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

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### ABSTRACT

Surveys of Australian and South African rivers revealed numerous *Phytophthora* isolates residing in clade 6 of the genus, with internal transcribed spacer (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 (antisilencing factor (ASF) and G protein alpha subunit (GPA)), and one mitochondrial locus (cytochrome oxidase c subunit I (coxI)) were amplified and sequenced to test 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 different hybrid types involving the three described parent species *Phytophthora amnicola*, *Phytophthora thermophila*, and *Phytophthora* taxon PgChlamydo. In all cases, only a single coxI allele was detected, suggesting that hybrids arose from sexual recombination. All the hybrid isolates were sterile in culture and all their physiological traits tended to resemble those of the maternal parents. Nothing is known regarding their host range or pathogenicity. Nonetheless, as several isolates from Western Australia were obtained from the rhizosphere soil of dying plants, they should be regarded as potential threats to plant health. The frequent occurrence of the hybrids and their parent species in Australia strongly suggests an Australian origin and a subsequent introduction into South Africa.

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### 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

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et al. 2001). Species of the oomycetes genus *Phytophthora*, which represents a large group of plant pathogens, are adapted for aquatic dispersal as they produce motile zoospores (Judelson & Blanco 2005). Often, multiple *Phytophthora* spp. are isolated in surveys of waterways (Hwang et al. 2008; Reeser et al. 2011; Hüberli et al. 2013). Not surprisingly, several *Phytophthora* spp. are involved with riparian tree diseases, such as *Phytophthora lateralis* (clade 8) that causes Port-Orford-cedar (*Chamaecyparis lawsoniana*) decline (Hansen et al. 2000), *Phytophthora alni* (clade 7) causing alder (*Alnus* spp.) decline (Brasier et al. 2004), and *Phytophthora ramorum* (clade 8) causing sudden oak death on oak (*Quercus* spp.) and tanoak (*Lithocarpus densiflorus*) (Rizzo et al. 2002).

*Phytophthora* spp. residing in internal transcribed spacer (ITS) clade 6 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.

*Phytophthora* clade 6 includes 24 taxa in three subclades, with several species not yet formally described (Crous et al. 2012; Kroon et al. 2012). It is hypothesized that this clade may contain between 28 and 84 extant species (Brasier 2009). In subclade I, *Phytophthora 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). *Phytophthora asparagi*, the only species in subclade III, causes disease on *Asparagus officinalis* in Australia, Europe, New Zealand, and USA (Förster & 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 subclade II, with the exception of *Phytophthora 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 subclade 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 *Phytophthora gonapodyides* that commonly infects feeder roots of various woody plants in the UK, Europe, and USA (Brasier et al. 1993). *Phytophthora megasperma* frequently causes root and collar rots of various agricultural and horticultural crops in temperate and subtropical regions of the world (Hansen et al. 1986; Brasier et al. 2003b). *Phytophthora 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 *Phytophthora cambivora* and a *Phytophthora 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 subspecies 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 *Phytophthora cactorum* and *Phytophthora nicotianae* and known as *Phytophthora × 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 *Phytophthora hedraiaandra*, described as *Phytophthora × serendipita*, were found in the Netherlands (Man in't Veld et al. 2007, in press). Experimental hybridization between *Phytophthora capsici* and *P. nicotianae* produced offspring that had a wider host range than either parental species (Ersek et al. 1995), reinforcing the view that hybridization can lead to novel or altered pathogenic capabilities.

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 those isolates with anomalous ITS sequence reads and to test the hypothesis of their hybrid nature using nuclear and mitochondrial molecular markers, as well as physiological and morphological traits. Furthermore, we considered the reasons why the ITS sequences have been difficult to read and the feasibility of using these sequences to differentiate between hybrids.

## Materials and methods

### 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 d. 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 10 s, rinsed in distilled water, and plated onto NARPH agar (Hüberli et al. 2000). Hyphal tips were excised from colonies, after the NARPH plates had been incubated for 3–5 d in the dark at room temperature, and transferred to 10 % V8 agar (V8A) (100 ml Campbell's V8 juice, 3 g CaCO<sub>3</sub>, 16 g agar, 900 ml distilled water) in Petri dishes.

Isolates from Tasmania and Victoria were obtained by filtration of 1 L 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 h 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 & Kassaby 1974). Regardless of the isolation technique, isolates were further subcultured 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.

#### Analysis of polymorphisms in ITS sequence data

*Phytophthora* isolates were grown for 2 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<sub>4</sub>Ac but were directly precipitated with 450 µl isopropanol.

The polymerase chain reaction (PCR) was used to amplify the ITS region of the ribosomal DNA (rDNA). PCR mixtures were set up so as to contain 1× PCR reaction buffer (Roche Diagnostics, Mannheim, Germany), 2 mM MgCl<sub>2</sub> (Roche Diagnostics, Mannheim, Germany), 2.5 units of FastStart Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany),

200 µM of each dNTP, 0.45 µM of each primer (Table 2), 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 °C for 4 min followed by 35 cycles of denaturation at 95 °C for 30 s, primer annealing at 55 °C for 45 s, and extension at 72 °C for 60 s. This was followed by an additional extension step of 72 °C for 4 min after which samples were held at 4 °C. 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<sup>®</sup>-T Easy Vector System (Promega, Madison, Wisconsin, USA). Competent JM109 *Escherichia coli* cells were transformed with recombinant plasmids and plated on Luria-Bertani (LB) agar (10 g Tryptone, 5 g Yeast Extract, 5 g NaCl and 15 g Agar) amended with 100 mg/l ampicillin, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 80 mg/l 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside plates. Plates were incubated overnight at 37 °C and recombinant transformant colonies were identified using blue/white screening. Colony PCRs 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 & 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<sup>®</sup> 3100 Genetic Analyser (Applied Biosystems, Foster City, California, USA). For the ITS region, ten 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 *Phytophthora amnicola* (Crous et al. 2012), *Phytophthora thermophila* (Jung et al. 2011), and *Phytophthora* taxon PgChlamydo (Brasier et al. 2003a). All three of these species occur in Australia, but only *P. taxon PgChlamydo* occurs in South Africa. In order to validate the consensus sequence for each of the putative parental species and to accurately identify which ITS sequences among the hybrid isolates were recombinant, the level of intraspecific sequence variation within the ITS region was established. To this end, 50 amplicons of the type isolate of *P. thermophila* (CBS127954), 20 amplicons of the type isolate of *P. amnicola* (CBS131652), ten 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*.



Table 1 – Isolates used in this study.

Reference collection number <sup>a</sup>	Other collection numbers	Identity <sup>b</sup>	Substrate	Host	Location	Isolated by	Date	GenBank accession number		
								ASF	GPA	coxI
CBS131652	DH228	<i>Phytophthora amnicola</i>	Water	Stream baiting	Lake Jualbup, WA, Australia	D. Hüberli	2009	JQ936759		JQ029948
VHS19503		<i>P. amnicola</i>	Soil	<i>Patersonia</i> sp.	Pemberton, WA, Australia	VHS	2008	JQ936760		JQ029950
CBS129424	DH086	<i>P. fluvialis</i>	Water	Stream baiting	Moore River, WA, Australia	D. Hüberli	2009	JQ936761	JQ936733	JF701442
VHS17350		<i>P. fluvialis</i>	Water	Stream baiting	Badgingarra, WA, Australia	VHS	2007	JQ936762		JF701440
MUCC775	DH213	<i>P. fluvialis</i>	Water	Stream baiting	Moore River, WA, Australia	D. Hüberli	2009		JQ936734	JF701441
CBS309.62	PFF309	<i>P. fragariae</i>	Plant	<i>Fragaria × ananassa</i>	Scotland, United Kingdom	C.J. Hickman	1962	DQ092832	DQ092858	
MUCC776	TAS35	<i>P. gonapodyides</i>	Water	Stream baiting	TAS, Australia		2009	JQ936763	JQ936735	JN547642
MUCC761	SLPA72	<i>P. gonapodyides</i>	Water	<i>Eucalyptus obliqua</i> forest	Toolangi North State Forest, VIC, Australia	W.A. Dunstan	2008	JQ936764	JQ936736	HQ012850
VHS17085		<i>P. litoralis</i>	Soil	<i>Banksia</i> sp.	Hopetoun, WA, Australia	VHS	2007	JQ936766	JQ936738	HQ012864
CBS127953	VHS20763	<i>P. litoralis</i>	Soil	<i>Banksia</i> sp.	Ravensthorpe, WA, Australia	VHS	2008	JQ936765	JQ936737	HQ012866
DDS3432		<i>P. megasperma</i>	Soil	<i>Banksia</i> sp.	North Dinninup, WA, Australia	VHS	1992	JQ936768	JQ936740	HQ012867
VHS17183		<i>P. megasperma</i>	Soil	<i>Xanthorrhoea platyphylla</i>	Esperance, WA, Australia	VHS	2007	JQ936767	JQ936739	HQ012868
VHS17175		<i>P. asparagi</i>	Soil	<i>Banksia media</i>	Esperance, WA, Australia	VHS	2007			HQ012844
MUCC766*	SLPA121	<i>P. taxon PgChlamydo</i>	Water	Stream baiting	Yea Wetlands, VIC, Australia	W.A. Dunstan	2008	JQ936771	JQ936743	JN547652
VHS6595*		<i>P. taxon PgChlamydo</i>	Soil	Native forest	Manjimup, WA, Australia	VHS	1999	JQ936770	JQ936742	HQ012879
DDS3753*		<i>P. taxon PgChlamydo</i>	Soil	Native forest	Manjimup, WA, Australia	VHS	1995	JQ936769	JQ936741	HQ012878
IMI389731	P510	<i>P. taxon PgChlamydo</i>	Roots	<i>Pseudotsuga</i> sp.	Walley, British Columbia, Canada	P.B. Hamm & E.M. Hansen	1984	JQ936772		
VHS7474		<i>P. thermophila</i>	Soil	Native forest	Manjimup, WA, Australia	VHS	2000	JQ936773	JQ936752	HQ012871
VHS13530	CBS127954	<i>P. thermophila</i>	Soil	<i>Eucalyptus marginata</i>	Dwellingup, WA, Australia	VHS	2004	JQ936774	JQ936753	HQ012872
VHS3655		<i>P. thermophila</i>	Soil	Native forest	Quinninup, WA, Australia	VHS	1998			HQ012870
VHS16164		<i>P. thermophila</i>	Soil	<i>Banksia grandis</i>	Pemberton, WA, Australia	VHS	2006			HQ012875
VHS13567		<i>P. thermophila</i>	Roots	<i>E. marginata</i>	Dwellingup, WA, Australia	VHS	2004			HQ012873
VHS13761		<i>P. thermophila</i>	Soil	<i>E. marginata</i>	Dwellingup, WA, Australia	VHS	2004			HQ012874
CMW37727	J 2.2 C	A–PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2009	JQ890332	JQ890356	JQ890348
CMW37728	J 2.4 A	A–PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2009	JQ890333 JQ890334		JQ890349
CMW37729	J 2.23 A	A–PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2010	JQ890335 JQ890336	JQ890357	JQ890350
CMW37730	J 2.24 A	A–PG	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2010	JQ890337 JQ890338	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	W.D. Dunstan	2008	JQ936776 JQ936785	JQ936732 JQ936745	JQ936798

MUCC778*	SLPA56	PG-A	Soil	Track drain, native forest	Toolangi North State Forest, VIC, Australia	W.D. Dunstan	2008	JQ936777 JQ936786	JQ936746	JQ936799
MUCC779*	SLPA57	PG-A	Soil	Native forest	Toolangi North State Forest, VIC, Australia	W.D. Dunstan	2008	JQ936778 JQ936787	JQ936747	JQ936800
CMW37731	J 1.3 A	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2009	JQ890340 JQ890341	JQ890359	JQ890352
CMW37732	J 4.2 D	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2009	JQ890342 JQ890343	JQ890360	JQ890353
CMW37733	J 4.9 A	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. Nagel	2010	JQ890344 JQ890345	JQ890361	JQ890354
CMW37734	J 5.11 C	T-A	Water	Stream baiting	Crocodile River, Roodepoort, South Africa	J.H. 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
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 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
ASF1	ASF-E1-1F	ACCAACATCACCGTGTGGAC	Ioos et al. (2006)
	ASF-E2-2R	CGTTGTTGACGTAGTAGCCAC	Ioos et al. (2006)
coxI	FM84	TTTAATTTTGTAGTCTTTTGC	Martin & Tooley (2003)
	FM83	CTCCAATAAAAAATAACCAAAAATG	Martin & Tooley (2003)
	FM50	GTTTACTGTTGGTTTAGATG	Martin & Tooley (2003)
	FM85	AACTTGACTAATAATACCAAA	Martin & Tooley (2003)
GPA1	GPA-E1-1F	GGACTCTGTGCGTCCCAGATG	Ioos et al. (2006)
	GPA-E2-1R	ATAATTGGTGTGCAGTGCCGC	Ioos et al. (2006)
ITS	ITS6	GAAGGTGAAGTCGTAACAAGG	Cooke et al. (2000)
	ITS4	TCCTCCGCTTATTGATATGC	White et al. (1990)
pGEM <sup>®</sup> -T Easy plasmid	T7	TAATACGACTCACTATAGGG	
	SP6	ATTTAGGTGACACTATAGAA	

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. An 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 and 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 more than one parent species.

#### Phylogenetic relationships of nuclear and mitochondrial genes

Although the ITS region is the most frequently used locus for phylogenetic inference, it is not particularly well suited for studies on 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 allopolyploid 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.

To elucidate the parentage of the putative hybrid isolates, two single copy nuclear genes and one mitochondrial gene region were sequenced and subjected to phylogenetic analyses. The antisilencing 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 (coxI) as the mitochondrial gene. These loci were amplified by PCR using the same DNA as 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 the ITS region, whereas for amplification of the coxI locus the annealing temperature was changed to 65 °C and the length of time for the cycled primer annealing and extension steps was increased to 60 s and 2 min, 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 °C for 30 s, the annealing temperature for 45 s, and 72 °C for 60 s. The annealing temperature in the above cycle was initially 65 °C, but was lowered by 0.2 °C per cycle for 25 cycles, followed by 15 cycles of 60 °C.

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, after which they 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 coxI 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 (Kato 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 Markov Chain (MC<sup>3</sup>) algorithm. In order to determine the optimal evolutionary model, each locus was subjected to hierarchical likelihood ratio tests (hLRT) using MrModeltest 2.2 (Nylander 2004). Bayesian analyses were done using MrBayes 3.1 (Ronquist & Huelsenbeck 2003) and each analysis was run for 5 000 000 generations. Tracer 1.4 (Rambaut & Drummond 2004) was used to determine burn-in values prior to parameter and tree summarization.

### Colony morphology and growth rates

In order to compare the putative 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 putative hybrid isolates, as well as for *Phytophthora* taxon PgChlamydo (VHS6595, VHS3753, MUCC766), *Phytophthora amnicola* (VHS19503 and CBS131652), and *Phytophthora thermophila* (VHS7474, CBS127954, VHS3655, and VHS16164) (Table 1). Colony growth patterns were described from 7-d-old cultures grown at 20 °C 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 centres of 90 mm V8A plates and incubated at 20 °C for 24 h in the dark. The growth that occurred during the 24 h incubation was noted on each plate following to the method described by Hall (1993). Plates were then transferred to incubators set at 15 °C, 20 °C, 25 °C, 30 °C, 32.5 °C, 35 °C, 37.5 °C. Three replicate plates were used for each isolate at each temperature. After 5–7 d, the radial growth of each culture was measured along two perpendicular axes and the mean radial growth rates (mm per day) were calculated and plotted against temperature. Plates, incubated at a high temperature and where no growth was observed, were moved to a 20 °C incubator to establish their viability.

### Morphology of sporangia and gametangia

Besides colony characteristics, dimensions of selected morphological characters were measured to further compare the putative 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 *Phytophthora thermophila* (Jung et al. 2011) and *Phytophthora amnicola* (Crous et al. 2012). Sporangia and hyphal swellings produced on V8A were measured using the methods described by Jung et al. (1999). Sporangia were produced by flooding 15 × 15 mm V8A agar pieces, taken from the growing margins of 7-d-old colonies, so that their surfaces were covered with distilled water in 90 mm Petri dishes. These were incubated at room temperature around 22 °C in natural daylight. The water was decanted and replaced after 2 and 8 h. Two millilitres of diluted nonsterile soil extract was added to the replaced water at 8 h. The soil extract was made from 20 g of rhizosphere soil (collected beneath a planted *Quercus* sp.) suspended in 200 ml distilled water, incubated for 24 h at 20 °C, filtered through cheesecloth, and refiltered through Whatman no. 1 paper.

After 15–24 h, dimensions and characteristic features were measured on 50 mature sporangia and 25 exit pores and zoospore cysts chosen at random per isolate. Likewise, after 3–7 d, 25 hyphal swellings were also measured. All measurements were made at 400× magnification (BX51, Olympus). In order to stimulate the formation of gametangia, isolates were paired with *Phytophthora cinnamomi* tester strains of the A1 (CMW29606, CMW29607) and A2 (CMW29597, CMW29598) mating type. Paired cultures were incubated at 20 °C in the dark for 2–4 weeks. Cultures were monitored throughout this period for the presence of sexual structures.

## Results

### 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).

#### Intraspecific polymorphisms of *Phytophthora amnicola*, *Phytophthora thermophila*, and *Phytophthora* 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 (Supplementary Material Table A.1). 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. 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 %.

*Phytophthora thermophila* produced ITS sequences that were 819 bp in length. Among 50 cloned amplicons of the *P. thermophila* type isolate (CBS127954) there were three high frequency SNPs (Supplementary Material Table A.2); a T–A transversion at position 464 and C–T transitions at positions 513 and 573.



**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 <sup>a</sup>	41	44	59	71	110	148	162	171	180	184	457	476	477	518	519	555	699	746	748	750	751	752	753	CODE <sup>b</sup>	
Isolates with <i>P. amnicola</i> and <i>P. taxon</i> PgChlamydo sequences																										
<i>P. amnicola</i> 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	
<i>P. amnicola</i> consensus-2		C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	A	
PgChlamydo consensus		C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
CMW37727-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
CMW37727-2	2	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	A	A	A	-	-	-	-	T	
CMW37727-3	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	-	-	-	-	T	
CMW37727-4	5	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	A	
CMW37728-1	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
CMW37728-2	9	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
CMW37729-1	4	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
CMW37729-2	2	C	T	T	C	G	G	A	-	T	T	G	C	G	T	T	C	C	A	A	A	-	-	-	T	
CMW37729-3	2	C	A	C	C	A	A	A	-	T	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
CMW37730-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
CMW37730-2	1	C	A	C	C	A	A	A	-	T	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
CMW37730-3	1	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
CMW37730-4	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
MUCC777-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC777-2	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	-	-	-	-	T	
MUCC777-3	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	C	
MUCC777-4	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
MUCC778-1	6	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC778-2	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	-	-	-	-	T	
MUCC778-3	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
MUCC779-1	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC779-2	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC779-3	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	G	G	G	-	-	-	-	C	
MUCC779-4	6	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
MUCC774-1	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC774-2	1	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	A	A	C	T	T	T	T	
MUCC774-3	1	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC774-4	1	C	A	C	C	A	A	A	-	T	T	A	G	T	T	C	C	G	G	G	-	-	-	-	C	
MUCC774-5	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	G	G	G	-	-	-	-	C	
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	T	
MUCC774-7	1	C	T	T	C	G	G	C	T	A	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC774-8	1	C	T	T	C	G	G	C	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC774-9	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
Isolates with <i>P. thermophila</i> and <i>P. taxon</i> PgChlamydo sequences																										
PgChlamydo consensus		C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
<i>P. thermophila</i> consensus		T	A	C	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C
MUCC783-1	2	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	A	G	A	-	-	-	-	C	
MUCC783-2	8	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
MUCC784-1	2	T	A	C	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C
MUCC784-2	8	C	A	C	C	A	A	A	-	T	T	G	C	G	C	T	C	G	G	G	-	-	-	-	C	
Isolates with <i>P. amnicola</i> and <i>P. thermophila</i> sequences																										
<i>P. amnicola</i> 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	
<i>P. amnicola</i> consensus-2		C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
<i>P. thermophila</i> consensus		T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C	
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	
CMW37731-3	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C	
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	
CMW37732-3	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	C	
MUCC780-1	9	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
MUCC780-2	1	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	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	
CMW37733-2	1	T	A	C	T	G	G	C	-	A	A	A	C	G	T	T	C	C	A	A	A	-	-	-	T	
CMW37733-3	1	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	C	
CMW37733-4	1	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	C	A	A	A	-	-	-	-	T	
CMW37733-5	1	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	A	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	
CMW37734-1	4	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
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	
CMW37734-3	1	T	A	C	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
CMW37734-4	3	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C	
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	
MUCC781-3	2	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	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	
MUCC781-5	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	T	
MUCC782-1	3	C	T	T	C	G	G	C	T	A	T	A	G	T	T	C	C	A	A	A	-	-	-	-	T	
MUCC782-2	1	T	A	C	T	G	G	C	T	A	T	A	C	G	T	T	C	C	A	A	A	-	-	-	T	
MUCC782-3	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	C	A	A	A	-	-	-	-	T	
MUCC782-4	1	C	T	C	T	G	G	C	T	A	T	A	C	G	T	T	C	C	A	A	A	-	-	-	T	
MUCC782-5	1	T	A	C	T	G	G	C	-	A	A	A	C	G	T	T	C	C	A	A	A	-	-	-	T	
MUCC782-6	2	T	A	C	T	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C	
VHS22715-1	7	C	T	T	C	G	G	C	T	A	T	A	C	G	T	T	C	C	A	A	A	-	-	-	T	
VHS22715-2	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	C	T	A	A	A	-	-	-	-	T	
VHS22715-3	1	C	T	T	C	G	G	C	T	A	T	A	C	G	T	T	C	T	A	G	A	-	-	-	C	
VHS22715-4	1	C	T	T	C	G	G	C	-	A	A	A	C	G	T	C	T	A	G	A	-	-	-	-	C	
VHS5185-1	6	C	T	T	C	G	G	C	T	A	T	A	C	G	T	T	C	C	A	A</						

Each of these variable sites appeared in approximately 30 % of ITS copies and was linked. Additionally, rare SNPs occurred at a frequency of approximately 0.09 %.

*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 (Supplementary Material Table A.3). These two high frequency SNPs were not linked. There were also very few rare SNPs (frequency <0.02 %).

#### Interspecific polymorphisms and comparisons of putative hybrid isolates

Interspecific variation in the form of SNPs and indels was seen in the alignment between the consensus ITS 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 *Phytophthora 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 *Phytophthora thermophila* or *Phytophthora* taxon PgChlamydo. The consensus sequences for *P. amnicola* differed from that of *P.* taxon PgChlamydo by 15 SNPs and the one indel. There were ten 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 putative 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 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.

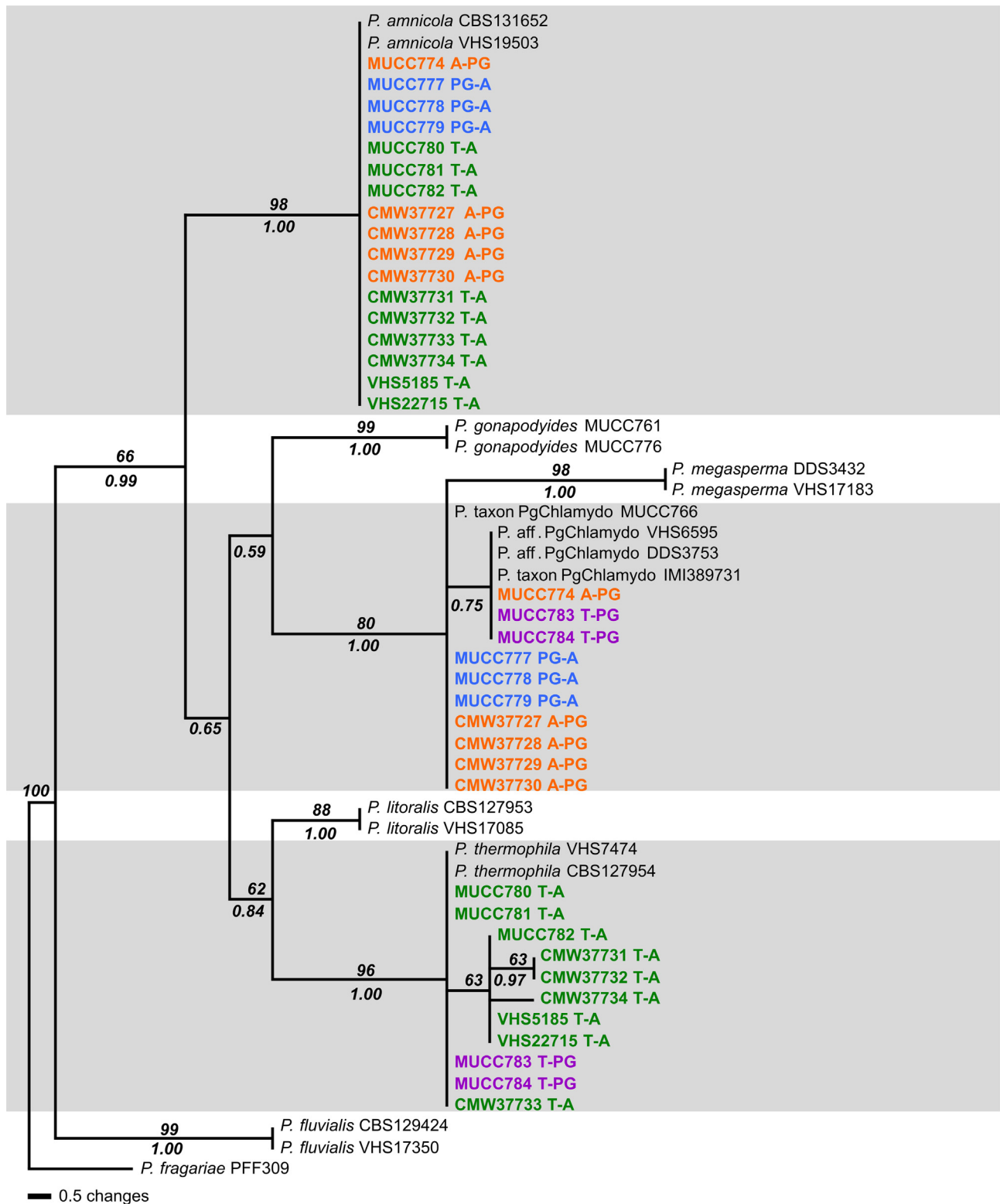
#### Phylogenetic relationships of nuclear and mitochondrial genes

Data matrices and trees have been deposited at TreeBASE under study accession number S12996 and are available at <http://purl.org/phylo/treebase/phyloids/study/TB2:S12996>. 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 (Fig 1). 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.

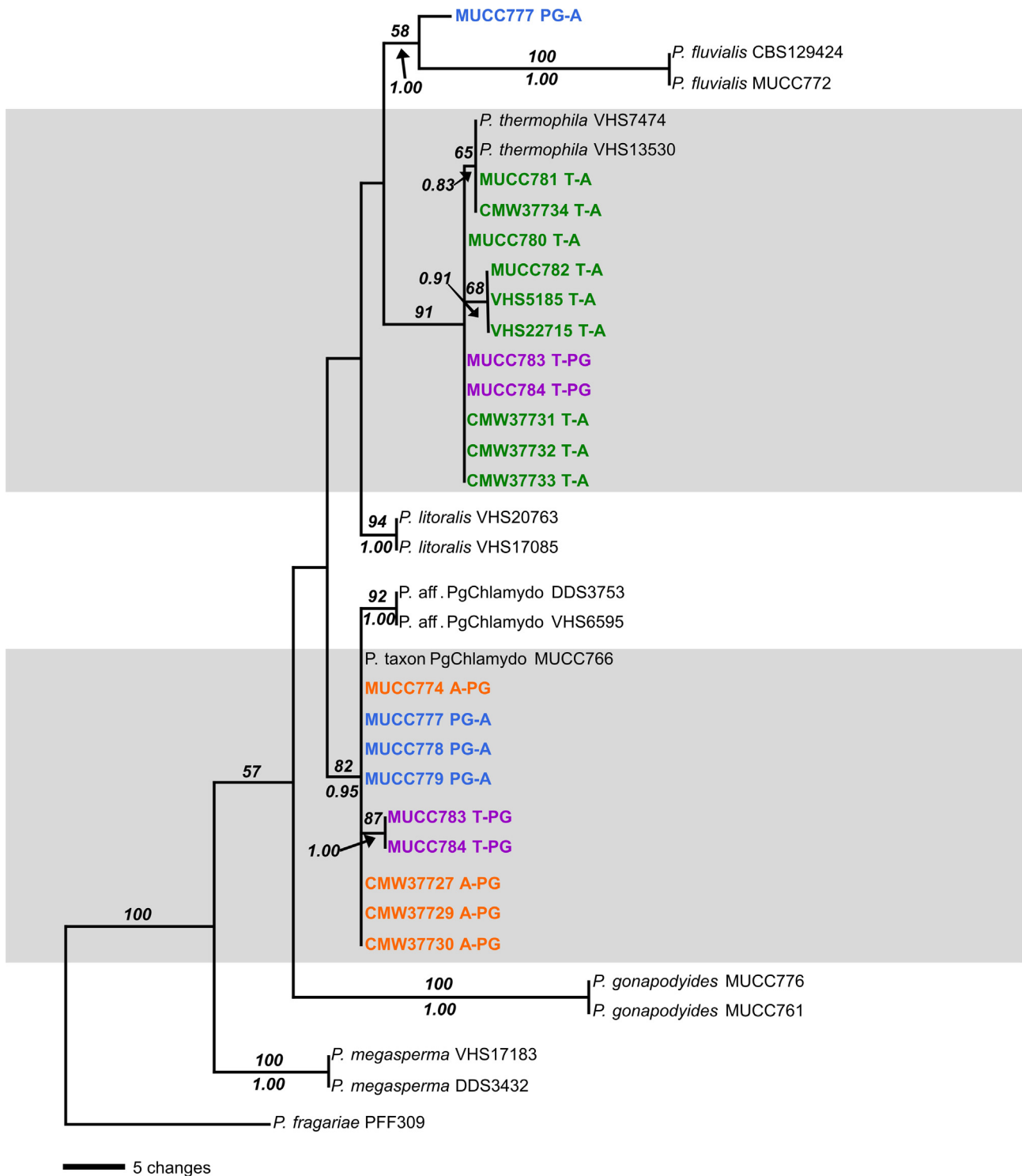
The GPA1 sequence alignment was 306 characters in length with 71 parsimony informative characters. Four equally parsimonious trees resulted from the analysis were generated of 141 steps (CI = 0.79, RI = 0.91, RC = 0.71) and there were only small differences in the terminal branches. All species resided in well supported clades (Fig 2). 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 *coxI* 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 (Fig 3). 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 (Fig 1) the three reference species, *Phytophthora amnicola*, *Phytophthora thermophila*, and *Phytophthora* taxon PgChlamydo each resided in a well supported clade. Each isolate of these three species had a single ASF-like allele. The isolates with dual ITS profiles, 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



**Fig 1 – Phylogenetic tree based on the ASF locus of *Phytophthora* spp. generated by a MP 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

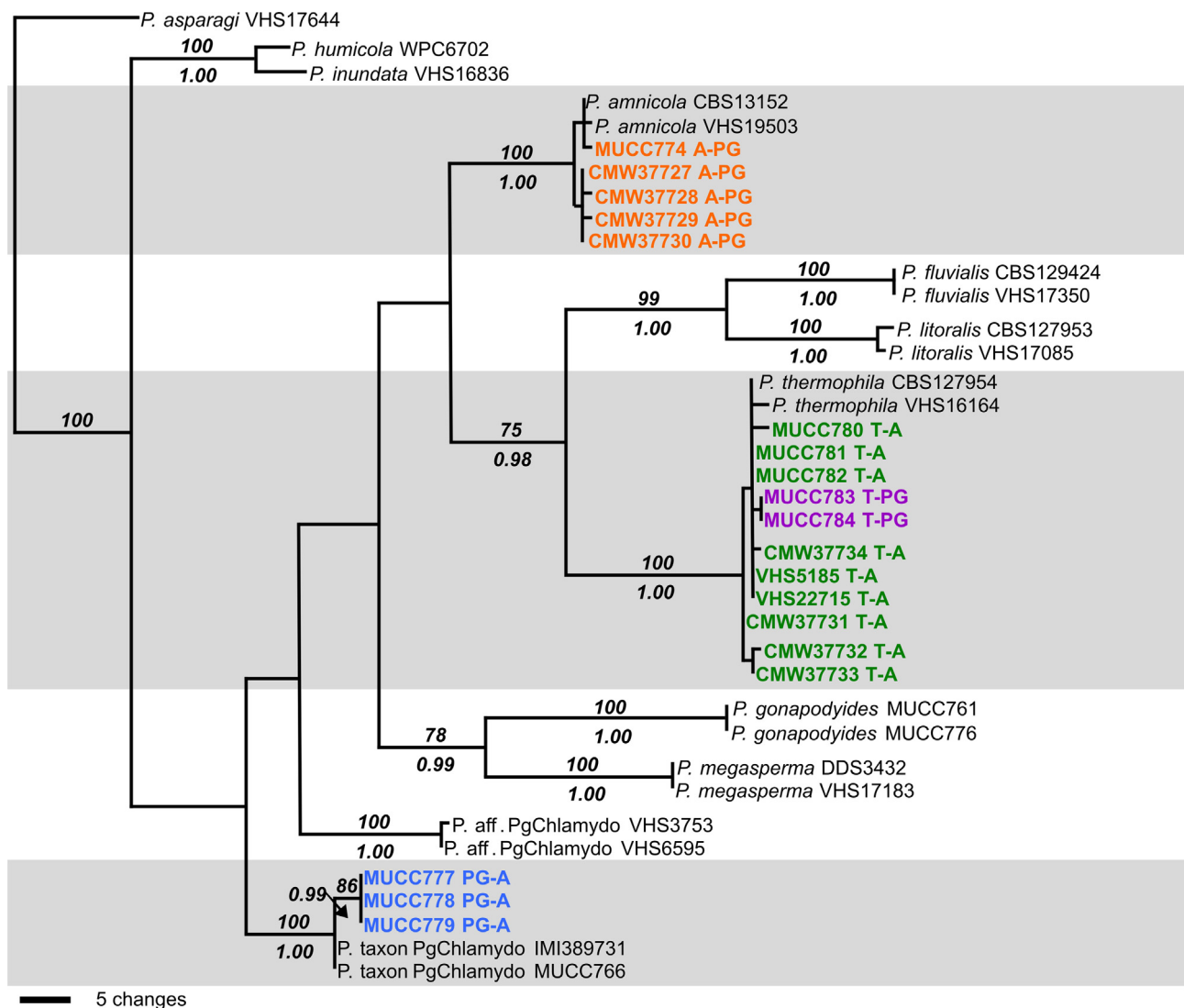


**Fig 2 – Phylogenetic tree based on the GPA locus of *Phytophthora* spp. generated by a MP heuristic search. Bootstrap support values appear above and posterior probabilities below branches. *Phytophthora fragariae* is used as an outgroup taxon. Hybrid taxa are indicated in colour: orange = A-PG, blue = PG-A, green = T-A, and purple = T-PG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)**

(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





**Fig 3** – Phylogenetic tree based on the *coxI* locus of *Phytophthora* spp. generated by a MP 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

two different parental species. Furthermore, the hybrid groups identified using the ASF-like phylogeny supported the same hybrid groups comprised the same isolates, as those identified by the 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 (Fig 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 (MUCC783 and MUCC784) that were identified by the ITS polymorphism analysis and ASF-like phylogeny to be hybrids of *P. thermophila* and *P. taxon PgChlamydo*, 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 *Phytophthora 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 *coxI* phylogeny (Fig 3), the three reference species, *P. amnicola*, *P. thermophila*, and *P. taxon PgChlamydo*, each resided in a well supported clade and isolates of each species had a single *coxI* allele. The hybrid isolates all had a single *coxI* 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 *coxI* 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 *coxI* allele grouping with those from *P. amnicola*, while another subset (MUCC777, MUCC778, and MUCC779) had a *coxI* allele that grouped with that from *P. taxon PgChlamydo*. The *coxI* phylogeny could not identify any isolates as hybrids, but it did indicate that the *coxI* 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.

#### Nomenclatural status of hybrids

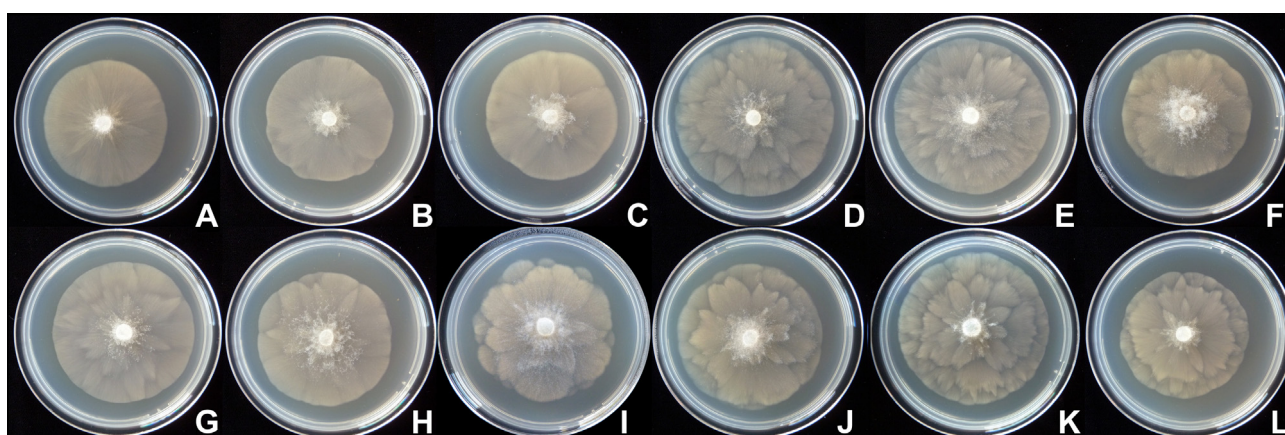
Four hybrid groups were identified from the phylogenetic analyses of ASF-like, GPA1, and *coxI* and from the polymorphism comparison of the ITS region. These hybrid taxa were represented by hybrid formulae and since the identity of the maternal parent could be established, the recommendation of the International Code of Nomenclature for algae, fungi, and plants (ICN) article H.2A.1 can be followed, where the name of the maternal parent precedes that of the male. The proposed terminology for these hybrids are as follows: *Phytophthora amnicola*×*Phytophthora taxon PgChlamydo* (A–PG), *P. taxon PgChlamydo*×*P. amnicola* (PG–A), *Phytophthora thermophila*×*P. amnicola* (T–A), and *P. thermophila*×*P. taxon PgChlamydo* (T–PG) (see Table 1).

#### Colony morphology and growth rates

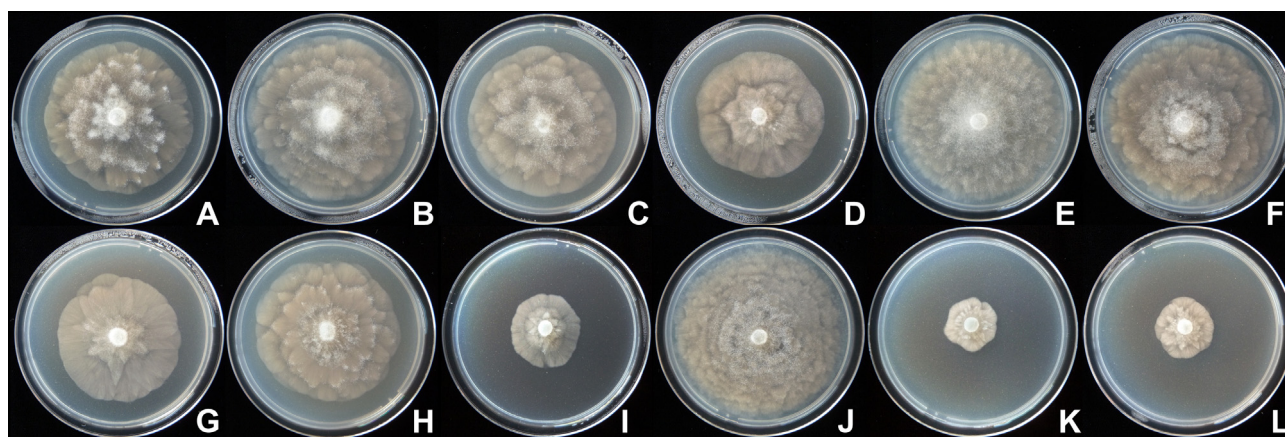
On V8A, all isolates and their parental species produced little to no aerial mycelia but differences in growth patterns could be observed between the reference species and hybrid groups (Fig 4). Isolates of *Phytophthora taxon PgChlamydo* grew uniformly without any distinct pattern, those of *Phytophthora amnicola* were densely petaloid to stellate and *Phytophthora thermophila* produced faintly petaloid colonies. Considerable variation was seen in the colony morphology of the hybrids, but overall, they 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 (Fig 5). Isolates of *P. taxon PgChlamydo* produced colonies with a rosaceous growth form, *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 patterns that tended to resemble that of the maternal parent more than the paternal parent. 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 (Fig 6). *Phytophthora 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,



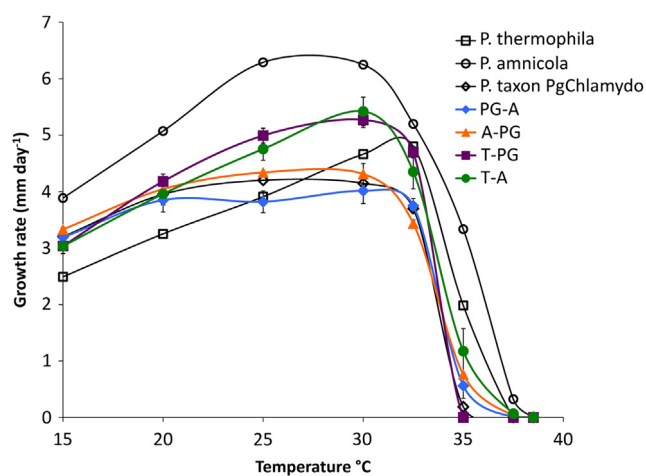
**Fig 4** – Colony morphology of four hybrid taxa on V8A compared with their known parental *Phytophthora* species. (A) *P. taxon PgChlamydo* (VHS6595), (B, C) *P. taxon PgChlamydo*×*P. amnicola* (MUCC778 and MUCC779), (D) *P. amnicola* (CBS131652), (E) *P. amnicola*×*P. taxon PgChlamydo* (MUCC774), (F) *P. taxon PgChlamydo* (MUCC766), (G,H) *P. thermophila*×*P. taxon PgChlamydo* (MUCC783 and MUCC784), (I) *P. thermophila* (VHS16164), (J–L) *P. thermophila*×*P. amnicola* (MUCC780, MUCC782, and VHS22715).



**Fig 5** – Colony morphology of four hybrid taxa on CA compared with known parental *Phytophthora* species. (A) *P. taxon PgChlamydo* (VHS6595), (B,C) *P. taxon PgChlamydo* × *P. amnicola* (MUCC778 and MUCC779), (D) *P. amnicola* (CBS131652), (E) *P. amnicola* × *P. taxon PgChlamydo* (MUCC774), (F) *P. taxon PgChlamydo* (MUCC766), (G,H) *P. thermophila* × *P. taxon PgChlamydo* (MUCC783 and MUCC784), (I) *P. thermophila* (CBS127954), (J–L) *P. thermophila* × *P. amnicola* (MUCC780, MUCC782, and VHS22715).

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 of that hybrid. Both isolates of A–PG and PG–A had a tempera

ture–growth relationship similar to that of the isolate representing *P. taxon PgChlamydo* with a broad optimum from 20 °C to 32.5 °C. Isolates of PG–A were able to maintain viability up to 35 °C, whereas A–PG isolates were more variable and the maximum temperature at which they could survive was 35 °C for some isolates and 37.5 °C for others. Isolates of T–A had a profile



**Fig 6** – Graph illustrating the average radial growth rate (in mm d<sup>-1</sup>) over temperature (°C) of isolates representing the parental species *Phytophthora amnicola*, *P. taxon PgChlamydo*, and *P. thermophila*, and the four hybrid taxa. Bars indicate standard errors of the means.

similar to that of *P. thermophila* with a clear optimal temperature. However, while the optimum for *P. thermophila* was 32.5 °C, the optimum for T–A isolates was 30 °C. 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.

#### Morphology of sporangia and gametangia

Isolates of all four hybrid species produced nonpapillate sporangia similar to those of *Phytophthora amnicola*, *Phytophthora thermophila*, and *Phytophthora 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 commonly observed for all the reference species and hybrids. The exception to this was of T–PG isolates which 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 *Phytophthora cinnamomi* tester isolates of either mating type.

#### Discussion

*Phytophthora* isolates collected from water and rhizosphere soil in Australia and South Africa, with highly polymorphic or unsequenceable ITS gene regions, were shown to represent four distinct interspecific hybrids between *Phytophthora amnicola*, *Phytophthora thermophila*, and *Phytophthora taxon PgChlamydo*. Analysis of interspecific polymorphic sites within the ITS region demonstrated the hybrid nature of these isolates

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

	<i>P. thermophila</i>	<i>P. amnicola</i>	<i>P. taxon PgChlamydo</i>	PG–A	A–PG	T–A	T–PG
No. of isolates	5	2	3	3	1	4	2
<b>Sporangia</b>	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 ± 6.3 × 25.7 ± 3.9	62 ± 9.0 × 35.3 ± 5.6	57.7 ± 7.4 × 35.5 ± 4.1	56.2 ± 9.6 × 34.2 ± 6.6	39.1 ± 5.5 × 27.1 ± 4.5	48.2 ± 8.3 × 30.3 ± 4.7	48.5 ± 7.7 × 31.5 ± 3.5
Total range (µm)	29.0–64.8 × 15.6–39.3	39–78 × 17–43	34.9–79.3 × 23.5–49.9	31–93.4 × 18–50.4	26.6–56.4 × 17.5–41.2	30.4–74.8 × 8.8–45.7	31.8–69.7 × 23.7 × 39
Isolate means (µm)	44.2–46.8 × 24.1–26.6		55.7–60.5 × 32.5–38.3	52.2–63 × 30.1–39.1		39.8–55.1 × 28.2 × 33.2	
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	
<b>Exit pores</b>							
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	
<b>Proliferation</b>	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested	Internal extended & nested
<b>Hypthal swellings</b>	Globose or elongated, partly catenulate	Ellipsoid to irregular catenulate hypthal 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	–		
<b>Chlamydospores</b>	Globose, radiating hyphae	–	Globose, radiating hyphae	Chlamydospore-like globose swellings but no true chlamydospores			
Mean diam (µm)	41.5 ± 14.7		41.0 ± 11.7				
<b>Sexual system</b>	Sterile or silent homothallic	Sterile	Sterile	Sterile	Sterile	Sterile	Sterile
<b>Maximum temperature (°C)</b>	35	37.5	35	>35 < 37.5	>35 < 37.5	>35 < 37.5	35
<b>Optimum temperature (°C)</b>	33	25–32.5	20–32.5	20–32.5	20–30	30	25–30



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 (*coxI*) enabled further differentiation of the hybrid isolates based on maternal species. In general, the colony morphology of the hybrids resembled that of the maternal parent, although substantial variation was observed in the growth patterns of isolates within some hybrid groups. For temperature–growth relationships and morphology, hybrids exhibited characteristics intermediate 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 *coxI* loci was the most effective for discriminating between the four hybrid groups. The ASF-like locus could be used effectively to identify both parental lineages without complication and the *coxI* locus was useful to identify the maternal species. Similarly 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 because the locus could not be amplified for *P. amnicola* and the *P. amnicola* allele could not be amplified in any of the hybrids involving that species (A–PG, PG–A or T–A). However, it could successfully identify both parental lineages from T–PG. The ASF-like and GPA1 loci both have been used previously without complication to characterize the *Phytophthora alni* hybrids (Ioos et al. 2006).

The absence of a *P. amnicola* allele for the GPA1 locus could 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 with a hybrid of *P. amnicola* to the effect that the GPA locus becomes homozygous for the *P. amnicola* allele in the resultant progeny. Noninheritance of alleles, due to meiotic nondisjunction, has been reported for *Phytophthora cinnamomi* (Dobrowolski et al. 2002) and *Phytophthora nicotianae* (Förster & Coffey 1990) and this phenomenon could explain the above observations. Alternatively, this could also be explained by gene conversion disparity where one allele is always lost (Chamnanpant 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 was 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 *coxI* 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 homoplasmic state. Interspecific somatic hybridization would combine two diploid genomes resulting in an allopolyploid hybrid that is also heteroplasmic for the mitochondrial genome. Heteroplasmy is rapidly reduced to homoplasmy through the random segregation of mitochondrial genomes (Chen & Butow 2005). Little is known, however, about the occurrence and mechanism of parasexual processes such as somatic hybridization in *Phytophthora*. It has been shown previously that *Phytophthora* × *pelgrandis* and *Phytophthora* × *serendipita* arose from sexual hybridization because both these hybrids exhibited biparental inheritance of nuclear genes and uniparental inheritance of mitochondrial genes (Bonants et al. 2000; Man in't Veld et al. 2007, in press; Hurtado-Gonzales et al. 2009). In contrast with the relatively simple situation observed in *P. pelgrandis* and *P. serendipita*, the *Paa* hybrid did not exhibit an obvious biparental inheritance pattern of nuclear genes as it possessed three alleles for nuclear loci. However, when we consider *Pam* and *Pau*, 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 selfsterile (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 nonsterile 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 might account for the apparent 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 and have the potential to cross with other hybrids (i.e. a hybrid swarm) or for 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 & Sekelsky 2010). If, however, these hybrids are capable of sexual reproduction in nature, both meiotic and mitotic recombination would occur. Although recombination gave rise to significant variation between the rDNA subunits, the nonrecombined parental-type subunits remained. This was also shown with *Paa* (Brasier et al. 1999), which 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. ×pelgrandis* (Hurtado-Gonzales *et al.* 2009) or *P. ×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 is 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 intraindividual rDNA sequence heterogeneity exist (Sang *et al.* 1995; Campbell *et al.* 1997). This high level of sequence heterogeneity suggests 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 nonhybrid species.

The number 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 had clear recombination in their ITS regions. 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 *coxI* 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.

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 (Nagel 2012) and probably has a global distribution (Hansen *et al.* 2007). Currently, the origin of all three species is unknown. By extension, it is also not known whether the reported hybrids represent a natural phenomenon between endemic species or whether they are the result of novel contact between endemic and introduced species. However, 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, where they maintain their presence through asexual reproduction. 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.

## Conclusions

Our observations that multiple hybridization events occurred and continue to occur in nature have important implications for plant pathology and ecosystem management. They reinforce 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 instance of the growing threat posed by hybrid fungi (Brasier 2000) to biodiversity, forestry, and agriculture. This is in addition to the well-known threat that is posed directly by the introduction of any pathogenic *Phytophthora* species into noninfested 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 *Phytophthora thermophila* and *Phytophthora* 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) the precautionary principle 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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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.funbio.2013.03.004>.

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