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Evolutionary relationships among *Phytophthora* species deduced from rDNA sequence analysis

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Sequence analysis of the internal transcribed spacer (ITS) regions I and II of ribosomal DNA were used to deduce evolutionary relationships among 15 *Phytophthora* species. Analysis of papillate, semi-papillate and non-papillate species showed that sporangium papillation has phylogenetic significance, with the three morphological groups each forming separate clusters. Within the *P. megasperma* species complex, separate evolutionary lines were identified for *P. medicaginis*, *P. trifolii*, and *P. sojae* formerly regarded as formae speciales of *P. megasperma*, confirming their recent reclassification as biological species. rDNA sequence analysis was able to distinguish *P. cryptogea* and *P. drechsleri* isolates indicating a valid basis for speciation. *P. macrochlamydospora* from soybean, which has only been observed in Australia, was closely related to *P. sojae*, indicating possible common ancestory.

The oomycete genus *Phytophthora* is represented by over 50 morphological species, almost all of which are serious plant pathogens. Some *Phytophthora* species are host-specific (e.g. *P. sojae* Kaufm. & Gerd. infecting soybean) while others have a broad host range (e.g. *P. cinnamomi* Rands infecting a wide range of hosts including many crops and ornamentals). Moreover, different species can cause the same disease on a particular host (i.e. *P. palmivora* E. J. Butler, *P. megakarya* Brasier & M. J. Griffin, *P. capsici* Leonian and *P. citrophthora* (R. E. Sm. & E. H. Sm.) Leonian causing black pod disease on cocoa).

Current classification systems in *Phytophthora* rely heavily on morphological characters. These include characteristics of the sporangium which can be either papillate (Waterhouse Groups I–II), semi-papillate (Waterhouse Groups III–IV), or non-papillate (Waterhouse Groups V–VI), the mating system, and the form of antheridial attachment (Waterhouse, 1963). A high level of variation in these characters within and between species is frequently observed thus making classification based on these criteria unreliable (Waterhouse, Newhook & Stamps, 1983). Due to this variation, the species boundaries of *P. cryptogea* Pethybr. & Laff., *P. drechsleri* Tucker and *P. megasperma* Drechsler species complex are uncertain (Brasier, 1983; Brasier, 1991; Erwin, 1983; Hansen, 1991).

The original description of *P. megasperma* (Drechsler, 1931) only included isolates producing unusually large oospores. However, Tompkins, Tucker & Gardner (1936) expanded the description to include isolates with smaller oospores. Six discrete groups of *P. megasperma* were proposed, three host-specific and three non-host-specific. The three host specific groups, *P. megasperma* f. sp. *medicaginis* Kuan & Erwin, *P. megasperma* f. sp. *glycinea* Kuan & Erwin, and *P. megasperma* f. sp. *trifolii* R. G. Pratt infect the legumes alfalfa, soybean, and clover, respectively (Kuan & Erwin, 1980; Pratt, 1981). Hansen & Maxwell (1991) proposed that these legume-infecting groups be classified as the separate species *P. medicaginis* E. M. Hansen & Maxwell, *P. sojae*, and *P. trifolii* E. M. Hansen & Maxwell, respectively. The non-host-specific *P. megasperma* species are classified as *P. megasperma* Douglas fir, apple/cherry and an undefined broad host range group. Nuclear and mitochondrial DNA (mtDNA) restriction fragment length polymorphisms (RFLPs) of *P. megasperma* isolates revealed nine molecular groups, five host-specific and four non-host-specific. Of the host-specific groups identified, three corresponded to *P. medicaginis*, *P. sojae*, and *P. trifolii*, plus possible Douglas fir and Asparagus groups (Förster *et al.*, 1988; Förster & Coffey, 1993).

P. cryptogea (Pethybridge & Lafferty, 1919) and *P. drechsleri* (Tucker, 1931) were described initially based on limited morphological and growth characteristics determined from a single isolate of each species (Tucker, 1931). Subsequent classification using additional isolates revealed that either no differences could be detected or that intermediate types existed (Flowers, Erwin & Hendrix, 1973; Bumbieris, 1974; Halsall, 1976; Klisiewicz, 1977; Shepherd, 1978; Stanghellini & Kronland, 1982; Ho & Jong, 1986). High levels of genetic variation were identified using mtDNA RFLPs and isozyme patterns (Mills, Förster & Coffey, 1991) which led them to suggest that the observed genetic variation represents more species than the two currently described.

Recently, a new *Phytophthora* species, *P. macrochlamydospora* J. A. G. Irwin, was described from Australia (Irwin, 1991). Like *P. sojae*, this species causes root rot of soybean but is distinguished morphologically by its production of large (up to 90 μm) thick-walled chlamydospores. Oospores have not been observed for this taxon, leaving its taxonomic status and relationship to other *Phytophthora* spp. unknown.

The complexity and overlap of morphological charac-

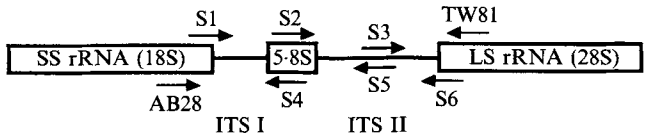

Fig. 1. Schematic representation of the rDNA of *Phytophthora*. The open boxes represent the genes for the ribosomal subunits. The arrows represent the positions of the PCR primers (AB28 and TW81) used for amplification of the ITS rDNA and the internal sequencing primers (S1–6).

teristics within *Phytophthora* may be due partly to the use of morphological traits which give unnecessary species distinctions or to the presence of hybrids (Brasier, 1991; Brasier & Hansen, 1992). For example, *P. cryptogea*, and *P. drechsleri* represent taxa with similar morphology which might not necessarily reflect true evolutionary relationships between these species. Ultimately, a natural classification system for *Phytophthora* must be based on evolutionary relationships to define biological species (interbreeding natural populations) which at the same time is useful for practical plant pathology. To this end, nucleotide sequence comparisons of ribosomal DNA (rDNA) can be used to analyse real phylogenetic relationships over a wide range of taxonomic levels (Pace, Olsen & Woese, 1986).

rDNA contains a mosaic of highly conserved and variable regions that enable inter- and intra-specific comparisons (Hibbett, 1992). Classification based on these rDNA sequences has great potential to clarify the evolutionary relationships between *Phytophthora* species. For example, analysis of the ITS I and ITS II regions (Fig. 1) of the rDNA of five *Phytophthora* spp. revealed a close evolutionary relationship between cacao isolates of *P. capsici* and *P. citrophthora*. It also demonstrated a common lineage for *P. palmivora* and *P. megakarya*, with *P. cinnamomi* being only distantly related (Lee & Taylor, 1992).

In this paper we employ rDNA sequence analysis to address several issues concerning *Phytophthora* taxonomy; (i) to reveal evolutionary relationships between papillate, semi-papillate, and non-papillate species; (ii) to determine whether current speciation within the *P. megasperma* species complex is justified; and (iii) to provide an evolutionary placement for the newly described *P. macrochlamydospora*. Besides providing an evolutionary basis for the taxonomy of this agronomically important genus of plant pathogens, sequence information from rDNA can be useful for the development of DNA-based diagnostic procedures.

MATERIALS AND METHODS

Fungal isolates

In this investigation 23 isolates representing 15 *Phytophthora* species were used (Table 1). Ex-type strains or representative isolates were obtained where possible.

Isolation of genomic DNA

Isolates were maintained on 20% clarified V-8 juice agar (Ribeiro, 1978) at 25 °C. After 5–7 d, mycelium was scraped from the plates and inoculated into 200 ml of sterile 20% clarified V-8 juice in a 500 ml flask. Cultures were grown for 5–7 d at 25° on an orbital shaker (150 rpm). Mycelium was harvested by vacuum filtration through Miracloth (Calbiochem Inc, Australia) and then stored at −70° until used for DNA extractions. DNA was extracted from the frozen fungal mycelium by a method modified from Raeder & Broda (1985) by the addition of 10 mg ml^{-1} RNase A (Sigma, Australia) and incubation at 37° for 1 h after the isopropanol precipitation. This was followed by precipitation with 2 volumes of 100% ethanol and centrifugation at 13000 ***g*** for 10 min. The pellet was rinsed with 70% ethanol, dried under vacuum and resuspended in TE buffer (pH 8·0). DNA was stored −20°.

PCR amplification

The region of the ribosomal repeat from the 3′ end of the 18S gene (through ITS I, the 5·8S gene, and ITS II) to the 5′ end of the 28S gene (Fig. 1) defined by the primers TW81 and AB28 (Table 2) (Howlett *et al.*, 1992) was amplified by the polymerase chain reaction (PCR). PCR was performed in a 50 µl reaction containing 50–100 ng of genomic DNA, 100 ng of each primer, 67 mM Tris-HCl (pH 8·8), 16·6 mM $(NH_4)_2SO_4$, 0·45% Triton X-100, 200 µg ml^{-1} gelatin, 5 mM $MgCl_2$, 200 µM of each dATP, dCTP, dGTP and dTTP (Biotech Ltd, Australia) and three Units *Tth plus* DNA Polymerase (Biotech Ltd, Australia). Mineral oil (30 µl, Sigma, Australia) was overlaid on the reaction mix and the tube was briefly centrifuged. The reaction was performed in a Perkin Elmer DNA Thermocycler 4800 for 35 cycles of denaturation at 94° for 30 s, annealing at 60° for 45 s and extension at 72° for 2 min, with an initial denaturation of 3 min at 94° before cycling and a final extension of 10 min at 72° after cycling. A portion (3 µl) of the amplified products was electrophoresed in an 0·8% agarose gel in Tris-Acetate-EDTA (TAE) buffer (Sambrook, Fritsch & Maniatis, 1989). The presence of a single bright band (850–950 bp) for each sample was a check for successful amplification. The PCR product was purified directly from the remainder of the PCR amplification reaction (47 µl) using the Wizard PCR Preps Purification system (Promega Corporation, Australia). All purified DNA samples were stored at −20°.

Cloning of the ITS

The purified amplification products were ligated into the *Eco*R V site of pGEM-T vector using the pGEM-T vector system I (Promega Corporation, Australia). The recombinant plasmids were used to transform *Escherichia coli* DH5α using the Gene Pulser apparatus and protocol (Bio-Rad Laboratories Inc, Australia). Recombinant colonies were identified by the colour selection after overnight growth at 37° on MacConkey

Table 1. Geographic origin and host of *Phytophthora* isolates used. Ex-type or authentic isolates are indicated by a T or A after isolate number. ITS I and II sizes obtained from sequence data are shown in base pairs (bp)

	Isolate	Host	Origin	ITS I	ITS II	Account no.[a]
P. cactorum	UQ1318	*Panax quinquefolius*	U.S.A.	218	415	L41357
P. cinnamomi	UQ734	*Allocasuarina* sp.	Australia	234	436	L41374
P. citricola	DAR 34210[b]	soil	Australia	184	417	L41375
P. cryptogea	UQ754	soil	Australia	208	431	L41376
P. drechsleri	IMI 22165[c]	*Westringua* sp.	Australia	207	432	L41377
P. iranica	CBS 374·72[d](A)	*Solanum* sp.	Iran	215	417	L41378
P. macrochlamydospora	IMI183280[c](T)	*Glycine max*	Australia	184	455	L41367
P. macrochlamydospora	DAR52299[b](A)	*Glycine max*	Australia	184	455	L41368
P. macrochlamydospora	DAR52301[b](A)	*Glycine max*	Australia	184	455	L41369
P. macrochlamydospora	DAR49431[b](A)	*Glycine max*	Australia	184	455	L41370
P. macrochlamydospora	T10446[e](A)	*Glycine max*	Australia	184	455	L41371
P. macrochlamydospora	T10456[e](A)	*Glycine max*	Australia	184	455	L41372
P. macrochlamydospora	T10455[e](A)	*Glycine max*	Australia	184	455	L41373
P. medicaginis	UQ125	*Medicago sativa*	Australia	205	431	L41379
P. megasperma	Pm12[f]	*Cicer arietinum*	Spain	206	430	L41380
P. megasperma (Asparagus)	EP1[g]	*Asparagus officinalis*	U.S.A.	227	432	L41381
P. megasperma (Douglas fir)	P1330[h]	*Pseudotsuga menziesii*	U.S.A.	227	432	L41382
P. nicotianae	IMI354395[c]	*Banksia* sp.	Australia	229	416	L41383
P. palmivora	P80[g]	*Cocos* sp.	Jamaica	213	414	L41384
P. sojae	9933R15[e]	*Glycine max*	Australia	184	455	L41385
P. syringae	DAR66142[b]	*Cymbidium* sp.	Australia	224	420	L41386
P. trifolii	P3356[g]	*Trifolium vesuculosum*	U.S.A.	205	430	L41387
P. vignae	UQ136	*Vigna unguiculata*	Australia	233	433	L41388
Achyla bisexualis	CBS102·50[d]	water	U.K.	174	336	L41389

[a] Sequences have been deposited in GenBank.
[b] Obtained from Department of Agricultural, Rydalmere, NSW, Australia.
[c] Obtained from International Mycological Institute, Egham, U.K.
[d] Obtained from Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.
[e] Obtained from M. J. Ryley, Queensland Department of Primary Industries, Toowoomba, Australia.
[f] Obtained from A. Trapero-Casas, Universidad de Cordoba, Cordoba, Spain.
[g] Obtained from D. C. Erwin, Department of Plant Pathology, University of California, Riverside, U.S.A.
[h] Obtained from C. M. Brasier, U.K. Forestry Commission, United Kingdom.

Table 2. Sequence of PCR and direct cycle sequencing primers[a]

Primer	Sequence (5′-3′)
TW81	GCGGATCCGTTTCCGTAGGTGAACCTGC
AB28	GCGGATCCATATGCTTAAGTTCAGCGGGT
S1	CCGTAGGTGAACCTGCGGAGG
S2	GCACATCGATGAAGAACGCTG
S3	GCTCGAAAAGCGTGACGT
S4	CCCGGAAGTGCAATATGCG
S5	GCCGAAGCCACCCATACCG
S6	GCTTAAGTTCAGCGGGTAATC

[a] Positions of primers are shown in fig. 1.

agar (FSE, Australia) supplemented with 100 μg ml^{-1} ampicillin (Progen, Australia). Plasmid DNA was isolated from *E. coli* cultures using the Wizard Minipreps DNA Purification System (Promega Corporation, Australia) and restricted with the restriction endonucleases *Pst* I and *Sac* II as recommended by the manufacturer (New England Biolabs, Australia) to verify the presence of cloned inserts. For sequencing, plasmid DNA was isolated from *E. coli* cultures using the Wizard Maxipreps DNA Purification System (Promega Corporation, Australia) and stored at −20°.

DNA sequencing

Cloned PCR products of eight isolates, *P. megasperma* (Asparagus and Douglas fir), *P. vignae* Purss, *P. drechsleri*, *P. trifolii*, *P. medicaginis*, *P. macrochlamydospora* (IMI183280), and *P. sojae*, were sequenced using double stranded DNA with the M13 Forward and Reverse sequencing primers following the protocol supplied with the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems Inc, Australia). Sequencing was conducted on an Applied Biosystems Model 373A DNA Sequencer (Applied Biosystems Inc, Australia) as recommended by the manufacturer. In order to sequence both strands, internal primers were synthesized on the Oligo 1000 DNA Synthesizer (Beckman, Australia). These primers, listed in Table 2, were used for direct double stranded DNA cycle sequencing of the remaining isolates using purified ITS amplified PCR products.

Analysis of DNA sequences

The sequence data was checked between complementary strands and the resulting sequences were aligned using the computer software package, CLUSTAL V (Higgins, Bleasby & Fuchs, 1992). The alignment of all sequences was checked visually. Nucleotide variation of these sequences was analysed by the maximum-parsimony method of the Phylogenetic Analysis Using Parsimony (PAUP) program 3·1·1 (Swofford, 1991). *Achlya bisexualis* Coker & Couch was included as an outgroup for comparisons. In parsimony analysis, gaps introduced to improve alignments were scored as ambiguities (missing information). The statistical method of bootstrapping

was applied to obtain an estimate of error, where the taxa are held constant and nucleotides are resampled by random replacement to obtain a new data set. A phylogeny is constructed from each data set; the frequency of each assemblage constructed by resampling is the bootstrap value (Felsenstein, 1988).

RESULTS

PCR amplification and sequence alignment

Primers TW81 and AB28 were used to amplify the entire ITS I, 5·8S gene, and ITS II regions as a single fragment from all isolates (Fig. 1, Table 1). The amplified products ranged in size from 854 bp (*P. citricola* Sawada) to 923 bp (*P. cinnamomi*). ITS I varied in size from 184 to 234 bp with ITS II between 415 and 455 bp for the *Phytophthora* spp. (Table 1). Of the 24 ITS rDNA nucleotide sequences examined, 19 were unique. Of the seven *P. macrochlamydospora* isolates, six had identical sequences (DAR52299, DAR52301, DAR49431, T10446, T10456, and T10455) with IMI183280 being different. Only DAR52299 was used together with IMI183280 for phylogenetic analysis. Multiple alignment using CLUSTAL V, followed by visual adjustment of all 19 unique sequences revealed very little divergence close to the ends of the 18S and 28S genes, and within the 5·8S gene. Sequences are deposited in GenBank (Table 1).

PAUP analysis

The consensus length after alignment of the ITS rDNA region, including the 5·8S gene was 875 bp. For maximum-parsimony analysis, only the ITS I (consensus length after alignment 246 bp) and ITS II (consensus length after alignment 468 bp) regions were used as the data set. Maximum-parsimony analysis using the Branch and Bound algorithm of PAUP 3·1·1 generated two trees of length 1339 steps. The most parsimonious tree (Figure 2) was determined by applying the 50% majority rule (strict consensus).

Papillate, semi-papillate, and non-papillate **Phytophthora** *spp. relationships*

When *A. bisexualis* was used as an outgroup, the *Phytophthora* spp. formed a monophyletic group with 274 nucleotide differences between the most divergent species. The papillate and semi-papillate *Phytophthora* spp. (Waterhouse Groups

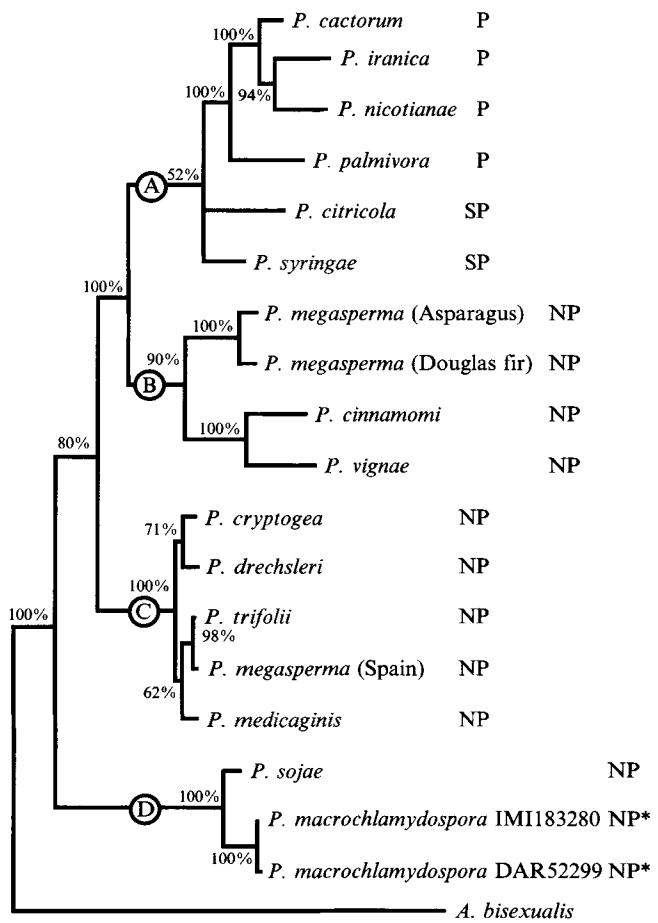

Fig. 2. Single most parsimonious phylogram generated from branch and bound algorithm in PAUP 3·1·1. The percentages are the frequencies with which a given branch appeared in 500 bootstrap replications. P, SP, and NP, represent the sporangial classes, Papillate, Semi-papillate and Non-papillate. NP* indicates non-papillate previously classified as semi-papillate. A–D indicate the four main branches Horizontal separation between nodes of the tree is proportional to phylogenetic distance. The tree was rooted by making *A. bisexualis* the outgroup.

I–IV) formed a separate group (Group A) (Fig. 2) with an average of 208 nucleotide differences to the non-papillate species. The papillate species *P. cactorum* (Lebert & Cohn) Schröt., *P. iranica* Ershad, *P. nicotianae* Breda de Haan, and *P. palmivora* with an average of 74 nucleotide differences, formed a distinct but closely related group to the semi-papillate species *P. citricola* and *P. syringae* (Kleb.) Kleb. with 85 nucleotide differences. There was an average of 142 nucleotide differences between the papillate and semi-papillate groups. The non-papillate *Phytophthora* spp. (Waterhouse Groups V–VI) formed three groups (Groups B–D) which had an average of 210 differences between the most divergent species. One group comprised *P. megasperma* (Asparagus and Douglas fir) with 28 nucleotide differences, and another included *P. cinnamomi* and *P. vignae* with 85 nucleotide differences (Group B). This group (Group B) was related to the papillate and semi-papillate group (Group A) with an average of 157 nucleotide differences between the groups. A second non-papillate group (Group C) comprised *P. cryptogea* and *P. drechsleri* with 20 differences, and *P. trifolii*, *P. megasperma* (Spain), and *P. medicaginis* with an average of 17 differences. The third and most distantly related non-papillate group (Group D) contained *P. sojae* and *P. macrochlamydospora* isolates which had 28 nucleotide differences.

P. megasperma *species complex*

Phylogenetic analysis revealed that isolates from the *P. megasperma* species complex were present in each of the three distinct non-papillate groups. The genetic distance between related *P. megasperma* isolates from Asparagus and Douglas fir (28 nucleotide differences) was similar to that between the related species *P. trifolii* and *P. medicaginis* (28 nucleotide differences). The Spanish *P. megasperma* isolate from chickpea was closely related to *P. trifolii* having only five nucleotide differences. Of the three host-specific groups formally classified within the *P. megasperma* species complex, *P. trifolii* and *P. medicaginis* are phylogenetically closely related with 28 nucleotide differences, where *P. sojae* is only distantly related, having an average of 200 nucleotide differences to other *P. megasperma* isolates.

P. macrochlamydospora

The *P. macrochlamydospora* isolates represent a highly monomorphic group, with low intraspecific variation; six isolates have identical sequences and IMI183280 has only one nucleotide change. This species was shown to be phylogenetically related to *P. sojae* with 28 nucleotide differences and was represented in the most divergent non-papillate group (Group D, Fig. 2).

DISCUSSION

The classification of *Phytophthora* has relied primarily on analysis of morphological characteristics with less emphasis on physiological characteristics such as host specialization. A more reliable classification system which reflects evolutionary rather than phenotypic relationships is required. The first aim of this investigation was to establish whether sporangium morphology, in particular papillation, reflects a relevant evolutionary basis for the division of *Phytophthora* into the major species groups of Waterhouse (1963). Our study has clearly shown that sporangium morphology has phylogenetic significance, with the three groups (papillate, semi-papillate, and non-papillate) each forming separate clusters based on rDNA sequence homology.

The second aim of this work, to infer evolutionary relationships between members of the *P. megasperma* species complex (both host-specific and non-host-specific) and other non-papillate *Phytophthora* spp., revealed unexpected evolutionary relationships. For example, *P. megasperma* (Douglas fir and Asparagus), *P. trifolii*, and *P. sojae*, previously regarded as members of the *P. megasperma* species complex, all showed separate evolutionary lineages. The third aim, evolutionary placement of *P. macrochlamydospora*, revealed that it was evolutionarily closely related to the other soybean pathogen, *P. sojae*.

Our DNA sequence information also provides a basis for delineation of biological species within the *P. megasperma* species complex, which does not represent a closely related group. The Asparagus, Douglas fir, and Spanish isolates were located in rDNA groups B and C (Fig. 2) while the host-specific isolates formally given formae speciales status, *P. medicaginis*, *P. trifolii* and *P. sojae*, were in groups C and D. These data are consistent with several separate lines of evolution for the *P. megasperma* species complex, one of the possibilities proposed by Brasier (1992). It would seem that host-specific forms have co-evolved in centres of origins with their respective hosts, rather than arising more recently from a common ancestor since the advent of modern agriculture as proposed by Hansen (1987*a*, *b*). Based on host range, colony morphology, and protein pattern profiles, Hansen proposed that an ancestral *P. megasperma* gave rise to two evolutionary lines, one representing the host-specific *P. megasperma* (plus a possible Douglas fir group), the other representing the broad host range and apple/cherry groups (Hansen *et al.*, 1986; Hansen, 1987*a*, *b*). However, our results do not support this hypothesis. While we found a close relationship between the host-specific *P. medicaginis* (alfalfa) and *P. trifolii* (clover), indicating a probable common ancestor, *P. sojae* (soybean) was only distantly related. This is in agreement with previously established relationships between these groups determined by mtDNA RFLP analysis (Förster & Coffey, 1993).

Based on protein pattern and mtDNA RFLP groupings of *P. megasperma* isolates from Douglas fir and Asparagus, it has been proposed that these might represent new host-specific groups within the *P. megasperma* species complex (Hansen *et al.*, 1986; Förster & Coffey, 1993). Our data support this suggestion. We found the genetic distance between the *P. megasperma* isolates from Douglas fir and Asparagus to be comparable to that exhibited between the species *P. trifolii* and *P. medicaginis*. A large number of isolates from the proposed centres of divergence would need to be examined to establish these new host-specific groups.

The only *P. megasperma* isolate that exhibited a close relationship to an existing species was the Spanish isolate from chickpea. Pathogenicity studies with this isolate showed

that it induced disease symptoms similar to those caused by *P. medicaginis* on chickpea and lucerne in Australia (Trapero-Casas *et al.*, 1992). That study also showed the Spanish isolate to be pathogenic on alfalfa and *Trifolium* spp., although it was more specific to chickpea than were the Australian *P. medicaginis* isolates. The Spanish and Australian chickpea isolates are morphologically distinguishable on the basis of sporangial apex shape and oospore wall thickness (Liew & Irwin, 1994). Our rDNA sequence analysis indicates a close relationship between the Spanish isolate and the isolates of *P. trifolii* and *P. medicaginis*.

The major problem in distinguishing *P. cryptogea* and *P. drechsleri* is the lack of definitive morphological characters. The sporangial size and shape range previously used to identify these species was shown to be a continuum (Ho & Jong, 1986). Isolates in these species were reclassified with intermediate-type isolates designated *P. cryptogea*, and thermotolerant isolates that grow well at 35° designated *P. drechsleri* (Ho & Jong 1991). The representative isolates of both species examined in this study show a significant level of genetic diversity that is comparable to that exhibited between *P. medicaginis* and *P. trifolii*. A previous report using isozyme and mtDNA RFLP analyses has indicated that at least seven genetically distinct groups exist within *P. cryptogea* and *P. drechsleri* (Mills *et al.*, 1991). Further rDNA sequence analysis of more isolates would be required to substantiate these subdivisions.

The other non-papillate species examined, *P. cinnamomi* and *P. vignae*, although related, form a distinct subgroup. These species can be readily distinguished by morphological characters and host-range; whilst *P. vignae* infects cowpeas and adzuki beans, *P. cinnamomi* infects a wide range of hosts (Tsuchiya, Yanagawa & Ogoshi, 1986; Zentmyer, 1981).

Our phylogenetic data show a close relationship between the semi-papillate *P. macrochlamydospora* and the non-papillate *P. sojae*. This result was unexpected since it contradicted our finding that non-papillate species are distinct from the more closely related papillate and semi-papillate species. To address this discrepancy, the sporangial morphology of *P. macrochlamydospora* was re-examined. A large number of sporangia produced from many isolates revealed that the sporangia were predominantly non-papillate (Crawford & Irwin, unpublished data), rather than semi-papillate as initially described based on a limited number of sporangia produced from one isolate (Irwin, 1991).

Recent biochemical and genetic evidence, such as mtDNA RFLPs, protein and isozyme patterns, and DNA sequence data, has revealed the limitations of a classification scheme for *Phytophthora* based on morphological characteristics, as exemplified by *P. megasperma* species complex, *P. cryptogea* and *P. drechsleri*. Reclassification employing a combination of both morphological and molecular characteristics, as suggested by Hansen (1991), may be more accurate. Molecular characters have advantages over morphological characters since they reflect evolutionary relatedness and are not subject to environmental influences such as culturing conditions. Nucleotide sequence data offers many variable and largely independent characters which can be compared directly with data collected by other researchers. Genetic analysis of fungal species will provide diagnostic primers/probes that will enable both rapid and reliable classification of new and existing isolates. The phylogenetic tree based on rDNA sequence analysis presented here should provide a framework for future comparative studies of the genus *Phytophthora*.

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