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Phytophthora parsiana sp. nov., a new high-temperature tolerant species

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ABSTRACT

As part of a study to examine the phylogenetic history of the taxonomically challenging species *Phytophthora cryptogea* and *P. drechsleri*, a distinct monophyletic group of isolates, previously described as *P. drechsleri* or *P. cryptogea*, were characterised. Analysis of their rDNA ITS sequences indicated that these isolates were distinct from *P. drechsleri*, *P. cryptogea*, and all members of *Phytophthora* ITS clades 1–8, clustering instead alongside basal groups previously described as clades 9 and 10. This group comprised six isolates all of which were isolated from woody plants, such as pistachio (*Pistacia vera*, Iran and USA), fig (*Ficus carica*, Iran), and almond (*Prunus dulcis*, Greece). Analysis of sequence data from nuclear (β-tubulin and translation elongation factor 1α) and mitochondrial (cytochrome c oxidase subunit I) genes confirmed the ITS-based analysis as these isolates formed a distinct monophyletic group in all NJ trees. The isolates were fast growing with a relatively high optimum growth temperature of 30 °C and, in most cases, rapid colony growth even at 37 °C. The isolates produced complex colony patterns on almost all media, especially corn meal agar (CMA). Phylogenetic analysis and examination of all the other morphological and physiological data lead us to infer that this taxon has not been described previously. As this taxon was first isolated and described from Iran we propose that this taxon be formally designated as *Phytophthora parsiana*.

Introduction

In 1991 a *Phytophthora* was isolated from the decaying crown of a fig (*Ficus carica*) tree from Bushehr Province, Iran. The non-papillate isolate was able to grow at up to 39 °C and was identified as a high-temperature isolate of *P. cryptogea* (Banihashemi & Ghaisi 1993). A *Phytophthora* isolated from the crown of pistachio (*Pistacia vera*) trees in Kerman Province, Iran in 1992 and 1993 (Z. Banihashemi, unpubl. data) again showed a superficial resemblance to *P. cryptogea* and had maximum growth temperature of 39 °C. MacDonald *et al.* (1992) reported *Phytophthora* isolates similar to *P. cryptogea* that were recovered from the trunk and branches of pistachio (Chico, CA). Again growth of these cultures at high temperatures was reported but their identification was not finalised.

The first step in a recent study to examine the evolutionary history of the taxonomically challenging species *P. cryptogea* and *P. drechsleri*, isolates involved assembling all isolates from the authors collections described as either *P. cryptogea* or *P. drechsleri*. It is clear that many isolates of these taxa have been misdiagnosed (for example Ho & Jong 1986; Mills *et al.* 1991), so a pre-screen of the collection was conducted on the basis of ITS sequence analysis. A group of six isolates shared a distinct and previously unreported ITS sequence

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clearly distinct from either *P. cryptogea* or *P. drechsleri*, and formed a monophyletic group in a basal clade more closely related to clades 9 and 10 *sensu* Cooke *et al.* (2000). These six isolates comprised the abovementioned plus an isolate from almond (*Prunus dulcis*) in Greece. They were considered to belong to a new taxon and were informally designated as *Phytophthora* sp. C10 (Mostowfizadeh-Ghalamfarsa 2005).

The use of DNA sequence data has clearly benefited taxonomic and phylogenetic studies in recent years with several publications examining a broad range of taxa in the genus *Phytophthora* based on several different nuclear and mitochondrial target regions (Cooke *et al.* 2000; Kroon *et al.* 2004; Martin & Tooley 2003). This is coupled with marked increases in *Phytophthora* accessions in international DNA sequence databases, such as GenBank. None of these studies or database accessions, however, contains representatives of the *Phytophthora* sp. C10 taxa. The present paper thus reports a multiple gene genealogy approach for phylogenetic reconstruction of *Phytophthora* sp. C10 to ascertain its relationship with other phytophthoras and describes *Phytophthora* sp. C10 as a new species formally named as *P. parsiana*. The study also reports on the morphological and physiological traits and temperature relations of this taxon.

Material and methods

Organisms and cultural conditions

Details of the *Phytophthora* isolates examined in this study are listed in Table 1. The isolates were sourced from the culture collections of the authors. Isolates were stored on corn meal agar (CMA; Sigma, Poole, UK) slopes at 15 °C. Routine stock cultures for research studies were grown on French bean agar (FBA; ground French beans 30 g l^{-1}, agar 15 g l^{-1}) at 20 °C.

Colony morphology and growth rate

The isolates were grown at 20 °C on CMA, clear V8-juice agar [CV8; 100 ml V8 juice (Campbell's, New Jersey, USA), 900 ml distilled water, 15 g agar], malt extract agar (MEA; Sigma, Poole, UK), potato–dextrose agar (PDA; Sigma, Poole, UK), and hemp seed agar (HSA; extract of 60 g ground hemp seed, 900 ml distilled water, 15 g agar). Petri dishes (9 cm diam) containing 20 ml of the test media were inoculated with 5 mm diam discs cut from the edge of a 5–10-d-old culture. The discs were placed upside down in the centre of each plate, and the plates were incubated in the dark. Colony morphology was noted after 8 d. Growth rate measurements were made after the onset of growth along two lines intersecting at right angles at the centre of the inoculum. Growth rate (mm d^{-1}) was recorded on all media after 5 d. For temperature–growth relationships, CMA plates were inoculated using three replicate plates per isolate and incubated at 5, 10, 15, 20, 25, 30, 35, 37, and 40 °C. Growth rate was recorded 5 d after the onset of linear growth. Tests were repeated twice for the range of 30–37 °C.

Sporangial morphology

One disc (10 mm diam), cut from the growing edge of a 7-d-old-culture grown on CV8 at 20 °C in the dark, was placed in a 9 cm Petri dish and flooded, just over its surface, with non-sterile soil extract (100 g soil flooded with 1 l distilled water for 24 h at room temperature, and then filtered). After incubation at 20 °C in the dark for 48–72 h, dimensions and characteristic features of 50 fully mature sporangia, chosen at random, were determined at ×400 magnification for each isolate.

Breeding system and morphology of oogonia, oospores, and antheridia

Oospores were produced in dual culture with either A1 (IMI 268688) or A2 (IMI 207770) mating types of *Phytophthora nicotianae* on HSA (amended with 30 mg β-sitosterol l^{-1}) plates using 0.2 μm polycarbonate membrane to prevent gametangia of the different species from mixing. For isolates that did not produce oospores the test was repeated using A1 (02B-05) and A2 (02-B10) mating types of *P. infestans* on amended HSA plates. For each isolate, 50 oogonia, oospores, and antheridia, chosen at random, were measured from four to six-week-old cultures grown at 20 °C in the dark on amended HSA. Measurements were made at ×400 magnification.

Pathogenicity

As part of the procedure to discriminate *Phytophthora cryptogea*, *P. drechsleri*, and *P. erythroseptica* from other taxa, all

Table 1 – Origins of isolates studied and their GenBank sequence accession numbers

Isolate code no.	IMI no.	Host	Location	Year isolated	Isolated by	GenBank accession no.[a]			
						ITS	*TUB*	*ELO*	*COX*
SCRP237	395362	*Prunus dulcis*	Greece	1976	INRA[b]	AY659736	AY659743	AY659750	AY659756
SUC7	395327	*Pistacia vera*	USA	1992	*Z. Banihashemi*	AY659737	AY659744	AY659751	AY659757
SUC19	395328	*Pistacia vera*	Iran	1992	*Z. Banihashemi*	AY659738	AY659745	AY659752	AY659758
SUC25	395329	*Ficus carica*	Iran	1991	*Z. Banihashemi*	AY659739	AY659746	AY659753	AY659759
SURf6	395330	*Pistacia vera*	Iran	1993	*Z. Banihashemi*	AY659740	AY659747	AY659754	AY659760
SURf17	395331	*Pistacia vera*	Iran	1993	*Z. Banihashemi*	AY659741	AY659748	AY659755	AY659761

a *COX*, cytochrome c oxidase subunit I; *ELO*, translation elongation factor 1α; ITS, Internal transcribed spacers; *TUB*, ß-tubulin.
b Name of INRA Antibes researcher not known.

Table 2 – Variation in morphological, physiological, and colony characteristics of isolates applied in this study

Character	Isolates					
	SCRP237	SUC7	SUC19	SUC25	SURf6	SURf17
Colony characteristics on						
Corn meal agar						
Pattern	Ros	Ros	Ros	Ros	Ros	Ros
Growth rate[a]	1.3	4.2	4.4	5.3	5.1	5.1
Clear V8-juice agar						
Pattern	Cry	Cry	Cry	Ros	Cry	Cry
Growth rate	3.1	3.5	3	3.9	3	5.1
Malt extract agar						
Pattern	Ros	Ros	Ros	Uni	Ros	Ros
Growth rate	2	3.8	2.8	3.1	2.8	3.3
Hemp seed agar						
Pattern	Cry	Uni	Uni	Uni	Ros	Ros
Growth rate	3.4	4.2	3.4	5	5.1	3.1
Potato–dextrose agar						
Pattern	Cry	Ros	Cry	Ros	Ros	Ros
Growth rate	0.9	2.3	2.4	2.3	3	5.4
Hyphae						
Average diam (μm)	5	5	5	5	5	5
Swellings in water	+	+	+	+	+	+
Swellings on agar	+	+	+	+	+	+
Chlamydospore						
Average diam (μm)	–	–	27	37.5	–	–
Sporangia						
Papilla	–	–	–	–	–	–
Average length (μm)	45.3	47.8	44.2	44.3	56.8	43.5
Range length (μm)	25–67	28–83	27–58	30–68	38–83	28–58
Average breadth (μm)	29.3	26.2	29.9	27.2	39.5	23.8
Range breadth (μm)	17–44	13–38	12–44	20–43	29–58	15–35
Length: breadth ratio	1.55	1.83	1.48	1.63	1.44	1.83
Shape(s)	Op	El,Op	El,Op	El,Op, Ds	El,Op	El,Op
Tapered base	+	–	+	+	+	+
Caducity	–	–	–	–	–	–
Proliferation	P/N	P/N	P/N	P/N	P/N	P/N
Sympodial	–	–	–	–	–	+
Average pore diam (μm)	8	8	11	8	9	7
Mating behaviour	S	S	A1	A1	A1	S
Oogonia						
Average diam (μm)			27.2	29.7	25.9	
Range (μm)			13–40	23–40	17–38	
Tapered base			+	+	+	
Oospores						
Average diam (μm)			27.2	22.8	25.5	
Range (μm)			13–40	15–33	17–38	
Plerotic			+	+	+	
Aplerotic			–	+	+	
Oospore wall						
Average diam (μm)			2	5	3	
Antheridia						
Average diam (μm)			7.7	14.4	13.1	
Shape(s)			Sph	Sph	Sph	
Inception			Amph	Amph	Amph	
Pathogenicity						
Potato pink rot	–	–	–	–	–	–

a Average radial growth rate at 20 °C (mm d^{-1}). +, Feature occurring frequently; –, feature not observed; A1, A1 mating type; Amph, amphigynous; Cyl, cylindrical; Ds, distorted shapes; El, ellipsoid sporangia; N, nested proliferation; Op, obpyriform sporangia; P, proliferating isolate; Rec, rectangular; Ros, rose shaped; S, sterile; Sph, spherical; Uni, uniform Cry, chrysanthemum.

isolates were evaluated for their ability to cause pink-rot symptoms on potato tubers. Tubers were washed and steeped in 10 % (w/v) sodium hypochlorite for 15 min, rinsed with sterile water, and dried. A 7 mm diam plug was removed from the tuber and a 5 mm mycelial disc cut from a CMA culture was inserted before re-sealing with the original potato plug. The cut was sealed with Nescofilm (Bando Chemical Industries, Kobe, Japan) to avoid desiccation, and the potatoes were incubated in the dark for 5 d at 20 °C. The tubers were cut open and exposed to the air for 30 min before observations were recorded.

DNA extraction

Isolates were grown in 20 ml still culture of pea broth (boiled extract of 125 g frozen green peas in 1000 ml distilled water, pH 6.2) at 20 °C. After vacuum filtration, the mycelium was freeze-dried for extended storage at −20 °C. DNA was extracted from mycelium using a Puregene DNA extraction kit, Flowgen (Lichfield, England).

DNA amplification and sequencing

DNA of the ITS regions were amplified using the universal primers ITS6 and ITS4 (Cooke *et al.* 2000; White *et al.* 1990) (Table 3). ITS6 is a version of ITS5 (White *et al.* 1990) modified by comparison against 18S sequences of *Phytophthora* to improve the amplification of DNA from *Oomycota* (Cooke & Duncan 1997). Fragments of the translation elongation factor 1 alpha gene (*ELO*) and the ß-tubulin (*TUB*) gene were amplified using, ELONGF1 and ELONGR1, TUBUF2 and TUBUR1 (Kroon *et al.* 2004) primers, respectively. No introns were present in these regions (Table 3). The region containing the mitochondrial cytochrome c oxidase subunit I (*COX*) gene fragment was amplified using, COXF4N and COXR4N (Kroon *et al.* 2004) primers (Table 3).

Amplifications were performed in a Primus 96 plus thermocycler (MWG-BIOTEC, Ebersberg, Germany). The PCR mixture contained: 10–20 ng template DNA, 1 μM of each primer, 100 μM of dNTPs, 0.4 U *Taq* DNA polymerase (Promega, Southampton, England), 1.5 mM $MgCl_2$, 2.5 μl of 10 × PCR buffer, 100 mM bovine serum albumin (BSA), in a reaction volume of 25 μl. For mtDNA gene amplification, the magnesium chloride ($MgCl_2$) concentration was raised to 3.5 mM. Successful amplification was confirmed by gel electrophoresis (1 h at 70 V) on 1 % agarose gels in 1 × Tris–borate–ethylenediamine tetraacetate (TBE) buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light. The PCR conditions for all regions are summarised in Table 4.

Sequencing of amplified product

The amplification products of all six *Phytophthora parsiana* isolates were purified through Wizard Prep columns (Promega, Southampton, England) to remove excess primers and nucleotides. PCR products were sequenced in forward and reverse orientation using the primers used for amplification and a dye terminator cycle sequencing kit (BigDye sequencing kit, Applied Biosystems, Warrington, UK) on an ABI377-96 automated sequencer (Applied Biosystems) according to the manufacturer's instructions.

Table 3 – The primers used in this study and their characters

Target DNA	Primer	Orientation	Primer sequence	Accession number[a]	Primer location[b]	T_m[c] (°C)	GC[d] (%)	Size[e] (bp)	Authors
ITSs 1, 2 and 5.8S gene of rDNA	ITS6	Forward	5′ GAA GGT GAA GTC GTA ACA AGG 3′			57.9	47.6	860	Cooke *et al.* (2000)
	ITS4	Reverse	5′ TCC TCC GCT TAT TGA TAT GC 3′			55.3	45		White *et al.* (1990)
ß-tubulin	TUBUF2	Forward	5′ CGG TAA CAA CTG GGC CAA GG 3′	U22050	570–589	68.5	60	989	Kroon *et al.* (2004)
	TUBUR1	Reverse	5′ CCT GGT ACT GCT GGT ACT CAG 3′		1538–1558	61.3	57.1		
Translation elongation factor 1α	ELONGF1	Forward	5′ TCA CGA TCG ACA TTG CCC TG 3′	AJ249839	180–199	69.5	55	972	Kroon *et al.* (2004)
	ELONGR1	Reverse	5′ ACG GCT CGA GGA TGA CCA TG 3′		1132–1151	69.7	60		
Cytochrome c oxidase subunit I	COXF4N	Forward	5′ GTA TTT CTT CTT TAT TAG GTG C 3′	U17009	9126–9147	53.2	31.8	972	Kroon *et al.* (2004)
	COXR4N	Reverse	5′ CGT GAA CTA ATG TTA CAT ATA C 3′		10076–10097	50.6	31.8		

a Reference to the GenBank accession containing the DNA sequence, on which the primer is based.
b Reference to the location of the primer within the original DNA sequence.
c Melting temperature of the primer.
d GC-content of the primer.
e Average amplicon length.

Table 4 – PCR conditions for genes studied

Gene[a]	Initial denaturation	Number of cycles	Denaturation	Annealing	Extension	Final extension
ITS	95 (120)[b]	30	95 (20)	55 (25)	72 (50)	72 (600)
TUB	94 (120)	35	94 (20)	60 (25)	72 (50)	72 (600)
ELO	94 (120)	35	94 (20)	60 (25)	72 (50)	72 (600)
COX	94 (120)	35	94 (20)	52 (25)	72 (50)	72 (600)

a ITS, ITS 1, 2 and 5.8S gene of rDNA; *TUB*, ß-tubulin; *ELO*, translation elongation factor 1α; *COX*, cytochrome c oxidase subunit I.
b Temperature in degrees Celcius (time in seconds).

Phylogenetic analysis

A multiple gene genealogy approach, as well as single gene comparisons, were applied in the study of the phylogenetic relationships. *Phytophthora parsiana* sequences generated in this study were compared with those of other taxa obtained from GenBank (Table 5). A preliminary alignment of sequences was made using ClustalX (Thompson *et al.* 1997) with subsequent visual adjustment. The alignments of each of the four regions and a concatenated single alignment of all regions were analysed by both distance-based and ML methods in PHYLIP (Felsenstein 1993). The transition–transversion parameter was estimated using the PUZZLE program (Strimmer & von Haeseler 1996). This parameter was used in the PHYLIP DNAML (Felsenstein & Churchill 1996) and DNADIST (Felsenstein 1993) programs. The robustness of the DNAML tree was tested using 500 BS trials. The trees were drawn using Treeview (Page 1996). The GenBank accession numbers of *Phytophthora* species used for phylogenetic studies are listed in Table 5.

Results

Sequence data and phylogenetic analysis

From *ca* 120 isolates that were pre-screened with the ITS sequencing approach, the six isolates studied (Table 1) were clustered in a distinct monophyletic group distantly related to the majority of taxa in ITS clades 1–8 (Fig 3). For each of these six isolates, fragments of two additional nuclear genes and one mitochondrial gene were sequenced (Table 1). Phylogenetic trees based on both distance-based and ML analysis of the four individual loci showed gene to gene concordance in all the trees observed (data not shown). The *Phytophthora parsiana* clade was resolved as monophyletic in the four individual gene trees, as well as combined genes tree (Fig 4). The rRNA gene ITS sequences of six *P. parsiana* isolates and two isolates from other researchers had an average of 96.2 % identity with a range of 94.2–99.6 % identity between different isolates and only an average of 75.3 and 76.6 % identity with their closest matches *P. insolita* and *P. polonica*, respectively, over an *ca* 768 bp sequence run.

Pathogenicity

None of the isolates produced the characteristic pink-rot symptoms in potato tubers.

Taxonomy

Phytophthora parsiana Mostowfizadeh, D.E. Cooke & Banihashemi, **sp. nov.**

MycoBank no.: MB 511268

Etym.: Named after the Farsi name for Persia, the region from which it was first isolated and described.

Phytophthora parsiana differt ab aliis speciebus nonpapillatis per suam combinationem characterum. Temperaturae crescentiae, optima 30 °C, maxima 35–37 °C. Hyphae hyalinae, plerumque non-septatae, sed paucis septis veteribus, 5 μm latae. Sporangia in mediis solidis non formata, sed in cultura liquida, terminalia, proliferatione internalia, persistentia, non-papillata, ellipsoidea vel obpyriformia, in medio 47 × 29.7 μm (25–83 × 12–58 μm), ratio longitudinis ad latitudinem 1.6 (1.4–1.8). Systema sexus heterothallica et infructuosus. Oogonia diam. 25.9–29.7 μm. Oosporae apleroticae vel plerotic, globosae 25.2 μm diametro mediocritate. Antheridia simper amphigynosa. Region 'rDNA ITS' cum polymorphismis unicis sequentiae (GenBank AY659736–AY659741).

Typus: Iran: *Bushehr Province*: Shabankareh, isol. ex *Ficus carica* crown, 1991, *Z. Banihashemi*, SUC25 (X-2-91). (IMI 395329 – holotypus).

Colonies with complex patterns on almost all agar media, especially CMA (rose-shaped to chrysanthemum) with uniform morphology in some cases and particularly on hempseed agar (Table 2; Fig 1), growth on CMA between 10–37 °C with optimum at 30 °C (Fig 5), optimum rate of growth on average 8 mm d^{-1}. *Hyphae* hyaline, normally nonseptate, 5 μm wide, with botryose to globose hyphal swellings both on agar and in water. *Chlamydospores* thin walled (averger wall diameter *ca* 1.5 μm) present in some isolates with mean diam ranging from 27–37.5 μm. *Sporangiophores* simple and unbranched, rarely sympodial. *Sporangia* not produced on solid media but formed in liquid culture, terminal, with internal proliferation (nesting), persistent (non-caducous), non-papillate on average 47 × 29.3 μm (range 25–83 × 12–58 μm) length:breadth ratio on average 1.6 (range 1.4–1.8), ellipsoid to obpyriform rarely with distorted shapes (Table 2; Fig 2), often tapered base, sporangial apical pores on average 8.5 μm (range 7–11 μm).

Heterothallic or *sterile*, classic heterothallics or sterile and could not produce or induce any sexual organs (Table 2). *Oogonia* not ornamented in culture, spherical, tapered base, average diameter 27.6 μm (range 13–40 μm). *Oospores* smooth, spherical, plerotic to aplerotic, average diameter 25.2 μm (range 13–40 μm), wall on average 3.3 μm thick (range 2–5 μm). *Antheridia* single, terminal, amphigynous, spherical, average width 11.7 μm (range 7.7–19.2 μm; Table 2; Fig 2).

Table 5 – GenBank accession numbers of isolates of *Phytophthora* spp. used for phylogenetic studies

Species	ITS[a]	*TUB*[b]	*ELO*[c]	*COX*[d]
Phytophthora arecae	AF266781	AY564049	AY564105	AY564164
P. boehmeriae	DQ297406[e]	AY564050	AY564106	AY564165
P. botryose	AF266784	AY564051	AY564107	AY564166
P. cactorum	AF266772	AY564052	AY564108	AY564167
P. captiosa	DQ297405[e]	NA	NA	NA
P. cinnamomi	AF266764	AY564054	AY564110	AY564169
P. citricola	AF266784	AY564055	AY564111	AY564170
P. citrophthora	AF266785	AY564056	AY564112	AY564171
P. clandestine	AJ131989	AY564057	AY564113	AY564172
P. colocasiae	AF266786	AY564058	AY564114	AY564173
P. cryptogea	AF266796	AY564059	AY564115	AY564174
P. drechsleri	AF266798	AY564060	AY564116	AY564175
P. erythroseptica	AF266797	AY564061	AY564117	AY564176
P. fallax	DQ297398[e]	NA	NA	NA
P. fragariae var. *fragariae*	AF266762	AY564062	AY564118	AY564177
P. fragariae var. *rubi*	AF266761	AY564064	AY564120	AY564179
P. gallica	DQ286726[f]	NA	NA	NA
P. gonapodyides	AF266793	AY564066	AY564122	AY564181
P. heveae	AF266770	AY564067	AY564123	AY564182
P. humicola	AF266792	AY564069	AY564125	AY564184
P. idaei	AF266773	AY564070	AY564126	AY564185
P. ilicis	AJ131990	AY564071	AY564127	AY564186
P. infestans	AF266779	AY564035	AY564093	AY564150
P. inflate	AF266789	AY564072	AY564128	AY564187
P. insolita	AF271222	AY564073	AY564129	AY564188
P. iranica	AJ131987	AY564074	AY564130	AY564189
P. katsurae	AF266771	AY564075	AY564131	AY564190
P. kernoviae	AY940661[g]	NA	NA	NA
P. lateralis	AF266804	AY564076	AY564132	AY564191
P. macrochlamydospora	L41373[h]	NA	NA	NA
P. megakarya	AF266782	AY564078	AY564134	AY564193
P. megasperma	AF266794	AY564079	AY564135	AY564194
P. mirabilis	AF266777	AY564038	AY564095	AY564153
P. multivesiculata	AF266790	AY564080	AY564136	AY564195
P. nicotianae	AF266776	AY564081	AY564137	AY564196
P. palmivora	AF266780	AY564082	AY564138	AY564197
P. phaseoli	AF266778	AY564044	AY564101	AY564159
P. polonica	DQ396409[i]	NA	NA	NA
P. pseudotsugae	AF266774	AY564084	AY564140	AY564199
P. quininea	DQ275189[i]	AY564085	AY564141	AY564200
P. ramorum	AY540491[j]	AY564092	AY564149	AY564208
P. richardiae	AF271221	AY564086	AY564142	AY56420
P. sinensis	AF266768	AY564087	AY564143	AY564202
P. sojae	AF266769	AY564047	AY564104	AY564162
P. syringae	AF266803	AY564088	AY564144	AY564203
P. tentaculata	AF266775	AY564089	AY564144	AY564203
P. vignae	AF266766	AY564090	AY564146	AY564205
Phytophthora sp. MN1	DQ486672[k]	NA	NA	NA
Phytophthora sp. Kunnunara	EF437222[l]	NA	NA	NA
Pythium aphaniderrmatum	AF271227	AY564048	NA	AY564163

NA, Not available.
a ITS, ITS 1, 2 and 5.8S gene of rDNA (Cooke *et al.* 2000).
b *TUB*, ß-tubulin (Kroon *et al.* 2004).
c *ELO*, translation elongation factor 1α (Kroon *et al.* 2004).
d *COX*, cytochrome c oxidase subunit I (Kroon *et al.* 2004).
e Dick *et al.* (2006).
f Unpubl. data (Jung T, Nechwatal J & Mendgen K).
g Brasier *et al.* (2005).
h Crawford *et. al.* (1996).
i Belbahri *et al.* (2006).
j Giltrap *et al.* (2004).
k Schwingle *et. al.* (2007).
l Unpubl. data (Cunnington J).

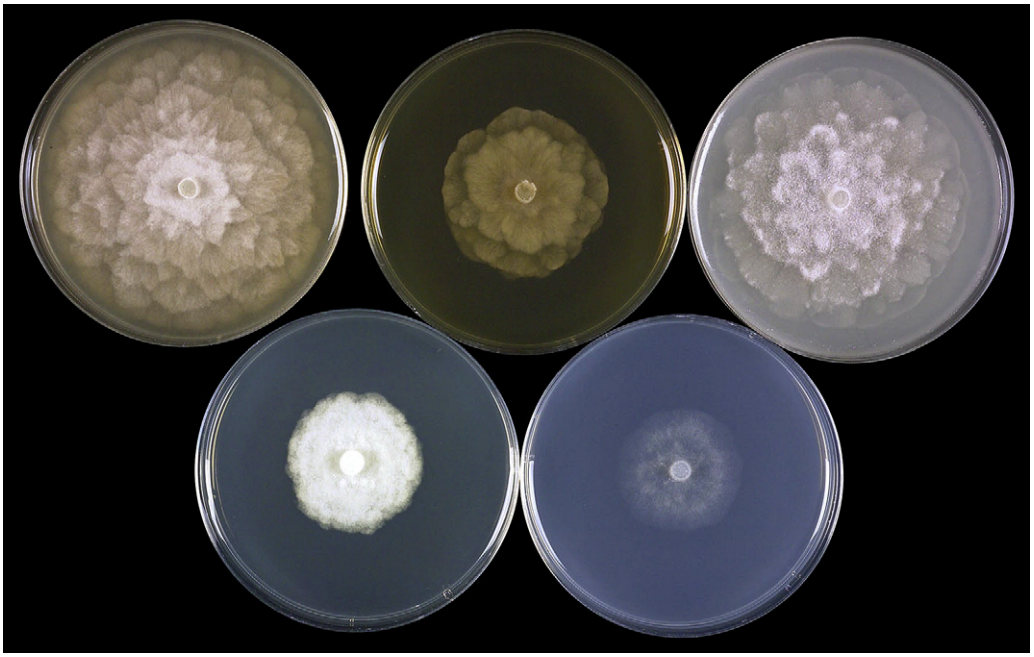

Fig 1 – Colony morphology of isolate SCRP237 of *Phytophthora parsiana* after 10 d at 20 °C; top: (from left to right) CV8, MEA, and HSA; bottom: (from left to right) PDA and CMA.

Habitat: Isolated from the crowns of *Ficus carica*, *Pistacia vera* crowns, branches and soil surrounding, and *Prunus dulcis*.

Discussion

In this study a new species of *Phytophthora* is described that is morphologically similar to *P. cryptogea* and *P. drechsleri*, but clearly unrelated based on multi-gene phylogenetic analysis. The six isolates reported to date form a closely related phylogenetic group, distinct and distantly related from the majority of described species in ITS clades 1–8 (Cooke *et al.* 2000). The clade comprising the newly described species, *P. parsiana* is most closely related to taxa at the base of the ITS-based tree alongside those in ITS clades 9 and 10. They are distinct from all the species studied in recent comprehensive phylogenetic analyses (Cooke *et al.* 2000; Kroon *et al.* 2004; Martin & Tooley 2003) and more recently described species that also group in this basal position, such as *P. polonica* (Belbahri *et al.* 2006), *P. gallica* (GenBank accession no. DQ286726), *P. kernoviae* (Brasier *et al.* 2005), *P. fallax* and *P. captiosa* (Dick *et al.* 2006).

Phylogenetic reconstruction based on the ITS sequences revealed a distinct monophyletic group of isolates clustered in a clade that shared a recent common ancestor with *Phytophthora* ITS clades 1–8 *sensu* Cooke *et al.* (2000). This group comprised six isolates, most of which were isolated from woody plants (Table 1). ITS clade 9 (represented by *P. richardiae*) and 10 (represented by *P. insolita*) were basal to this group and to its sister clades 1–8 in the ITS tree (Fig 3). As these isolates did not belong to any major ITS Clades and their ITS sequence indicated they were most closely related to *P. insolita*, they were informally designated as *Phytophthora* sp. C10 (i.e. similar to ITS clade 10, Mostowfizadeh-Ghalamfarsa 2005).

Three other isolates have recently been reported that, based on ITS sequence data, are either additional representative isolates of *P. parsiana* or closely related sister taxa. First, an isolate reported from a leaf of *Malus* cv. 'Snowdrift' (crabapple) in a nursery in Minnesota (USA) (Schwingle *et al.* 2007, GenBank accession no. DQ486672) very closely matches that of SCRP237 (99 % nucleotide identity) in this study. Second, a *Phytophthora* species isolated from irrigation water, Kununurra, Australia, (James Cunnington, Department of Primary Industries, Knoxfield Centre, Australia; GenBank accession no. EF437222), which was close to SURf17 (99 % nucleotide identity). Last, an additional isolate recovered by a different research group from a pistachio tree in Kerman Province, Iran, was also shown to match *P. parsiana* (Mansoureh Mirabolfathy, Plant Pests and Diseases Research institute, Tehran, pers. comm.) which was most closely related to SURf17 (98.2 % nucleotide identity). Although detailed morphological studies are needed to confirm the identity of these isolates as *P. parsiana*, a broad global distribution of this taxon is suggested.

Analysis of the other sequence datasets (*TUB*, *ELO* and *COX*) supports the ITS data as these isolates formed a distinct monophyletic group in each NJ tree (data not shown). In *TUB* and *ELO* phylograms, *P. insolita* and *P. richardiae* appeared to share the most recent common ancestor with *P. parsiana* isolates; whereas in the mtDNA *COX* tree, the *P. parsiana* isolates clustered into a distinct basal clade. This may be because of a different rate of mutation within the mtDNA region or could be related to the fact that mtDNA is uniparentally inherited (Whittaker *et al.* 1994) and indicative of a distinct 'maternal' lineage of this taxon. Combining all four datasets for a multi-gene genealogy revealed a monophyletic *P. parsiana* clade that is a sister taxon of *P. insolita*

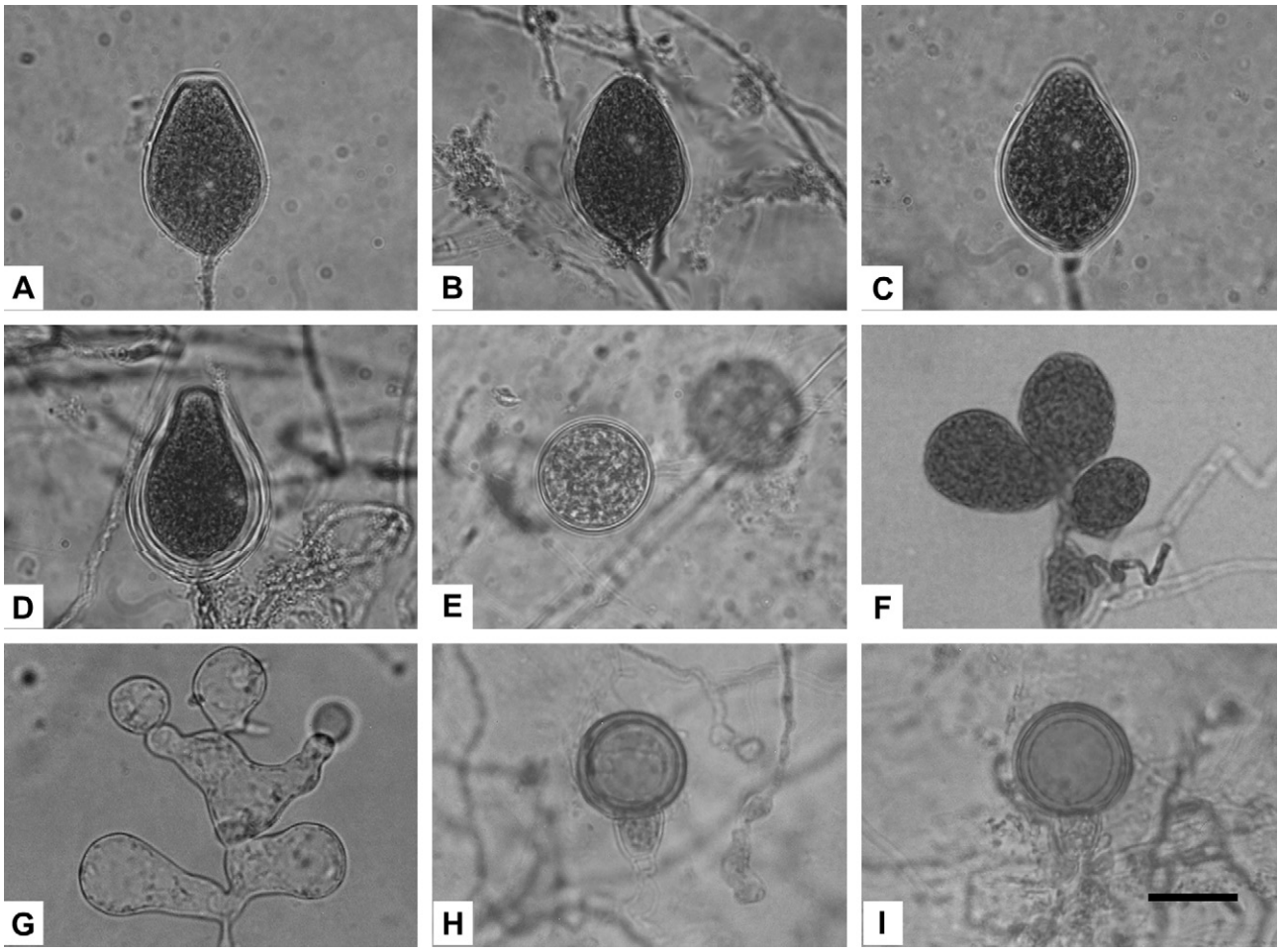

Fig 2 – Range of sporangial morphology [(A–B) SCRP237, (C) SUC19], internal proliferation of sporangium [(D) SCRP237], chlamydospore [(E) SUC25], hyphal swellings [(F–G) SURf17] and characteristics of the oospores and antheridia [(H–I) SURf6] of *Phytophthora parsiana*. Bar = 20 μm.

and that both species share a recent common ancestor with *P. richardiae* and *P. quininea* clade (Fig 4).

Although genetic diversity among *P. parsiana* isolates was apparent, no particular pattern or consistency in the differences was observed between the different gene phylograms. According to the combined gene tree, isolate SCRP237 (*Prunus dulcis*, Greece) was basal to other isolates. Isolates SURf6, SURf17, and SUC19 (*Pistacia vera* isolates, Iran) shared a recent common ancestor, and isolates SUC7 (*Pistacia vera*, USA) and SUC25 (*Ficus carica*, Iran) appeared as sister taxa.

P. parsiana isolates produce colonies with complex patterns on almost all different types of agar media. Hyphae normally contain abundant, aggregate, botryose to globose hyphal swellings both on agar and in water. Sporangia are non-papillate, persistent (non-caducous), with notable apical pores, which proliferate internally (nesting). Heterothallic or sterile mating systems are observed among isolates. Oospores are plerotic to aplerotic with amphigynous antheridia attached on the base of oospores. Morphological characters of this species indicated some overlaps with high-temperature tolerant *Phytophthora* species of ITS clades 6, 7 and 8 (Cooke *et al.* 2000). The majority of the species in ITS clades 6–8 *sensu* Cooke *et al.* 2000, are soil-borne, producing non-papillate, non-caducous and proliferating sporangia, and in some cases, are high-temperature tolerant; thus *P. parsiana* could easily be mistaken for one of these species. Most of the *P. parisana* isolates were initially misidentified as 'high-temperature *P. cryptogea*' based on sporangial morphology and the high upper temperature limit for growth, which was more than 35 °C. The closest taxa to *P. parsiana* in the ITS tree are ITS clades 9 and 10 *sensu* Cooke *et al.* 2000. In clades 9 and 10 species are soil-borne or air-borne plant pathogens and have either papillate or semi-papillate sporangia. There are some high-temperature tolerant species, such as *P. polonica* and *P. insolita* (Belbahri *et al.* 2006; Cooke *et al.* 2000), but these are genetically distinct from *P. parsiana*. The only major character that could separate these clades from *P. parsiana* is the homothallic behaviour of all of the ITS clades 9 and 10 species. It also seems that most of the taxa in ITS clades 9 and 10 are pathogens on a particular host, whereas *P. parsiana* has a multiple host range.

The optimum growth temperature of all isolates was 30 °C and isolates were fast growing and could grow well even at 37 °C. The exception to this was SCRP237 with a growth rate markedly lower across the whole range and an upper limit

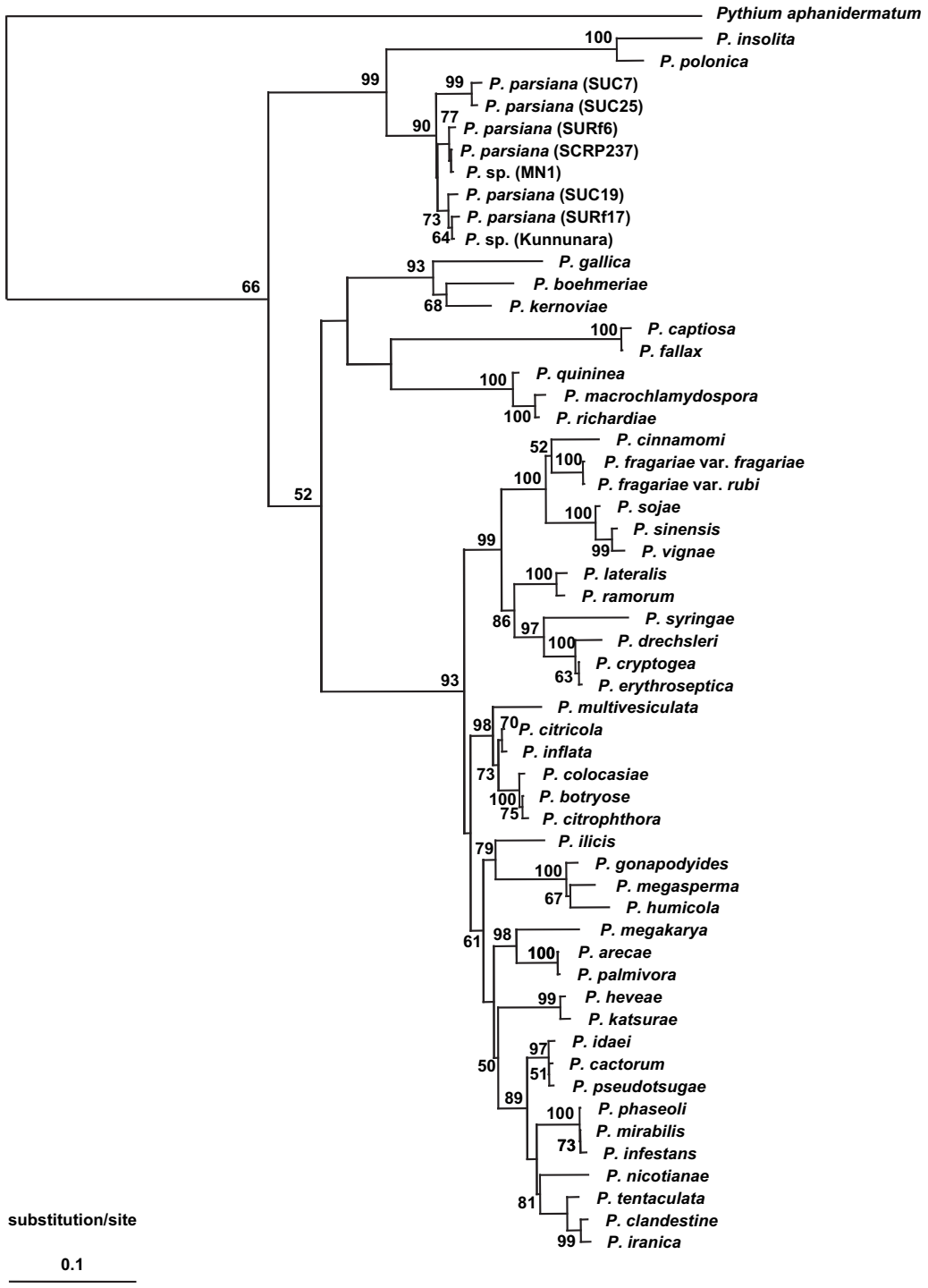

Fig 3 – Detailed phylogram of 49 *Phytophthora* taxa and six *P. parsiana* isolates. The numbers within parentheses indicate the isolate numbers. The phylogram was constructed by DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the genomic rRNA. The numbers at the branch points indicate the percentages of BS values $\geq$ 50 %.

for growth of 35 °C (Fig 5). This isolate has been in culture since 1976 and this long period of storage may have affected its growth rate.

Isolates SUC19, SUC25, and SURf6 were classically heterothallic, whereas other isolates (SCRP237, SUC7, and SURf17) did not form any sexual organs in several different experiments. Such variation in mating behaviour is not unprecedented amongst *Phytophthora* species; members of ITS clade 6 also exhibit such traits (Brasier *et al.* 2003).

Isolates produced complex colony patterns on almost all media specially CMA (Table 2; Fig 1). As most of the non-papillate, high-temperature tolerant species produce uniform

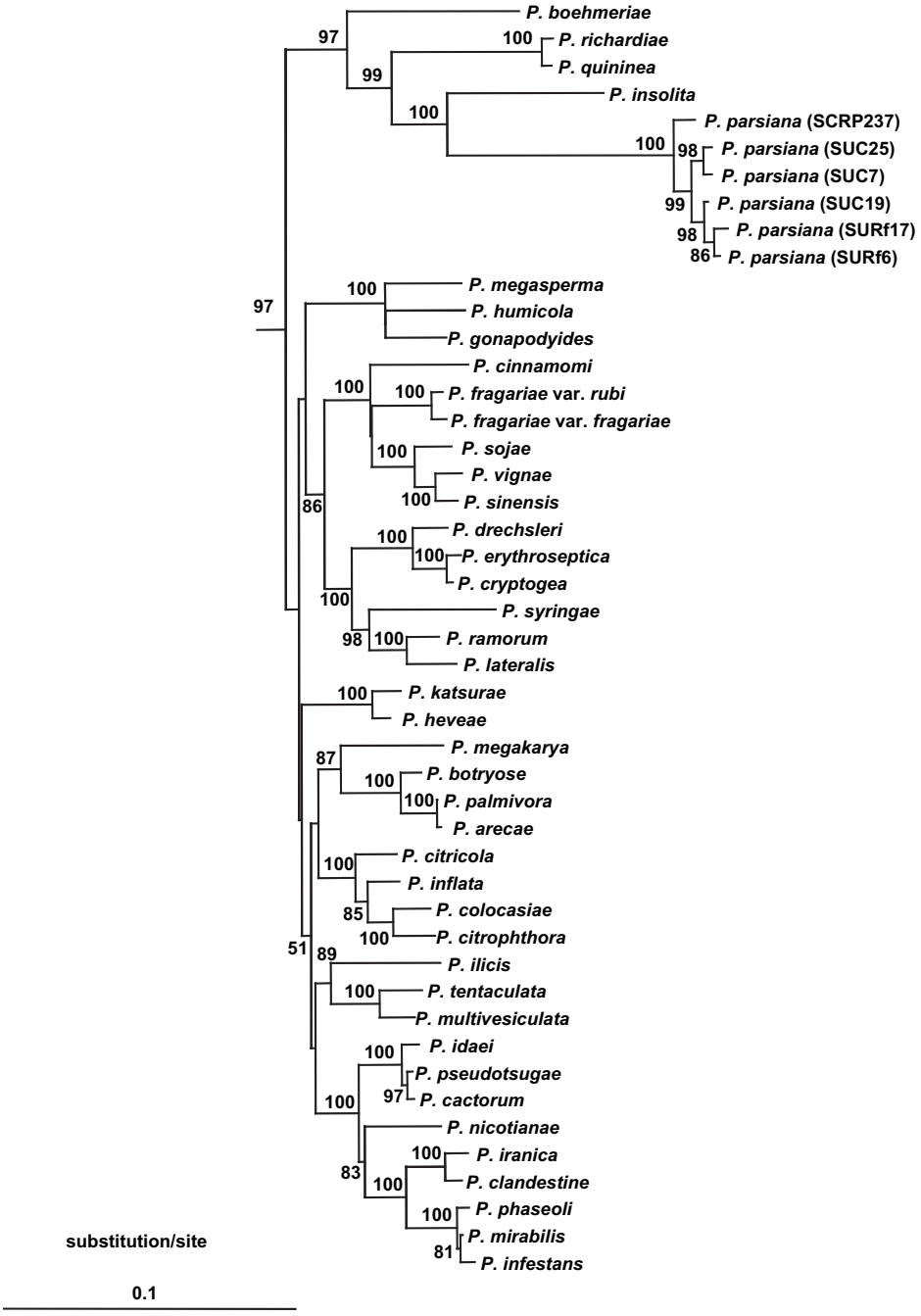

Fig 4 – Combined genes phylogram of 41 *Phytophthora* taxa and six *P. parsiana* isolates. The numbers within parentheses indicate the isolates numbers. The phylogram was constructed after DNA distance-based analysis of the of the combined ITS1, 5.8S subunit, and ITS2 regions of the rDNA (Cooke *et al.* 2000), ß-tubulin, translation elongation factor 1α and cytochrome c oxidase subunit I (Kroon *et al.* 2004) genes. The numbers at the branch points indicate the percentages of BS values ≥ 50 %.

colonies on CMA (Mostowfizadeh-Ghalamfarsa 2005), production of rose-shaped to chrysanthemum colonies, especially on CMA, could be a characteristic trait for discrimination of this new species.

P. parsiana isolates did not induce pink-rot in potato tubers. This may form an additional reliable physiological trait to discriminate *P. parsiana* from other morphologically similar species, such as *P. cryptogea* and *P. drechsleri* that, along with *P. erythroseptica*, have been demonstrated to produce a distinctive pink tuber rot (Mostowfizadeh-Ghalamfarsa *et al.* 2006).

Phylogenetic analysis of various genes and analysis of all other data lead us to infer that *P. parsiana* is a distinct and newly described species. However, there were taxonomic challenges exacerbated perhaps by the scarcity of isolates

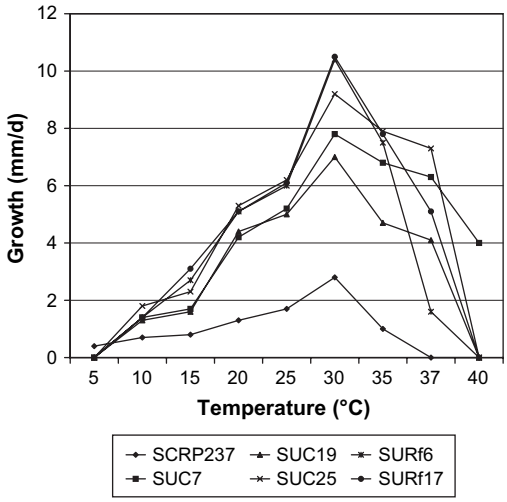

Fig 5 – Average radial growth rate of different *Phytophthora parsiana* isolates on CMA at 5–40 °C.

and lack of background information on the host range, pathogenicity, and origin of the species. Relatively little is known of any of the 11 *Phytophthora* species found outside of the better characterised ITS clades 1–8 taxa (Cooke *et al.* 2000). The *P. parsiana* sequence data clearly indicates a distinct evolutionary origin of this taxon, but the sequence diversity between isolates was unexpected. Multiple isolates of the recently described species, *P. fallax* and *P. captiosa* in New Zealand, for example, were uniform in ITS sequence. However, sequence diversity within *Phytophthora* species is not unprecedented. For example, variation within *P. megasperma* (Brasier *et al.* 2003) and *P. cryptogea* (Förster *et al.* 2000; R.M.-G. unpubl. data) have been reported. Similarly, the apparent absence of a pattern between the sequence and morphological and reproductive trait variation was also unexpected. Although some characters, such as the high upper temperature limit for growth, characteristic colony patterns, and production of hyphal swellings both on agar and in water, could be useful traits for taxonomic purposes, it seems that the only reliable means of identification are DNA sequence comparisons. The range of distinctive morphological traits is relatively restricted in *Phytophthora*, so this may be an increasingly common phenomena as phylogenetic analysis of DNA sequences are increasingly used, either in support of or, on occasion, as the only means of discriminating and describing morphologically similar species.

Since this taxon was first described from *Ficus carica* in the south of Iran by Banihashemi in 1991 (Banihashemi & Ghaisi 1993), it was proposed that it be formally designated with reference to the Farsi name for Persia as *Phytophthora parsiana*. Although detailed pathogenicity studies have not been conducted, the diseases caused by *P. parsiana* on commercially important trees, such as pistachio, almond, and fig and their worldwide distribution emphasises the necessity of a comprehensive study of their origin, diversity and geographical distribution.

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