Plant Pathology (2011) **60**, 1055–1068 Doi: 10.1111/j.1365-3059.2011.02463.x

Two novel and potentially endemic species of *Phytophthora* associated with episodic dieback of Kwongan vegetation in the south-west of Western Australia

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Two novel homothallic species of *Phytophthora* causing dieback of Kwongan vegetation in south-west Western Australia are described here as *Phytophthora arenaria* sp. nov. and *Phytophthora constricta* sp. nov. DNA sequencing of the ITS rDNA and *cox*1 gene confirmed that *P. arenaria* and *P. constricta* are unique species residing in ITS clades 4 and 9, respectively. *Phytophthora arenaria* has been isolated from vegetation occurring on the northern sandplains which are warmer and drier than the southern sandplains from which *P. constricta* has been predominantly isolated, and both species appear morphologically and physiologically well adapted to the ecosystems in which they occur. Both species have been associated mainly with dead and dying *Banksia* species and the pathogenicity of both *P. arenaria* and *P. constricta* to *Banksia attenuata* was confirmed in this study. The combination of unique DNA sequences, including considerable variation in *cox*1 sequence data, thick oospore walls and physiological characteristics that appear to be adaptations favouring survival in the harsh Kwongan ecosystem suggest that these species may be endemic to Western Australia.

Keywords: Mediterranean climate, natural ecosystems, pathogens, phylogeny, *Phytophthora arenaria*, *Phytophthora constricta*

Introduction

The sclerophyllous shrubland occurring on the nutrient-impoverished sandplains of south-west Western Australia (WA) is referred to by the local aboriginal term, Kwongan (Beard, 1976). Most plant species occurring in Kwongan vegetation belong to the families Myrtaceae, Proteaceae, Papillionaceae, Epacridaceae and Asteraceae (Lamont *et al.*, 1984). Kwongan ecosystems occur on dry sites unable to support woodland or forest and are extremely fire-prone (Beard & Pate, 1984). Whilst generally consisting of shrubs approximately 1 m or less in height, Kwongan vegetation may also contain dominant tall shrubs other than *Eucalyptus*, or nondominant scattered trees (Beard & Pate, 1984). A number of distinct vegetation units, including heath, scrub-heath with or without low trees, mallee-heath, and thicket, occur in the Kwongan. A characteristic of Kwongan is its high levels of floristic endemism and species richness, the latter manifesting as both alpha diversity (species richness at a particular site) and beta diversity (species turnover between sites) (Lamont *et al.*, 1984). Thus, Kwongan vegetation is floristically diverse, occurs in a harsh Mediterranean environment and represents a complex ecosystem of significant conservation value. Analogous Mediterranean ecosystems include the Chaparral in California, and the Fynbos in South Africa (Beard, 1984).

In WA, the decline of forest and other ecosystems has often been attributed to disease caused by *Phytophthora cinnamomi*; however, many other *Phytophthora* taxa have been isolated from natural ecosystems in WA. A molecular re-evaluation of a large number of isolates from the collection of the Vegetation Health Service (VHS) of the Western Australian Department of Environment and Conservation (DEC) revealed nine potentially new taxa associated with dead and dying plants. Some of these taxa were previously assigned to known morphospecies such as *P. citricola*, *P. drechsleri* and *P. megasperma* (Burgess *et al.*, 2009).

Phytophthora multivora, the first of these new species to be described (Scott *et al.*, 2009) had previously been identified as *P. citricola*. *Phytophthora multivora* has been isolated in WA from natural forest and heathland stands for the last 30 years from beneath dead and dying

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Published online 27 April 2011

plants of 16 species from seven families. It is very widespread in south-west WA, with a distribution similar to *P. cinnamomi*, but is also active on calcareous soils, whereas *P. cinnamomi* is not. *Phytophthora elongata,* another recently described species, also historically misidentified as *P. citricola*, occurs mostly on the lateritic soils of the jarrah forest of WA (Rea *et al.*, 2010), but also occasionally on the sands of the Swan Coastal Plain. *Phytophthora elongata* has been primarily isolated from dead and diseased *Eucalyptus marginata* and *Corymbia calophylla* and from understorey species in the jarrah forest, with isolations dating back over 20 years. Two additional species recognized by Burgess *et al.* (2009) are associated exclusively with Kwongan vegetation on both the northern and southern sandplains in south-west WA. Based on their unique combination of morphological and physiological characters and ITS DNA and mtDNA sequences these taxa are described here as *Phytophthora constricta* sp. nov. and *Phytophthora arenaria* sp. nov.

Materials and methods

Isolation methods

Samples of soil and root material were baited with either *Eucalyptus sieberi* cotyledons (Marks & Kassaby, 1974) or young *Quercus ilex* and *Q. suber* leaves (Jung *et al.*, 1996) which were plated after 5 or 10 days onto P10VPH (Tsao & Guy, 1977) or NARPH (Hüberli *et al.*, 2000) selective agar, from which pure cultures were then isolated. Isolations from collar rot lesions of *Banksia hookeriana* and *B. sphaerocarpa* were made using the oak leaf baiting method (Jung, 2009).

Preparation of *Phytophthora* cultures

The isolates used in this study are detailed in Table 1. All isolates were initially grown on cornmeal agar (CMA; BBL Microbiology) plates prior to being subcultured twice onto *Phytophthora*-selective NARPH agar plates. They were then routinely maintained on V8 agar (V8A) plates. To ensure the vigour of isolates used in the pathogenicity trials, each isolate was passaged through an apple fruit (cv. Granny Smith) and reisolated after 6 days using NARPH. Isolates were then maintained on V8A plates.

DNA isolation, amplification and sequencing

The *Phytophthora* isolates were grown on half-strength potato dextrose agar (PDA) (Becton, Dickinson and Company; 19·5 g PDA, 7·5 g Difco agar and 1 L distilled water) at 20°C for 2 weeks. The mycelium was harvested and genomic DNA extracted as described previously (Andjic *et al.*, 2007). The region spanning the internal transcribed spacer ITS1-5·8S-ITS2 region of the ribosomal DNA and the mitochondrial *cox*1 gene were amplified and sequenced as described previously (Jung & Burgess, 2009).

Phylogenetic analysis

The ITS rDNA and *cox*1 gene were sequenced for the *P. constricta* and *P. arenaria* isolates used in this study. Species closely related to *P. arenaria* in ITS clade 4 and *P. constricta* in ITS clade 9 (Cooke *et al.*, 2000), as well as representative isolates from other clades within the genus, were either sequenced or obtained from GenBank (http://www.ncbi.nlm.nih.gov/). All sequences derived in this study were deposited in GenBank and accession numbers are shown in Table 1. ITS sequence data were assembled and manually edited as described previously (Jung & Burgess, 2009). There were no gaps in the *cox*1 alignment. The first 540 bp of the *cox*1 region were excluded to allow for alignment with the other sequences available on GenBank (leaving 742 characters).

Parsimony analysis was performed in PAUP (Phylogenetic Analysis Using Parsimony) v. 4.0b10 (Swofford, 2003) and Bayesian analysis with MRBAYES v. 3.1 (Ronquist & Heuelsenbeck, 2003) as described previously (Jung & Burgess, 2009). Alignment files and trees can be viewed on TREEBASE (http://www.treebase.org/).

Colony morphology, growth rates and cardinal temperatures

Colony morphology was described from 7-day-old cultures grown at 20°C in the dark on carrot agar (CA), V8A, malt extract agar (MEA) and half-strength PDA. CA was prepared as described previously (Ribeiro, 1978). Colony morphologies were described according to Brasier & Griffin (1979), Erwin & Ribeiro (1996) and Jung *et al.* (2003). Radial growth rate was recorded 4–6 days following the onset of linear growth along two lines intersecting the centre of the inoculum at right angles (Jung *et al.*, 1999). For temperature-growth studies, five isolates of *P. arenaria*, four of *P. constricta*, one of *P.* taxon arenaria-like, and one each of *P. cactorum* and *P. megasperma* (Table 1) were subcultured onto CA plates and incubated for 24 h at 20°C to initiate growth. Three replicates for each isolate were then transferred to incubators set at 10, 15, 20, 25, 30, 32·5 and 35°C, and radial growth was measured as above after 4–6 days. *Phytophthora constricta* and *P. megasperma* were also incubated at 4 and 22·5°C.

Morphology of sporangia and gametangia

Sporangia and gametangia of five isolates of *P. constricta*, 10 isolates of *P. arenaria* and one isolate of *P. megasperma* (Table 1) were produced on V8A and measurements were made as described previously (Jung *et al.*, 1999). Sporangia were obtained by flooding 5- × 5-mm agar squares taken from the growing margins of 3- to 5-day-old colonies in 90-mm Petri dishes with nonsterile soil extract (Rea *et al.*, 2010). Petri dishes were incubated in the dark at 20°C for 18–36 h and the solution was exchanged after 6–8 h. Dimensions and characteristic features of >50 mature sporangia chosen at random were

Table 1 Identity, date and location of isolation, host information and GenBank accession numbers for isolates of *Phytophthora* spp. considered in this study; all WA isolates were obtained from baiting of rhizosphere soil samples unless otherwise stated

Species	Isolate number[abc]	Location	Isolation date (month/year)	Host association	GenBank accession no.	
					ITS	*cox*I
Phytophthora arenaria	**DDS 1221**[c]	Kalbarri, Western Australia (WA)	06/1986	Kwongan heathland	EU593266	HQ013201
P. arenaria	VHS 9861 IMI 389662	Lancelin, WA	11/2001	*Banksia menziesii*	EU301118	
P. arenaria	**VHS 10154 IMI 389663**[bc]	Bunbury, WA	02/2002	*Banksia littoralis*	EU301114	HQ013202
P. arenaria	**VHS 15453**[bc]	Badgingarra,WA	04/2006	*Banksia attenuata*	EU301115	HQ013199
P. arenaria	**VHS 15489**[b]	Badgingarra,WA	04/2006	*B. attenuata*	HQ013216	HQ013200
P. arenaria	VHS 19931	Eneabba, WA	06/2008	*B. attenuata*	HQ013217	n.a.
P. arenaria	VHS 19950	Eneabba, WA	06/2008	*B. attenuata*	HQ013218	n.a.
P. arenaria	CLJO142	Cooljarloo, WA	01/2009	*Hibbertia hypericoides*	HQ013221	n.a.
P. arenaria	**CBS 125800 ENA 1**[b]	Eneabba, WA	02/2009	*Eucalyptus drummondii*	HQ013205	HQ013215
P. arenaria	**CBS 127950 ENA 3** (type)[b]	Eneabba, WA	02/2009	*E. drummondii*	HQ013219	HQ013203
P. arenaria	**ENA 4**	Eneabba, WA	02/2009	*E. drummondii*	n.a.	n.a.
P. arenaria	**ENA 9**	Eneabba, WA	02/2009	*B. attenuata*	n.a.	n.a.
P. arenaria	**ENA 12**	Eneabba, WA	02/2009	*Banksia sphaerocarpa*	n.a.	n.a.
P. arenaria	**ENA 18**	Eneabba, WA	02/2009	*Banksia hookeriana*, collar rot	HQ013220	HQ013204
P. cactorum	DCE 505[b]	Wisconsin USA	07/1992	*Panax quinquefolius*	n.a.	n.a.
P. cinnamomi	MU 9448[c]	Willowdale, WA	1994	*Eucalyptus marginata*	n.a.	n.a.
P. constricta	**DCE 177 MJS129**[bc]	Nannup, WA	07/1980	*Pinus radiata* (healthy), root	EU301147	HQ013212
P. constricta	DDS 3543	Fitzgerald River National Park, WA	09/1993	*Banksia falcata*	EU301143	n.a.
P. constricta	MJS186	Nannup, WA	08/1981	*P. radiata*, root	EU301148	n.a.
P. constricta	TCH003	Cape Arid, WA			EU301149	HQ013211
P. constricta	**VHS 15435**[bc]	Badgingarra, WA	04/2006	*B. attenuata*	HQ013228	HQ013210
P. constricta	**VHS 16125**[bc]	Fitzgerald River National Park, WA	08/2006	*Banksia cirsioides*	HQ013226	HQ013211
P. constricta	**VHS 16127**	Fitzgerald River National Park (Bell Track), WA	08/2006	Kwongan heathland	HQ013224	HQ013206
P. constricta	**CBS 125801** (type)[bc] **VHS 16130**	Fitzgerald River National Park, WA	08/2006	Kwongan heathland	HQ013225	HQ013207
P. constricta	VHS 16134	Fitzgerald River National Park, WA	08/2006	Kwongan heathland	HQ013227	HQ013208
P. fallax	VPRI 40606	Victoria, Australia		Rain gauge in *Eucalyptus regnans* forest	HQ013222	n.a.
P. fallax	SLPA61	Toolangi North State Forest, Victoria, Australia	10/2008	*Xanthorrhoea minor*	HQ013223	n.a.
P. megasperma	**VHS 17183**[b]	Esperance, WA	04/2007	*Xanthorrhoea platyphylla*	EU301166	n.a.
P. taxon arenaria-like	**VHS 16282**[bc]	Ravensthorpe, WA	08/2006	*Banksia media*	EU301117	HQ013198

n.a.: not assessed.

[a]Abbreviations of isolates and culture collections: CBS: Centraalbureau voor Schimmelcultures Utrecht, the Netherlands; IMI: CABI Bioscience (Imperial Mycological Institute), UK; VHS: Vegetation Health Service Collection, Department of Environment and Conservation, Perth, Australia; DDS: earlier prefix of VHS Collection; TCH: TC Hill, in VHS Collection; MJS: M.J.C. Stukely, in VHS Collection; DCE: E.M. Davison, in VHS Collection; VPRI: Primary Industries, Knoxfield, Australia; MU: Murdoch University Culture Collection; CLJO, ENA and SLPA: isolates from Cooljarloo, Eneabba and Sugarloaf Reservoir Reserve, respectively, in MU Collection. Numbers in bold are isolates used in the morphological studies.

[b]Isolates used in the growth rate studies.

[c]Isolates used in the pathogenicity trial.

determined at ×400 magnification (BH-Olympus) for each isolate. Dimensions and characteristic features of >50 mature oogonia, oospores and antheridia chosen at random were measured for each isolate at ×400 magnification at the surface of *c.* 15-mm squares cut from the centre of 14- to 22-day-old V8A cultures grown in the dark at 20°C. For each isolate the oospore wall index was calculated as the ratio between the volume of the oospore wall and the volume of the entire oospore (Dick, 1990).

Pathogenicity trials

The pathogenicity of four isolates of *P. constricta*, three isolates of *P. arenaria* and one isolate of *P.* taxon arenaria-like (Table 1) to *Banksia attenuata* was tested in a soil-infestation pot trial. *Banksia attenuata* occurs throughout the Kwongan from which both *P. constricta* and *P. arenaria* have been isolated in the field. *Phytophthora cinnamomi* isolate MU 9448 was included for comparison as it is known to kill this host in the field.

Pine plugs were used as the source of inoculum (Butcher *et al.*, 1984). The preparation of pine plugs, soil and pots, and the inoculation of the pots with pine plugs were carried out as described previously (Rea *et al.*, 2010). Colonization of the pine plugs by each isolate was confirmed prior to pot inoculation. Four 6-month-old *B. attenuata* seedlings (purchased from Oakford Tree Farms, WA) were planted in each pot in June 2008. Plants were watered daily to container capacity by hand and maintained in an evaporatively cooled glasshouse in which the temperature did not exceed 30°C.

In early October 2008, 40 *B. attentuata* plants (10 pots per isolate) were inoculated with colonized pine plugs. Noncolonized pine plugs were used as a control. Pots were organized in completely randomized blocks. Pots were first individually flooded in buckets for 16–20 h in mid-December 2008 (start of summer), and then in mid-January, mid-February and mid-March 2009 to stimulate the production of sporangia and pathogen spread and infection via zoospores. Between flooding events plants were watered daily to container capacity by hand. For the duration of the trial (233 days) plants were assessed daily. Mortality of seedlings was recorded, and immediately after death reisolations were made from necrotic roots and the root collar using NARPH.

The ability of *P. constricta* and *P. arenaria* to persist in the pine plug inoculum used in the pathogenicity trial was assessed at the end of the trial by removing all plugs from one randomly chosen pot per isolate and plating them, after surface sterilization, onto NARPH agar plates.

Results

Distribution of species

Phytophthora constricta has predominantly been isolated from the southern sandplains of WA. Of the 34 isolates from the VHS collection, 29 were from the southern sandplains and 22 (84·6%) of these originated from within the Fitzgerald River National Park. Other southern isolates came from Nannup and from Cape Arid. Of the isolates from the northern sandplains two originated from Badgingarra and Eneabba, and one from Jurien Bay. The origins of the other two isolates are uncertain. Isolations date back to 1980. Most isolates were associated with dead or dying proteaceous plant species from the genera *Banksia*, *Hakea*, *Adenanthos* and *Isopogon*. Two isolates were associated with the exotic *Pinus radiata* (one healthy and one dead plant) in separate plantations near Nannup.

Conversely, with the exception of one isolate from Bunbury (south-west coast) *P. arenaria* has been isolated exclusively from the northern sandplains. The geographic distribution of the 23 sequenced isolates from the northern sandplains ranges from Kalbarri to Gingin, ~580 and 84 km north of Perth, respectively, with most isolations occurring around Eneabba and Badgingarra. Most isolates were associated with dead or dying *Banksia* or *Eucalyptus* species (Fig. 1); however, isolates were also recovered in association with symptomless *Banksia* and *Eucalyptus* species. The first isolation of this taxon was from soil in native Kwongan vegetation near Kalbarri in 1986.

Phylogenetic analysis

The aligned ITS dataset consisted of 953 characters, of which 505 were parsimony informative. The dataset contained significant ($g1 = -0{\cdot}46$, $P < 0{\cdot}001$) phylogenetic signal. Heuristic searches resulted in six most parsimonious trees of 2096 steps (CI = 0·50, RI = 0·88). The topology of the Bayesian tree was very similar and is presented here (Fig. 2, TREEBASE 10794). With the exception of the isolate DDS 3543, which differed by 1 bp, ITS sequences of all isolates of *P. constricta* were identical and resided in a strongly supported terminal clade, separate from the closest species *P. captiosa* and *P. fallax* in clade 9 by at least 60 bp.

Figure 1 Wilted *Banksia hookeriana* in a Kwongan heathland on mineral sand near Eneabba, WA recently killed by root and collar rot caused by *Phytophthora arenaria*.

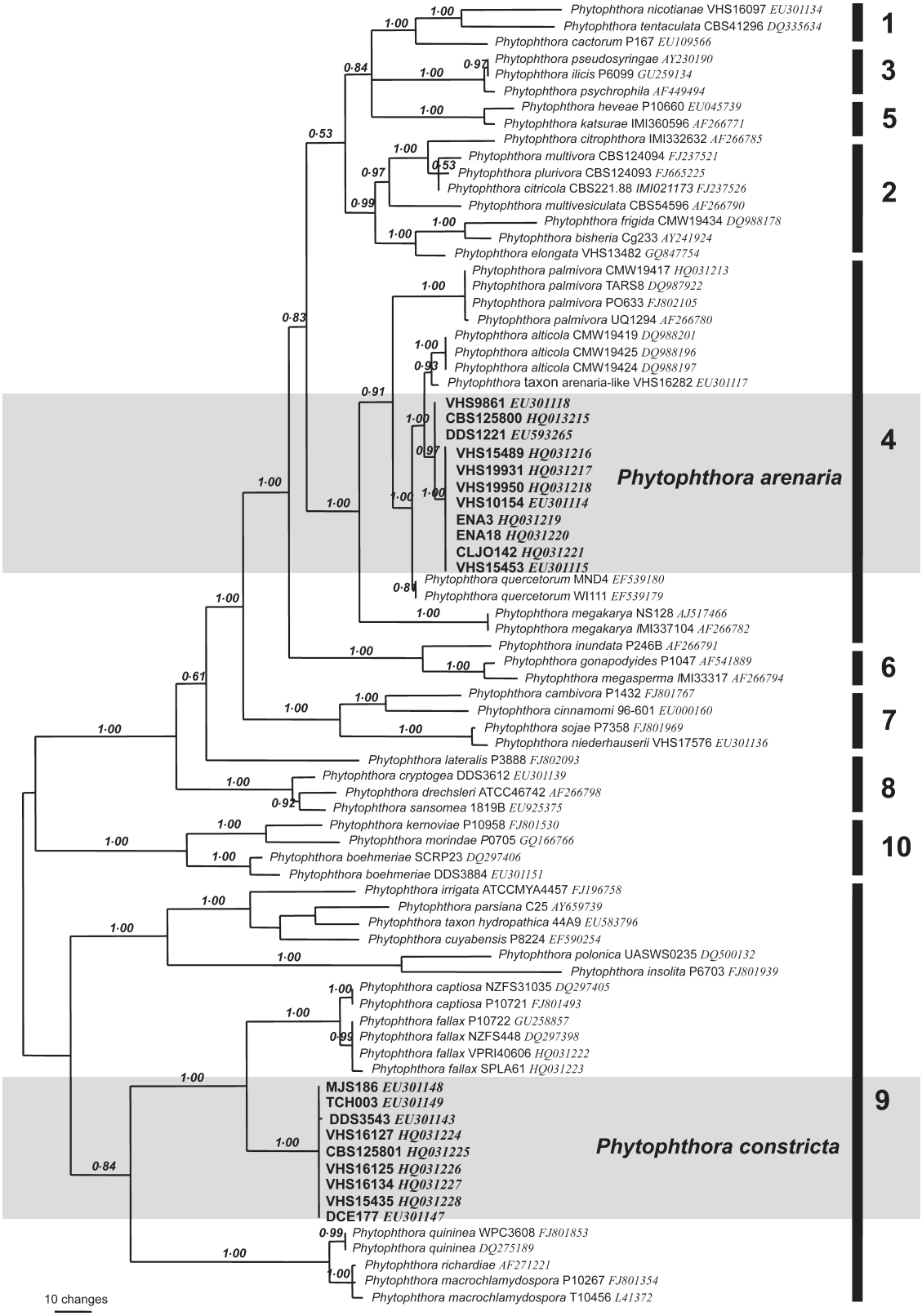


Figure 2 One of 12 most parsimonious trees of 703 steps based on analysis of rDNA ITS sequence data showing phylogenetic relationships between *Phytophthora arenaria* and related species from ITS clade 4, and *P. constricta* and related species from ITS clades 9 and 10. Numbers in italics represent posterior probability based on Bayesian analysis.

Phytophthora arenaria resided in clade 4 and was most closely related to *P. alticola* and *P. quercetorum*. Isolates designated as *P. arenaria* formed two clusters separated by 2 bp; one cluster was composed of isolates CBS 125800, VHS 9861, DDS 1221 and VHS 15489; the other of isolates CLJO142, CBS 127950, ENA 18, VHS 10154, VHS 15453, VHS 19931, and VHS 19950. Based on ITS sequence data, *P. arenaria* differed from *P. alticola* by 10–12 bp and from *P. quercetorum* by 5–7 bp.

The *cox*1 dataset consisted of 742 characters, of which 157 were parsimony informative. The dataset contained significant (g1 = −0·56, $P < 0·001$) phylogenetic signal. Heuristic searches resulted in 171 most parsimonious trees of 505 steps (CI = 0·40, RI = 0·75) (TREEBASE 10794). Because of the high level of homoplasy in the dataset the Bayesian analysis was more conservative and is presented here (Fig. 3, TREEBASE 10794). As in the ITS analysis, *P. constricta* allied with other clade 9 species. *Phytophthora arenaria* formed a loose attachment with clade 2 species and *P. palmivora*, the only clade 4 species for which *cox*1 sequence data were available. Both *P. constricta* and *P. arenaria* showed considerable variability in the *cox*1 region, with differences of up to 10 bp between isolates.

One isolate (VHS 16282), originally thought to be *P. arenaria*, was excluded because of differences in sequence data in both the ITS (4 bp) and *cox*1 (8 bp) gene regions and morphology; this taxon is referred to as '*P.* taxon arenaria-like'.

Pathogenicity

The first plant deaths in pots infested by *P. constricta* occurred on day 84, 24 days after the initial flooding.

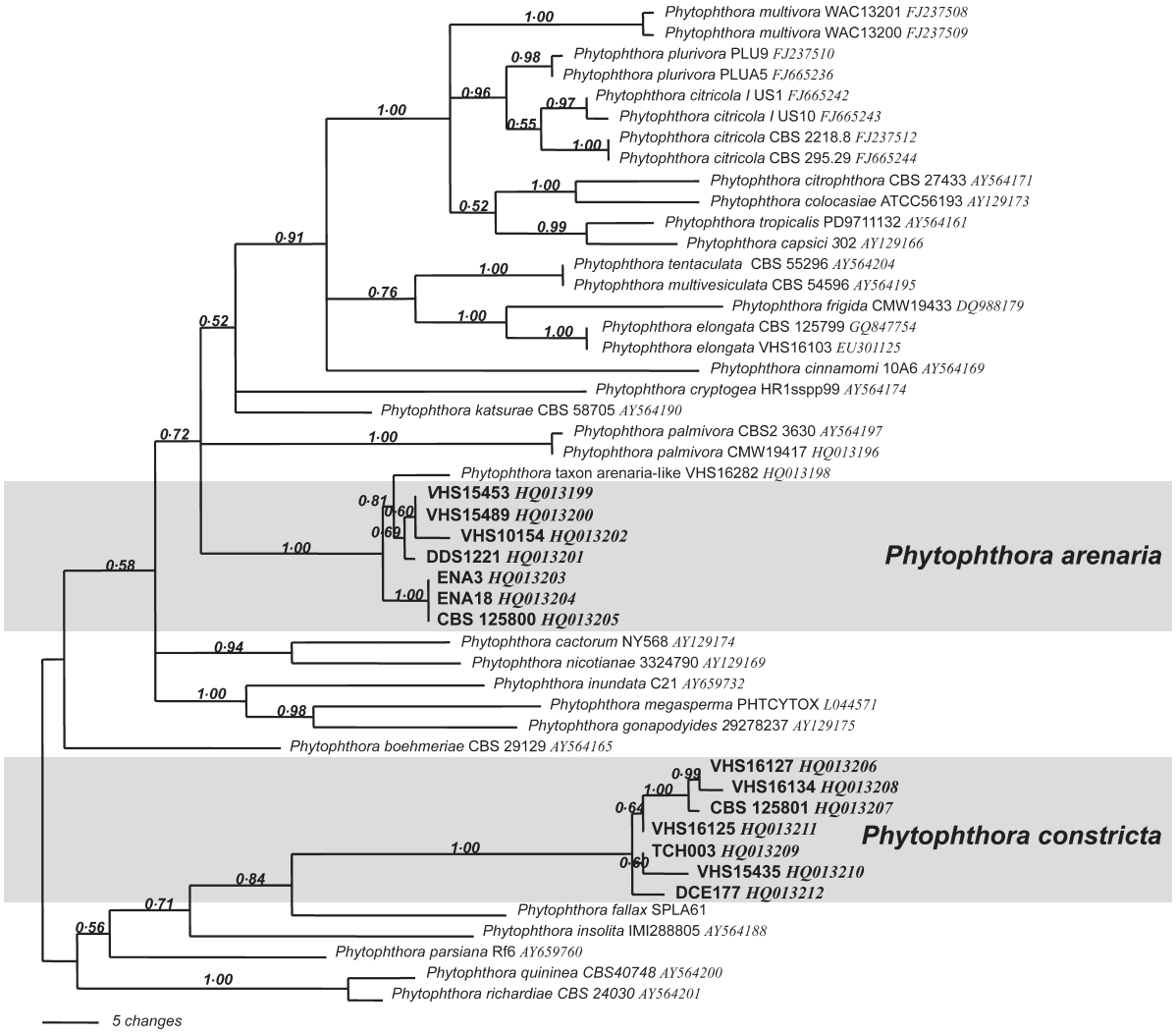


Figure 3 One of two most parsimonious trees of 242 steps based on analysis of mitochondrial gene *cox*I sequence, showing phylogenetic relationships between *Phytophthora arenaria* and related species from ITS clade 4, and *P. constricta* and related species from ITS clades 9 and 10. Numbers in italics represent posterior probability based on Bayesian analysis.

The isolates of *P. constricta* varied in their aggressiveness, killing 10% (DCE 177), 17·5% (VHS 15435), 12·5% (VHS 16125) and 25% (CBS 125801) of the seedlings, respectively, in three to six of 10 pots (average mortality 16·3%). The first plant deaths in pots infested by *P. arenaria* occurred on day 104, 40 days after the initial flooding and 12 days after the second flooding. The three isolates of *P. arenaria* also varied in their aggressiveness to *B. attenuata*, killing 10% (VHS 10154), 15% (DDS 1221) and 32·5% (VHS 15453) of the seedlings, respectively, in two to eight of 10 pots (average mortality 19·2%). The isolate of *P.* taxon arenaria-like killed 15% of seedlings in five of 10 pots. As expected, *P. cinnamomi* killed all *B. attenuata* seedlings, with the first and the last plants dying after 20 and 110 days, respectively. No noninoculated control plants died. *Phytophthora cinnamomi*, *P. constricta, P. arenaria* and *P.* taxon arenaria-like were reisolated from the roots and collars of all dead plants from pots infested by the respective isolates. In addition, all isolates could be reisolated at the end of the trial from the pine plug inoculum of randomly chosen pots. The glasshouse temperature recorded during the trial ranged from an overnight minimum of 9°C to a maximum of 30°C.

Taxonomy

Phytophthora arenaria A. Rea, M. Stukely & T. Jung, sp. nov.
MycoBank no.: MB 518792.

Etymology: Name refers to the association of this species with sandy soils.

Systema sexus homothallica; oogonia globosa ad subglobosa vel rare excentrica, saepe cum margine unduloso, maturitate frequenter pigmentatus aureo-fuscus ad aereus, in medio 24 μm (21–27 μm). Oosporae apleroticae, in medio 21 μm (19–24 μm), paries in medio 2·3 μm (1·6–3·0 μm). Antheridia semper paragynosa, in medio 11 × 8 μm (9–14 × 6–9 μm), frequenter cum appendicibus digitaliformibus. Sporangia abundantia in cultura liquida, persistentia, terminalia, interdum lateralia aut intercalaria, papillata, ovoidea vel obpyriformia, interdum distorta, bipapillata, tripapillata vel bilobata, saepe cum obturamento conspicuo basale et vacuola conspicua, frequenter cum apice arcuato vel acuto et appendicibus digitaliformis, in medio 32 × 24 μm (24–46 × 13–32 μm), ratio longitudo ad altitudinem in medio 1·4 (1·2–2·5). Sporangiophora simplicia; interdum inserta lateraliter ad sporangia, interdum cum inflationes ad basim sporangii. Chlamydosporae non observatae. Hyphae in cultura liquida saepe cum inflationibus catenulatis, globosis aut subglobosis. Temperaturae crescentiae in agaro 'CA', optima c. 30°C et maxima c. 32·5°C. Coloniae in agaro 'CA' radiatae cum mycelio aerio restricto. Regiones 'rDNA ITS' et '*cox*I' cum unica sequentia (GenBank HQ013219, HQ013203) (Fig. 4).

Typus: Western Australia, Eneabba, isolated from soil sample collected beneath symptomless *Eucalyptus drummondii* growing in native Kwongan vegetation, February 2009, T. Jung. Holotype: MURU 455 (dried culture on V8A in the herbarium of Murdoch University, Western Australia). Ex-type isolate: CBS 127950. GenBank accession numbers HQ013219, HQ013203.

Papillate persistent sporangia were abundantly produced in soil extract water on simple sporangiophores frequently with globose swellings close to the sporangial base (Fig. 4e). Sporangial apices were often pointed (Fig. 4b–d, f–h). Although predominantly ovoid (Fig. 4a,c), a range of sporangial shapes was observed including broadly ovoid (Fig. 4b,e,i), elongated ovoid (Fig. 4d), obpyriform (Fig. 4g,h), and asymmetrically mouse-shaped (Fig. 4f). Bipapillate (Fig. 4k), tripapillate and bilobed (Fig. 4l) sporangia were also observed. Sporangia often had special features including laterally displaced apices (Fig. 4d,f), a large vacuole (Fig. 4d,h,k–l), lateral attachment of the sporangium (Fig. 4b,c,i), a conspicuous basal plug (Fig. 4j), hyphal extensions (Fig. 4i) and intercalary formation of the sporangium (Fig. 4h). Sporangia from 10 isolates averaged 31·8 ± 4·6 μm in length and 23·7 ± 3·5 μm in width, with narrow exit pores (Fig. 4j) of 6·0 ± 1·0 μm and a length:breadth (l:b) ratio of 1·4 ± 0·17 (Table S1).

Phytophthora arenaria is homothallic and, with the exception of isolate VHS 10154, readily produced oogonia in single culture on CA and V8A, containing oospores which matured within 14–21 days. Isolate VHS 10154 produced gametangia, but only when agar squares were incubated in nonsterile soil extract water. Oogonia from nine isolates averaged 25·3 ± 2·2 μm, with isolate means ranging from 24·3 to 28·1 μm (Table S1). Oogonia often had a slightly wavy outer wall (Fig. 4o,p,r) and turned dark-brown to bronze-brown with maturity (Fig. 4o–r). Oogonia sometimes had a tapering base or were excentric. Oospores were aplerotic in all isolates and contained ooplasts when semi-mature to mature (Fig. 4n–r). Some oospores were excentric. Oospores averaged 22·3 ± 1·8 μm in diameter, with isolate means ranging from 21·4 to 23·9 μm (Table S1). Oospore walls were thick (2·3 ± 0·34 μm) (Fig. 4n–r). The oospore wall index was 0·50 ± 0·05 μm (Table S1), with isolate means ranging from 0·49 to 0·52 μm. Antheridia were exclusively paragynous and usually club-shaped, with some having finger-like projections (Fig. 4n,r), and were attached to the oogonium close to the oogonial stalk (Fig. 4n,p,r) or at an angle of up to 90° from the oogonial stalk (Fig. 4m). Antheridia averaged *c.* 11·2 ± 1·7 × 8·4 ± 1·3 μm. Catenulate, globose to subglobose hyphal swellings, some with radiating hyphae, were formed in nonsterile soil extract water (Fig. 4t).

Colony morphology, growth rates and cardinal temperatures. All *P. arenaria* isolates produced colonies with a radiate morphology on V8A and CA (Fig. 5). On MEA and PDA some isolates also produced radiate patterns (Fig. 5), while others showed irregular colony morphologies (DDS 1221, VHS 15453 and VHS 15489; not shown). Aerial mycelium was formed on CA, PDA and V8A (Fig. 5). *Phytophthora cactorum* produced radiate

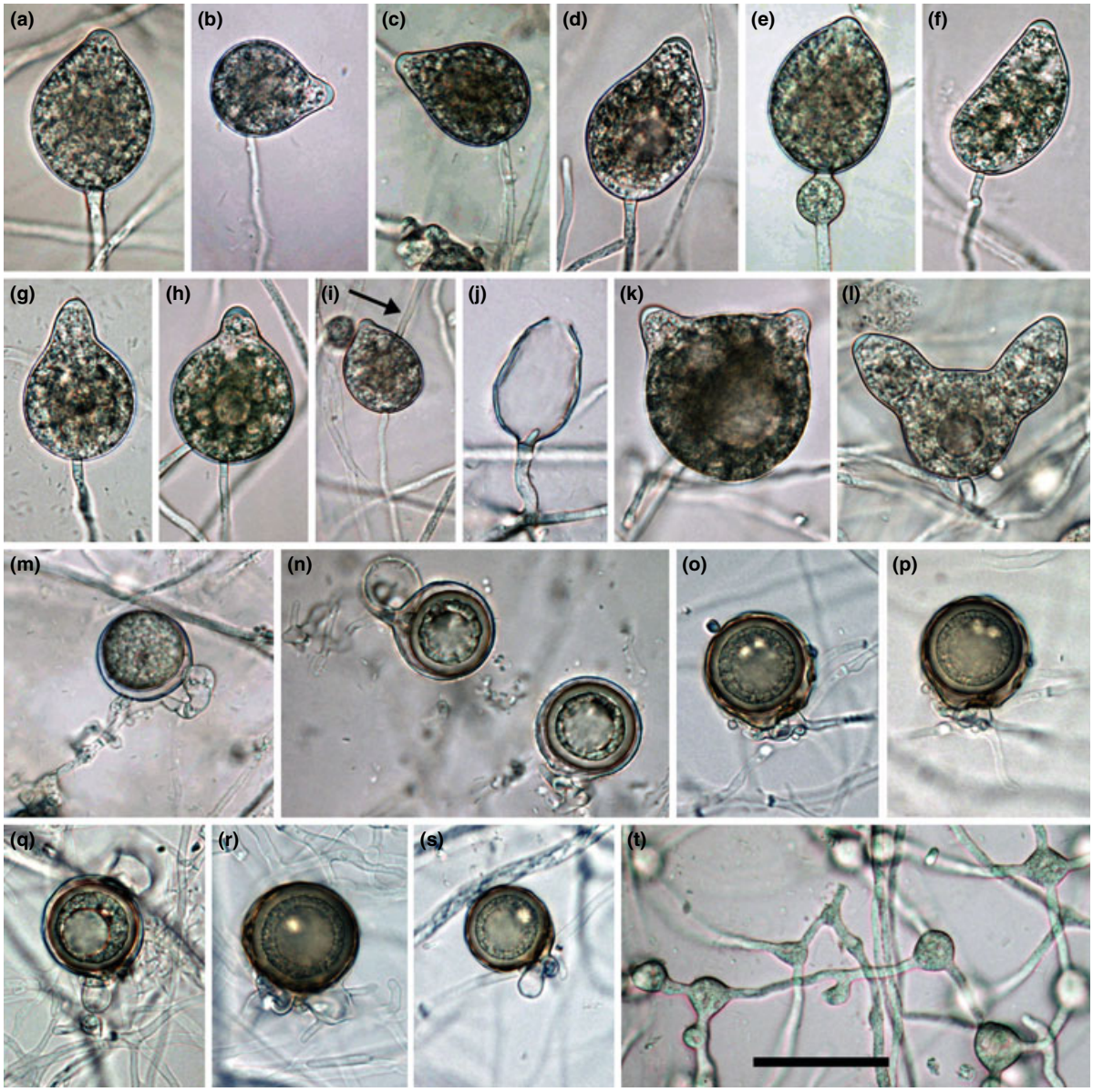


Figure 4 Morphological structures of *Phytophthora arenaria*. (a–l) Papillate sporangia formed on V8 agar flooded with soil extract; (a) ovoid; (b,c) ovoid, sporangiophore laterally attached; (d) elongated ovoid, with laterally displaced apex and large vacuole; (e) broadly ovoid, with globose swelling close to sporangial base; (f) mouse-shaped; (g,h) obpyriform, with pointed apex; (h) intercalary inserted; (i) ovoid, with hyphal extension (arrow), sporangiophore laterally attached; (j) empty ovoid sporangium after release of zoospores with conspicuous basal plug; (k) bipapillate, with large vacuole; (l) bilobed, with vacuole. (m–s) Oogonia with aplerotic oospores and paragynous antheridia in solid V8 agar; (m) juvenile oogonium with thin-walled oospore without ooplast; (n) semi-mature oogonia with aplerotic thick-walled oospores containing ooplasts and coarsely granulated cytoplasm; (o–s) mature oogonia with slightly wavy-edged, bronze-brown walls and thick-walled oospores containing ooplasts; (p,r) antheridia with finger-like projections. (t) Catenulate, globose to subglobose hyphal swellings, some with radiating hyphae, formed in soil extract. Scale bar = 50 μm.

to stellate colonies on all four media, growing slower than *P. arenaria* on V8A and PDA and faster on CA and MEA (Fig. 5).

Phytophthora arenaria isolates had varying mean radial growth rates on CA. At the optimum temperature of 30°C the average growth rate was 6·5 ± 0·49 mm per day. The maximum temperature for growth was 32·5°C (Table S1; Fig. 6). Isolates did not grow at 35°C. While this temperature was lethal for isolates VHS 10154, VHS 15453 and VHS 15489, all cultures of the isolates CBS 125800 and CBS 127950 incubated at 35°C for 4 days resumed growth when subsequently incubated at 20°C. *Phytophthora cactorum* grew at 10°C, whilst *P. arenaria* did not (Fig. 6).

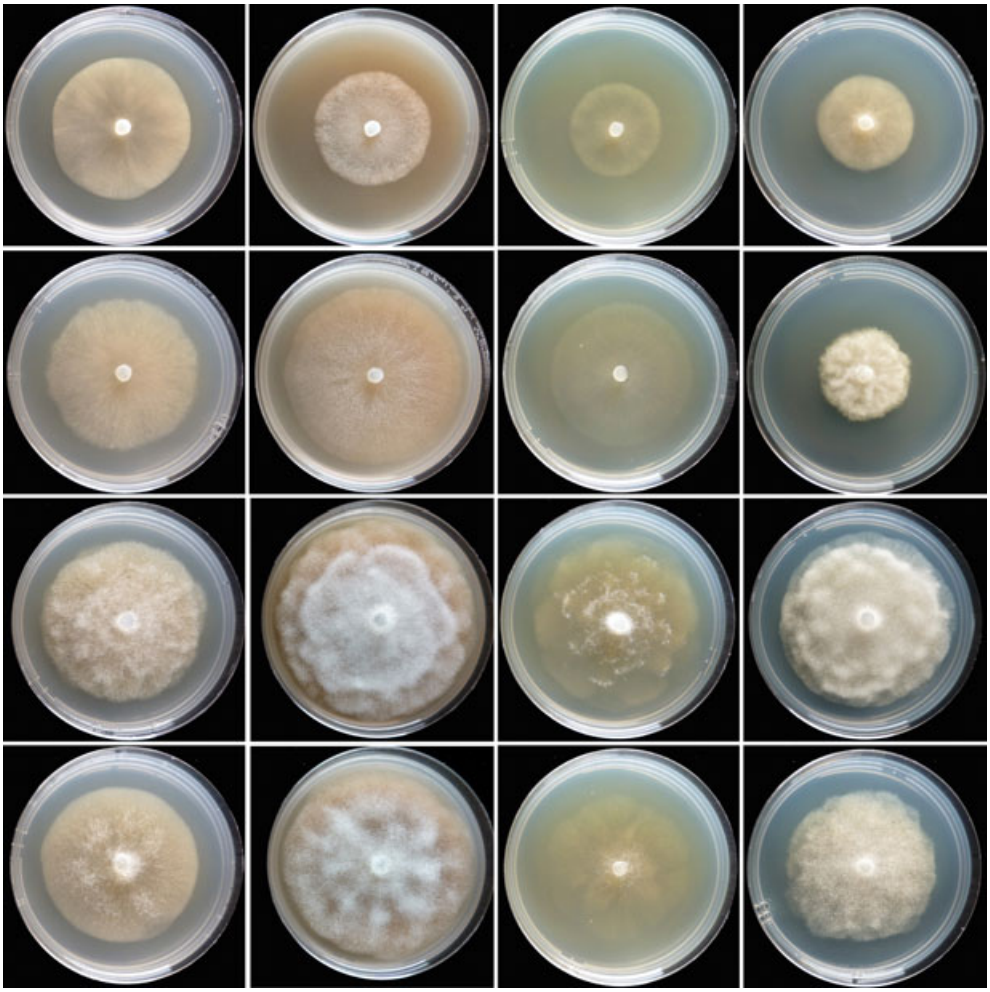


Figure 5 Colony morphology of isolate CBS 127950 (ex-type) of *Phytophthora arenaria* and DCE 505 of *P. cactorum*, isolate CBS 125801 (ex-type) of *P. constricta* and VHS 17183 of *P. megasperma* (from top to bottom) after 7 days' growth at 20°C on V8 agar, carrot agar, malt extract agar and potato dextrose agar (from left to right).

Notes. In a previous study, *P. arenaria* was referred to as *Phytophthora* sp. 1 (Burgess *et al.*, 2009). Morphologically, the closest species to *P. arenaria* are *P. cactorum* and *P. quercetorum*, all belonging to Waterhouse group I (Table S1). Phylogenetically, the closest species to *P. arenaria* are *P. quercetorum* and *P. alticola*. Major differences between *P. arenaria* and *P. cactorum* are the wavy outer oogonial wall in *P. arenaria* and the production of caducous sporangia in sympodia by *P. cactorum*. *Phytophthora arenaria* can easily be distinguished from *P. alticola* by the lack of amphigynous antheridia and caducous sporangia, and by its lower maximum temperature for growth (Maseko *et al.*, 2007; Table S1). *Phytophthora quercetorum* is separated from *P. arenaria* by having a lower optimum temperature for growth, thinner oospore walls and on average larger sporangia formed in sympodia (Balci *et al.*, 2008; Table S1).

The most closely related taxon to *P. arenaria*, represented by a single isolate (VHS 16282), was also associated with Kwongan vegetation in south-west WA. However,

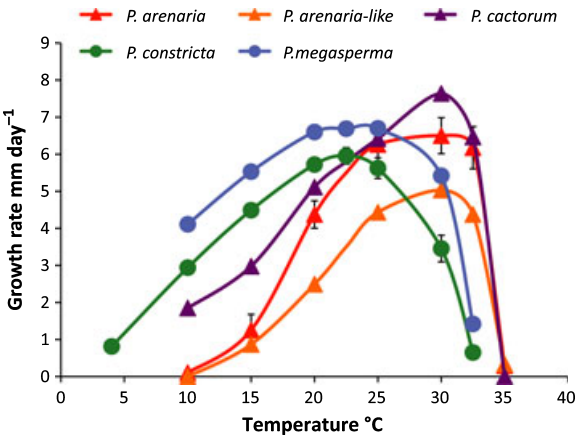


Figure 6 Radial growth rates of isolates of *Phytophthora arenaria*, *P.* taxon arenaria-like, *P. cactorum*, *P. constricta* and *P. megasperma* on carrot agar at different temperatures. For *P. arenaria* and *P. constricta* data presented is an average of several isolates and error bars are given.

this taxon was isolated at Ravensthorpe on the south coast of WA, which is geographically more than 600 km distant from the northern sandplains, where all but one of the isolates of *P. arenaria* were recovered. This taxon shares with *P. arenaria* the same cardinal temperatures for growth, showed similar aggressiveness towards *B. attenuata* and has overlapping ranges in almost all morphological characters (Table S1). However, on average it has markedly larger sporangia, a higher sporangial l:b ratio and a lower oospore wall index. In addition, this taxon differs from *P. arenaria* by a minimum of 4 and 8 bp in ITS and *cox*1 sequence, respectively. This taxon is sufficiently distinct that a separate species description may be warranted if more isolates were available. Meanwhile, this taxon is informally designated *Phytophthora* taxon arenaria-like.

Phytophthora constricta A. Rea, M. Stukely & T. Jung, sp. nov.
MycoBank no.: MB 518793.

Etymology: Name refers to the fragile constriction of most sporangiophores towards the sporangial base.

Systema sexus homothallica; oogonia terminales, in medio 49 μm (37–56 μm), globosa vel rare subglobosa ad elongata, interdum cum basim attenuata. Oosporae fere apleroticae aut interdum pleroticae, in medio 42 μm (32–49 μm), paries in medio 2·8 μm (1·3–3·5 μm). Antheridia singulares, terminalia, unicellularia, hyalina, globosa ad claviformes, in medio 16 × 13 μm (11–23 × 10–17 μm), semper paragynosa, interdum cum appendicibus digitaliformibus. Sporangiophora simplicia, fere cum constrictione extrema ad basim sporangii versus, interdum cum inflationibus ellipsoideis. Sporangia abundantia in cultura liquida, terminalia, nonpapillata, ovoidea ad obturbinata, in medio 66 × 54 μm (52–79 × 42–69 μm), ratio longitudo ad altitudinem in medio 1·23 (1·1–1·54). Proliferationes sporangiorum semper internae et niduformes. Chlamydosporae et inflationes hypharum non observatae. Temperaturae crescentiae in agaro 'CA', optima c. 22·5–25°C et maxima c. 32°C. Coloniae in agaro 'CA' petaloideae et cottoneae. Regiones 'rDNA ITS' et '*cox*I' cum unica sequentia (GenBank HQ013225, HQ013207).

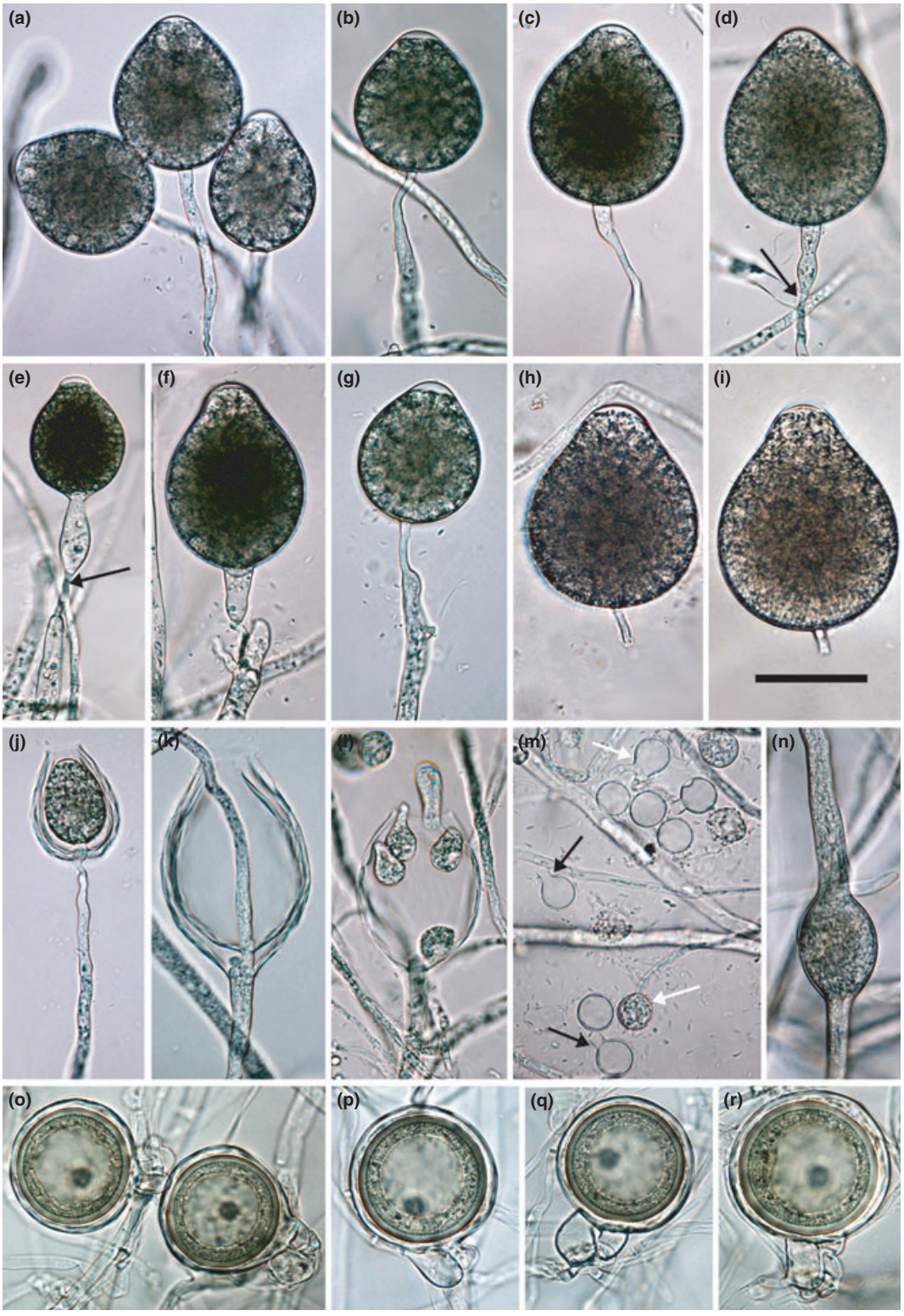
Typus: Western Australia, Fitzgerald River National Park, isolated from soil sample collected in dying native Kwongan vegetation, August 2006, M. Stukely. Holotype: MURU 454 (dried culture on V8A in the herbarium of Murdoch University, Western Australia). Ex-type isolates: CBS 125801 and VHS 16130. GenBank accession numbers HQ013225, HQ013207 (Fig. 7).

Nonpapillate to semipapillate persistent sporangia were abundantly produced in nonsterile soil extract water. Sporangial proliferation was internal, usually nested (Fig. 7j) or much less frequently extended (Fig. 7k). In all isolates most sporangiophores became constricted towards the base of the sporangium (Fig. 7b–g) and in some cases widening again (Fig. 7c–f). Sporangia easily broke from the sporangiophore at the site of the constriction under slight pressure (Fig. 7h,i). Sporangiophores of even thickness also occured (Fig. 7a). Sporangial apices were flat and broad (Fig. 7b–i). Sporangial shapes ranged from ovoid (Fig. 7a, d–g) or broadly ovoid to turbinate (Fig. 7b–c, h–i). Sporangia averaged 59·8 ± 8·7 μm in length and 48·8 ± 7·4 μm in width, with a l:b ratio of 1·2 ± 0·09 (Table S2). Zoospores were released through wide exit pores (Fig. 7l; 13·4 ± 2·7 μm). Release of a secondary zoospore from encysted zoopores (diplanetism) was regularly observed (Fig. 7m). Ellipsoid hyphal swellings were occasionally formed on sporangiophores (Fig. 7n).

Phytophthora constricta is homothallic and readily produced oogonia in single culture on CA and V8A, containing oospores which matured within 14–21 days. Oogonia from four isolates averaged 48·0 ± 4·8 μm (type isolate CBS 125801 49·0 ± 4·4 μm) with isolate means ranging from 46·0 to 50·4 μm (Table S2). Some oogonia had a tapering base (Fig. 7o). Elongated and slightly excentric oogonia were observed. Oospores were usually slightly aplerotic to nearly plerotic, and averaged 40·4 ± 4·3 μm in diameter, with isolate means ranging from 38·9 to 41·8 μm. Walls of 21-day-old oospores were moderately thick (2·9 ± 0·87 μm), often turning bronze-brown or golden-brown with maturity, and contained a clearly visible nucleus and a large ooplast at maturity (Fig. 7o–r). Oospore walls continued to thicken over the next 4–10 months. The oospore wall index for 14- to 21-day-old oospores was 0·36 ± 0·09 (isolate means ranging from 0·31 to 0·39; Table S2), whilst for 4- to 10-month-old oospores it was 0·52 ± 0·09 (isolate means ranging from 0·49 ± 0·08 to 0·58 ± 0·04; Table S2). Antheridia were exclusively paragynous, with some having a finger-like extension, and were attached to the oogonium either close to the oogonial stalk (Fig. 7o–r) or at an angle of up to 90°. Antheridia averaged 16·9 ± 2·4 × 13·3 ± 2·1 μm. No gametangia were observed for isolate DCE 177.

Colony morphology, growth rates and cardinal temperatures. All *P. constricta* isolates produced colonies with petaloid morphology on V8A, CA and PDA,

Figure 7 Morphological structures of *Phytophthora constricta*. (a–l) Ovoid to broadly ovoid sporangia formed on V8 agar flooded with soil extract; (a) nonpapillate, evenly wide sporangiophore; (b,c) nonpapillate to slighly semipapillate, with broad flat apex and conspicuous narrowing of the sporangiophore towards the sporangial base; (d,e) nonpapillate to semipapillate, with extreme constriction (arrows) and rewidening of the sporangiophore towards the sporangial base; (g) semipapillate, with conspicuous narrowing of the sporangiophore towards the sporangial base; (h,i) nonpapillate sporangia, which broke off under slight pressure at the conspicuous constriction of the sporangiophore; (j) nested proliferation; (k) nested and extended proliferation; (l) release of zoospores; (m) encysted zoospores germinating directly (white arrow) or empty after release of a secondary zoospore (=diplanetism; black arrows); (n) swelling on sporangiophore. (o–r) Oogonia with mature, slightly aplerotic to nearly plerotic bronze-brown oospores each containing a large ooplast and a dark nucleus, with paragynous antheridia, in solid V8 agar; (r) antheridia with finger-like projections. Scale bar = 50 μm.



and a faintly petaloid pattern on MEA. All isolates formed aerial mycelium on all four agar media and had cottony growth on CA and PDA (Fig. 5). Colonies of *P. megasperma* were radiate on CA, V8A, MEA and PDA, and less defined than those of *P. constricta*. Aerial mycelium was formed on CA, V8A and PDA (Fig. 5).

Phytophthora constricta had a mean radial growth rate on CA of 6·0 ± 0·22 mm per day at its optimum

temperature of 22·5°C. The maximum temperature for growth was 32·5°C (Fig. 6). Although growth at this temperature ceased after 2 days, 32·5°C was not lethal as isolates resumed growth when subsequently incubated at 20°C. *Phytophthora megasperma* had higher growth rates than *P. constricta* at all temperatures on CA, but at 20°C on V8A, MEA and PDA *P. constricta* was faster growing than *P. megasperma* (Fig. 6, Table S2).

Notes. In a previous study *P. constricta* was referred to as *Phytophthora* sp. 9 (Burgess *et al.*, 2009). Whilst phylogenetically distant, both *P. constricta* and *P. megasperma* belong to Waterhouse group V and share a number of morphological similarities (Table S2). The major differences between *P. constricta* and *P. megasperma* are the absence of both amphigynous antheridia and external sporangial proliferation, the characteristic constriction of the sporangiophore beneath the sporangium in *P. constricta* and different colony morphology on CA and MEA. *Phytophthora constricta* is phylogenetically closest to *P. fallax* and *P. captiosa*. However, there are many morphological differences and differences in colony morphology on CA (Table S2).

Discussion

Phytophthora constricta and *P. arenaria* are homothallic species associated with the Kwongan vegetation on the sandplains of south-west WA, predominantly isolated from dead and dying *Banksia* species and from the rhizosphere soil associated with such plants. Phylogenetic analysis of both the nuclear ITS rDNA and mitochondrial *cox*1 gene in this study show *P. constricta* and *P. arenaria* to be unique species residing in ITS clades 9 and 4, respectively, of Cooke *et al.* (2000). Both *P. constricta* and *P. arenaria* are easily distinguished from morphologically similar species on the basis of both ITS and *cox*1 sequence data, and by a range of morphological criteria (see Notes and Tables S1 and S2). The variability in the *cox*1 data for *P. constricta* and *P. arenaria* is comparable to that observed for another species recently described from south-west WA, *P. multivora* (Scott *et al.*, 2009). Such variability implies these species are either (i) endemic, or (ii) have been introduced on multiple occasions from a comparable ecosystem imposing selective pressures favouring adaptations suitable for survival in their current ecosystem, or (iii) were introduced long ago and have evolved *in situ*. In contrast, another recently described species from WA, *P. elongata,* is monomorphic in the *cox*1 region and is thought to be a recent introduction to the region (Rea *et al.*, 2010).

Over the last 5–6 million years, the biota of the Kwongan, and indeed of the entire south-west of WA, have coevolved in an increasingly arid climate geographically isolated from eastern Australia and unaffected by glaciation, significant geological disturbance or dramatic extinction events (Coates & Atkins, 2001). The high variation in the *cox*1 sequence data of *P. constricta* and *P. arenaria*, as well as the absence of any similar sequences at GenBank from other parts of the world, suggests endemism and coevolution with the flora. This scenario is in agreement with the results from the pathogenicity trials in which mortality of *B. attenuata* induced by *P. constricta* and *P. arenaria* was dependent on a flooding stimulus and showed a variation between isolates of 10–25% and 10–35%, respectively. In contrast, the introduced pathogen *P. cinnamomi* caused 100% mortality of seedlings and did not require a flooding stimulus to initiate death. This supports the hypothesis of host–pathogen coevolution. Furthermore, the impact on Kwongan vegetation of *P. constricta* or *P. arenaria* is distinct from that caused by the introduction of *P. cinnamomi*. Whereas *P. cinnamomi* forms a visible and indiscriminate path of destruction through entire plant communities, *P. constricta* and *P. arenaria* have a more limited impact, selectively killing species belonging predominantly to the family Proteaceae. Furthermore, the incidence of *P. constricta* or *P. arenaria* is usually episodic following extreme rainfall events, which is congruent with the results of the soil infestation trial, where flooding was required to cause disease. Although an ability to cause disease without flooding would surely be an advantage to an endemic pathogen in a dry region such as the sandplains of WA, it can be expected that host–pathogen coevolution has prevented this. However, it is also likely that host plants have not evolved thresholds of resistance against the high zoospore inoculum produced during episodic flooding events since these occur too rarely to become a major selective factor. The ecological role of endemic *Phytophthora* species in their native habitats is largely unknown (Jung *et al.*, 2003; Reeser *et al.*, 2007). For *P. arenaria* and *P. constricta* it may be speculated that the episodic mortality of susceptible Proteaceae species helps to maintain high spatial plant diversity in the Kwongan vegetation, which would otherwise be even more dominated by well-adapted species from this plant family.

Phytophthora arenaria and *P. constricta* appear well adapted to the ecosystems from which they have been isolated. *Phytophthora arenaria* has a high optimum temperature for growth of 30°C and a relatively high minimum of 10°C. This, coupled with a thick oospore wall and high oospore wall index, are life strategies favourable for growth and survival in the Kwongan on the warmer and drier northern sandplains, from which *P. arenaria* has predominantly been isolated. Most isolations of *P. arenaria* are from more than 200 km north of Perth in a region with a long-term mean summer rainfall of 9·8 mm and a mean daily maximum temperature in summer of 35·2°C (Australian Bureau of Meteorology, http://www.bom.gov.au/).

In contrast, *P. constricta* has markedly lower optimum and minimum temperatures for growth than *P. arenaria*, i.e. 22·5°C and 4°C, respectively, favourable for survival in the Kwongan of the southern sandplains along the cooler south coast of WA, from where it has predominantly been isolated. The long-term mean daily

maximum temperature in this region is 25–27°C in summer and 13–19°C in winter. These temperatures are within the range favourable for growth of *P. constricta*.

The characteristic constriction of the sporangiophore evident in all isolates of *P. constricta* indicates this species may be evolving to become partly caducous as has been observed in the recently described *P. pinifolia*, an ITS clade 6 species causing an epidemic of needle blight in *P. radiata* plantations in Chile (Durán *et al.*, 2008).

Cooke *et al.* (2000) proposed that species in ITS clades 1–5 have evolved towards an aerial habit and produce papillate, caducous sporangia, whilst species in ITS clades 6–8 have evolved towards a tendency to be soilborne with nonpapillate sporangia. No mention is made of the tendencies of species in ITS clades 9 and 10 with respect to caducity, sporangial apex or lifestyle, though a possible reassignment of species in these clades and removal from the genus *Phytophthora* was suggested. However, since the study of Cooke *et al.* (2000) a number of species belonging to ITS clades 9 and 10 displaying characteristics consistent with the genus *Phytophthora* have been described. Two of these species, *P. fallax* and *P. captiosa*, the closest phylogenetic relatives of *P. constricta*, are foliar pathogens spreading in the canopies of *Eucalyptus* trees in New Zealand, although they are noncaducous (Dick *et al.*, 2006). The mode of dispersal of these pathogens is still unknown, but they appear to be aerially dispersed by rain-splash. Clades 9 and 10 are basal to the *Phytophthora* phylogeny and represent an independently evolving ancestral lineage that diverged prior to the radiation of clades 1–8 (Dick *et al.*, 2006). The apparent tendency towards caducity in both *P. pinifolia* and *P. constricta* are, therefore, examples of convergent evolution.

An analogous nonpapillate, partially caducous species is *P. lateralis*, a soilborne pathogen infecting the roots and collars of susceptible hosts (Brasier *et al.*, 2010). Like its closest relatives, *P. foliorum, P. hibernalis* and *P. ramorum, P. lateralis* produces caducous sporangia with well-formed pedicels. However, *P. foliorum, P. hibernalis* and *P. ramorum* are aerially dispersed and infect above-ground plant tissues. Brasier *et al.* (2010) hypothesized a common papillate, caducous ancestor for all these species, with *P. lateralis* adapting to a soilborne habit while losing its papillum and retaining caducity. This compares interestingly with *P. constricta*, a nonpapillate soilborne species apparently evolving towards caducity.

Sporangia of *P. constricta* have been observed to break at the constriction of the sporangiophore under the slight pressure of a microscope slide cover slip; likewise, sporangia formed on superficial roots or stem lesions could be dispersed by rain-splash or wind, as is the case with caducous species such as *P. ramorum* (Rizzo *et al.*, 2002; Davidson *et al.*, 2008).

In summary, two novel species, *P. constricta* and *P. arenaria*, are presented here. Based on GenBank accession data these taxa have not been isolated elsewhere in the world, and appear to be restricted to the Kwongan vegetation of south-west WA. The diversity in *cox*1 sequence data indicates they have been evolving *in situ* for some time, and both morphological and physiological data suggest they are very well adapted to the ecosystems in which they occur. Since both *P. constricta* and *P. arenaria* require a flooding stimulus to cause mortality in their common host *B. attenuata*, they might be in equilibrium with the host species with which they have coevolved. However, long-term field studies in infested Kwongan stands are required to confirm this hypothesis.

Acknowledgements

The authors are grateful to the Cooperative Research Centre for National Plant Biosecurity, ACT, Australia for the financial support of the first author; Diane White (CPSM, Murdoch University, WA), Janet Webster and Juanita Ciampini (WA Department of Environment and Conservation) for technical assistance; William Dunstan (CPSM, Murdoch University, WA) for providing information on field observations; and Glevan Consulting, and the staff of the WA Department of Environment and Conservation (and its predecessors: the Department of Conservation and Land Management, and the Forests Department), for the collection of field samples.

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Supporting information

The following supplementary materials are available for this article:

Table S1. Comparisons of morphological characters and dimensions, and temperature-growth relations of *Phytophthora arenaria, P.* taxon arenaria-like, *P. cactorum*, *P. alticola* and *P. quercetorum*.

Table S2. Comparisons of morphological characters and dimensions, and temperature–growth relations of *Phytophthora constricta, P. megasperma s. str.*, *P. fallax* and *P. captiosa*.

Additional Supporting Information may be found in the online version of this article.

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