Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat

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The internal transcribed spacer regions (ITS1 and ITS2) of the ribosomal RNA gene repeat from *Phytophthora* species were amplified using the polymerase chain reaction and sequenced. Sequences from *P. cambivora*, *P. cinnamomi*, *P. citricola*, *P. cryptogea*, *P. drechsleri*, *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi*, *P. megasperma* var. *megasperma* and *P. nicotianae* were compared with published sequences and phylogenetic trees were produced. The resultant grouping of species generally agreed with groupings established using classical morphological criteria, primarily sporangial morphology. Amongst species with non-papillate sporangia two clades were evident, one consisting of *P. fragariae*, *P. cambivora* and *P. cinnamomi* and the other of *P. megasperma*, *P. drechsleri* and *P. cryptogea*. The latter three were placed in the tree between the non-papillate groups and the papillate and semi-papillate groups which formed three distinct clades. One group comprised *P. citricola*, *P. citrophthora* and *P. capsici*, another *P. megakarya* and *P. palmivora* and a third *P. pseudotsugae*, *P. cactorum*, *P. idaei*, *P. nicotianae* and *P. infestans*. More isolates of *P. megasperma*, *P. drechsleri* and *P. cryptogea* will need to be examined to settle more precisely the relationship of these species to the others. PCR amplification of ITS sequences using freeze-thawed mycelial scrapings from pure cultures growing on agar followed by digestion with restriction enzymes is a quick and easy way to compare and identify isolates without the need for laborious DNA extraction procedures. With improved technology, rapid automatic sequencing of PCR-amplified ITS regions is now possible and yields detailed information of relationships within the genus as well as allowing the design of species-specific PCR primers for diagnostic purposes.

Phytophthora contains mostly plant pathogenic species that attack roots, stem bases, growing points, fruit and foliage of many plant species in natural communities and cropping systems. Its systematics have been based on morphological and physiological criteria (Tucker, 1931; Waterhouse, 1970; Newhook, Waterhouse & Stamps, 1978). A tabular key, devised by Newhook *et al*. (1978) and revised by Stamps *et al*. (1990), splits the genus into six groups, with the most important criteria for separation being the form and nature of the sporangium (papillate, semi-papillate and non-papillate), and the attachment of the antheridium to the oogonium (amphigynous or paragynous).

The keys were always intended as ‘interim documents’ (Stamps *et al*., 1990), and the need for more detailed analysis of relationships within the genus is widely recognized (Brasier & Hansen, 1992; Stamps *et al*., 1990; Oudemans & Coffey, 1991*b*). The few suitable morphological characters available may not be the basis of a natural classification system and some species are undoubtedly polyphyletic with several or many relatively unrelated groups included within them. This is due, in part, to the paucity of suitable morphological characters; failure to adhere strictly to the morphological criteria used originally to describe certain species, for example the inclusion in *P. megasperma* of taxa with oospores consistently smaller than the sizes given in the original description by Drechsler (1931) (Tomkins, Tucker & Gardner, 1936); exclusion from consideration of characters other than morphology in describing species, e.g. physiology and pathogenicity; and when included, failure to correlate these and other characters with one another. *P. megasperma* has been shown to be just such an assemblage on the basis of pathogenicity, colony morphology and soluble protein patterns (Hansen *et al*., 1986), and mitochondrial and nuclear RFLPs (Förster & Coffey, 1993). Although some clades, such as *P. sojae*, have been elevated to species status, others that may well merit the same treatment remain within *P. megasperma*, distinguished only by the use of subordinate letters to designate the group to which they belong (Förster & Coffey, 1993). Similar studies have shown that *P. cryptogea* and the closely related *P. drechsleri* are also polyphyletic assemblages (Förster, Learn & Coffey, 1995). By contrast, other species such as *P. cactorum* (Cooke *et al*., 1996) and *P. nicotianae* (Oudemans & Coffey, 1991*a*, *b*; Lacourt *et al*., 1994) appear much more uniform.

Protein profiles (Brasier, Hamm & Hansen, 1993) and isozymes (Oudemans & Coffey, 1991*b*; Latorre *et al*., 1995) have been useful in resolving differences between isolates and species but in recent years perhaps most important have been the technological advances in molecular biology allowing examination of polymorphisms at the DNA level. The ribosomal RNA gene repeat (rDNA) has been used extensively to compare and relate taxa at many levels, from kingdoms (Gray, Sankoff & Cedergreen, 1984) to genera (Berbee & Taylor, 1992) and species (Lee & Taylor, 1992). Briard *et al*. (1995), studying the Pythiaceae, found little variation in the DNA sequences of the 28S rRNA gene of 15 *Phytophthora*

spp., in contrast to *Pythium*, eight species of which could be assigned to four distinct groups. The internal transcribed spacer regions (ITS1 and ITS2) which lie between the 18S and 28S genes lack a functional role (Nues *et al.*, 1994), which is thought to explain the high levels of sequence variation within them. Studies of ITS regions from rusts (Zambino & Szabo, 1993), *Colletotrichum* (Sherriff *et al.*, 1994) and *Alternaria* (Jasalavich *et al.*, 1995) have shown sequence variation at the interspecific level but generally low levels of intraspecific variation within well defined species. Lee & Taylor (1992) published the ITS1 and ITS2 sequences of the tropical *Phytophthora* species *P. palmivora*, *P. megakarya*, *P. capsici*, *P. citrophthora* and *P. cinnamomi* and showed excellent resolution at the species level.

In this study, previously published ITS sequences from *Phytophthora* species (Lee & Taylor, 1992; Cooke *et al.*, 1996) were compared with eight other species allowing a comparison of 16 species with representatives from each of the six groups defined by Waterhouse (1970). The aims of this study were to (1) confirm the presence of species-specific sequence variation in ITS1 and ITS2 regions across a broad range of *Phytophthora* species (2) assess the potential for species discrimination on the basis of ITS restriction fragment analysis (3) use such sequence information to examine phylogenetic relationships within the genus and (4) assess the potential of such regions for the development of species-specific PCR primers or DNA probes for use in diagnostics.

MATERIALS AND METHODS

Fungal material and culturing conditions

Phytophthora species used in this study are detailed in Table 1. All species were maintained at 5 °C on oatmeal agar and grown routinely at 20° on french bean agar. Mycelium for DNA extraction was grown at 20° in 20 ml still culture of a sucrose/asparagine/mineral salts broth containing 30 μg ml^{-1} β-sitosterol (Elliott, Hendrie & Knights, 1966). After vacuum filtration the mycelium was freeze dried for extended storage at −20°.

DNA extraction and PCR conditions

DNA was extracted using a Nucleon DNA extraction kit (Scotlab, Coatbridge, U.K.) according to the manufacturer's recommendations.

PCR amplification and sequencing of ITS1 and ITS2 regions of rDNA

Primers ITS1 and ITS2, and ITS3 and ITS4 (White *et al.*, 1990) were used to amplify ITS1 and ITS2 regions of rDNA respectively. The 50 μl reaction mixture contained 10 ng template DNA, 1 μM of each primer, 100 μM each dNTP, 5 μl 10 × PCR buffer, 1·5 mM $MgCl_2$, 2·5 Units *Taq* Polymerase (Life Technologies, Paisley, U.K.). The mixture was overlaid with 30 μl of sterile mineral oil and subjected to thermal cycling in a Techne PHC-3 cycler (Techne, Cambridge, U.K.). An initial denaturation step of 2 min at 95° was followed by 30 cycles of annealing for 30 s at 55°, extension for 1 min at 72° and denaturation for 30 s at 95° before a final extension step of 10 min at 72°. The reaction mixture (5 μl) was run on 1·5% agarose gels, stained with ethidium bromide and visualized under uv illumination to determine the number and size of DNA products amplified in the PCR.

Table 1. Species and isolates used in this study

	(Short name)[a]	Collection number		Country	Host	Source	EMBL accession number[b]	
		SCRI[c]	IMI				ITS1	ITS2
P. cactorum (Lebert & Cohn) J. Schröt.	(*cac*)	CAC23	—	England	Apple	HRI	Y08652	Y08653
P. cambivora (Petri) Buisman	(*cam*)	CAM1	296830	Scotland	Raspberry	SCRI	Y08654	Y08655
P. capsici Leonian	(*cap*)	CAPS	P255[d]	Sarawak	*Piper nigrum*	C. Brasier	—	—
P. cinnamomi Rands	(*cin*)	CIN8	342.72[d]	U.S.A.	*Persea*	CBS	Y08656	Y08657
P. citricola Sawada	(*cit*)	CIT2	313729	Ireland	Raspberry	SCRI	Y08658	Y08659
P. cryptogea Pethybr. & Laff.	(*cry*)	CRY3	045168	N. Zealand	Tomato	IMI	Y08660	Y08661
P. drechsleri Tucker	(*dre*)	DRE1	303922	Ireland	Raspberry	SCRI	—	Y08662
P. fragariae var. *rubi* Hickman	(*fvr*)	FVR11	355974	Scotland	Raspberry	SCRI	Y08665	Y08666
P. fragariae var. *fragariae* W. F. Wilcox & J. M. Duncan	(*fvf*)	FVF7	278659	England	Strawberry	C. J. Hickman	Y08663	Y08664
P. idaei D. M. Kenn.	(*ida*)	IDA1	—	Scotland	Raspberry	SCRI	Y08667	Y08668
P. infestans (Mont.) de Bary	(*inf*)	89/AF1		Wales	Potato	R. Shattock	Y08669	Y08670
P. megasperma Drechster	(*meg*)	MEG1	296828	Scotland	Raspberry	Authors	Y08671	Y08672
P. nicotianae Breda de Haan.	(*nic*)	NIC1	208688	Unknown	Unknown	IMI	Y08673	Y08674
P. palmivora (E. J. Butler) E. J. Butler	(*pal*)	PAL	P488[d]	Ivory Coast	Coconut	C. Brasier	—	—
P. pseudotsugae Hamm & E. M. Hansen	(*pse*)	PSE1	331662	U.S.A.	*Pseudotsuga*	P. Hamm	Y08675	Y08676

[a] The short names in brackets are used in Table 2.

[b] EMBL Nucleotide Database.

[c] The size of the *Phytophthora* collection at SCRI has necessitated a change in the numbering system for isolates. Some of these isolates have been described under different local numbers in previous publications.

[d] Not IMI number but the number given by those providing the isolate.

HRI – Horticulture Research International (D. C. Harris); SCRI – Scottish Crop Research Institute; CBS – Centraalbureau voor Schimmelcultures; IMI – International Mycological Institute.

Table 2. Sizes (bp) of ITS1 and ITS2 regions of *Phytophthora* species

	Phytophthora species (see Table 1 for full names)																
	pse	*cac*	*ida*	*inf*	*nic*	*megk*	*pal*	*ctr*	*cit*	*cap*	*meg*	*cry*	*dre*	*fvf*	*fvr*	*cam*	*cin*
ITS1	[ac]217	[a]218	[ac]218	[ac]221	[c]226	[c]214	[b]214	[b]176	[c]184	[b]176	[c]221	206	nt	231	231	[c]232	229
								[b]196									[b]223
ITS2	[a]413	[a]413	[a]413	[a]414	415	411	[b]414	[b]417	414	[b]417	425	427	427	437	437	437	436
								[b]427									[b]436

[a] From Cooke *et al.*; [b] from Lee & Taylor (1992); [c] some terminal nucleotides were not determined in these isolates, sequence homology has been assumed and the total ITS length calculated on that basis.

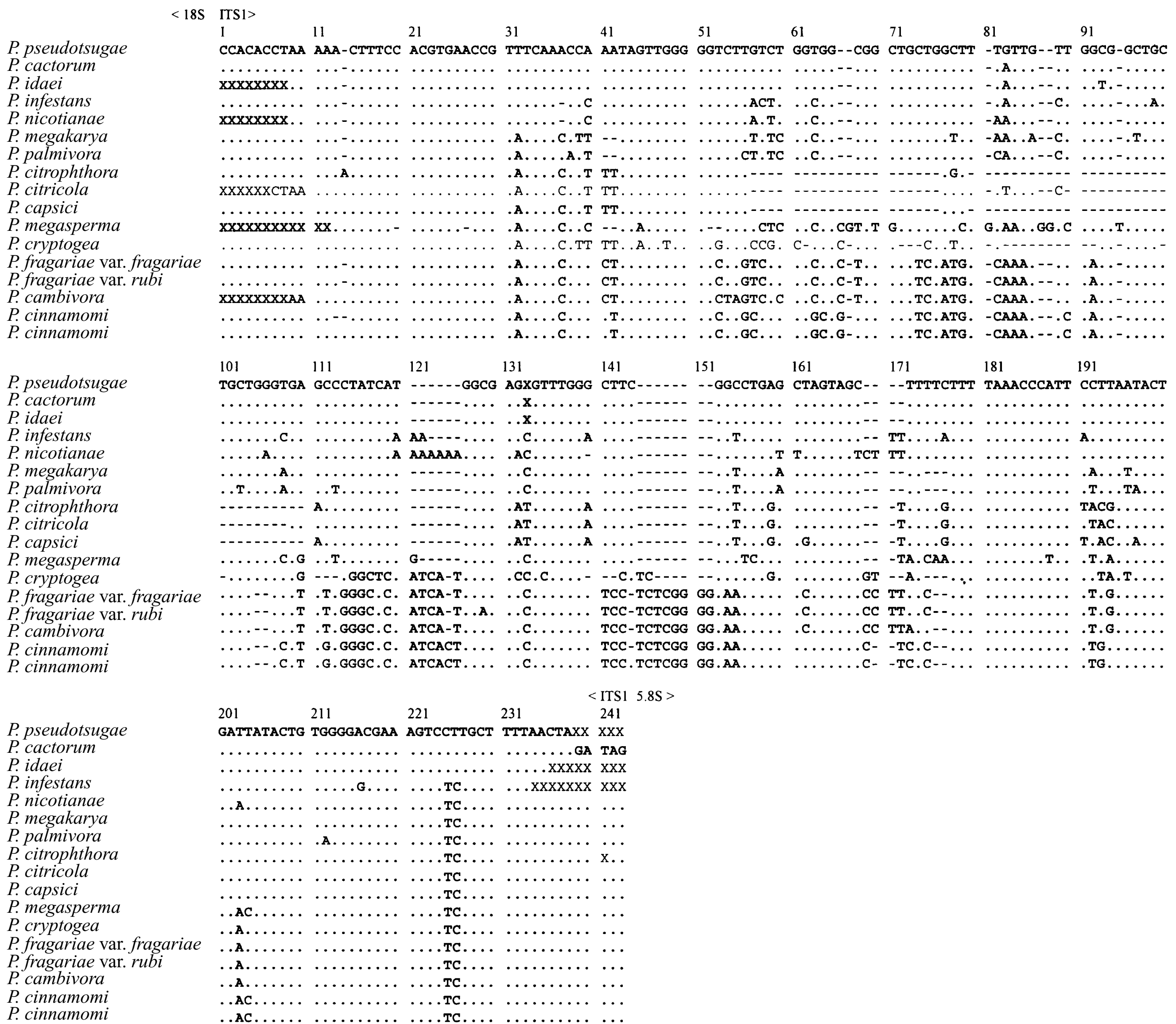

Fig. 1. Aligned DNA sequences of the ITS1 regions of 16 *Phytophthora* species. The start (>) and end (<) of the ITS1 region are indicated, as are the end of the 18S and start of the 5·8S subunits of the RNA gene repeat. The sequences have been arranged according to their phylogenetic relationship as determined by PHYLIP analysis. Those for *P. megakarya* Brassier & M. J. Griffin, *P. palmivora*, *P. citrophthora* (R. E. Sm & E. H. Sm.) Leonian, *P. capsici* and *P. cinnamomi* (second sequence) were taken from (Lee & Taylor, 1992) (**.** sequence identical to and – deletion from that of *P. pseudotsugae*; X, sequence not determined).

For the ITS1 region, only one strand of the double symmetric PCR product was sequenced. This was due to failure to sequence using primer ITS1, probably because of the primer folding and annealing to itself. Direct sequencing was carried out using the Sequenase PCR product kit according to the instructions of the manufacturer (Amersham International, Little Chalfont, U.K.) with the dideoxynucleotide chain-termination sequencing being initiated using primer ITS2. For

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                             <5.8 S  ITS2 >
                             1          11         21         31         41         51         61         71         81         91
P. pseudotsugae              CATCAAACTT GGCTTTCTTC CTTCCGTGTA GTCGGTGGA- GGAGATGC-C AGATGTGAAG TGTCTTGCGG CTGGT---TT TCGGACCGA- -CTGCGAGTC
P. cactorum                  .......... .......... .......... .........- ........-. .......... .......... .....---.. .........- -.........
P. idaei                     .......... .......... .......... .........- ........-. .......... .......... .....---.. .........- -.........
P. infestans                 ..A....... .......... .......... .........- ........-. .......... .......... T....---.. .........- -.........
P. nicotianae                ...T...... .A........ .......... .........- .......T-. .......... .........A T....---C. ........G- -.........
P. megakarya                 .......... ..T....... .......... .........- ...T....-. ........G. .......... ....C---C. ....GT..G- -.........
P. palmivora                 .......... ..T....... .....A.... ........T- ...TG...-. .......... .......... .....---C. .....T..G- -...T.....
P. citrophthora              .......... .......... .......... .........- ...TG...-. .......... .......... T.T..G--C. ....G....G G.........
P. citricola                 .......... .......... .......... .........- ...TG...-. .......... .......... ---..GTCC. ....GT..T- -.........
P. capsici                   .......... ....-..... .......... .........- .-.TG...-. .......... .........T G.T..--CC. ....GT..G- -.........
P. megasperma                ......C... ..T....... .......... .........- ...T....-. ...C...... ........TA GC...--C.. ...AGT.-TG C.G.T.....
P. cryptogea                 ..CT...... ....CC.... .......... .........T ..G..C..G. .......... .......... .....---C. ....T...G- -.........
P. drechsleri                ..CT...... ....CC.... .......... .........T ..G..C..G. .......... .......... .....---C. ....T...G- -.........
P. fragariae var. fragariae  .......... ....C..... .......... .........T ..G..C..-. ...C....G. .......... G...C---C. ....G.T.C- -.........
P. fragariae var. rubi       .......... ....C..... .......... .........T ..G..C..-. ...C....G. .......... G...C---C. ....G.T.C- -.........
P. cambivora                 .......... ....C..... .......... .........T ..G..C..-. ...C....G. .......... G...X---C. ....G.T.G- -.........
P. cinnamomi                 ......-... ....C..... .......... .........T ....G...-. ...C....G. .......... .GC..---C. ......T.G- -...T.....
P. cinnamomi                 .......... ....C..... .......... .........T ....G...-. ...C....G. .......... GC...---C. ......T.G- -...T.....

                             101        111        121        131        141        151        161        171        181        191
P. pseudotsugae              CTTTTAAATG TACTGAACTG TACT-TCTCT TTGCTCGAAA AGCG-TGGCG -TTGCTGGTT GTGGAGGCTG CTATTGTAGC AAGTT-GGCG ACCGGTTTGT
P. cactorum                  .......... .......... ....-..... .......... ....-..... -......... .......... .......... .....-.... ..........
P. idaei                     .......... .......... ....-..... .......... ....-..... -......... .......... .......... .....-..T. ..........
P. infestans                 .......... ....A..... ....-..... ......C... ..T.G....A C......... .....C.... .......... X....-.... ..........
P. nicotianae                .......... ....A..... ....-..... .......... ..T.G..... -......... .......... .......... X....-.... ..T.......
P. megakarya                 .C..G..... .......... ....-..... .......... ..T-AAA..- -.....T... .......... ..TG....A. C...C-.... ..TA......
P. palmivora                 ....G..... .......... ....-..... ......C... ...-G..... -......A.. .......... ..TGC..... C...CT.... ...A......
P. citrophthora              ....T..... .......... ....-..... .......... ..A-...T.. -......... .......... .CTGC..G.. C...C-.... ..........
P. citricola                 ....G..... .......... ....-..... .......... ....-...T. -......... .......... .CTGC..G.. C...C-.... ..........
P. capsici                   .......... .......... ....-..... .......... ....-...T. -......... .......... .CTGC..G.. C...C-.... ..........
P. megasperma                .......... .......... ....C..... .......... ...-G..... -......... .......... .CCG...G.. C...C-.... ..........
P. cryptogea                 ....G..... ....AC.... ....-..... .......... ...-G..A.. -......... .......... .CTG...G.. .T..C-.... ..........
P. drechsleri                ....G..... ....AC.... ....-..... .......... ...-G..A.. -......... .......... .CTG...G.. .T..C-.... ..........
P. fragariae var. fragariae  ....G..... .......... ....-..... .......... ..-CG..A.. -...T..... .......... .CTG...G.. C...C-.... ..........
P. fragariae var. rubi       ....G..... .......... ....-..... .......... ..-CG..A.. -...T..... .......... .CTG...G.. C...C-.... ..........
P. cambivora                 ....G..... .......... ....-..... .......... ..-CG..A.. -...T..... .......... .CTG...G.. C...C-.... ..........
P. cinnamomi                 ....G..... .......... ....-..... .......... ..-CG..A.. -......... .......... .CTG.A.G.. C...C-.... ..........
P. cinnamomi                 .C..G..... .......... ....-..... .......... ..-CG..A.. -......... .......... .CTG.A.G.. C...C-.... ..........

                             201        211        221        231        241        251        261        271        281        291
P. pseudotsugae              CTGCTGCGGC GTT-AATGGA AGAGTGTTCG ATTCGCGGTA TGGTTGGCTT CGGCTGAACA ATGCG-CTTA TTGGATGATT TTTCTGCTGT GGCGTGATGG
P. cactorum                  .......... ...-...... .......... .......... .....A.... .......... .....-.... .......T.. .......... ..........
P. idaei                     .......... ...-...... .......... .......... .......... .......... .....-.... .......T.. .......... ..........
P. infestans                 .......... ...-...... GA.A..C... .....T.... .......... .......... .....-.... ....G..... ..C....... ..........
P. nicotianae                .......... X..-...... .......... .....T.... .......... .......... ....AA.... .....C.T.. ..C....... ..........
P. megakarya                 ......T... ...-...... G......... .......... ..A....... .......... GA--AG.... ....GC.T.. ..C.....A. ....GT...A
P. palmivora                 ......T... ...-...... G......... .......... .......... .......... GA--CG.... ...A..AT.. C...A..... ..T.GT...A
P. citrophthora              .......... ...T...... G......... .......... .......... .......... G-...-.... ...T...C.. ..C....... ..........
P. citricola                 .......A.. ...T...... G......... .......... .......... .......... G-...-.... ...T...C.. ..C....... ..........
P. capsici                   .......... ...T...... G......... .......... .........X X......... G-...-.... ...T...C.. ..C....... ..........
P. megasperma                TA....T... ...----... G......... .......... .......... .......... ..-.TG.... ....G..C.. ..C...TCA. ....GT...A
P. cryptogea                 .......... ...T...... G......... .......... .......... .......... G-A..-.... ....G..C.. ..C....... ...TG.....
P. drechsleri                .......... ...T...... G......... .......... .......... .......... G-A..-.... ....G..C.. ..C....... ...TG.....
P. fragariae var. fragariae  .......... ...T...... G......... .......... .......... .......... .....-.... .....C.T.C ..C....... ....GT.C..
P. fragariae var. rubi       .......... ...T...... G......... .......... .......... .......... .....-.... .....C.T.C ..C....... ....GT.C..
P. cambivora                 .......... ...T...... G......... .......... .......... .......... .....-.... .....C.T.C ..C....... ....GT.C..
P. cinnamomi                 .......... ...T...... G......... .......... .......... .......... .A...-.... ...-...T.C ..C....... ....GT.C..
P. cinnamomi                 .......... ...T...... G......... .......... .......... .......... .A...-.... .......T.C ..C....... ....GT-C..

                             301        311        321        331        341        351        361        371        381        391
P. pseudotsugae              ACCGGTGAAC CATAGCTCAG TG--GCTTGG CTTTTGAATC GGCTTTGCTG -TTGCGAAGT AGAGTGGCGG CTTCGGCT-- -GTCGAGG-- ------G-TC
P. cactorum                  .......... .......... .T--...... .......... .......... -......... .......... ........-- -.......-- ------.-..
P. idaei                     .......... .......... ..--...... .......... .......... -.....G... .......... ........-- -.......-- ------.-..
P. infestans                 ..T....... ...G....TT .A--...... .A........ .......... -......... .......... ........-- -.C.....-- ------.-..
P. nicotianae                ..T....... ........G. ..--...... .........T .......... -......... ..G....A.. ......T.-- -.......-- ------.-..
P. megakarya                 .GTA...... .G...-.T.T GTGG...... .........G T...C-.... -.-....... .........A ...T..T.-- -.......-- ------.-..
P. palmivora                 T-T....... .G.....T.T GTGA...... .........T .......... -......... .......... ........-- -.......-- ------.-..
P. citrophthora              G.T....... .G.....GT. ..TG...... .......... .......... -......... .......... ........-- -.......-- ------.-..
P. citricola                 G.T....... .G.....AT. ..TG...... .......... .......... -......... .......... ........-- -.......-- ------.-..
P. capsici                   G.T....... .G.....GT. ..TG...... .......... .......... -......... .......... ........-- -.......-- ------.-..
P. megasperma                ..T....... .G.....GTX ..GT...... ........C. .........X --........ ..T....... ........-- -.......-- ------.G..
P. cryptogea                 ..T....... .G.....GT. CTAG...... ........C. ...GG..-.. G.-....... ..G...T.T. T.CT...GTA A.CT.G..TG GACGAG.G..
P. drechsleri                ..T....... .G.....GT. CTAG...... ........C. ...GG..-.. G.-....... ..G...T.T. T.CX...GCA A.CT.G..TG GACGAG.G..
P. fragariae var. fragariae  .T........ .G...G.GT. C.AG...... ........C. ...GG..T.. G.C....... ..G....... ........-- -.......-- ------.G..
P. fragariae var. rubi       .T........ .G.....GT. C.AG...... ........C. ...GG..T.. G.C....... ..G....... ........-- -.......-- ------.G..
P. cambivora                 .T........ .G.....GT. C.AG...... ........C. ...GG..T.. G.C....... ..G....... ........-- -.......-- ------.G..
P. cinnamomi                 .T........ .G.....GT. CTAG...... ........C. ...GG..T.. -.....TT.. ..G....... ........-- -.......-- ------.--.
P. cinnamomi                 .T........ .G.....GT. CTAG...... .G......C. ...GG..T.. -......... ..G....... ........-- -.......-- ------.--.
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Fig. 2. For caption see facing page.

the ITS2 region, both strands were sequenced in exactly the same way using the ITS3 and ITS4 primers to initiate sequencing. Electrophoresis of the sequencing products was carried out in 6% denaturing polyacrylamide gels. The eight sequences were aligned with the five from Lee & Taylor (1992) and five from Cooke *et al.* (1996) using the computer package CLUSTALV (Higgins, Bleasby & Fuchs, 1992). Modifications to the alignment were carried out by eye where necessary.

Phylogenetic analysis

All analyses were carried out using programmes within the PHYLIP version 3.5c package (Felsenstein, 1993). Only those

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                                                                        < ITS2  28S >
                            401        411        421        431        441        451        461
P. pseudotsugae             GATTCATTT- GGGAAA-TGT GTGTGTA--- -----CTTCG GTATG----- -----CATCT CAA
P. cactorum                 .........- ......-... .......--- -----..... .....----- -----..... .XX
P. idaei                    .........- ......-... .......--- -----..... .....----- -----..... ...
P. infestans                .........- ......T-.. -......--- -----..... .....----- -----..... ...
P. nicotianae               .........- .....-C.TA A......--- -----..... .....----- -----..... ...
P. megakarya                ...C.....- ....XXT--. .......--- -----..... .....----- -----..... .X.
P. palmivora                ...C.....- ....XXT--. ....A..--- -----..... .C...----- -----..... ...
P. citrophthora             ...C.....T ....XXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXX
P. citricola                ...C.....- ....------ ---------- ------..TG TGXACCTCGG XXXXXXXXXX XXX
P. capsici                  ...C.....T .....GT.-. .....-.--- -----..... ..GC.----- -----..... .X.
P. megasperma               ...C.....T ......C.T. ......GCGG -----..... TCG..CGCG- -----..... ...
P. cryptogea                ...C.....- ......CGT. ...XX----- -----..... .----CGCG- -----..... ...
P. drechsleri               ...C.....- ......CGT. ...XX----- -----X.... .----CGCG- -----..... ...
P. fragariae var. fragariae ...C.....- ...-.CT... ..C.C.G-CG GCGCG..... ..G..CTGCG GGTGG..... ...
P. fragariae var. rubi      ...C.....- ...-.CT... ..C.C.G-CG GCGCG..... ..G..CTGCG GGTGGXXXXX XXX
P. cambivora                ...C.....- .....CT... ..C.C.G-CG GCGCG...X. ..G..CTGCG GGTGG..CTC T..
P. cinnamomi                ...C.....- ..-..CTC-. ...XXXXXXX XXXXXXXXXX XXXXXXXXXX XXXXXXXXXX XXX
P. cinnamomi                ...C.....- .....CTC-. ....C.CTGC GXGCGA..T- ..G..CTTCT GGTGG..... ...
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Fig. 2. Aligned DNA sequences of the ITS2 regions of 17 *Phytophthora* species. The start (>) and end (<) of the ITS2 region are indicated, as are the end of the 5.8S and start of the 28S subunits of the RNA gene repeat. The sequences have been arranged according to their phylogenetic relationship as determined by PHYLIP analysis. Those for *P. megakarya*, *P. palmivora*, *P. citrophthora*, *P. capsici* and *P. cinnamomi* (second sequence) were taken from (Lee & Taylor, 1992) (**.** sequence identical to and – deletion from that of *P. pseudotsugae*; X, sequence not determined).

regions with complete sequence for all species were analysed. The more precise method of 'DNA maximum likelihood' was carried out on sequence data using five randomizations of sequence input order in the DNAML programme. Neighbour-joining methods were also applied to DNA distance matrices produced from the sequence data using the Kimura 2-parameter model in DNADIST. Neighbour-joining analysis was carried out using the NEIGHBOR program and the topology tested with 2000 bootstrap trials.

Quick-prep method

Small fragments of mycelium were removed from colonies growing on agar, placed in 100 µl of SDW and subjected to three cycles of rapid freeze-thawing from −80° to 60° in order to release the cell contents. One µl of this mixture was added to a 50 µl PCR reaction and the ITS regions were amplified as previously described. The forward primer, ITS6 (5′ GAAGGTGAAGTCGTAACAAGG 3′) was based on ITS5 (White *et al.*, 1990) but modified to allow more efficient amplification of *Phytophthora* species on the basis of 18S sequences of *P. fragariae* (Stammler, 1992) and *P. megasperma* (Auwera, Chapelle & Wachter, 1994). A sample of the PCR product (10 µl) was digested with the restriction enzymes *Taq* I, *Msp* I or *Alu* I according to the manufacturer's instructions (Life Technologies). Digestion products were electrophoresed on 3% NuSieve agarose (FMC BioProducts, ME, U.S.A.).

RESULTS

All primer combinations amplified a single product which was resolved by electrophoresis. Primers ITS1 and ITS2 gave one *ca* 300 bp band; primers ITS3 and ITS4, one *ca* 560 bp band.

Sequence alignments

Sequencing resolved the exact lengths of the ITS regions (Table 2). ITS1 lengths varied by as much as 49 bp, from 184 bp for *P. citricola* to 233 bp for *P. megakarya*. Less variation was evident in ITS2; only 26 bp separated *P. megakarya* at 411 bp and *P. cambivora* at 437 bp.

Accurate alignments of ITS1 and ITS2 were possible for the eight species with those already published (Figs 1, 2). Variations in sequence ranged from single base pair changes to multiple changes representing deletions and insertions. Most changes were present in several species but some single base pair changes were unique to a particular species. More sequence variation was evident in ITS1 with only a few short regions showing complete homology across all species examined, whereas in ITS2 the sequences were more conserved, with many regions of complete homology interspersed with more variable regions. The nucleotide sequence data (Figs 1, 2) have also been lodged in the EMBL Nucleotide Sequence Database under the accession numbers shown in Table 1.

Genetic distances

Within ITS1, interspecific genetic distances varied from 0·0047 to 0·2997 with a mean distance of 0·1826. Representatives of the same species, such as the two varieties of *P. fragariae* and the two isolates of *P. cinnamomi*, had the lowest genetic distances, less than 0·005. Within the group I species, the distances were also small, 0·005 between *P. cactorum* and *P. idaei*, for example. The largest distances of 0·29 and above were seen between the group of non-papillate species, comprising *P. fragariae* var. *fragariae*, *P. fragariae* var. *rubi*, *P. cambivora* and *P. cinnamomi*, and the papillate *P. infestans*.

The genetic distances calculated from the ITS2 sequences covered a narrower range of 0·0025 to 0·1906 with a mean value of 0·1035. As in ITS1, the lowest figures were for the intraspecific comparisons but the largest distances in this case were those between the closely related *P. cryptogea* and *P. drechsleri* and *P. megakarya*.

Phylogenetic analysis

A tree comparison using the package DNAML showed that the phylogenetic trees produced by 'Neighbour-joining' methods were not significantly different from those produced by the 'DNA maximum likelihood' method.

Table 3. Relationship of morphological features (from Stamps *et al.*, 1990) with groupings suggested by phylogenetic analysis of ITS DNA sequence data with the species grouped according to the ITS-based phylogenetic analysis

	Group	Papillae	Caducous	Sporangial proliferation	Antheridial attachment	Homo- or heterothallic
P. pseudotsugae	I	+	−	−	P	Hom
P. cactorum	I	+	+S	−	P	Hom
P. idaei	I	+	+S	−	P	Hom
P. infestans	IV	+SP	+S	−	A	Het
P. nicotianae	II	+	+Oc S	−	A	Het
P. megakarya	II	+	+M	−	A	Het
P. palmivora	II	+	+S	−	A	Het
P. citrophthora	III	+	++/−M	−		Het
P. citricola	III	+SP	−	−	P	Hom
P. capsici	II	+	+L	−	A	Het
P. megasperma	V	−	−	+	P/A	Hom
P. cryptogea	VI	−	−	+	A	Het
P. drechsleri	VI	−	−	+	A	Het
P. f. var. *fragariae*	V	−	−	+	A	Hom
P. f. var. *rubi*	V	−	−	+	A	Hom
P. cambivora	VI	−	−	+	A	Het
P. cinnamomi	VI	−	−	+	A	Het

Horizontal lines indicate separations into clades which deliberately have been left unnamed until confirmed in more complete phylogenies.

+ and − indicates the presence or absence of each character; SP, semi-papillate; S, M and L, small, medium or large sporangial pedicel; P, paragynous; A, amphigynous; Hom, homothallic; Het, heterothallic; Oc, occasional.

ITS1 sequences

Two clear groupings of species can be seen in the tree based on ITS1 sequences. Papillate and semi-papillate species (Table 3 and Fig. 3) formed a distinct and well defined group separated from a similarly well defined group of three non-papillate species (*P. cinnamomi*, *P. cambivora* and *P. fragariae*). However, the two remaining species, *P. megasperma* and *P. cryptogea*, were intermediate between these groups. Analysis of the sequence illustrates this intermediate position (Fig. 1). In some cases the sequence of *P. cryptogea* was homologous with the non-papillate species and in other cases homologous with some papillate species. *P. megasperma* showed more homology with the papillate and semi-papillate species than with the non-papillate species. Bootstrap analysis indicated that the main branches of the tree were well supported. The only main branch with a low bootstrap value was the point at which *P. megakarya* and *P. palmivora* join the main tree. Others involving *P. nicotianae*, *P. citricola* and *P. fragariae* lay at the tips of the branches and did not affect the overall phylogeny.

ITS2 sequences

The basic pattern of the species groupings seen in the analysis of ITS1 were mirrored in the ITS2 phylogenetic tree although the separation between papillate and non-papillate species was less pronounced (Fig. 3). In this case, *P. megasperma* was on the same branch as *P. palmivora* and *P. megakarya*. However, the low bootstrap value of 43·7 and the value of 30·6 for the neighbouring node is indicative of the difficulty of confirming the exact position of *P. megasperma* in the intermediate ground between the papillate and non-papillate species. The position of *P. nicotianae* is also different in the ITS2-derived tree, grouped with *P. infestans* but again with a low bootstrap value, which shows that it is merely its position in the clade that is in doubt, not whether it should be grouped with *P. infestans* and the other Group I species. The additional species, *P. drechsleri*, grouped closely with *P. cryptogea*.

Restriction fragment analysis

Sufficient DNA was present in mycelial extracts to allow efficient amplification of a 900 bp PCR product (results not shown). Individual digest products formed by restriction enzyme digestion usually could be resolved by electrophoresis in 3% NuSieve agarose. Species were placed into groups matching those determined by phylogenetic analysis of ITS sequences thus allowing similarities to be seen within and between groups (Fig. 4). The combination of three restriction enzymes resolved individual species in almost every case.

Band sizes were calculated according to the mobility of standards and the sum of all bands in each species calculated. In most cases the total of all bands added up to approximately 900 bp. Where the total was smaller, calculation of the theoretical restriction sites using the computer program MAP (part of the GCG package, Devereux, Haeberli & Smithies, 1984) showed that the discrepancy was due to fragments of equal sizes co-migrating on the gel. Where larger than expected, it was due to the occurrence of a polymorphism within the recognition site of the enzyme, e.g. in the *Msp* I digest patterns of *P. cambivora*. The combined size of the two extra bands not seen in the closely related *P. fragariae* and *P. cinnamomi* was approximately the same size as the largest (*ca* 400 bp) band. Subsequent sequencing of this and another isolate (results not shown) using an automated sequencer (Applied Biosystems model 373) revealed a polymorphism within the recognition site of the enzyme. Similar results were obtained with *P. capsici* and *P. drechsleri* when digested with *Msp* I.

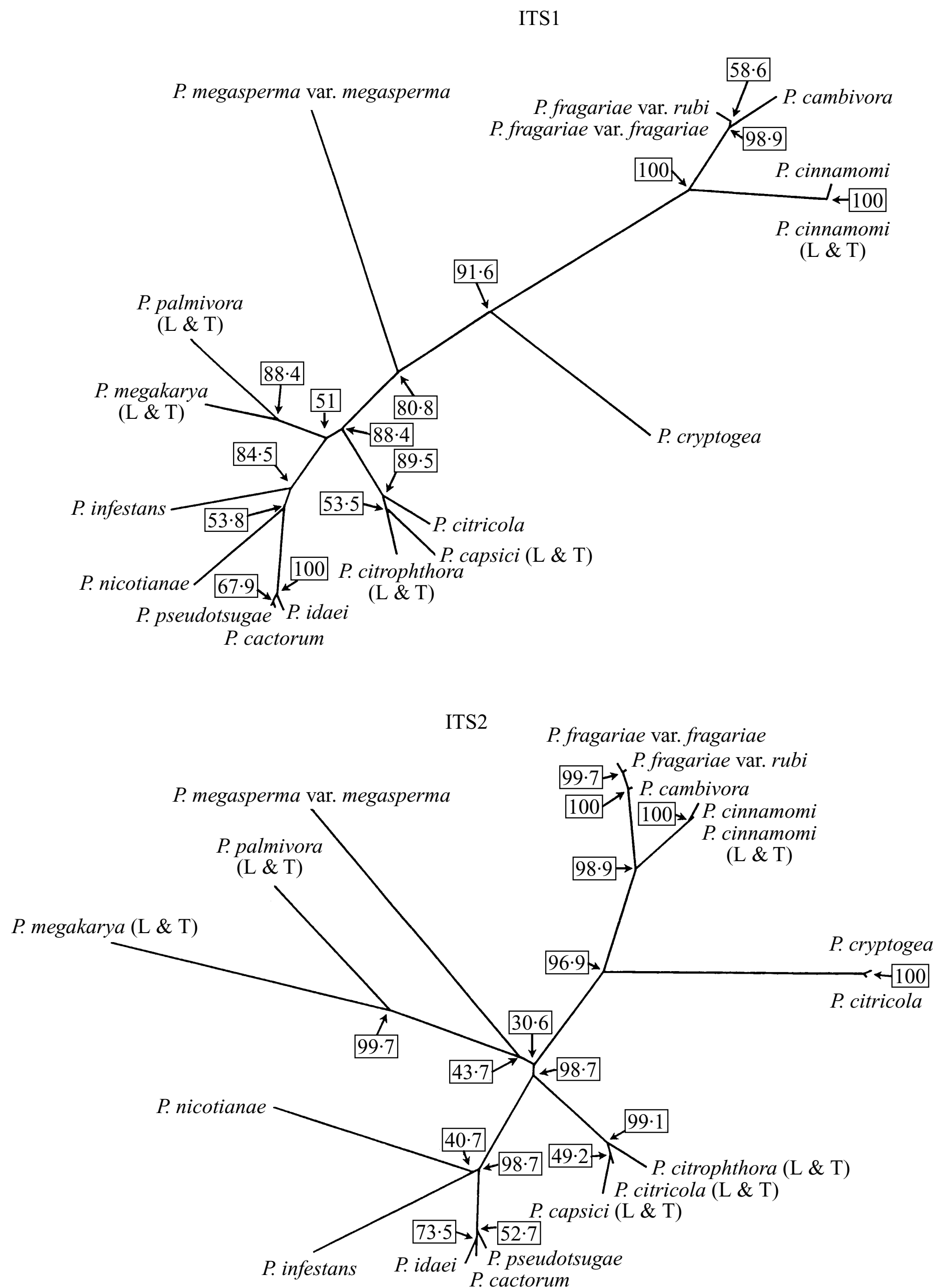

Fig. 3. Phylogenetic trees indicating the relationships between *Phytophthora* species based on sequences of ITS1 and ITS2. Unrooted trees created using neighbour-joining method (NEIGHBOR package) from genetic distance values created with the Kimura 2 parameter model (DNADIST package). Confidence limits of the branches, which are shown in boxes with arrows to indicate the branches to which they apply, were created in a bootstrap analyses using 2000 trials.

DISCUSSION

The addition of new ITS1 and ITS2 sequences for nine species to those already published (Lee & Taylor, 1992; Cooke *et al.*, 1996) confirms their utility in identifying species, determining natural groupings of species within the genus and gaining an understanding of their evolution. As with *Alternaria* (Jalasavich *et al.*, 1995) the ITS1 sequences were shorter and more polymorphic than ITS2 sequences but the resultant phylogenetic trees were very similar.

A clear grouping of species according to ITS sequence divergence was evident and it matched, to some degree, the broad classification based on type of papilla. However, a separation of semi-papillate and papillate species was not evident and the papillate and semi-papillate species found within groups I–IV (Waterhouse, 1970) were all grouped in the same clade, distinct from the clade consisting of the non-papillate species from Groups V and VI (Fig. 3). Papilla type therefore may be a sound criterion for classifying *Phytophthora* species. In a study of the Pythiaceae, Briard *et al.* (1995), likewise, found that in *Pythium*, there was a strict correlation between groups of species as defined by sequences of 28S rRNA and groups defined by the form of the sporangium.

Waterhouse's groups were further refined on the basis of antheridial attachment and much recent analysis of the genus has been based on this and whether species are homo- or

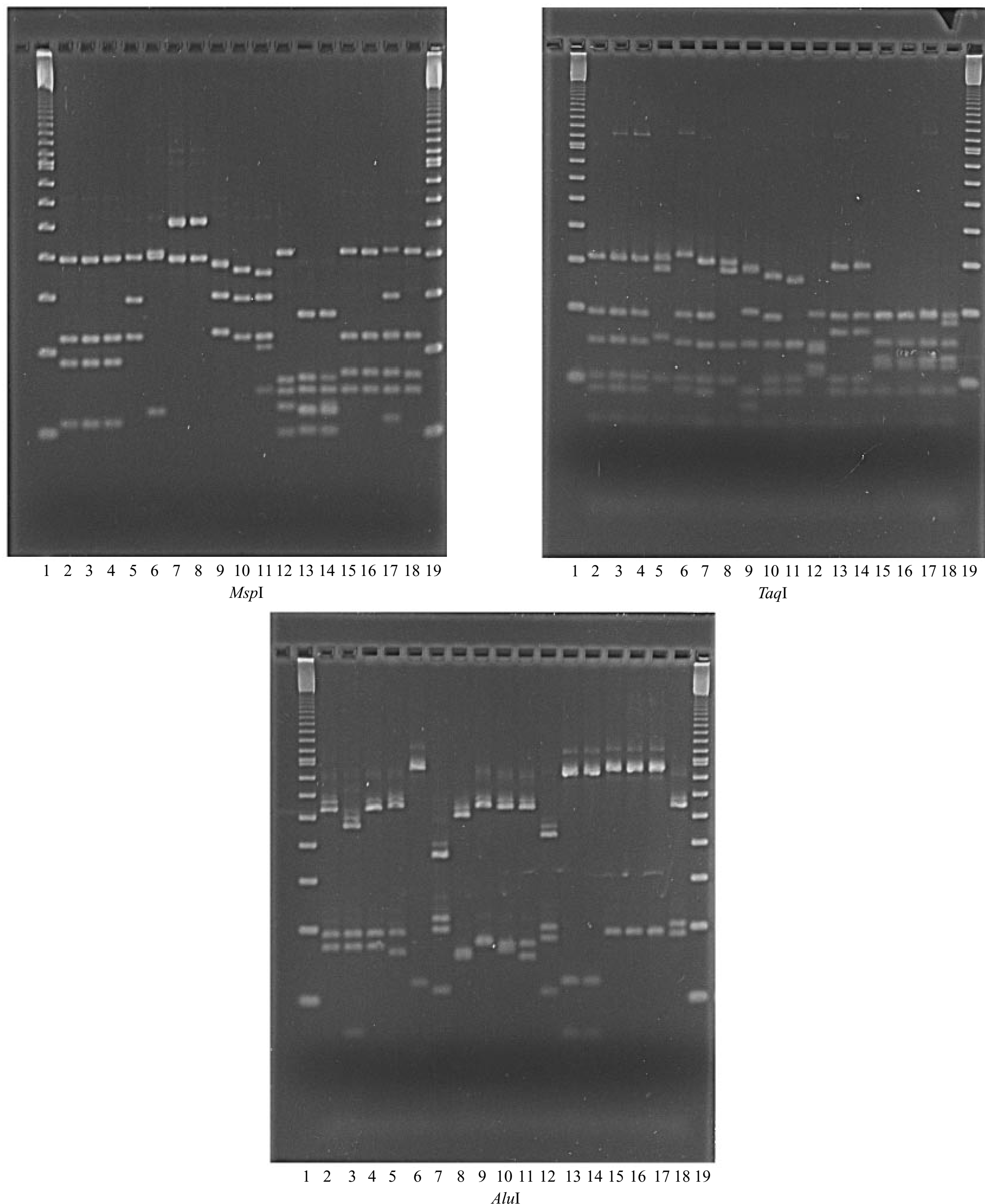

Fig. 4. PCR amplification products of the ITS1/5.8S/ITS2 region of the RNA gene repeat of 17 *Phytophthora* spp. (primers ITS6 and ITS4, see Materials and Methods for further details) digested with the DNA restriction enzymes *Msp* I, *Taq* I or *Alu* I and then separated on 3% agarose gels: Lanes 1 and 19, 100 bp ladder; 2, *P. pseudotsugae*; 3, *P. cactorum*; 4, *P. idaei*; 5, *P. infestans*; 6, *P. nicotianae*; 7, *P. megakarya*; 8, *P. palmivora*; 9, *P. citrophthora*; 10, *P. capsici*; 11, *P. citricola*; 12, *P. megasperma*; 13, *P. cryptogea*; 14, *P. drechsleri*; 15, *P. fragariae* var. *fragariae*: 16, *P. fragariae* var. *rubi*; 17, *P. cambivora*; 18, *P. cinnamomi*.

heterothallic. Phylogenies based on ITS sequences suggest that antheridial attachment and homo- and heterothallism cannot be taken as indicators of close phylogenetic relationships. Homo- and heterothallic species were spread throughout the genus and paragynous and amphigynous species were placed in the same clade. Förster *et al.* (1995) reached a similar conclusion. The lack of correlation with the ITS trees suggests that antheridial attachment and sexuality are under relatively

simple genetic control or have evolved more than once and that their significance may have been overplayed.

Papillate and semi-papillate species

Group I species were tightly clustered. Only a few well conserved differences in ITS sequences distinguished the morphologically similar *P. idaei*, *P. cactorum* and *P. pseudotsugae* from each other, although they could be separated by randomly amplified polymorphic DNA (Cooke *et al*., 1996). Isozyme analysis also has confirmed *P. cactorum* as a tightly defined species (Oudemans & Coffey, 1991*a*). *P. nicotianae* and *P. infestans* were closely related to these Group I species and isozyme data have also shown *P. nicotianae* to be a well defined homogenous species (Oudemans & Coffey, 1991*b*), so the single isolate examined here is likely to be fairly representative. Further evidence for an association of *P. nicotianae* with *P. cactorum* comes from phylogenetic analysis of isozyme data (Oudemans & Coffey, 1991*b*). Limited variation was seen amongst isolates of *P. infestans* using RFLP analysis of mtDNA (Carter *et al*., 1990). Thus it appears that species in this group are well characterized and stable on morphological characters as well as isozyme and DNA markers. Their relationships are confirmed by high bootstrap values in both phylogenetic trees in this paper, corroborating evidence from a study of ribosomal 28S D2 domain sequence divergence within the Pythiaceae (Briard *et al*., 1995).

Another distinct group of papillate species consisted of *P. capsici*, *P. citricola* and *P. citrophthora*. The close relationship between *P. capsici* and *P. citrophthora* has been shown by isozyme (Oudemans & Coffey, 1991*b*) and ITS sequence analyses (Lee & Taylor, 1992). Isozyme and mtDNA variation amongst many isolates of *P. citricola* resolved many genetic subgroups that varied little in morphology and indicated a close relationship with *P. capsici* and *P. citrophthora* (Oudemans, Förster & Coffey, 1994). The inclusion of *P. citricola*, a semi-papillate Group III species, serves to indicate that the division between semi- and papillate species is not phylogenetically significant. From this study it appears the affinities within the group are unclear as *P. capsici* grouped with *P. citrophthora* in the ITS1 tree and with *P. citricola* in the ITS2 tree. Low levels of intraspecific ITS variation within *P. citrophthora* were noted by Lee & Taylor (1992), resulting in a difficulty in separating *P. capsici* and *P. citrophthora*. Although the situation appears to have been clarified in a more recent analysis of ITS1 sequences (Förster *et al*., 1995) further studies on this group of species is justified in order to define the species boundaries more clearly or, if necessary, remove them.

Two other Group II species, *P. megakarya* and *P. palmivora*, formed a distinct cluster separate from *P. citrophthora*, *P. capsici* and *P. cinnamomi* (Lee & Taylor, 1992). These two species still grouped together in this study, although the position of their group was slightly different between the ITS1 and ITS2 trees.

Non-papillate species

There was a clear division between the relatively tight cluster of *P. fragariae* var. *rubi*, *P. fragariae* var. *fragariae*, *P. cambivora* and *P. cinnamomi* and the other species. The isolates of *P. cryptogea* and *P. drechsleri* grouped closely together but the *P. megasperma* sequence was markedly different and this species was placed in an intermediate position between the papillate and non-papillate groups.

P. fragariae has two varieties, one causes red core of strawberry (Hickman, 1940), the other raspberry root rot. The latter was variously identified as *P. erythroseptica sensu lato* (Converse & Schwartze, 1968) and *P. megasperma* (Duncan, Kenndy & Seemüller, 1987), and only recently described as *P. fragariae* var. *rubi* (Wilcox *et al*., 1992). ITS sequence homology of the two varieties accords with other molecular evidence based on ribosomal RFLPs (Stammler, Seemüller & Duncan, 1993) and mtDNA analysis (Förster and Coffey, 1992) and confirms their varietal status. Specific primers for *P. fragariae* based on the ITS sequences given in this paper gave an identical amplification product in PCR with all isolates and races of both varieties collected worldwide that have been tested to date (Cooke, Duncan & Unkles, 1995) but not with any other species; strong support for the view that variation at the ITS level is very limited.

The species most closely related to *P. fragariae* was *P. cambivora*, a species identified as being similar to *P. fragariae* by Hickman (1940) and reflected in whole protein profiles (Wilcox *et al*., 1992) and RFLPs (Stammler *et al*., 1993). Again *P. cambivora*-specific primers amplified all eight isolates in the collection at SCRI indicating a lack of variation in its ITS sequences, although it must be noted that most of these isolates came from raspberry. A similar lack of variation has been noted in *P. cinnamomi* by Lee & Taylor (1992).

In contrast, *P. cryptogea*, *P. drechsleri* and *P. megasperma* are all known to be highly polymorphic species, each previously having been classified into many groups (Hamm & Hansen, 1981; Hansen & Maxwell, 1991; Mills, Förster & Coffey, 1991; Förster & Coffey, 1993). As only one isolate of each was included in this study, it would be premature to assign a definitive position to them before examining more isolates.

Thus the reliability that can be placed on the use of single isolates varies from species to species and with present perceptions that taxonomists hold of particular species. This has practical consequences for the design of species-specific diagnostic probes (Lee, White & Taylor, 1993) and PCR primers (Cooke, Duncan & Lacourt, 1995).

Sequencing large numbers of isolates of each species is desirable but time-consuming and costly, although automatic sequencing of PCR products is now available and should hasten the production of even more detailed phylogenies that include other species not examined in this study. Alternatively, large numbers of isolates could be screened routinely by digesting ITS amplification products by restriction enzymes and comparing the electrophoretic pattern of the subsequent fragments. Inevitably, differences based on single base-pair changes would be missed. Nevertheless, this approach could be used to determine the broad affinities of large numbers of isolates as well as identifying those isolates requiring closer examination by sequencing. Sequence analysis has an advantage over RAPD or RFLP data in that the database of sequences can be expanded with the inclusion of new species without the need for standardized protocols, the effectiveness

of which can vary from laboratory to laboratory. Sequences of *P. cinnamomi* obtained in this work agreed completely with the sequences for the same fungus already published by Lee & Taylor (1992).

In this study, sequencing and/or digest patterns also revealed polymorphisms within the ITS regions of individual isolates of *P. cambivora*, *P. capsici* and *P. cryptogea*. In each case, the equal intensities of restriction digest bands of both ITS variants suggested similar frequencies for each form of the polymorphism. Further analysis with cloning and automatic sequencing (results not shown) suggested that these polymorphisms may be located on different chromosomes and could have arisen through hybridization, although more work is required to confirm this.

The phylogenetic trees correlated with sporangial morphology and may be useful in testing the hypothesis of Gaümann (Gaümann & Wynd, 1952) of an evolutionary advance in the Peronosporales from soilborne, unspecialized *Pythium* species through *Phytophthora* to the specialized downy mildews adapted to an aerial environment. Excluding sexuality and antheridial attachment would split *Phytophthora* into two broad groups: non-papillate, largely soilborne, and papillate/semi-papillate with some species adapted to an aerial environment. If ITS data can be obtained for the downy mildews and combined with existing data for *Pythium* (Briard *et al*., 1995) and those presented here, Gaümann's hypothesis could be examined more rigorously.

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