot is also a major constraint of avocado production include Cameroon (Huguenin *et al.* 1975), Ethiopia (Shumeta 2010) and Kenya (Onsando and Gathungu 1985), but little is known regarding this disease from the rest of Africa.

Phytophthora cinnamomi is one of the most serious and widespread plant pathogens in the world (Hardham 2005). It causes severe losses in a number of agriculturally important crops across the globe, including avocado (*Persea americana*, Lauraceae, Laurales) (Wager 1942), *Macadamia* spp. (Proteaceae, Proteales) (Zentmyer and Storey 1961) and pineapple (*Ananas comosus,* Bromeliaceae, Poales) (Rohrbach and Schenck 1985). *Phytophthora cinnamomi* infections result in serious crop losses of avocado in Africa. In South Africa, losses are estimated as R 45 million or 10% of the annual gross value of avocados (Bekker 2007). Estimates for other African countries are scarce, but it is widely stated that root rot is a serious disease of avocados in African countries (Griesbach 2005; Shumeta 2010).

Phytophthora cinnamomi is heterothallic with the A2 mating type the more common of the two, globally (Zentmyer 1976). Both mating types occur in South Africa in agricultural and native environments, although the A1 mating type is restricted to the South Western Cape, whereas the A2 mating type occurs in both the South Western Cape as well as the Mpumalanga province of South Africa (Von Broembsen 1989a; Linde *et al.* 1997). In Kenya both mating types are also present on *Macadamia* spp. (Mbaka *et al.* 2010). Information on mating type distribution of *P. cinnamom* in South Africa needs to be reassessed and it is severely limited elsewhere in Africa.

Most contemporary evidence suggests that the centre of origin of *P. cinnamomi* is New Guinea (Zentmyer 1988). Criteria to assess the likelihood that a region is a centre of origin include the presence of resistant indigenous plant species, presence of both mating types in a near equal ratio and the presence of *P. cinnamomi* in undisturbed environments. The Western Cape of South Africa was at one time regarded as a possible centre of origin of *P. cinnamomi* (Von Broembsen and Kruger 1985; Zentmyer 1988) due to its presence in undisturbed native Fynbos vegetation. This hypothesis seems unlikely given data on the biogeographical distribution of *P. cinnamomi* mating types in South Africa, which indicated that only the A1 mating type is found in fynbos of the Western Cape, while the A2 mating type is found in cultivated crops across the country (Von Broembsen 1989a). Although both mating types are present in natural forest (Von Broembsen 1989a), later population studies of South African *P. cinnamomi* isolates revealed a low level of gene diversity, indicating that *P. cinnamomi* is an introduced organism and, although both mating types occur in the same locations, that sexual reproduction rarely occurs in South African populations (Linde *et al.* 1997; Linde *et al.* 1999).

Although *P. cinnamomi* is best known from avocado, it occurs on several other important crops in Africa, but these have received less attention. In the Western Cape of South Africa it

causes crown and root rot of grapevines during the 1970's (van der Merwe *et al.* 1972). Although other *Phytophthora* spp. were also involved (Table 1), *P. cinnamomi* was shown to be the most virulent species on grapevines (Marais 1979; 1980). More recently, *P. cinnamomi* and other *Phytophthora* spp. have become much less common on grapevines in South Africa, probably due to widespread use of chemical control measures (van Coller *et al.* 2005). *P. cinnamomi* also occurs on *Macadamia* spp. in South Africa (Verbeek 1972) and Kenya (Mbaka *et al.* 2009; Mbaka *et al.* 2010), as well as on pineapple in South Africa (Malan 1954) and Ghana (Donkoh and Agboka 1995), but little further research has been done on these important hosts. *P. cinnamomi* has also been encountered from various other countries, including the Republic of Congo, Democratic Republic of Congo, Republic of Guinea, Côte d'Ivoire, Morocco, Uganda, Zambia and Zimbabwe (Zentmyer *et al.* 1976; Zentmyer 1988), although the hosts from which it was isolated were not recorded.

There has been much focus on the prevention and amelioration of *Phytophthora* root rot disease on avocado trees in Africa. A very strong emphasis is placed on integrated disease management of avocado. The four most important factors of integrated control of *P. cinnamomi* include disease free nurseries, resistant rootstocks, responsible use of chemical fungicides and appropriate cultural practises (Kotzé 1985). Of these, resistant rootstocks play a very important role in conferring a measure of tolerance against root rot. In South Africa the most sought-after rootstock characteristic is tolerance against root rot and so the two most widely used rootstocks are Duke 7 and Merensky II/Dusa (Bijzet 1999; Donkin 2003; Wolstenholme 2003). In Kenya, root rot resistance does not affect rootstock choice as much as in South Africa, and a wider variety of rootstocks are thus used (Griesbach 2005).

The most common types of chemical control measures for root rot of avocado is phosphonate based methods and metalaxyl application (Griesbach 1985; Kotzé 1985; Onsando and Gathungu 1985). *P. cinnamomi,* like other *Phytophthora* spp., could gain resistance to metalaxyl after continuous exposure (Darvas and Becker 1984) and thus cautious use of the chemical is advised. Phosphonate based methods include surface applications such as trunk paints and leaf sprays (Duvenhage 2001) and trunk injection with Fosetyl-Al (Darvas *et al.* 1983) and phosponic acid (Darvas and Bezuidenhout 1987). Trunk injection is the most effective form of chemical control of *P. cinnamomi* on avocado, although *P. cinnamomi* also develops resistance against phosphonates after prolonged exposure (Duvenhage 1999). Recently, potassium silicate was shown to effectively control root rot when applied as a soil drench (Bekker 2007).

3. *Phytophthora* spp. in plantation forestry

Forests represent a valuable natural resource for Africa and they are an important source of timber and non-timber forest products (Sunderland and Ndoye 2004). Only 2.3 % of Africa's total forest area consists of planted forests of which only 38% is planted to non-native tree species. Thirty percent of Africa's forest area is primarily for production of timber and non timber forest products (FAO 2010). The majority of timber harvested in Africa, be it from natural or planted forests, is used as fuel wood and only about 10% for industrial purposes. Countries with planted forests produce a significantly larger proportion of industrial wood. Plantations of non-native trees are a statistical minority and their distribution is irregular since only certain countries rely almost exclusively on non-native trees for plantations (FAO 2010). Of these, several countries have extensive plantations with the most prominent plantation species belonging to the genera *Pinus*, *Eucalyptus*, *Acacia* and *Cupressus* (Pohjonen and Pukkala 1990; Ciesla *et al.* 1995; Directorate Forestry Technical and Information Services 2010; Lemenih and Woldemariam 2010; Delgado-Matas and Pukkala 2011a; b)

Plantations of exotic tree species generally perform very well in suitable climates, compared to those of indigenous tree species. A key factor for their success is the absence of their natural enemies (Keane and Crawley 2002; Mitchell and Power 2003) that enables such trees to grow quicker and remain healthy for longer than native trees. However, a steady increase of pests and diseases has been observed (Wingfield *et al.* 2008). These include several *Phytophthora* spp. that are associated with plantation tree species.

Black Wattle (*Acacia mearnsii*) plantations make up approximately eight percent of the total forestry land usage in South Africa and the trees are mainly used for tannin production and pulpwood (Directorate Forestry Technical and Information Services 2010). In the 1960's, a serious disease of these trees known as black butt disease, appeared in South African plantations, characterized by blackened bases of the tree trunks, copious gum exudation as well as mottled lesions occurring on the stems of young trees (Zeiljemaker 1971). Initially, *P. nicotianae* was shown to be the cause of this disease (Zeiljemaker 1971), but two additional species, *P. boehmeriae* and *P. meadii* were later shown to be associated with the same symptoms (Roux and Wingfield 1997). Black butt disease is also present in Kenya and Tanzania, but the *Phytophthora* sp. involved has not been identified (Roux *et al.* 2005).

Pines and eucalypts provide the backbone of the South African forestry industry and make up approximately 50% and 40%, respectively, of the total forestry land area planted of South Africa (Directorate Forestry Technical and Information Services 2010). Several *Phytophthora*

spp. are associated with diseases of these trees. *P. cinnamomi* causes root and collar rot of both pines and eucalypts in plantations (Linde *et al.* 1994b). In forestry nurseries this pathogen also causes damping off of pine and eucalypt seedlings and the pathogen has resulted in nursery quarantine and the destruction of countless seedlings in the past (Donald and von Broembsen 1977). Until twenty years ago, *P. cinnamomi* was the only species known to cause disease in pine and eucalypt plantations. Thereafter two species, *P. boehmeriae* and *P. nicotianae*, were identified as also causing disease on several *Eucalyptus* species (Linde *et al.* 1994b). *P. nicotianae* in particular, became so prevalent that it was more consistently recovered from dying eucalypts than *P. cinnamomi* (Maseko *et al.* 2001). More recently, two new species, *P. alticola* and *P. frigida*, were found to cause root and collar rot of cold tolerant eucalypts in the Kwazulu-Natal province (Maseko *et al.* 2007).

The main focus of control of *Phytophthora* infestations of forestry trees is on production of healthy seedlings. Strong emphasis is placed on disease-free nurseries in South Africa and this relies on sanitary cultural practices and chemical control (Viljoen *et al.* 1992). The choice of tree species within plantations is also affected by their tolerance to *Phytophthora* infections. For example, in South Africa, *Eucalyptus fraxidoides* and *E. fastiga* are no longer planted due to their susceptibility to *P. cinnamomi* (Linde *et al.* 1994a).

4. *Phytophthora* spp. in native environments

There has been very little research on *Phytophthora* in native environments in Africa. This is evident when comparing the number of *Phytophthora* species from crops to those occurring on indigenous plants or in native environments (Table 1). Even among *Phytophthora* spp. identified from indigenous plants, many were isolated from plants cultivated as food crops (Fontem *et al.* 2004a), medicines and cosmetics (Bezuidenhout *et al.* 2010) or for the flower trade (Von Broembsen and Brits 1985b).

Recently, there has been an increase in focus on *Phytophthora* spp. associated with native ecosystems internationally (Balci and Halmschlager 2003; Brasier *et al.* 2003; Hüberli *et al.* 2010; Jung *et al.* 2011). This has occurred because a number of very serious *Phytophthora* diseases have emerged in native woody ecosystems. Yet surveys for these organisms in native African environments have yet to be made. The Cape Floristic Region (CFR) in South Africa has received the most attention relating to *Phytophthora* spp. (Von Broembsen 1984a; b; Bezuidenhout *et al.* 2010) of all the native habitats in Africa. This was initially motivated by the dramatic death of the iconic Proteaceae in natural ecosystems (Van Wyk 1973). Numerous species are now known to occur in the CFR including *P. capensis*, *P. cinnamomi*, *P. citricola*, *P. cryptogea*, and *P. dreschleri* which are found in rivers and river catchments

(Von Broembsen 1984a; 1989b; Bezuidenhout *et al.* 2010). Investigation into diseased cultivated buchu (*Agathosma* spp., Rutaceae, Sapindiales) also revealed that multiple *Phytophthora* species are involved (Bezuidenhout *et al.* 2010), including a new but as of yet undescribed taxon in the *P. citricola* complex, *Phytophthora* taxon emzansi. Additionally, *P. capensis* was described as a new species following the re-examination of isolates also from buchu and previously regarded as *P. citricola* (Oudemans *et al.* 1994; Bezuidenhout *et al.* 2010). Apart from *P. capensis*, the above mentioned *Phytophthora* spp. all have a cosmopolitan distribution and diverse host range.

Phytophthora cinnamomi is the most commonly encountered *Phytophthora* in the CFR. It is associated with root rot and decline of several native Bruniaceae (Lamiales), Ericaceae (Ericales) and Proteaceae (Proteales) species of Fynbos (Van Wyk 1973; Von Broembsen 1984b; Von Broembsen and Kruger 1985). *P. cinnamomi* is also a serious problem for the cut flower industry, where it causes root rot of cultivated Proteaceae (Von Broembsen and Brits 1985b). Additionally, *P. cinnamomi* is associated with the decline of Stinkwood (*Ocotea bullata*, Lauraceae, Laurales) trees in native forests of the Eastern Cape of South Africa (Von Broembsen *et al.* 1986).

There are few studies reporting on *Phytophthora* spp. from native ecosystems in other parts of Africa. As mentioned earlier, in Cameroon and Kenya *P. infestans* infects several native Asteraceae and Solanaceae (Nattrass and Ryan 1951; Fontem *et al.* 2004a). *P. megakarya* occurs on several native tree species in cacao plantations in Cameroon and Ghana (Table 1) (Opoku *et al.* 2002; Holmes *et al.* 2003). A single instance of *P. hevea* has been recorded from Cameroon, where it was isolated from an unidentified fallen fruit in a native forest (Holmes *et al.* 2003).

Control and management strategies against *Phytophthora* spp. in native ecosystems are virtually non-existent in Africa. However, the situation is not as severe as is seen in other native ecosystems of the world, where species such as *P. cinnamomi* in Western Australia (Shearer *et al.* 2004) and *P. ramorum* in California, USA (Garbelotto *et al.* 2003) cause enormous damage. The only attempt to control *Phytophthora* infections are in the Protea cut-flower industry of South Africa where emphasis is placed on excluding *P. cinnamomi* from cultivation areas, using suppressive and sanitary cultural practices, applying chemical control and exploiting host resistance (von Broembsen and Brits 1985a). Metalaxyl is not used due to severe phytotoxic reactions of the cultivated plants, and thus Fosetyl-Al sprays are used to treat and prevent *Phytophthora* infections.

5. Conclusions

Diseases caused by *Phytophthora* species have a major socio-economic impact on the countries and people of the African continent. Foremost among these impacts are the loss of agricultural produce. Late blight, caused by *P. infestans* severely affects potato and tomato yields and this has a considerable impact as these two crops are widely grown in Africa for subsistence and commercial purposes. Black pod disease of cacao is responsible for substantial losses of cacao, of which Africa is the largest producer. These losses impact directly on the lives of resource-poor farmers, who are dependent on cacao as a source of income. Avocado production is also severely affected by *P. cinnamomi* and can result in extreme economic losses.

Phytophthora disease control and management strategies of agricultural crops receive some attention, but these are lacking in commercial forestry and native environments. Integrated management strategies combining chemical control, good cultural practices, host resistance and biological control are widely emphasized. Chemical control should be applied with caution as overuse can lead to an increase in resistance in *Phytophthora* populations. Improved disease control strategies would result in better crop yields, improved livelihoods and economic benefit for African farmers and countries.

Although forestry is an important industry, little is known about the impact of *Phytophthora* on native or non-native trees. Several of the most commonly planted trees, including non-native acacias, eucalypts and pines, are affected by various *Phytophthora* species. *P. cinnamomi* is a significant threat to commercial forestry and also surrounding native ecosystems. In addition to *P. cinnamomi*, *P. nicotianae* has emerged as a serious pathogen disease of eucalypts and pines and other *Phytophthora* species also appear to be involved. Vigilance is required for the early detection of possible new *Phytophthora* species or known pathogenic species from elsewhere in the world.

Compared to the research on agronomic crops, *Phytophthora* in native habitats has received limited attention. Although this bias is understandable, there is much room for further investigation. Africa has several regions of plant mega-diversity and endemism and likely harbours several new *Phytophthora* species. Thus far the majority of *Phytophthora* species on native and indigenous plant hosts have been found in the Cape Floristic Region of South Africa. In just the last decade, the number of described *Phytophthora* species has more than doubled, but only three of these new species descriptions originated from Africa. This lack of species discovery is due to the limited number of studies investigating species diversity, a focus on economically important species, and a lack of funding and trained researchers in

the field. There is consequently a great need for comprehensive studies on *Phytophthora* species diversity, especially in native habitats in Africa. This is especially important because novel *Phytophthora* species may be a threat to important non-native or native plants elsewhere in the world.

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Table 1: *Phytophthora* species occurrence in Africa

Species	Host	Country	References
Agriculture			
Phytophthora cactorum	*Citrus* spp., *Malus domestica*, *Vitis vinifera*	South Africa	Wager (1941), van der Merwe and Matthee (1973), Marais (1979; 1980) and Tewoldemedhin *et al*. (2011)
	Malus domestica	Tunisia	Boughalleb *et al*. (2006)
P. capsici	*Capsicum* spp., *Cumus melo*, *Curcurbita* spp, *Solanum lycopersicum*	South Africa	Thompson *et al.* (1994), Labuschagne *et al.* (2000), Labuschagne *et al.* (2003) and Meitz *et al*. (2010)
	Capsicum sp.	Nigeria	Alegbejo *et al*. (2006)
P. cinnamomi	*Annanas comosus, Macadamia sp., Persea americana, Pyrus communis, Vitis* spp.	South Africa	Wager (1941), Malan (1954), van der Merwe *et al*.(1972), Verbeek (1972) , Marais (1979; 1980), von Broembsen (1984b) and Lonsdale *et al.* (1988)
	Persea americana	Various countries	Huguenin *et al*. (1975), Onsando and Gathungu (1985) and Shumeta (2010)
	Macadamia spp.	Kenya	Mbaka *et al*. (2009)
P. citricola	*Citrus* spp.	South Africa	von Maltitz and von Broembsen (1985) and Bezuidenhout *et al*. (2010),
P. citrophthora	*Citrus* spp.	South Africa	Doidge and Bottomley (1931), Wager (1931; 1941) and Schutte and Botha (2008)
	Citrus spp.	Liberia	Muzik (1952)
P. cryptogea	*Citrus* spp., *Vitis vinifera*	South Africa	Marais (1979; 1980) and Thompson *et al.* (1995)
P. drechsleri	*Brassica oleracea*, *Medicago sativa*	South Africa	Thompson (1987; 1988) and Thompson and Phillips (1988)
P. infestans	Solanaceous crops	Various Countries	Doidge and Bottomley (1931), Wager (1941), Baka (1997), Sedegui *et al.* (2000) and McLeod *et al* (2001)
P. medicaginis	*Medicago sativa*	South Africa	Thompson (1987; 1988)
P. megakarya	*Theobromea cacao*	Various Countries	Brasier and Griffin (1979), Appiah *et al*. (2004b); Ndoumbe-Nkeng *et al*. (2004) and Pokou *et al* (2008)
P. megasperma	*Vitis vinifera*	South Africa	Marais (1980)
P. multivora	*Medicago sativa*	South Africa	Bezuidenhout *et al*. (2010)

Species	Host	Country	References
P. nicotianae	*Chamaecytisus palmensis*, *Citrus* spp., *Musa* sp., *Nicotiana tabacum*, *Rheum rhaponticum, Solanum lycopersicum, Vitis vinifera*	South Africa	Doidge and Bottomley (1931), Wager(1931; 1935), Lamprecht *et al* (1974), Marais (1979; 1980), Thompson (1981), Ferreira *et al* (1991), Botha (1993) and Thompson *et al*. (1995)
	Nicotiana tabacum, Solanum betacae	Ghana	Dade (1940)
	Passiflora edulis	Kenya	Mbaka *et al*. (2006)
	Agave spp., *Citrus* sp., *Hibuscus cannabinus*	Tanzania	Ebbels and Allan (1979), Nsolomo and Venn (1994)
	Capsicum annuum, *Malus domestica*	Tunisia	Allagui *et al*. (1996), Allagui *et al*. (2000), Darine *et al*. (2007) , Boughalleb *et al*. (2008) and Saadoun and Allagui (2008)
	Nicotiana tabacum	Zimbabwe	Hopkins (1931)
P. palmivora	*Agave* spp., *Cocos nucifera, Hevea brasiliensis, Musa* sp., *Persea americana, Theobromea cacao*	Various Countries	Ashby (1929), West (1938) Dade (1940), Esenam (1971), Ebbels and Allan (1979), Nsolomo and Venn (1994), Appiah *et al.* (2003) and Pokou *et al*. (2008)
P. porri	*Allium cepa*	South Africa	von Maltitz and von Broembsen (1984)
P. syringae	*Citrus* sp.	South Africa	Doidge and Bottemley (1931) and Wager (1941)
	Citrus sp.	Libya	Naim *et al* (1967)
P. niederhauseri	*Vitis spp.*	South Africa	Spies *et al*. (2011)
Forestry			
P. boehmeriae	*Acacia mearnsii, Eucalyptus* spp.	South Africa	Linde *et al*. (1994b) and Roux and Wingfield (1997)
P. cinnamomi	*Eucalyptus* spp., *Pinus* spp.	South Africa	Donald and von Broembsen (1977), Wingfield and Knox-Davies (1980) and Linde *et al.* (1994b)
P. meadii	*Acacia mearnsii*	South Africa	Roux and Wingfield (1997)
P. nicotianae	*Acacia mearnsii, Eucalyptus* spp.	South Africa	Zeiljemaker (1971), Linde *et al*. (1994b), Roux and Wingfield (1997) and Matseko *et al*. (2001)
P. alticola	*Eucalyptus* spp.	South Africa	Maseko *et al*. (2007)
P. frigida	*Eucalyptus* spp.	South Africa	Maseko *et al*. (2007)

Species	Host	Country	References
Ornamental/Exotic			
P. cactorum	*Antirrhinum majus*, *Centauri moschata*, *Dianthus caryophyllus*, *Verbena* sp.	South Africa	Mes (1934), Wijers (1937) and Wager (1941)
P. cinnamomi	*Araucaria angustifolia, Banksia* spp., *Casuarina cunninghamiana, Cedrus deodara, Centauri* sp., *Chamaecyparis lawsoniana, Cryptomeria japonica, Rhododendron* sp., *Telopea speciosissima, Thuja* sp.	South Africa	von Broembsen (1984b)
P. cryptogea	*Godetia* sp.	South Africa	Wager (1941)
P. infestans	*Petunia* × *hybrida*	South Africa	Mcleod and Coertze (2006)
P. nicotianae	*Delphinium* sp., *Gypsophila paniculata, Trichocaulon* sp.	South Africa	Wager (1941)
P. palmivora	*Mimusops elengi*	Ghana	Dade (1940)
Native plants and habitats			
P. capensis	*Curtisia dentata,* rivers, *Olea capensis*,	South Africa	Oudemans *et al* (1994), Bezuidenhout *et al* (2010)
P. cinnamomi	Bruniaceae, Ericaceae and Proteacae, *Agasthoma spp., Cliffortia spp., Curtisia dentate, Nymania capensis, Ocotea bullata, Priestleya* sp., rivers, *Widdringtonia* spp.	South Africa	van Wyk (1973), von Broembsen (1984a; 1984b), von Broembsen and Brits (1985b), von Broembsen and Kruger (1985), von Broembsen *et al* (1986), Linde *et al*. (1999) and Bezuidenhout *et al*. (2010)
P. citricola	Rivers	South Africa	von Broembsen (1989b)
P. cryptogea	*Agathosma* spp., *Osteospermum* sp., rivers	South Africa	von Broembsen (1989b), Mcleod and Coertze (2007) and Bezuidenhout *et al*. (2010)
P. drechsleri	*Agathosma* spp., Rivers	South Africa	von Broembsen (1989b) and Bezuidenhout *et al*. (2010)
P. hevea	Unknown	Cameroon	Holmes *et al*. (2003)
P. infestans	*Aspalia africa*, *Solanecia biafrae*, *Solanum* spp.	Cameroon Kenya	Nattrass and Ryan (1951) and Fontem *et al*. (2004a)
P. megakarya	*Dracaena mannii, Funtumia elastic*, Irvingia spp, *Ricinodendron heudelotii, Sterculia tragacantha*	Cameroon Ghana	Opoku *et al*. (2002) and Holmes *et al*. (2003)

Species	Host	Country	References
P. multivora	*Agathosma* spp., *Ocotea bullata*	South Africa	Bezuidenhout *et al* (2010)
P. nicotianae	*Agathosma* spp., *Cotyledon* sp.	South Africa	Wager (1931) and Bezuidenhout *et al*. (2010)
P. taxon emzansi	*Agathosma* spp.	South Africa	Bezuidenhout *et al*. (2010)

SUMMARY

Rivers and riparian ecosystems often reveal the simultaneous presence of a large number of *Phytophthora* spp. In South Africa a small number of studies have focused on *Phytophthora* diversity in rivers, and these were restricted to the Western Cape Province. The studies conducted in this thesis investigated the *Phytophthora* diversity within the Crocodile River (West) and it's associated riparian ecosystem in the Gauteng Province. Furthermore, a large number of the isolates retrieved during the sampling period were suspected to be hybrids that were also found independently by researchers in Australia. The first objective of this study was to characterize these suspected hybrids together with the similar isolates originating from Australia. The second objective was to characterize the *Phytophthora* spp. diversity present in the Crocodile River using molecular techniques.

Four novel interspecific *Phytophthora* hybrids were characterized from the riparian systems in Australia and South Africa. Three known species were identified as the parental species of these hybrids. All but one of the hybrids exhibited evidence of recombination within their ITS regions. Patterns of nuclear and mitochondrial diversity suggested that these hybrids were most likely formed through sexual hybridization. A high diversity of *Phytophthora* spp. was discovered in the Crocodile River. Eight species were identified in this study, namely *P. lacustris*, *P.* taxon PgChlamydo, *P. citrophthora*, *P. multivora*, *P. plurivora* and a new taxon *P.* taxon Sisulu-river. Six of the species are reported for the first time in South Africa. Two of the novel hybrid species described in Chapter Two were also detected in the Crocodile River.

The presence of sexual hybrids between apparently sterile species raises questions as to how sterility is experimentally determined within *Phytophthora*. It is also curious how these hybrids came to be present on two different continents. Species from Clade 6 were dominant in this study and this is consistent with their purported role as aquatic saprobes living off freshly fallen leaves and other plant debris. The results of this study suggest that the *Phytophthora* diversity of riparian ecosystems in South Africa is high and they emphasise the need for more extensive studies to be conducted in a greater number of locations.