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Redesignation of *Phytophthora* taxon Pgchlamydo as *Phytophthora chlamydospora sp. nov.*

Everett M. Hansen[1], Paul Reeser[1], Wendy Sutton[1], and Clive M. Brasier[2]

[1]Oregon State University, Corvallis, Oregon, USA, and
[2]Forest Research, Alice Holt Lodge, Farnham, Surrey GU10 4LH, UK

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Corresponding author: Everett M. Hansen, hansene@science.oregonstate.edu. April 29, 2015.

Abstract: A new species, *Phytophthora chlamydospora*, is described. *P. chlamydospora,* previously known informally as *P.* taxon Pgchlamydo, is found in streams and wet soil worldwide and is a pathogen of some riparian tree species. It is self-sterile, and produces persistent non-papillate sporangia, usually on unbranched sporangiophores. Clamydospores are formed most regularly at warmer temperatures. *Phytophthora chlamydospora* is classified in ITS Clade 6.

Key words: *Phytophthora, chlamydospora,* new species, Pg chlamydo, streams, soil.

Introduction: *Phytophthora* species are well known as damaging pathogens of agricultural crop plants (Erwin and Ribeiro 1996) and forest trees (Hansen 2007) and there is an increasing awareness of *Phytophthora* diversity in woodland and riparian ecosystems. In recent years surveys for *Phytophthora* species in forest soils and streams, coupled with the advent of molecular phylogenetic methods, have revealed numerous new species (Brasier 2009). In riparian ecosystems especially a considerable number of previously undescribed taxa falling within *Phytophthora* major ITS Clade 6, the '*P. megasperma/P. gonapodyides* Clade', have been identified. Many of them are slow-growing, sexually sterile and able to survive at comparatively high temperatures (Brasier et al. 1993, 2003; Jung et al. 2011; Hansen et al. 2012; Nechwatal et al. 2012; Nagel et al. 2013).

Among the latter is a relatively slow growing sexually sterile *Phytophthora* with internally proliferating sporangia first isolated from waterlogged roots of a street-side ornamental *Prunus* in Gloucestershire, UK in 1971 (Brasier et al. 1993). On the basis of its morphology and habitat it was tentatively assigned to *P. gonapodyides* Petersen (Buisman), a weak root parasite and a common saprotroph on plant detritus in ponds and rivers. During the 1980s similar isolates from Britain and North America were collected and compared. All exhibited comparable colony patterns and sporangial dimensions. All isolates were sexually sterile, yet capable of inducing gametangial formation (selfing) in A2 isolates of heterothallic *Phytophthora* species. However four isolates including the UK *Prunus* isolate and three isolates from nurseries in Oregon and Washington, USA produced large thin-walled chlamydospores in culture. On this basis it was considered this group of isolates could be a separate taxon from *P. gonapodyides* (Brasier et al. 1993).

The subsequent development of ITS-based molecular phylogenies of *Phytophthora* isolates (White et al. 1990; Cooke et al. 2000;) prompted a comparative investigation of phenotypes and ITS types among a wide range of unidentified or uncertain isolates resembling *P. gonapodyides* (Brasier et al. 2003). In this study the chlamydospore-forming and non-chlamydospore-forming '*P. gonapodyides*' groups of Brasier et al. (1993) formed separate phylogenetic lineages. To facilitate scientific communication pending a formal description, the chlamydospore-forming group was informally designated *Phytophthora* taxon Pgchlamydo. Since 2003 *P.* taxon Pgchlamydo has been identified from many locations worldwide including natural and semi-natural ecosystems and a range of woody hosts (Table 1). It is therefore desirable that the species is formally recognised. In this paper we describe it as *P. chlamydospora sp. nov.*, and review current knowledge of its genetic variation, behaviour and distribution and that of the many ecologically similar species in the Clade.

Materials and Methods: Observations of morphology, growth, and pathogenicity were compiled from previously published sources (Brasier et al. 1993, 2003; Greslebin et al. 2005; Hansen and Delatour 1999; Reeser et al. 2008; Yakabe et al. 2007), supplemented by new data as noted.

DNA sequence information was generated and analyzed for a representative group of five isolates (Table 2). DNA was extracted from *Phytophthora* isolates growing on corn meal agar using a CTAB buffer with ethanol precipitation protocol (Winton and Hansen 2001). ITS, ß-tubulin, and the COX spacer DNA regions were amplified with appropriate primers (Table 3) and sequenced, as described in Reeser et al. (2011). Sequences were aligned with Clustal X 2.1 (Thompson et al. 1997). Sequences were

compared to closely related reference isolates in the OSU *Phytophthora* collection, those in the validated database *Phytophthora* ID (Grunwald et al. 2011) (http://phytophthora-id.org/) and those available at GenBank. The phylogenetic tree was generated with Clustal X and displayed with TreeView (Win32).

Results:

TAXONOMY

Phytophthora chlamydospora Brasier and Hansen *sp. nov*.

MycoBank no.: MB 809175

Etymology: "chlamydospora" refers to the distinguishing chlamydospores formed especially at higher temperatures.

Type: P236, isolated in 1971 from ornamental *Prunus* in Cheltenham, Gloucestershire, U.K. by C.M Brasier and R.G Strouts (Forest Research Advisory Service Record 72/445/82). OSC # 153153. Isotypes IMI389736, ATCC28765. GenBank ITS AF541890, COX spacer KF750598, β-tubulin KF750602.

Description

Sporangia formed in water on simple, unbranched (occasionally sympodial) sporangiophores; obpyriform or ovoid, often somewhat elongated, non-papillate, persistent (non-caducous), with internal proliferation; average 56 µm by 36 µm, length: breadth ratios from 1.5 to 1.7 (Figures 5-8) (Brasier et al. 1993). Clumps of large globose to subglobose hyphal swellings in branched chains usually formed in water. Chlamydospores formed in agar media, often scarce at 22 C but usually abundant at 28 C. Chlamydospores are mostly intercalary but lateral, terminal and sessile chlamydospores are observed (Figures 1-4).

Gametangia have not been observed in single isolate culture i.e. *P. chlamydospora* appears to be self-sterile. Some isolates exhibit a relic of heterothallism, remaining self- sterile but acting as a 'silent' A1 compatibility type, inducing gametangial formation in A2 isolates of other *Phytophthora* species when paired directly with them or via a polycarbonate membrane (Brasier et al 1993; 2003).

Temperature optimum mostly ranges from 25–28 C (one isolate 30C). Maximum temperature for growth 36 or 37 C. Growth rate on carrot agar at 25 C 3.2 – 4.1 mm/d (Brasier et al. 2003). Colonies on carrot agar petaloid, very similar to *P. gonapodyides* (Brasier et al. 1993, 2003) (Figure 9).

Phylogeny

Five isolates previously identified as *P.* taxon Pgchlamydo, including the type isolate, (Table 2) were examined and shown to be monophyletic within *Phytophthora* Clade 6 (Figure 10) using Clustal X 2.1 for Neighbor Joining analysis (based on 1000 bootstraps). The isolates grouped into three multilocus genotypes for the nuclear ITS and β-tubulin regions and the mitochondrial COX spacer region (Table 4).Their ITS sequences are nearly identical, although 'double peaks' are variously present at three base positions. The COX spacer sequences formed two haplotypes, differing at four bases. β-tubulin sequences of the five isolates were more variable, with 20 polymorphic bases. In each gene region, however, the isolates clustered in the same terminal clade (data not shown).

Notes

Phytophthora chlamydospora previously was known informally as *P.* taxon Pgchlamydo (Brasier et al. 2003). It also corresponds to isozyme group "K" in the analysis of '*P. drechsleri* and *P. cryptogea*' by Mills et al. (1991). *P. chlamydospora* is found in streams and wet soil worldwide (Table 1).

Phytophthora chlamydospora is rather nondescript in culture. It long has been confused with *P. gonapodyides.* Because chlamydospores

may not be formed at lower temperatures this distinguishing feature is not always available. Sporangia usually form rapidly (within 24 hours) in water but are not easily distinguished from those of other non-papillate species. *Phytophthora chlamydospora* can be mis-identified as *P. lateralis* because of its combination of large, sometimes laterally-attached chlamydospores and non-papillate sporangia, but tolerates much higher growth temperatures. *Phytophthora chlamydospora* is itself sterile, although it may induce gametangia in A2 isolates of heterothallic species. Without carefully controlled mating tests to determine which isolate is producing gametangia, it often has been assumed to be heterothallic and consequentally misidentified as *P. drechsleri* or *P. cryptogea* because of its colony morphology and non-papillate sporangia. Clumps of large globose to subglobose hyphal swellings often are observed in water and can be a useful identifying feature. These clumps are sometimes large enough to be visible to the naked eye. Isolates examined from China, Europe, North America and Argentina have been indistinguishable morphologically.

Phytophthora chlamydospora occasionally is recovered from cankers on trees and roots (Reeser et al. 2008, Navarro et al. 2014, Sims et al. 2014) and foliage of horticultural nursery stock (Jung and Blaschke 2004, Yakabe et al. 2009). It has been associated with root rot of Port-Orford cedar (*Chamaecyparis lawsoniana*) in German nurseries, where it was initially misidentified as *P. lateralis* (Hansen et al. 1999), and with root rot and stem cankers of *Abies* species in nurseries and Christmas tree plantations, where it was originally misidentified as *P. drechsleri* (Brasier et al. 1993).

Several multilocus genotypes have been identified; isolates with sequences identical to the type isolate are most numerous, and present in both Europe and western North America. This multilocus genotype lacks double peaks in both ITS and β-tubulin gene regions. The second most frequently encountered multilocus genotype is also found in both Europe and North America. The third genotype was found in 5 isolates from Argentina. Isolates with further variations in COX spacer and β-tubulin were also identified, but not fully characterized. Despite this variation, *P. chlamydospora* can be distinguished from all other known *Phytophthora* species by the similarity of its DNA sequences of the ITS, COX spacer, and β-tubulin gene regions. *Phytophthora pinifolia* and *P. borealis* are sister species to *P. clamydospora* in ITS, *β*-tubulin and COX phylogenies, with modest bootstrap support. In the ITS region, the type isolates of *P. pinifolia* and *P. borealis* differ from P 236, type of *P. chlamydospora* by 2 indels and 6 base changes, and 7 indels and 13 base changes, respectively. The three species are readily distinguished by growth rate, optimum and maximum temperature, and colony morphology, as well as chlamydospore formation. In addition, *P. pinifolia* exhibits a tendency to cauducity in culture, not observed in *P. chlamydospora*.

Discussion: A significant proportion of the known species in *Phytophthora* major Clade 6 grow slowly, tolerate high temperatures, are sexually sterile and are associated with riparian conditions (Brasier et al. 1993, 2003). Examples include *P. gonapodyides, P. riparia, P. borealis, P. fluvialis, P. pinifolia*, *P. amnicola, P. litoralis* and *P. lacustris*. In these regards *P. chlamydospora* is fairly typical. Its growth at its optimum is slow and its maximum temperature for growth of 36 – 37 C is at the high end of the spectrum for Phytophthoras. It does not produce gametangia under normal culture conditions. It frequently is encountered in streams, rivers, and irrigation water, and from adjacent riparian soils in temperate forests in western North and South America, Europe, Asia, Africa, and Australia (Table 1). Indeed it could be the second most broadly distributed *Phytophthora* species, after *P. gonapodyides*.

The ecological and evolutionary significance of the somewhat specialized combination of characters exhibited by Clade 6 species has been extensively discussed and is broadly considered to be an adaptation to riparian conditions (Brasier et al. 1993, 2003. Jung et al. 2011; Nechwatal et al. 2012; Nagel et al. 2013). It has been suggested that slow growth rate and high temperature tolerance are associated with saprotrophy on submerged plant debris, and with warm summer conditions at the margins of water bodies. In regard to the latter it should be noted that *P. chlamydospora* is something of an exception among the sterile species in the Clade in producing asexual chlamydospores. These could be an adaptation to seasonal drying of marginal bodies of water. In some circumstances they could conceivably give *P. chlamydospora* an advantage over local ecologically similar species such as *P. gonapodyides*.

Regarding the frequent sexual sterility of these organisms it has been suggested that a more homogeneous aquatic environment may favor inbreeding and clonality over genetic heterogeneity, and loss of sexual reproduction in favor of enhanced zoospore formation. Indeed it is notable that the Clade 6 taxa that do readily undergo sexual reproduction in culture are also mainly inbreeding species, in that they are predominantly self-fertile (homothallic) taxa, examples being *P. humicola*, *P. rosacearum*, *P. gibbosa, P. gregata, P. thermophila, P. bilorbang,* and *P.* taxon paludosa. Since more new taxa currently are being identified in Clade 6 than in any of the predominantly terrestrial *Phytophthora* clades, it has also been suggested that the combination of aquatic life styles and inbreeding and sterility could have led to the emergence of numerous locally adapted clonal populations that effectively have become unique taxa (Brasier 2009). The main taxonomic units in Clade 6 may tend to be somewhat different genetically from those observed in the more terrestrial *Phytophthora* clades, and the species concept applied within the Clade might also need to be different.

There is some evidence that 'sterile' Clade 6 species sometimes may undergo sexual reproduction under as-yet unknown conditions in nature. Brasier et al. (2003) reported a single observation of oogonial formation (abundant oogonia with amphigynous antheridia) when two isolates of *P. gonapodyides* were paired in culture. However when the same two isolates were paired together subsequently in multiple repeat tests no oogonia were obtained. Nagel et al. (2013) have produced molecular and phenotypic evidence for local emergence of hybrid swarms between *P. chlamydospora* and *P. amnicola* and *P. chlamydospora* and *P. thermophila* in Australia and South Africa. Despite the sterility in culture of the parent species of the hybrids their evidence also indicates the hybrids may have originated from sexual recombination. In the present study two different nucleotide bases (a double peak) were recorded at some base positions in the ITS and β-tubulin sequences of *P. chlamydospora*. These might reflect past hybridization events.

Apart from its riparian habitat *P. chlamydospora* has been associated with what may be largely opportunistic root and foliar infections of woody nursery stock, root infections of *Abies* and *Pseudotsuga* in Christmas tree plantations, root rot of ornamental *Prunus* and *Chamaecyparis*; and, in one instance, with stem cankers on tanoak (*N. densiflorus*) in natural forest (Table 1). Artificial inoculations of several plant species have confirmed that *P. chlamydospora* can be pathogenic on stems and roots (Navarro et al. 2014), Reeser et al. 2008, Yakabe et al. 2009). However, true status of *P. chlamydospora* as a pathogen in the natural environment has yet to be established. Where it is locally abundant in streams there is usually no visible evidence of disease in adjacent vegetation (P. Reeser and E. M. Hansen, unpublished observations). We

suggest this should not necessarily be taken as evidence that it is not behaving as a pathogen in these situations, however. First because pathogens often cause only limited damage to the hosts with which they have coevolved (Hansen et al. 2012). Second, potentially highly-damaging Phytophthoras, such as *P. ramorum, P. kernoviae* and *P. cinnamomi*, can cause asymptomatic root and foliar infections (Denman et al. 2006; Fichtner et al. 2011; Crone et al. 2013). It is therefore possible that *P. chlamydospora* and other ecologically similar Clade 6 species may cause limited and ephemeral root infections on tree and understory species.

Many of the points discussed above are largely theoretical and speculative owing to the paucity of available information. It is clear therefore that much additional, detailed research is needed on the ecology, pathology and breeding behavior of *P. chlamydospora* and similar species in the Clade.

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Table 1. Some worldwide records of *P. chlamydospora* (*P.* taxon Pgchlamydo).

Country/State or Province	**Substrate**	**Reference**	**GenBank #**
Argentina/Chubut	Streams	Greslebin et al. 2005	AY787024
Australia/Western Australia	Soil	Burgess et al. 2009	EU301160
Canada/British Columbia	*Pseudotsuga menziezii* roots	Hamm & Hansen 1987	AF541902
China/Yunnan	Streams	Huai Wenxia et al. 2013	JQ730724
France/Lorraine	Water	Hansen & Delatour 1999	AF541901
Germany	*Chamaecyparis lawsoniana* seedling	Hansen et al. 1999	
Germany	*Alnus* seedling soil	Jung and Blaschke 2004	
South Africa	Streams and soil	Oh et al. 2013	KC855172
Spain/Asturias	*Rhododendron* leaves	Morajelo et al. 2009	EU194442
United Kingdom/ Surrey	*Prunus* roots	Brasier et al. 1993; 2003	AF541890
USA/Oregon	Streams	Reeser et al. 2011	HM004224
USA/Oregon	*Notholithocarpus densiflorus* canker	Reeser et al. 2008	
USA/Minnesota	*Taxus*	Schwingle et al. 2007	DQ486671
USA/California	Woody ornamentals	Yakabe et al. 2009	

Table 2. Isolates of *P. chlamydospora* used for phylogenetic analysis and Genbank accession numbers.

Isolate	**Source**	**Substrate**	**Collector**	**Genbank Accessions**		
				ITS	**COX spacer**	**ß-tubulin**
AG29	Argentina	Water	Greslebin	KF750595	KF750596	KF750604
Haye3.1	France	Water	Hansen & Delatour	AF541901	KF750599	KF750603
WA5.1-072003	Oregon	Water	Hansen lab	HM004224	KF750597	KF750601
P236 (type)	England	Root	Brasier lab	AF541890	KF750598	KF750602
133	British Columbia (Canada)	Root	Hansen lab	AF541902	KF750600	KF750605

Table 3. DNA regions and PCR primers used for genetic analysis of *P. chlamydospora*.

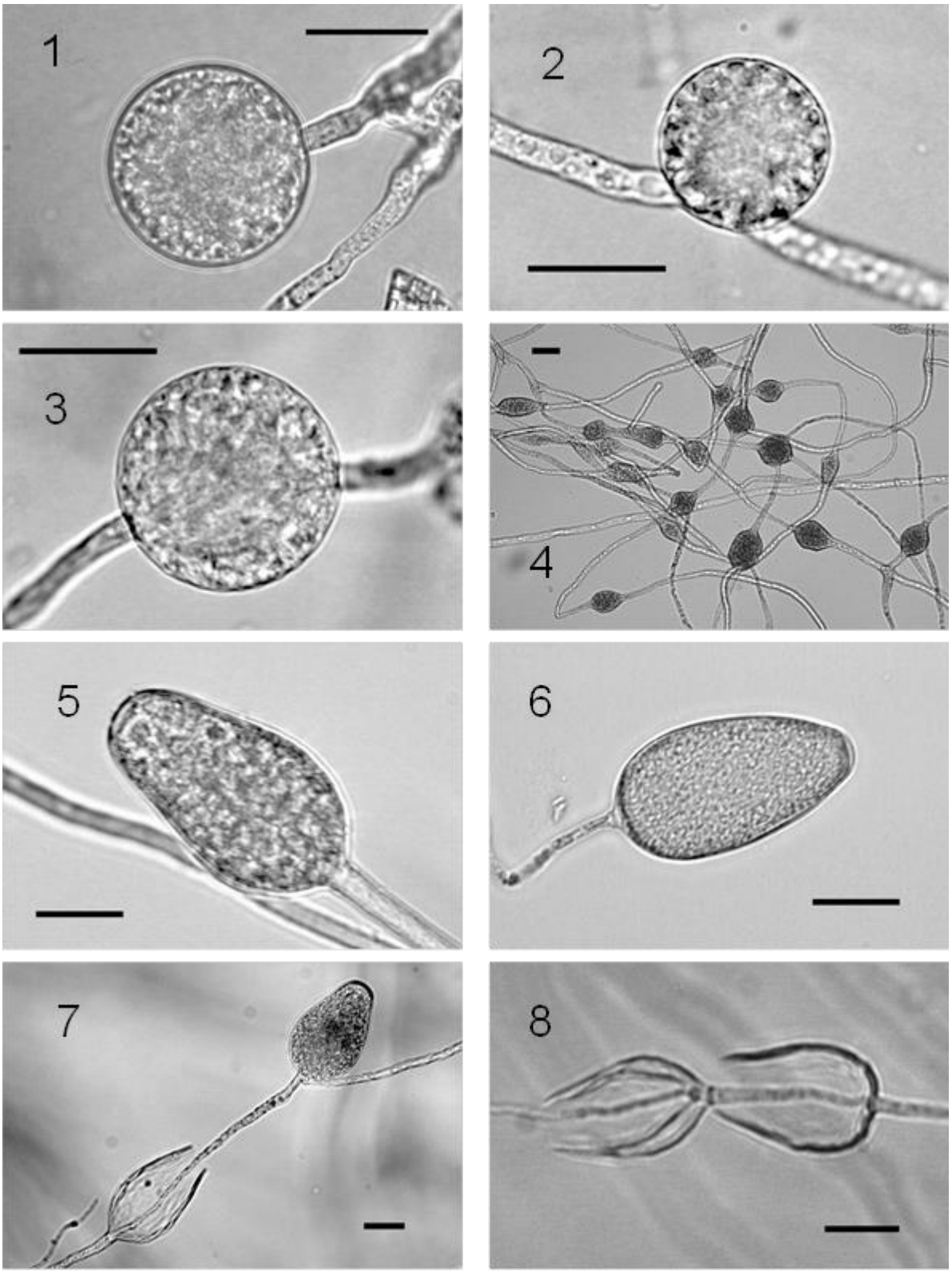
GENE OR DNA REGION	**PRIMERS**	**LENGTH AMPLIFIED**	**CITATION**
ITS rDNA	ITS4 and ITS5	~820bp	White et al. 1990
COX spacer	FMPH8 and FMPH10	~340bp	http://www.ars.usda.gov/Research/docs.htm?docid=8737
β Tubulin	β-TubF1 and β-TubR1	~990bp	Kroon et al. 2004

Table 4. Base table showing polymorphic bases by position for β-tubulin, ITS, and COX spacer gene regions for five isolates of *P. chlamydospora*.

B tubulin	102	105	180	276	342	354	429	450	462	486	519	561	571	594	621	634	667	702	726	759
P236-TYPE	C	G	G	T	A	C	T	C	A	C	G	G	T	C	T	T	C	T	C	C
133	C	G	G	T	A	C	T	C	A	C	G	G	T	C	T	T	C	T	C	C
HAYE3.1	C	R	R	Y	A	C	Y	C	R	Y	G	S	Y	C	T	Y	C	T	C	Y
WA5.1	C	R	R	Y	A	C	Y	C	R	Y	G	S	Y	C	T	Y	C	T	C	Y
AG29-2	Y	G	G	Y	M	Y	Y	Y	R	C	R	S	Y	M	Y	Y	Y	Y	Y	M

ITS	171	653	666
P236-TYPE	C	G	T
133	C	G	T
Haye3.1	Y	G	Y
WA5.1	Y	G	Y
AG29	C	K	T

COX spacer	6	51	86	106
P236-TYPE	T	T	A	C
133	T	T	A	C
AG29	T	T	A	C
Haye3.1	G	C	G	A
WA5.1	G	C	G	A



Figs. 1 – 8. Structures of *Phytophthora chlamydospora*. Figs. 1 – 3. Chlamydospores in agar. Fig. 4. Hyphal swellings in water. Figs. 5 – 8. Sporangia in water, showing subsporangial elongation in Fig. 7 and internal proliferation in Figs. 7 and 8. Bar is 20 μm. Photos Paul Reeser

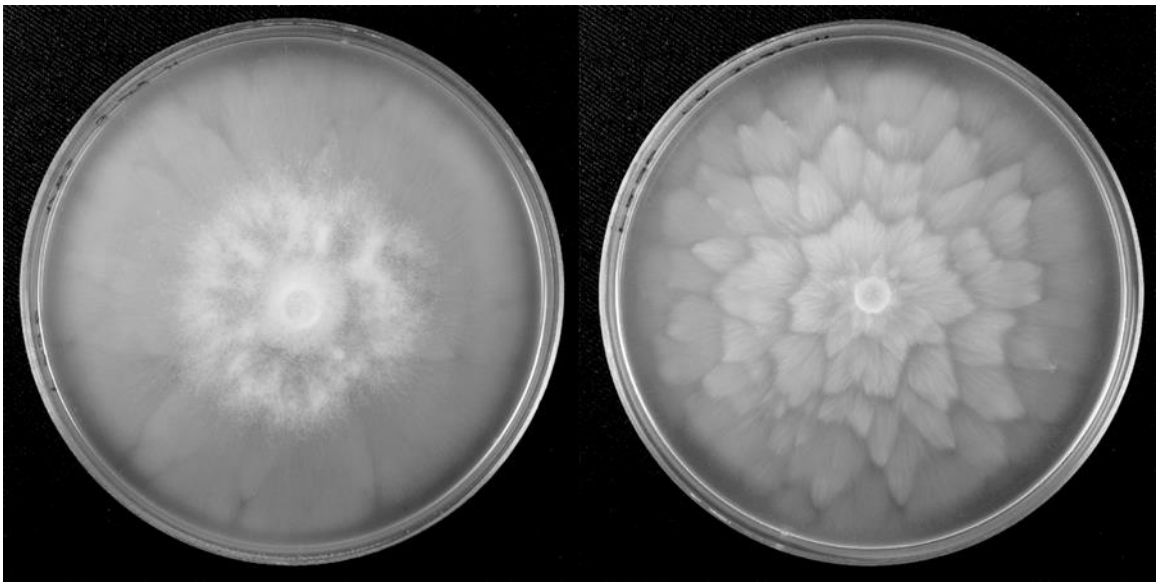


Figure 9. *Phytophthora chlamydospora* isolate P236 on V8 agar (left) and carrot agar (right). Photos Paul Reeser

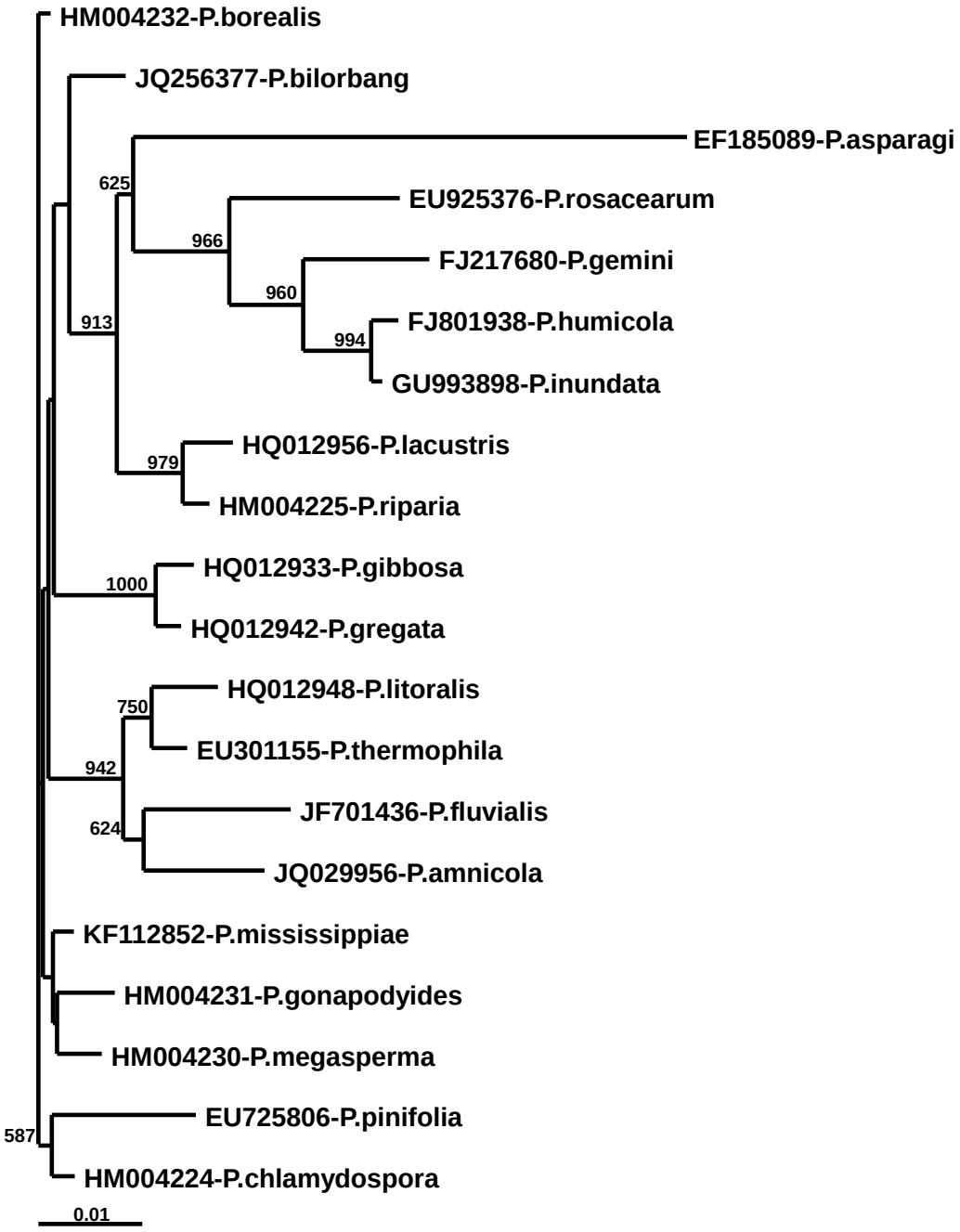


Figure 10. ITS phylogeny of *P. chlamydospora* in *Phytophthora* ITS Clade 6 aligned with ClustalX 2.1. The Neighbor Joining tree was generated by Clustal X and displayed with TreeView (Win32). Numbers on nodes represent bootstrap support values >500.