# Re-evaluation of *Phytophthora* Species Isolated During 30 Years of Vegetation Health Surveys in Western Australia Using Molecular Techniques

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**ABSTRACT**

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For 30 years, large-scale aerial photography has been used to map the extent of Phytophthora dieback disease in native forests in the southwest of Western Australia, with validation of the observations involving routine testing of soil and root samples for the presence of *Phytophthora cinnamomi.* In addition to *P. cinnamomi*, six morpho-species have been identified using this technique: *P. citricola, P. megasperma, P. cryptogea, P. drechsleri, P. nicotianae,* and *P. boehmeriae.* In recent years, many new *Phytophthora* species have been described worldwide, often with similar morphology to existing species; thus, as many of the isolates collected in Western Australia have been difficult to identify based on morphology, molecular identification of the morpho-species is required. Based on amplification of the internal transcribed spacer (ITS) region of the rDNA gene, sequence data of more than 230 isolates were compared with those of existing species and undescribed taxa. *P. inundata, P. asparagi, P.* taxon PgChlamydo*, P.* taxon personii, and *P.* taxon niederhauserii were identified based on sequence data. Phylogenetic analysis revealed that nine potentially new and undescribed taxa can be distinguished. Several of the new taxa are morphologically indistinguishable from species such as *P. citricola, P. drechsleri,* and *P. megasperma*. In some cases, the new taxa are closely related to species with similar morphology (e.g., P.sp.4 and *P. citricola*). However, the DNA sequences of other new taxa such as P.sp.3 and P.sp.9 show that they are not closely related to morphologically similar species *P. drechsleri* and *P. megasperma,* respectively. Most of the new taxa have been associated with dying *Banksia* spp., while P.sp.2 and P.sp.4 have also been isolated from dying *Eucalyptus marginata* (jarrah). Some taxa (P.sp.3, 6, and 7) appear to have limited distribution, while others like P.sp.4 are widespread.

The southwest of Western Australia (WA) supports one of the richest regions of floristic biodiversity in the world, with more than 5,700 described native plant species. *Eucalyptus marginata* (jarrah) is the dominant overstory tree species in much of the forest in this region. The introduced root and collar pathogen *Phytophthora cinnamomi* has had a major impact on the jarrah forest ecosystem (31,32,43). Shearer et al. (30) estimated that 2,284 of the plant species are susceptible to the pathogen, while many other species are affected indirectly through the loss of the overstory. *P. cinnamomi* reduces the diversity of plant species on infested sites. The altered vegetation structure in turn affects the biodiversity of fauna (17). In WA, there has been, and continues to be, active management of Phytophthora dieback spread through extensive mapping of affected areas and then quarantining of dieback free areas (32). Infested areas of high conservation value are also treated with salts of phosphonic acid (active compound, the anion $HPO_3^{2-}$) to reduce the impact and rate of spread of the pathogen (18).

Extensive vegetation health survey in the WA jarrah forest has been in continuous operation since 1978 (12,34). The mapping of the extent of Phytophthora dieback disease is based on shadowless color aerial photography (34). Validation of mapping involves collecting soil and root samples from beneath dying, *Phytophthora*-sensitive native plants known as "indicator species" and the testing of them for the presence of *Phytophthora* spp. (34). Samples have also been collected from dying plants in native heathlands and other forest types, and from dying trees in *Pinus radiata* plantations. *Phytophthora* isolates are obtained by standard baiting procedures (24) at the Vegetation Health Service (VHS) laboratory of the Department of Environment and Conservation, where isolates are identified from morphological characters where possible, and representatives are added to the VHS culture collection. *P. cinnamomi* is easily identified by its morphological characteristics. However, about 10 to 15% of baited samples return a positive result for *Phytophthora* species other than *P. cinnamomi.* Six other *Phytophthora* spp., *P. citricola, P. megasperma, P. cryptogea, P. drechsleri, P. nicotianae,* and *P. boehmeriae,* have been isolated and identified using morphological characters (10,35).

Cooke et al. (8) determined the phylogenetic relationships among 50 *Phytophthora* species based on internal transcribed spacer (ITS) sequence data, and identified 10 phylogenetic clades that did not correspond to previous morphological groupings (40,41). The contradiction between morphological characteristics and molecular phylogenetics indicates that the traditional morphological features used to classify *Phytophthora* spp. have evolved numerous times within the genus and that morphological classification alone does not result in natural assemblages. Since Cooke et al. (8), most descriptions of new *Phytophthora* spp. have included a molecular phylogeny where the new species is compared not only with those species with the most similar morphology, but also with those that are phylogenetically the closest (e.g., 4,6,22,26). Additional gene regions have been sequenced for most *Phytophthora* spp. (3,23,25). However, for an initial identification, sequencing the ITS region and conducting a BLAST search on GenBank coupled with phylogenetic analysis provides a valid identification. New taxa can also be identified in this manner, although further gene regions might be required to distinguish cryptic species.

In addition to the *Phytophthora* species identified by morphology, the VHS has acquired many isolates that could not be identified, or for which the morphological identification seemed to be misleading. Thus, the ITS gene region of more than 230 isolates, including both unidentified

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isolates and those previously identified by morphological characters, was sequenced to confirm isolate identity, and the results are reported here.

## MATERIALS AND METHODS

**Collection of isolates.** Soil and root samples were collected from beneath dying, *Phytophthora*-sensitive indicator species in native ecosystems in the southwest of WA. Samples were baited with *Eucalyptus sieberi* cotyledons (24) that were plated after 5 days and 10 days onto NARPH (21), or $P_{10}$VPH (39) selective media, from which a pure culture of *Phytophthora* was then isolated. In some cases, plant roots were surface-sterilized in 70% ethanol for 30 s, followed by four rinses in distilled water, and plated directly onto selective media. Cultures derived from earlier research and surveys have been incorporated into the present VHS collection, retaining the original abbreviations that designate their origin: MJS = M. J. C. Stukely; TCH = T. C. Hill; HSA = Hart, Simpson & Associates; DCE = E. M. Davison; and DDS = earlier prefix of the VHS collection.

**Morphological identification.** In general, morphological examination and identification were conducted when the isolates were initially collected. Isolates with IMI codes (Tables 1 and 2) were sent to the Imperial Mycological Institute, Kew, England for identification. Some isolates have been re-examined recently. In all cases, the same procedure is followed. Pure cultures of *Phytophthora* grown on 10% V8 juice agar were identified to species based on the morphology of their sporangia, oogonia, oospores, and related structures (15), or to the most likely Waterhouse Group (40) if all of the required structures were not formed. Sporangia were examined on squares cut from actively growing cultures 4 to 7 days old and suspended in nonsterile

**Table 1.** Identity (based on morphology and internal transcribed spacer [ITS] sequence data), host, location, and GenBank accession numbers for representative *Phytophthora* isolates from Western Australia, excluding isolates from Cooke's clade 6[a]

| Culture identification[b] | Morphology identification | Sequence identification | Host | Location | Cooke's clade[c] | GenBank accession no. |
|---|---|---|---|---|---|---|
| **DDS3453** | ***P. nicotianae* (A2)** | ***P. nicotianae*** | ***Chamaelaucium* sp.** | **Perth (Marangaroo)** | **Clade 1b** | **EU301133** |
| **VHS16097** | ***P. nicotianae* (A1)** | ***P. nicotianae*** | ***Xanthorrhoea platyphylla*** | **Ravensthorpe** | **Clade 1b** | **EU301134** |
| VHS16099 | *P. nicotianae* (A1) | *P. nicotianae* | *Dryandra cirsioides* | Ravensthorpe | Clade 1b | EU301135 |
| VHS13502 | *P. citricola* | P.sp.2 | *Corymbia calophylla* | Dwellingup | Clade 2 | EU301119 |
| VHS13523 | *P. citricola* | P.sp.2 | *Eucalyptus marginata* | Dwellingup | Clade 2 | EU301120 |
| **VHS13559** | ***P. citricola*** | **P.sp.2** | ***E. marginata*** | **Dwellingup** | **Clade 2** | **EU301121** |
| VHS13607 | *P. citricola* | P.sp.2 | *E. marginata* | Dwellingup | Clade 2 | EU301122 |
| VHS13615, WAC13038 | *P. citricola* | P.sp.2 | *E. marginata* | Dwellingup | Clade 2 | EF121961 |
| **VHS13663** | ***P. citricola*** | **P.sp.2** | ***E. marginata*** | **Dwellingup** | **Clade 2** | **EU301123** |
| VHS13694 | *P. citricola* | P.sp.2 | *E. marginata* | Dwellingup | Clade 2 | EU301124 |
| VHS13713, WAC13039 | *P. citricola* | P.sp.2 | *E. marginata* | Dwellingup | Clade 2 | EF121962 |
| **VHS16103** | ***P. citricola*** | **P.sp.2** | ***Banksia grandis*** | **Pemberton** | **Clade 2** | **EU301125** |
| **DCE10, IMI133316** | ***P. citricola*** | **P.sp.4** | **(ex Agriculture Dept 1980)** | | **Clade 2** | **EU301126** |
| **DDS1450, IMI329674** | ***P. citricola*** | **P.sp.4** | **Soil, native forest** | **Walpole** | **Clade 2** | **EU301127** |
| **DDS3458** | ***P. citricola*** | **P.sp.4** | ***Patersonia* sp.** | **Walpole** | **Clade 2** | **EU301128** |
| DDS3571 | *P. citricola* | P.sp.4 | *Conospermum* sp. | Perth (Kings Park) | Clade 2 | EU301129 |
| **HSA1210** | ***P. citricola*** | **P.sp.4** | ***Gastrolobium spinosum*** | **Coorow** | **Clade 2** | **EU301130** |
| **MJS278** | ***P. citricola*** | **P.sp.4** | ***E. marginata*** | **Jarrahdale** | **Clade 2** | **EU301131** |
| VHS15445 | *P. citricola* | P.sp.4 | *B. attenuata* | Badgingarra | Clade 2 | EU301132 |
| **VHS10154, IMI389663** | **Waterhouse Group 1** | **P.sp.1** | ***B. littoralis*** | **Bunbury** | **Clade 4** | **EU301114** |
| VHS15453 | Unidentified | P.sp.1 | *B. attenuata* | Badgingarra | Clade 4 | EU301115 |
| VHS15485 | Unidentified | P.sp.1 | *B. attenuata* | Badgingarra | Clade 4 | EU301116 |
| VHS16282 | *P. citricola*-like | P.sp.1 | *B. media* | Ravensthorpe | Clade 4 | EU301117 |
| **VHS9861, IMI389662** | **Waterhouse Group 1** | **P.sp.1** | ***B. menziesii*** | **Lancelin** | **Clade 4** | **EU301118** |
| **DDS1221** | ***P. citricola*-like** | **P.sp.1** | **Soil, native bush** | **Kalbarri** | **Clade 4** | **EU593265** |
| VHS17576 | Unidentified | P. taxon niederhauserii | *B. prionotes* | Lancelin | Clade 7b | EU301136 |
| **VHS17577** | **Unidentified** | **P. taxon niederhauserii** | ***B. prionotes*** | **Lancelin** | **Clade 7b** | **EU301137** |
| DDS3606 | *P. cryptogea* (A2) | *P. cryptogea* | *Daviesia* sp. | Fitzgerald River NP | Clade 8 | EU301138 |
| **DDS3612** | ***P. cryptogea* (A2)** | ***P. cryptogea*** | ***D. cirsioides*** | **Fitzgerald River NP** | **Clade 8** | **EU301139** |
| MJS046 | *P. cryptogea* (A2) | *P. cryptogea* | *Pinus radiata* | Nannup | Clade 8 | EU301140 |
| **TCH010** | **Unidentified** | ***P. cryptogea*** | ***B. ilicifolia*** | **Mogumber** | **Clade 8** | **EU301141** |
| **VHS14620** | **Waterhouse Group 6** | ***P. cryptogea*** | ***X. gracilis*** | **Fitzgerald River NP** | **Clade 8** | **EU301142** |
| DDS3543 | *P. megasperma* | P.sp.9 | *D. falcata* | Fitzgerald River NP | Clade 9 | EU301143 |
| DDS3565 | *P. megasperma* | P.sp.9 | *Adenanthos cuneata* | Fitzgerald River NP | Clade 9 | EU301144 |
| DDS3584 | *P. megasperma* | P.sp.9 | *B. baxteri* | Fitzgerald River NP | Clade 9 | EU301145 |
| DDS3601 | *P. megasperma* | P.sp.9 | *D. cirsioides* | Fitzgerald River NP | Clade 9 | EU301146 |
| DCE177, MJS129 | *P. megasperma* | P.sp.9 | *P. radiata* (H)-1980 | Nannup | Clade 9 | EU301147 |
| **MJS186** | ***P. megasperma*** | **P.sp.9** | ***P. radiata*** | **Nannup** | **Clade 9** | **EU301148** |
| **TCH003** | ***P. megasperma*** | **P.sp.9** | **Soil, native heathland** | **Cape Arid** | **Clade 9** | **EU301149** |
| **VHS16134** | ***P. megasperma*** | **P.sp.9** | **Soil** | **Fitzgerald River NP** | **Clade 9** | **EU301150** |
| **DDS3884** | ***P. boehmeriae*** | ***P. boehmeriae*** | ***Persoonia longifolia*** | **Nannup** | **Clade 10** | **EU301151** |

[a] Isolates in bold were included in the phylogenetic study (Fig. 1).

[b] Abbreviations of isolates and culture collections: IMI = CABI Bioscience (Imperial Mycological Institute), UK; WAC = Department of Agriculture and Food Western Australia Plant Pathogen Collection, Perth, Australia; VHS = Vegetation Health Service; DDS = earlier prefix of VHS collection. The following collections have been incorporated into the VHS collection: MJS = M. J. C. Stukely; TCH = T. C. Hill; HSA = Hart, Simpson & Associates; DCE = E. M. Davison.

[c] Cooke et al. (8).

soil extract in daylight for ~24 h. Gametangia were examined in cultures 7 to 10 days old where they were produced in pure culture; for heterothallic isolates, cultures were paired with tester isolates of known mating type and examined at intervals for up to 3 months.

**DNA isolation, amplification, and sequencing.** The *Phytophthora* isolates were grown on half-strength potato dextrose agar (PDA) (Becton, Dickinson and Company, Sparks, MD, USA; 19.5 g of PDA, 7.5 g of agar, and 1 liter of distilled water) at 20°C for 2 weeks, and the mycelium was harvested and placed in a 1.5-ml sterile Eppendorf tube. Harvested mycelium was frozen in liquid nitrogen, ground to a fine powder, and genomic DNA was extracted as described previously (2). The region spanning the ITS1-5.8S-ITS2 region of the ribosomal DNA was amplified using the primers ITS-6 (5′ GAA GGT GAA GTC GTA ACA AGG 3′) (8) and ITS-4 (5′ TCC TCC GCT TAT TGA TAT GC 3′) (42). The PCR reaction mixture, PCR conditions, the clean-up of products, and sequencing were as described previously (2).

**Phylogenetic analysis.** In order to compare *Phytophthora* isolates used in this study with other closely related *Phytophthora* spp., additional sequences were obtained from GenBank. Sequence data were initially assembled using Sequence Navigator version 1.01 (Applied Biosystems, Foster City, CA) and aligned in Clustal X (38). Manual adjustments were made visually by inserting gaps where necessary. All sequences derived in this study were deposited in GenBank, and accession numbers are shown in Tables 1 and 2.

Isolates representing each of the known species and undescribed taxa obtained in this study were compiled in a single dataset, and parsimony analysis was performed in PAUP version 4.0b10 (D. L. Swofford. 2003. Phylogenetic analysis using parsimony (*and other methods). Sinauer Associates, Sunderland, MA). The most parsimonious trees were obtained using heuristic searches with random stepwise addition in 100 replicates, with the tree bisection-reconnection branch-swapping option on and the steepest-descent option off. Maxtrees were unlimited, branches of zero length were collapsed, and all multiple, equally parsimonious trees were saved. Estimated levels of homoplasy and phylogenetic signal (retention and consistency indices) were determined (20). Branch and branch node support was determined using 1,000 bootstrap replicates (16).

Bayesian analysis was conducted on the same individual and combined dataset as that used in the parsimony analysis. First, MrModeltest v.2.5 (J. A. A. Nylander. 2004. Evolutionary Biology Centre, Uppsala University) was used to determine the best nucleotide substitution model. Phylogenetic analyses were performed with

**Table 2.** Identity (based on morphology and internal transcribed spacer [ITS] sequence data), host, location, and GenBank accession numbers for representative *Phytophthora* isolates from Cooke's clade 6 from Western Australia (WA)[a]

| Culture identification[b] | Morphology identification | Sequence identification | Host | Location | Cooke's clade[c] | GenBank accession no. |
|---|---|---|---|---|---|---|
| **DCE444, TCH012, IMI329666** | ***P. drechsleri* (A1)** | **P.sp.3** | ***Banksia attenuata*** | **Badgingarra** | **Clade 6** | **EU301152** |
| **DDS3760** | ***P. drechsleri*** | **P.sp.3** | **Soil - gravel pit - forest** | **Manjimup** | **Clade 6** | **EU301153** |
| **MJS148** | **Unidentified** | **P.sp.3** | ***Pinus radiata*** | **Nannup** | **Clade 6** | **EU301154** |
| VHS13530 | Unidentified | P.sp.3 | *Eucalyptus marginata* | Dwellingup | Clade 6 | EU301155 |
| VHS13567 | Unidentified | P.sp.3 | *E. marginata* | Dwellingup | Clade 6 | EU301156 |
| VHS13761 | Unidentified | P.sp.3 | *E. marginata* | Dwellingup | Clade 6 | EU301157 |
| **VHS16164** | **Waterhouse Group 6** | **P.sp.3** | ***Banksia grandis*** | **Pemberton** | **Clade 6** | **EU301158** |
| **DCE68, INH116468** | ***P. megasperma* var. *sojae*** | **P.sp.7** | **Soil, Lupin bait in 1965** | **Byford (Hwy)** | **Clade 6** | **EU301171** |
| **MJS235** | **Unidentified** | **P.sp.7** | ***P. radiata*** | **Nannup** | **Clade 6** | **EU301172** |
| **MJS238** | **Unidentified** | **P.sp.7** | ***P. radiata*** | **Nannup** | **Clade 6** | **EU301173** |
| **VHS9854** | ***P. megasperma*** | **P.sp.7** | ***Xanthorrhoea preissii*** | **Lancelin** | **Clade 6** | **EU301174** |
| **DDS3642** | ***P. drechsleri* (A1)** | **P.sp.8** | **Water, pear bait** | **Albany** | **Clade 6** | **EU301176** |
| **VHS2713** | **Unidentified** | **P.sp.8** | **Soil** | **Walpole** | **Clade 6** | **EU301175** |
| **TCH009, IMI329669** | ***P. megasperma* var. *sojae*** | **P.sp.10** | ***B. prionotes*** | **Badgingarra** | **Clade 6** | **EU593265** |
| **VHS17779** | ***P. megasperma*** | **P.sp.10** | ***B. grandis*** | **Perth (Bold Park)** | **Clade 6** | **EU593263** |
| **VHS17085** | **Unidentified** | **P.sp.11** | ***Banksia* sp.** | **Hopetoun** | **Clade 6** | **EU593262** |
| **VHS19173** | **Waterhouse Group 6** | **P.sp.11** | ***X. preissii*** | **Wilga** | **Clade 6** | **EU869199** |
| **DDS3599** | ***P. megasperma*** | **Unique** | ***X. platyphylla*** | **Fitzgerald River NP** | **Clade 6** | **EU593258** |
| **VHS16108** | **Waterhouse Group 6** | **Unique** | **Soil, native bush** | **Fitzgerald River NP** | **Clade 6** | **EU593259** |
| **VHS16115** | **Waterhouse Group 6** | **Unique** | **Soil, native bush** | **Fitzgerald River NP** | **Clade 6** | **EU593260** |
| **VHS17350** | **Waterhouse Group 6** | **Unique** | **Water, native bush** | **Badgingarra** | **Clade 6** | **EU593261** |
| **VHS5185** | **Unidentified** | **Unique** | **Soil, native bush** | **Pemberton** | **Clade 6** | **EU593264** |
| **VHS14801** | **Unidentified** | **P. taxon personii** | ***Grevillea mccutcheonii*** | **Busselton** | **Clade 6** | **EU301169** |
| **DDS3753** | **Unidentified** | **P. taxon PgChlamydo** | **Soil, native forest** | **Manjimup** | **Clade 6** | **EU301160** |
| **VHS6595** | **Unidentified** | **P. taxon PgChlamydo** | **Soil, native forest** | **Manjimup** | **Clade 6** | **EU301159** |
| **VHS17175** | **Waterhouse Grp. 5 or 6** | ***P. asparagi*** | ***B. media*** | **Esperance** | **Clade 6** | **EU301167** |
| **VHS17644** | **Waterhouse Grp. 5 or 6** | ***P. asparagi*** | ***Lomandra sonderi*** | **Perth (Murdoch)** | **Clade 6** | **EU301168** |
| **DDS1540** | **Unidentified** | ***P. inundata*** | ***Adenanthos cuneata*** | **Esperance** | **Clade 6** | **EU301161** |
| **MJS262** | **Unidentified** | ***P. inundata*** | ***B. littoralis*** | **Grimwade** | **Clade 6** | **EU301162** |
| VHS13920, WAC12880 | Unidentified | *P. inundata* | *X. preissii* | Wilga | Clade 6 | EU301163 |
| VHS16932 | Unidentified | *P. inundata* | *X. preissii* | Rocky Gully | Clade 6 | EU301164 |
| **VHS2910** | **Unidentified** | ***P. inundata*** | **Soil, native forest** | **Mount Barker** | **Clade 6** | **EU301165** |
| **VHS17183** | ***P. megasperma*** | ***P. megasperma*** | ***X. platyphylla*** | **Esperance** | **Clade 6** | **EU301166** |

[a] Isolates in bold were included in the phylogenetic study (Fig. 2).

[b] Abbreviations of isolates and culture collections: IMI = CABI Bioscience (Imperial Mycological Institute), UK; WAC = Department of Agriculture and Food Western Australia Plant Pathogen Collection, Perth, Australia; VHS = Vegetation Health Service; DDS = earlier prefix of VHS collection. The following collections have been incorporated into the VHS collection: MJS = M. J. C. Stukely; TCH = T. C. Hill; HSA = Hart, Simpson & Associates; DCE = E. M. Davison.

[c] Cooke et al. (8).

MrBayes v. 3.1 (28) applying a general time reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Two independent runs of Markov Chain Monte Carlo (MCMC) using 4 chains were run over 1,000,000 generations. Trees were saved each 1,000 generations, resulting in 10,001 trees. Burn-in was set at 50,001 generations (i.e., 51 trees), well after the likelihood values converged to stationary, leaving 9,950 trees from which the consensus trees and posterior probabilities were calculated.

## RESULTS

**Morphological identification.** The morphology of many of the *Phytophthora* isolates was examined when they were first isolated and the morphological identification initially provided (Tables 1 and 2). *P. nicotianae, P. cryptogea*, *P. boehmeriae, P. citricola, P. megasperma,* and *P. drechsleri* were identified, and several other isolates were placed in Waterhouse groups 1, 5, and 6. However, there were also many isolates that could not be assigned to a known species or even into a Waterhouse morphological group due to the lack of useful morphological features (usually oogonia).

**Phylogenetic analysis.** After an initial phylogenetic analysis containing all sequenced *Phytophthora* isolates from WA, the dataset was split into two. The first dataset included known species and undescribed taxa from ITS clades 1, 2, 4, 7, 8, 9, and 10, and the second included known species and undescribed taxa from ITS clade 6 (*sensu* Cooke et al. 8). Due to the size of the first dataset, only three representative isolates were included for each of the undescribed *Phytophthora* taxa from WA. In addition, only those known *Phytophthora* spp. that were phylogenetically close to the undescribed taxa were included in the analysis. Included in the second dataset were isolates of undescribed *Phytophthora* taxa from ITS clade 6 with sequences similar to isolates from the current study, including representatives of all undescribed *Phytophthora* taxa considered by Brasier et al. (4).

The first dataset consisted of 909 characters, of which 472 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1,000 random trees ($P < 0.01$, g1 = –0.78). Heuristic searches resulted in 8 most parsimonious trees of 1,260 steps (CI = 0.61, RI = 0.92) (Fig. 1, TreeBASE SN4084). The topology of the Bayesian tree was very similar (TreeBASE SN4084). Isolates obtained in this study resided in ITS clades 1, 2, 4, 7, 8, 9, and 10. Several isolates corresponded to known species (*P. nicotianae,* clade 1; *P. cryptogea,* clade 8; and *P. boehmeriae,* clade 10). *P.* taxon niederhauserii (clade 7) was identified solely on the 100% similarity to GenBank sequences, as there is no formal description for this species. The ITS analysis also revealed four undescribed *Phytophthora* taxa (P.sp.1, clade 4; P.sp.2 and P.sp.4, clade 2; and P.sp.9, clade 9). In each case, the phylogenetic analysis indicated that these new taxa were distinct from described species and reside in well supported terminal clades.

The second dataset consisted of 911 characters, of which 408 were parsimony informative. The dataset contained significant phylogenetic signal compared to 1,000 random trees ($P < 0.01$, g1 = –1.54). Heuristic searches resulted in 1,000 most parsimonious trees of 849 steps (CI = 0.70, RI = 0.89) (Fig. 2, TreeBASE SN4084). The topology of the Bayesian tree was very similar (TreeBASE SN4084). Isolates obtained in this study resided in ITS clade 6. Only a single isolate (VHS17183), originally identified as *P. megasperma* on morphological characters, had 100% similarity to sequences available for *P. megasperma* on GenBank. Others isolates thought to be *P. megasperma* based on morphological characters all represented undescribed taxa in clade 6 and clade 9 (Tables 1 and 2). Several other isolates corresponded to *P. inundata,* both on morphology and ITS sequence data. Two isolates, VHS17175 and VHS17644, had almost identical ITS sequence to the recently described *P. asparagi* (29). Several more isolates have been identified only by their 100% similarity to sequences available on GenBank for the as yet undescribed taxa *P.* taxon personii and *P.* taxon Pgchlamydo.

The ITS analysis revealed three undescribed *Phytophthora* taxa (P.sp.3, P.sp.8, and P.sp.11) and five unique isolates (DDS3599, VHS16108, VHS16115, VHS17350, and VHS5185) (Fig. 2, Table 2). The sequences from isolates in P.sp.3 were all identical (including sequences from isolates not included in the phylogenetic study) and were a close match to *Phytophthora* isolate 3267 of unknown origin. P.sp.8 was represented by two isolates (VHS2713 and DDS3642) with identical sequence and was a closest sequence match to three unique isolates from WA (VHS17350, VHS5185, and VHS16108). P.sp.11 was also represented by two isolates (VHS 17085 and VHS 19173) and was closest to another unique isolate from WA (VHS16115). Together, these isolates, P.sp.3, 8, 11, and four of the unique isolates from WA and *Phytophthora* isolate 3267, form a clade with 74% bootstrap support and a Bayesian probability of 0.90.

Also identified in clade 6 was P.sp.7, which formed a well supported clade with *P.* taxon raspberry isolates P1049 and P896. These unidentified isolates had been collected from raspberries in Australia and had been used in previous phylogenetic studies (4,6). Two isolates were thought to represent P.sp.10; however, when sequences of other undescribed *Phytophthora* isolates were included, the clustering of these isolates was less resolved. The unknown *Phytophthora* isolates closest to P.sp.10 are from *Malus* in the United States (P462), *Prunus* in Switzerland (P532), and SCRP223 of unknown origin. A single unique WA isolate DDS3599 had identical ITS sequence to an unknown *Phytophthora* isolate 4 FFL-2008 from Hungary (Fig. 2).

**Distribution of *Phytophthora* spp. in WA.** The known associations of *Phytophthora* species with dead and dying native plant species in WA are shown in Table 3. Besides *P. cinnamomi,* the most commonly isolated species in WA are *P. cryptogea, P. inundata,* P.sp.2, P.sp.3, P.sp.4, and P.sp.9. *P. cryptogea* and P.sp.4 have the largest host range and the widest distribution. The distribution of P.sp.9 and P.sp.1 is limited to the Midwest, West, and South Coast Land Management Regions of WA (Table 3, Fig. 3). Other *Phytophthora* spp. were isolated too infrequently to draw any conclusions on their distribution.

## DISCUSSION

Using molecular techniques to re-evaluate unidentified isolates from the VHS collection of *Phytophthora* isolates from natural ecosystems in WA, *P. inundata, P. asparagi,* and several named but as yet undescribed taxa, *P.* taxon PgChlamydo, *P.* taxon personii, and *P.* taxon niederhauserii, were identified for the first time. The presence of other species, *P. nicotianae, P. boehmeriae, P. cryptogea,* and *P. megasperma,* was confirmed. In addition, based on phylogenetic analysis, nine undescribed *Phytophthora* taxa were recognized.

*P. inundata*, described in Europe in 2003 (6), was identified based on phylogenetic analysis from several locations in the southwest of WA, where it has been associated with dying native plants (36). Some of these isolates were recovered in the 1980s. *P. boehmeriae* has been reported from most continents except North America. It is best known as the causal agent of brown rot of citrus and boll rot of cotton (15) and has also been implicated in gummosis of *Acacia mearnsii* in South Africa and Brazil (14). In WA, it was isolated from soil baits at only one site, collected beneath a dead, mature *Persoonia longifolia* (10). *P. asparagi* has just been described as a pathogen of asparagus in Michigan, USA (29). *P. asparagi* has been recorded previously in Australia from diseased agave in the Royal Botanic Gardens in Melbourne (9), but it is intriguing that isolates with identical ITS sequences have also been isolated from natural ecosystems in WA.

*P. cryptogea* has a cosmopolitan distribution and causes disease on many hosts (33). When sequencing of the VHS isolates

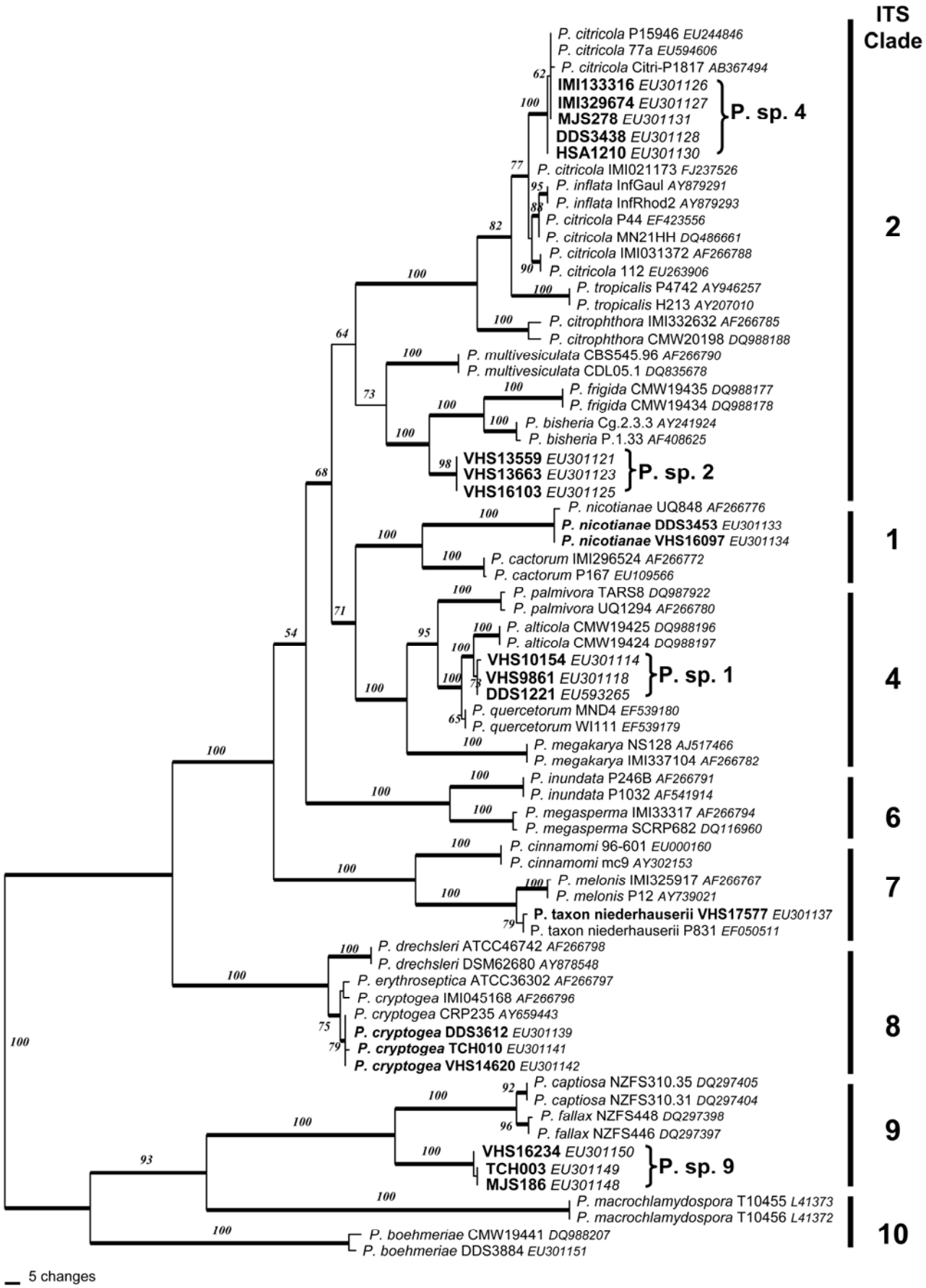


**Fig. 1.** One of the eight most parsimonious trees of 1,260 steps resulting from analysis of the internal transcribed spacer (ITS) sequence data of *Phytophthora* isolates. Bootstrap values of the branch nodes are given in italics. Branches with posterior probabilities resulting from Bayesian analysis of greater than 0.85 are thickened. Isolates from this study are in bold. The ITS clade as designated by Cooke et al. (8) is indicated on the right.

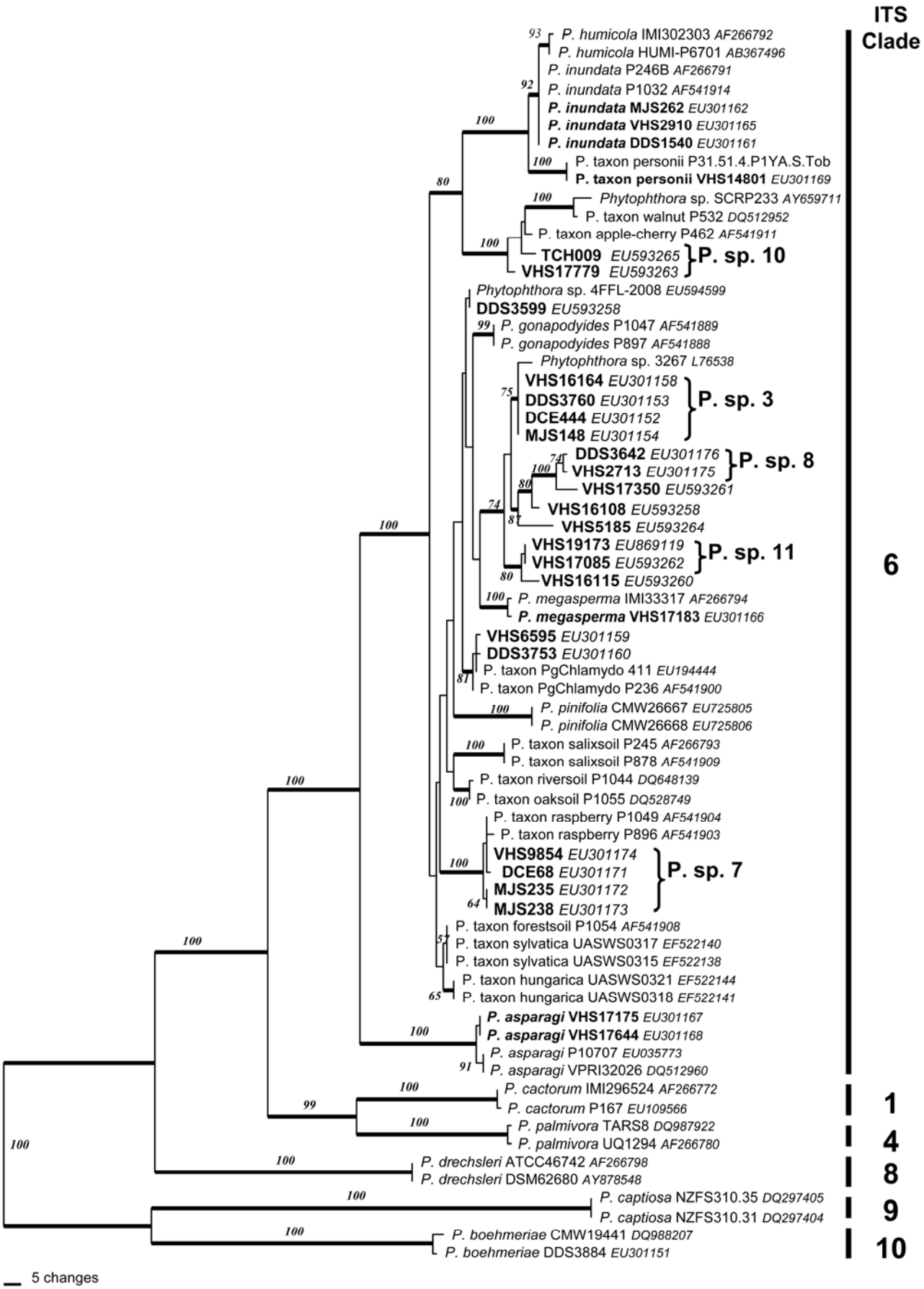


**Fig. 2.** One of the 1,000 most parsimonious trees of 849 steps resulting from analysis of the internal transcribed spacer (ITS) sequence data of *Phytophthora* isolates. Bootstrap values of the branch nodes are given in italics. Branches with posterior probabilities resulting from Bayesian analysis of greater than 0.85 are thickened. Isolates from this study are in bold. The ITS clade as designated by Cooke et al. (8) is indicated on the right.

commenced, a large group of isolates with identical sequences were observed which had the closest sequence match to *P. cryptogea.* Initially, it was thought to be a distinct species, and was named P.sp.5. However, as additional sequence information appeared on GenBank, the WA isolates clustered within *P. cryptogea sensu lato.* This complex is currently undergoing extensive revision, which will result in the description of several new species (M. D. Coffey, *personal communication*). The isolates from WA may reside in one of these new species. *P. cryptogea* is commonly isolated in WA, but its role in the natural environment is currently unclear.

*P. megasperma* has previously been reported from dying native vegetation in WA (35). However, after molecular re-evaluation, many isolates thought to be *P. megasperma* (ITS clade 6) based on morphology actually represent a new taxa belonging to ITS clade 9, and have been provisionally named P.sp.9. Other isolates represent the new taxa P.sp.7 and P.sp.10 (ITS clade 6). Phylogenetic analysis resolved only a single, recent, WA isolate (VHS17183) as a "true" *P. megasperma*. The earlier identification of *P. megasperma* on morphological characters alone was thus not reliable, and the ITS sequence data confirm that the *P. megasperma* morpho-species in WA represent a complex of several distinct taxa.

As with *P. megasperma,* so few isolates of *P.* taxon Pgchlamydo have been recorded among the VHS collection, that their role in the landscape is considered minor. In Europe, *P.* taxon Pgchlamydo is closely related to and inhabits a similar niche to *P. gonapodyides* (5). *P. gonapodyides* is common in North America and Europe and has been reported from Australia, New Zealand, and Chile, where it is predominantly known as a minor root and seedling pathogen with a restricted host range (15). Brasier et al. (4) suggests *P. gonapodyides* and some other taxa in ITS clade 6 may play a significant role in the breakdown of green plant litter, especially in waterways.

Hardy and Sivasithamparam (19) reported *P. cactorum, P. citricola, P. cryptogea, P. drechsleri, P. megasperma,* and *P. nicotianae* from container-grown nursery plants in WA. Davison et al. (11) reported several *Phytophthora* species on nursery plants imported into WA: *P. nicotianae, P. palmivora, P. drechsleri, P. citrophthora, P.* taxon parvispora, and *P.* taxon niederhauserii. These were intercepted prior to placement in WA nurseries. Among the VHS collection were isolates of *P. cryptogea, P. nicotianae,* and *P.* taxon niederhauserii and an isolate of *P. megasperma,* indicating that these species are estab-

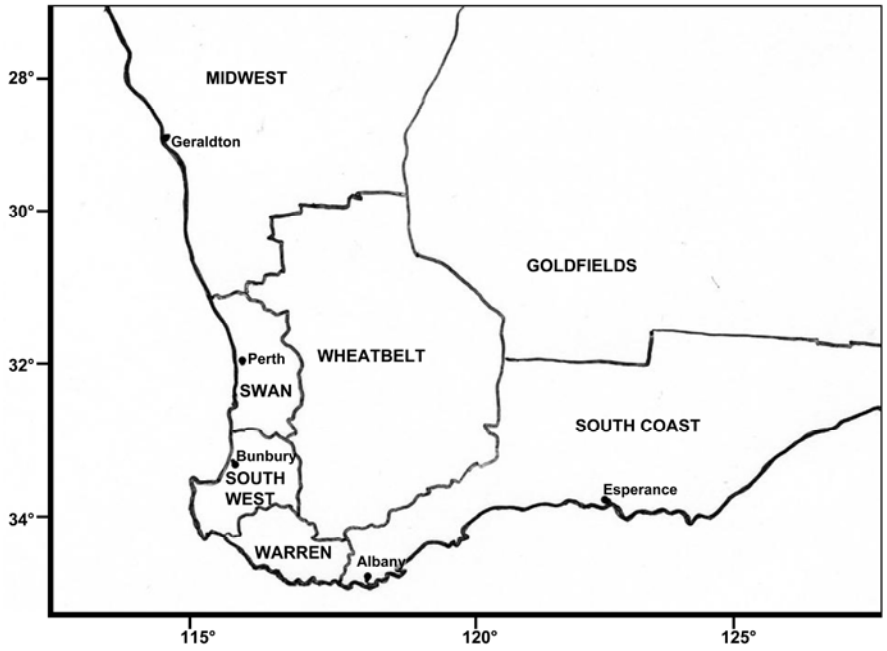


**Fig. 3.** Map of the southwest of Western Australia showing Department of Environment and Conservation Land Management Regions.

**Table 3.** Numbers of isolates of each *Phytophthora* species identified from the Vegetation Health Service (VHS) collection (excluding *P. cinnamomi*), including host and distribution across the southwest of Western Australia based on Land Management Regions (Fig. 3)

| ***Phytophthora* species** | **No. isolates** | **Host species** | **Distribution (region)** |
|---|---|---|---|
| *P. nicotianae* | 3 | *Chamaelaucium* sp., *Xanthorrhoea platyphylla, Dryandra cirsioides* | Swan, South Coast |
| *P.* taxon personii | 1 | *Grevillea mccutcheonii* | South West |
| *P. asparagi* | 2 | *Banksia media, Lomandra sonderi* | Swan, South Coast |
| *P.* taxon PgChlamydo | 2 | Soil, native forest | Warren |
| *P. inundata* | 15 | *Banksia littoralis, Xanthorrhoea preissii, Adenanthos cuneata* | South West, Warren, Wheatbelt, South Coast |
| *P. megasperma* | 1 | *X. platyphylla* | South Coast |
| *P.* taxon niederhauserii | 2 | *Banksia prionotes* | Midwest |
| *P. cryptogea* | 28 | *Banksia grandis, B. ilicifolia, Dryandra cirsiodes, Xanthorrhoea gracilis, X. platyphylla, Patersonia* sp., *Crowea angustifolia, Isopogon buxifolius, Daviesia* sp.; *Pinus radiata* (plantation) | Midwest, Swan, South West, Warren, Wheatbelt, South Coast |
| *P. boehmeriae* | 1 | *Persoonia longifolia* | South West |
| P.sp.1 | 10 | *Banksia menziesii, B. littoralis, B. attenuata, B media* | Midwest, South West, South Coast |
| P.sp.2 | 21 | *Eucalyptus marginata, Corymbia calophylla, B. grandis, Dryandra squarrosa, Patersonia xanthina* | Swan, South West, Warren |
| P.sp.3 | 15 | *E. marginata, B. attenuata, B. grandis; Pinus radiata* (plantation) | Midwest, Swan, South West, Warren |
| P.sp.4 | 64 | *E. marginata, B. attenuata, B. grandis, B. littoralis, B. menziesii, B. prionotes, Conospermum* sp., *Leucopogon verticillatus, X. gracilis, Podocarpus drouyniana, Patersonia* sp., *Bossiaea* sp., *Gastrolobium spinosum*; *Pinus radiata* (plantation) | Midwest, Swan, South West, Warren, Wheatbelt, South Coast |
| P.sp.7 | 4 | *X. preissii*; *Pinus radiata* (plantation) | Midwest, Swan, South West |
| P.sp.8 | 3 | Soil and water baits, native forest | Midwest, Warren, South Coast |
| P.sp.9 | 23 | *B. attenuata, B. baxteri, D. cirsioides, D. falcata, A. cuneata, Isopogon* sp.; *Pinus radiata* (plantation) | Midwest, South West, South Coast |
| P.sp.10 | 2 | *B. prionotes, B. grandis* | Midwest, Swan |
| P.sp.11 | 2 | *Banksia* sp., *X. preissii* | South West, South Coast |

lished in natural ecosystems. The other species reported in nursery plants have not been collected from natural ecosystems in WA.

P.sp.4 is morphologically very similar to *P. citricola,* and it resides within the *P. citricola* complex in clade 2. However, there is strong bootstrap support for the separation of these isolates into a new taxa. P.sp.4 is the most commonly isolated *Phytophthora* taxa in WA after *P. cinnamomi*. It has been found on 14 native host plant species representing seven families, from a wide range of locations, as well as on *Pinus radiata,* an introduced tree species in WA. Until recently, P.sp.4 was thought to be *P. citricola,* but after sequencing 73 isolates, not a single isolate was found with ITS sequence corresponding exactly to *P. citricola sensu stricto.* It has long been recognized that *P. citricola* is most likely a species complex (27). The widespread nature of this undescribed taxa in WA and the lack of consistent association with "high impact" disease suggests this species may be indigenous to the region. Interestingly, three sequences that are identical to P.sp.4 have been recently submitted to GenBank: EU244846 from Spain, EU594606 from Hungary, and AB367494 from Japan.

P.sp.2 has been isolated from the lower stem and roots of dying 1- to 2-year-old jarrah (*Eucalyptus marginata*) seedlings in rehabilitated open-cut bauxite mine pits in the WA jarrah forest since 2003, with unusually large numbers of isolations in the summer of 2004–05 (37). It has also been isolated from similar aged *Corymbia calophylla* in the same sites (M. J. C. Stukely, *unpublished data*) and from undisturbed jarrah forest on several occasions since the mid-1980s (37). P.sp.2 is morphologically similar to *P. citricola,* but most closely related to the recently described *P. bisheria* and *P. frigida,* the former from plants in the Rosaceae on three continents (1) and the latter from *Eucalyptus* in South Africa (26). The pathogenicity of P.sp.2 to jarrah is currently under investigation.

P.sp.1 has been isolated only from dead and dying *Banksia* spp. in coastal areas of the southwest of WA. It has a morphology similar to *P. cactorum*, but has the closest ITS sequence match to *P. alticola* and *P. quercetorum*, the former isolated and described from *Eucalyptu*s in South Africa (26) and the latter from *Quercus* in the United States. P.sp.9 has been recovered from six WA native hosts, but not from *Eucalyptus* spp. Morphologically, P.sp.9 resembles *P. megasperma,* and for several years, the death of young *Pinus radiata* in plantations in the Donnybrook Sunklands (Blackwood Plateau) in WA was attributed to *P. megasperma* (7). However, molecular re-evaluation places these isolates in ITS clade 9, most closely related to the newly described *P. captiosa* and *P. fallax* from *Eucalyptus* in New Zealand (13). Over 20 isolates of P.sp.9 have been recovered from across the southwest of WA, and studies are currently underway to determine the pathogenicity of both P.sp.1 and P.sp.9 toward *Banksia* spp.

Many new undescribed taxa and unique isolates were recognized within ITS clade 6. Among the isolates in the VHS collection was a single isolate, originally designated P.sp.6, which corresponds to *P.* taxon personii (Z. G. Abad, *personal communication*). The most commonly encountered taxa in WA from ITS clade 6 is P.sp.3. This taxa is morphologically very similar to *P. drechsleri* and *P. cryptogea,* and has been isolated from soil associated with dying *E. marginata* and *Banksia* spp., as well as *P. radiata*. There is very little sequence variation among P.sp.3 isolates, and it is widely distributed in WA. In the phylogenetic analysis, there were eight other isolates from WA which formed a strongly supported clade together with P.sp.3. Four of these isolates were unique and did not correspond to any sequence currently on GenBank. The other four isolates fell into two subclades designated P.sp.8 and P.sp.11. The role of these ITS clade 6 isolates in WA ecosystems is unclear. P.sp.3 is commonly isolated from stream baiting within natural ecosystems (*unpublished data*), and perhaps the other undescribed species in this clade will also be found in water bodies once more baiting is conducted.

In conclusion, based on phylogenetic analysis, nine potentially new and undescribed taxa of *Phytophthora* can be distinguished among isolates from natural ecosystems in the southwest of WA. Several of these undescribed taxa are morphologically indistinguishable from known species such as *P. citricola, P. megasperma,* and *P. drechsleri*. In some cases, the new taxa are closely related to known species with similar morphology (e.g., P.sp.4 and *P. citricola*). However, the DNA sequences of other new taxa show that they are not closely related to morphologically similar species (e.g., P.sp.3 and *P. drechsleri*, P.sp.9 and *P. megasperma*). Further work is planned to describe the new taxa and their relationships, and to test their pathogenicity, so that an estimate of the level of threat they pose to native vegetation can be made. It is not known if these new *Phytophthora* taxa are endemic or introduced, and thus their quarantine status within Australia and elsewhere remains unknown.

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