



Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada

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Key words

COI
ITS rDNA
LSU rDNA
Oomycete
Peronosporales
Pythium
taxonomy

Abstract Three new species of *Pythium*, namely, *P. oopapillum*, *P. emineosum* and *P. camurandrum* are presented in this paper based on morphological descriptions and molecular phylogenetic characterisation. These new species were isolated from various ecological regions in Canada. They have unique morphological features in the genus *Pythium*, and form distinct clades in maximum parsimony analyses, which are also supported by maximum likelihood phylogeny using general time reversible model (GTR), and Bayesian inference (BI) phylogeny using Markov Chain Monte Carlo (MCMC) analysis methods. A comparative study of the new species with closely related taxa, their clade positions, and morphological features are described in this paper.

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INTRODUCTION

Pythium is a diverse genus comprised of approximately 140 recognised saprobic and parasitic species. The parasitic species affect both plants (Harrison 1989, Chang et al. 1994, Hou et al. 1997) and animals (Dick 1990, Barr 1992). These pathogenic species have a devastating impact on crops of economic importance worldwide. Proper identification and characterisation of *Pythium* species is extremely important in understanding the biology and evolutionary relationships among these species.

The lack of distinctive morphological structures is one of the major limitations in the taxonomic identification of *Pythium*. Although non-morphological methods are receiving more attention in the identification or detection of *Pythium* species e.g., Tambong et al. (2006), a complete analysis of both morphology and molecular data is still essential for the description of new species in the genus *Pythium*. There are some examples of *Pythium* species, e.g. *P. aphanidermatum* and *P. deliense* that share identical or near identical Internal Transcribed Spacer (ITS) regions, though exhibit clear but subtle differences in their morphology (van der Plaats-Niterink 1981, Lévesque & de Cock 2004). Hence, study of more than one molecular marker is useful for distinguishing *Pythium* species.

DNA barcoding is the utilisation of DNA sequence data for the characterisation and identification of species. The cytochrome c oxidase subunit 1 (COI) has been successfully used as a genetic marker for identification and to delimit species boundaries in animals (Hebert et al. 2003) whereas, ITS has been the equivalent marker in *Peronosporales* (Matsumoto et al. 1999, 2000, Cooke et al. 2000, Lévesque & de Cock 2004).

Both ITS (Cooke et al. 2000) and COI (Martin & Tooley 2003) are used as barcodes for *Phytophthora* species; one of the closest relatives of the genus *Pythium*, because of their high interspecific and low intraspecific variation. These genetic regions are also useful for examining relationships of other

closely related oomycetes. The large subunit (LSU) of ribosomal DNA contains highly divergent regions D1–D3, and is used as molecular marker in recognition of *Pythium* species (Lévesque & de Cock 2004).

Morphological studies and DNA barcoding of nuclear and mitochondrial barcodes of a large number of *Pythium* strains from collections of the Centraalbureau voor Schimmelcultures (CBS) and the Department of Agriculture, Ottawa, Mycology (DAOM) was performed for the identification and description of *P. oopapillum*, *P. emineosum* and *P. camurandrum*. These three new species of *Pythium* were isolated from the soil of four different regions in Canada. A molecular phylogeny of these based on ITS, COI and LSU with the closely related *Pythium* species were performed.

Morphological descriptions and comprehensive comparative analyses of the morphological characters were also performed. This is the second report of new *Pythium* species of Canadian origin to be included in the clades B, E and F (Lévesque & de Cock 2004).

MATERIALS AND METHODS

Fungal isolations

Isolates were obtained from existing culture collections except for the two strains (CBS 124056, Lev 3133) that were isolated in 2008 from soil using the hemp-seed (*Cannabis sativa*) baiting technique (Bala et al. 2006). The number of isolates per species used in this study is provided in Table 1.

Growth in culture and morphological characterisation

The isolates were grown and maintained on Potato-carrot agar (PCA), Cornmeal agar (CMA) and Sabouraud dextrose agar (SDA) for morphological studies. PCA was prepared by boiling 20 g of carrots and 20 g of potato in 1 L of distilled water, followed by adding 15 g of agar (Difco) to the extract with sterilisation for 20 min by autoclaving. CMA (Difco) and SDA (Difco) were prepared according to the manufacturer instructions. Water cultures were prepared following the methods of de Cock & Lévesque (2004). Autoclaved grass blades and hemp-seed halves were added to the oomycetes colonies growing on

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Table 1 Sequence length, GenBank accession and culture collection numbers as well as distribution of the strains.

Features	<i>P. oopapillum</i>		<i>P. emineosum</i>		<i>P. camurandrum</i>	
	ITS	COI	ITS	COI	ITS	COI
Sequence length	779–780 bp	727 bp	851–921 bp	717–727 bp	941bp	610 bp
GenBank accession number	FJ655174 FJ655175 FJ655176 FJ655177	FJ655178 FJ655179 FJ655180 FJ655181	GQ244427 GQ244428	GQ244423 GQ244424	GQ244426	GQ244425
CBS accession numbers	CBS 124053, CBS 124054, CBS 124055, CBS 124056		CBS 124057, CBS 124058		CBS 124059, CBS 124096	
Country of origin	Canada		Canada, United Kingdom		Canada, The Netherlands	
Numbers of isolates per species	Four		Two		Two	

agar. Asexual and sexual reproductive bodies were abundantly produced on the grass blades and hemp-seed halves in water. The sexual structures were observed for 2–30 d. Fifty measurements were taken for sporangia, oogonia, oospore and oospore wall, and averages were calculated. The cardinal temperatures were determined on PCA and growth was measured every 24 h. The PCA plates inoculated with each isolate were incubated at 5–40 °C with intervals of 5 °C. When the growth arrested at high temperatures, the culture was returned to room temperature to check if growth resumed, indicating if the culture was still alive. Microscopic slides were prepared in distilled water with asexual and sexual reproductive structures observed under a compound light microscope (Nikon). Images were captured using high-resolution digital camera (DXM 1200, Nikon). Examinations under standardised, calibrated magnification were performed using a computer-based software system (NIS-Elements version D, Nikon Canada Inc.). Nomenclatural novelties and descriptions were deposited in MycoBank (www.Mycobank.org; Crous et al. 2004).

DNA extraction and PCR

DNA was extracted following either the protocols of Möller et al. (1992) from mycelium prepared in pea broth (de Cock et al. 1992), or from mycelium grown in potato-dextrose broth (Lévesque et al. 1998). The ITS and D1–D3 LSU regions were amplified using universal eukaryotic primers Un-Up18S42 (5'-CGTAACAAGGTTTCCGTAGGTGAAC-3') (Lévesque & de Cock 2004) and Un-Lo28S1220 (5'-GTTGTTACACACTCCTTAGCGGAT-3') designed for a high annealing temperature. COI amplification was performed using the forward primer Oom-COI-Lev-up 5'-TCAWCWGMATGGCTTTTTTCAAC-3' and the reverse primer FM85-mod 5'-RRHWACKTGACTDATRATACAAA-3' modified from Fm85 of (Martin & Tooley 2003).

Sequencing and phylogenetic analysis

The primers used for PCR were also used for sequencing. For rDNA, Un-Up28S40 (GCATATCAATAAGCGGAGGAAAAG) and Un-lo28S576B (CTCCTTGGTCCGTGTTTCAAGACG), Un-Up28S577 (CGTCTTCAAACACGGACCAAGGAG) and Un-lo28S22 (GTTTCTTTTCCCTCCGCTTATTGATATG) were used as internal sequencing primers (Schurko et al. 2003). Sequencing reactions were prepared using the Big Dye Terminator (BDT) v2 protocol (Applied Biosystem, Foster City, CA). Sequencing of the PCR product was done on an Applied Biosystems Prism Genetic Analyzer model 310. An alignment using Muscle software v3.41 (Edgar 2004) and maximum parsimony analysis using heuristic search by stepwise addition of 100 random replicates and bootstrapping with 1 000 replicates were performed with PAUP v4.0b10 software (Swofford 2002). The PhyML (Guindon & Gascuel 2003) program with General Time Reversible (GTR) model was run to obtain maximum likelihood trees and nonparametric ML bootstraps were cal-

culated with 1 000 replicates. MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003) was used to generate Bayesian inferences (BI) with Markov Chain Monte Carlo (MCMC) methodology and posterior probabilities of the phylogenies. The program was run for 400 000 generations and sampled every 100 generations. TreeView was used to view ML and Bayesian trees.

RESULTS

Taxonomy

Pythium (Clade B in Lévesque & de Cock 2004)

Pythium oopapillum Bala, de Cock & Lévesque, *sp. nov.*
— MycoBank MB512818; Fig. 1, 2

Hyphae praecipuae 4.5 µm diam. Coloniae in agaro *Potator carotae* (PCA) chrysanthemale. Sporangia filamentosa. Oogonia intercalaria, globosa, subterminalia 17.5–24.5 µm diam. Antheridia monoclinata vel diclinata raro hypogynata. Oosporae singulae, apertoticae vel pleroticae, globosae, subglobosae 14.5–18 µm diam, paries 0.8–3 µm crassus, papillis. Augmentum chrysanthemale quotidianum 17 mm ad 25 °C in agaro *Potator carotae* (PCA). Temperatura minima crescentis 5 °C, optima 30 °C maxima 30 °C.

Etymology. Name refers to the presence of papilla on the oospore.

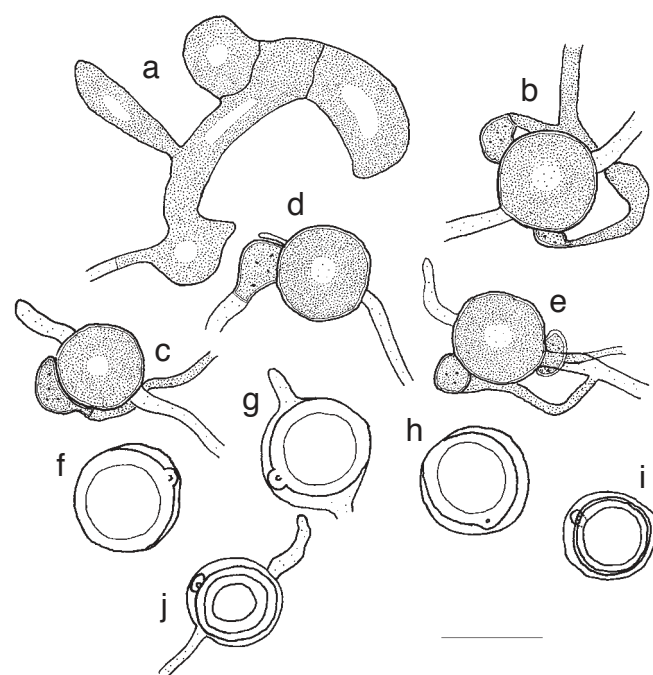


Fig. 1 *Pythium oopapillum*. a. Filamentous inflated sporangium (constrictions shown); b–e. antheridia and oogonia; f–j. oospores. — Scale bar = 10 µm.

Main *hyphae* up to 4.5 μm diam. *Colonies* on PCA show a vague chrysanthemum pattern with an average radial growth of 5 mm/d at 5 °C, 10 mm/d at 10 °C, 14 mm/d at 20 °C, 17 mm/d at 25 °C, 22 mm/d at 30 °C and no growth at 35–40 °C. The mycelium grows easily on PCA, CMA and SDA producing plentiful of oogonia, antheridia and oospores. Vesicles and zoospores are formed on sterile grass blades in water cultures at 15–30 °C. *Sporangia* are filamentous inflated, consisting of lobate elements which give rise to vesicles containing 5–24 zoospores. Vesicles and zoospores are produced plentifully in water cultures at 15–30 °C. Hyphal bodies developed in water culture, PCA, CMA or SDA are spherical, terminal as well as intercalary. At times, they germinate directly through germ tubes to produce a new mycelium. The *zoospores* develop within vesicles in water cultures within the first two days of inoculation at 15–25 °C but in a week when incubated at 30 °C. Encysted zoospores usually measure from 7–9 μm diam. *Oogonia* are mostly intercalary, occasionally subterminal, smooth, globose

17.5–24.5 μm (av. 21.5 μm) diam. *Antheridia* 1–2(–4) per oogonium, monoclinal or diclinal, mostly terminal on branched antheridial stalks, club-shaped, making apical or lateral contact, rarely hypogynous. *Oospores* aplerotic or nearly plerotic, mostly thick-walled and provided with a papilla, globose, subglobose, occasionally ellipsoidal, 14.5–18 μm (av. 16.4 μm) diam; wall 0.8–3 μm (av. 1.9 μm) thick. The papilla present on the oospore measures up to 6.5 μm in length and 2 μm in width.

Specimens examined. CANADA, Alberta, Barrhead, from hydroponic cucumber (*Cucumis sativus*), Mar. 1989, K.F. Chang, holotype culture in liquid nitrogen DAOM BR632, culture ex-type CBS 124053; Alberta, from cucumber (*Cucumis sativus*), Sept. 1989, S.F. Huang, culture DAOM BR641 = CBS 124054; Ontario, Ottawa, Central Experimental Farm, from wheat roots, June 1975, D.J.S. Barr, culture DAOM BR180 = CBS 125055; Central Experimental Farm, soil baiting (Bala et al. 2006) from field with Alfalfa (*Medicago sativa*) and corn (*Zea mays*) rotation, May 2008, K. Bala, culture CBS 124056 = CEF72; Ottawa, Parliament Hill, May 2008, roots of diseased tulips (*Tulipa* sp.), K. Bala, culture Lev3133 (KBF2).

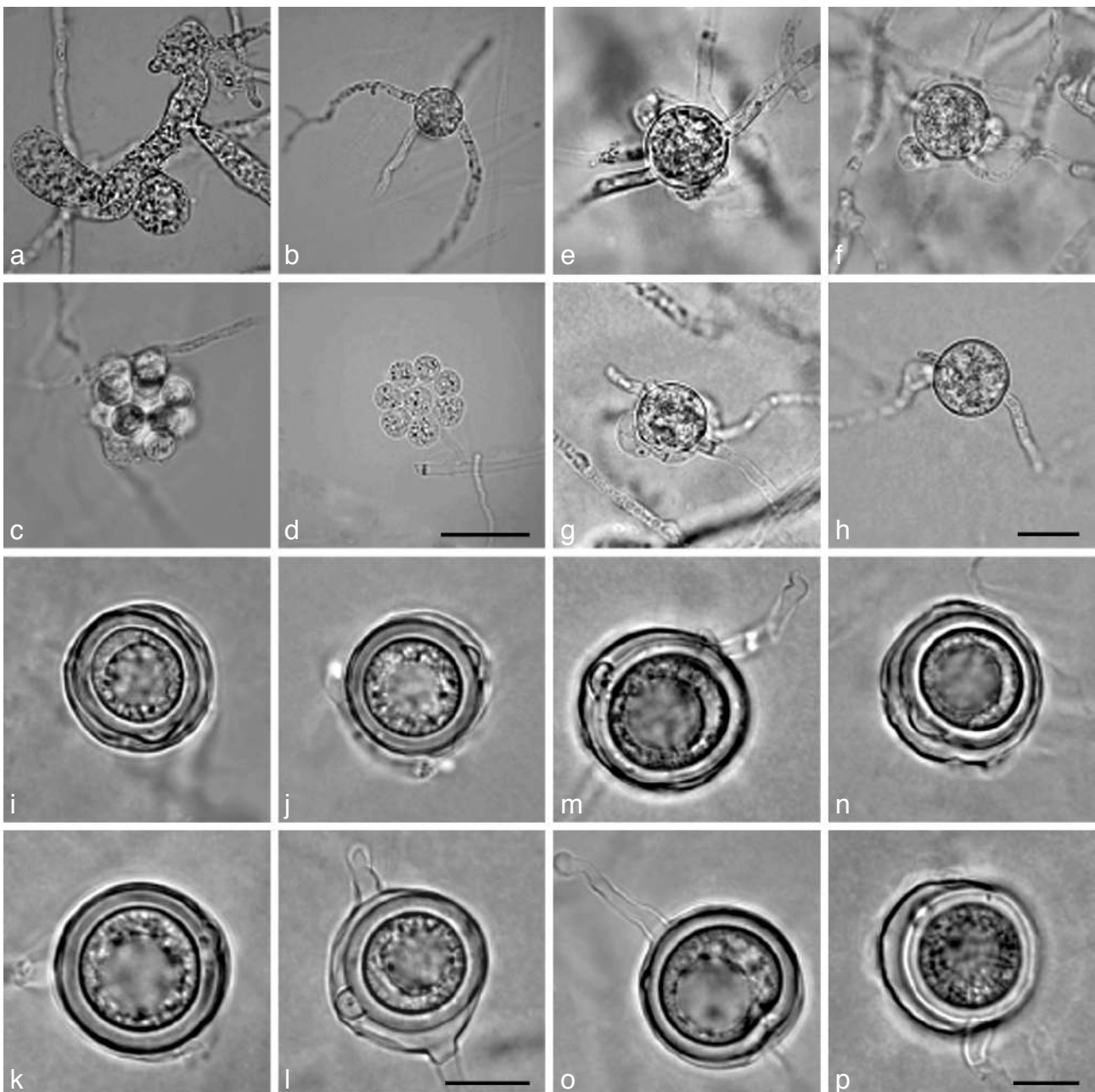


Fig. 2 Asexual and sexual reproduction in *P. oopapillum*. a. Filamentous inflated (lobate) sporangium; b. globose hyphal body germinating directly to give rise to mycelium; c, d. vesicles containing zoospores; e. antheridial cells on branched stalks making apical contact with intercalary oogonium; f. monoclinal antheridia; g. lateral attachment of antheridium to subterminal oogonium; h. diclinal antheridium and subterminal oogonium; i, j. oospores; m, n. spherical thick-walled aplerotic oospores provided with papillae; k. nearly plerotic oospore provided with papilla; m–p. spherical thick-walled oospores provided with papillae. — Scale bars: a–h = 20 μm ; i–p = 10 μm .

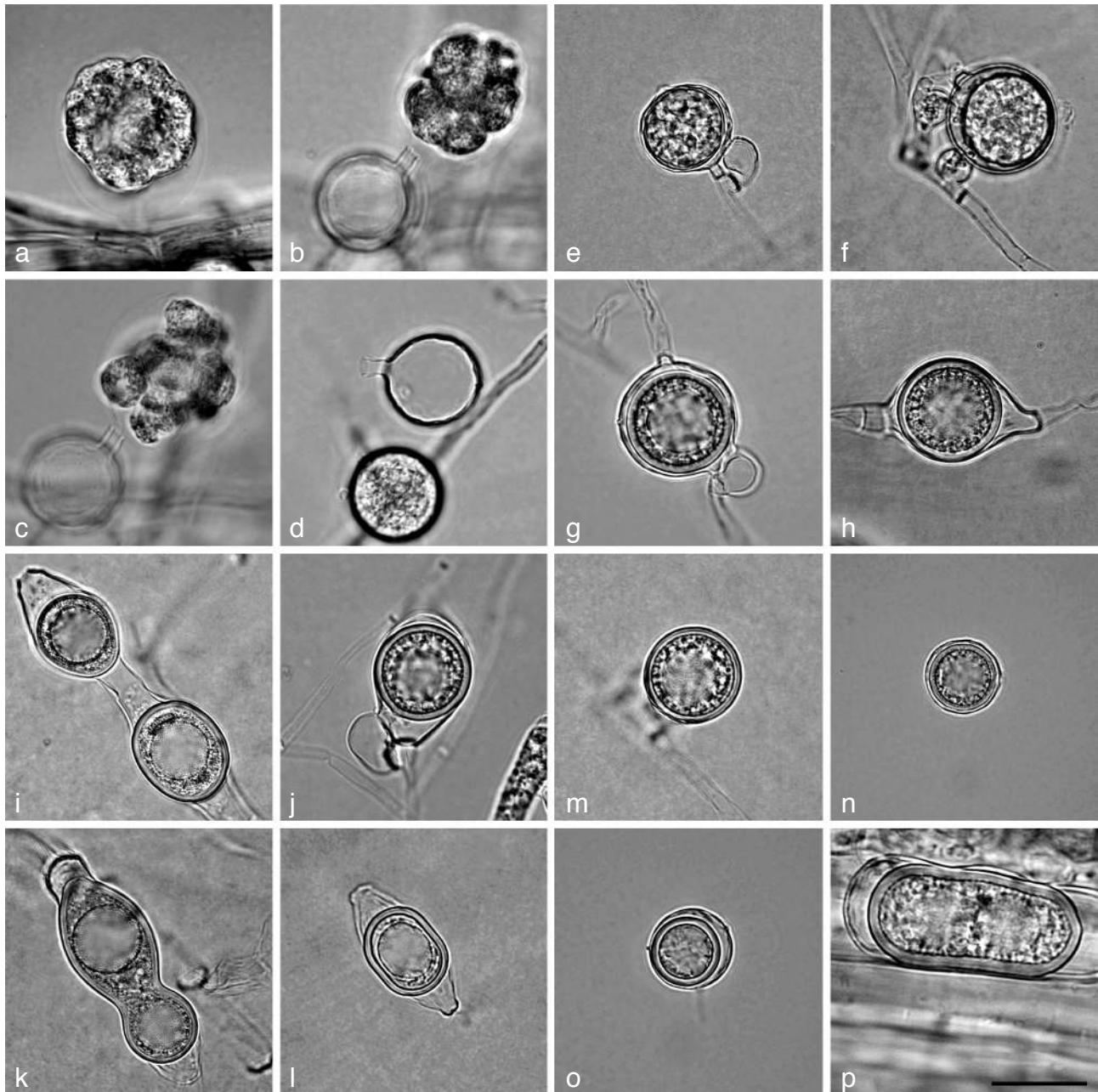


Fig. 3 Asexual and sexual reproduction in *P. emineosum*. a. Vesicle containing developing zoospores; b, c. empty sporangium with a vesicle containing zoospores; d. empty sporangium with a monoclinous antheridium; e. monoclinous antheridial cell arising just below oogonium and making apical contact; f. intercalary antheridia; g. intercalary oogonium with monoclinous antheridium; h. intercalary oogonium provided with hypogynous antheridium; i–l. plerotic oospores; k. peanut-shaped oospore in an elongated oogonium; l. cylindrical oospore; m, n. plerotic oospore; o. aplerotic oospore; p. an elongated oogonium containing elongated oospore. — Scale bars = 10 μ m.

Pythium (Clade F in Lévesque & de Cock 2004)

Pythium emineosum Bala, de Cock & Lévesque, *sp. nov.*

— MycoBank MB514186; Fig. 3, 4

Hyphae hyaline 5–7 μ m crassa. Coloniae in agaro *Potatori carotae* (PCA) chrysanthemale. Sporangia et zoosporae observata. Oogonia globosa, cylindrica vel elongata, terminalia vel intercalaria, 13.6–28.3 μ m diam. Antheridia monoclinata vel diclinata vel hypogynata. Oosporae singulae vel duae, apleroticae vel pleroticae, globosae, 11.9–24.4 μ m diam, paries 0.4–1.2 μ m crassus. Incrementum chrysanthemale quotidianum 15 mm ad 25 °C in agaro *Potatori carotae* (PCA).

Etymology. Name refers to the morphological features of oogonia and oospores that are distinct from the other members of clade F.

Main *hyphae* up to 5–7 μ m wide. *Colonies* on PCA show a broad chrysanthemum pattern with an average radial growth of 15 mm/d at 25 °C. The oomycete grows easily on PCA, CMA and SDA and produces reproductive structures abundantly on

sterile grass blades in water at 15–30 °C. *Sporangia* globose, 12.6–32 μ m diam. Vesicles and zoospores produced plentifully at room temperature. Encysted zoospores usually 7–9 μ m diam developed in water cultures at 15–25 °C within the first two days. *Oogonia* mostly intercalary, occasionally terminal, smooth-walled, globose or cylindrical and peanut-shaped. Globose ones 13.6–28.3 μ m diam, elongated ones 28.8 μ m (av.) in length and 21.4 μ m (av.) in width. *Antheridia* usually 1–3 per oogonium, monoclinous, sessile or hypogynous, or diclinous, occasionally intercalary. *Oospores* 1–2 per oogonium, double oospores very common, elongated, cylindrical, plerotic and aplerotic, 11.9–24.4 μ m diam, wall 0.4–1.2 μ m. The most distinct characteristics of this species are the presence of peanut-shaped oogonia, elongated oogonia, double oospores and various types of antheridial contacts with the oogonia and growth rate of 15 mm/d. All these features together make this species quite distinct from its closely related species.

Fig. 4 *Pythium emineosum*. a. Sporangium; b, c. antheridia fertilising with oogonium; d–f. oogonia containing oospores; d, h. peanut-shaped and elongated oogonia containing double oospores; g, j. oospores. — Scale bar = 10 μ m.

Specimens examined. CANADA, British Columbia, Surrey, Juniper (*Juniperus communis*) roots exhibiting rot, Nov. 1984, C. Holbrook, holotype culture in liquid nitrogen DAOM BR479, culture ex-type CBS 124057. — UNITED KINGDOM, Berkshire, Reading, isolated from soil, 1981, M.S. Ali-Shtayeh, strain DAOM BR836 = CBS 124058 = IMI 308275.

Pythium (Clade E in Lévesque & de Cock 2004)

Pythium camurandrum Bala, de Cock & Lévesque, *sp. nov.*
— MycoBank MB514187; Fig. 5, 6

Hyphae praecipae 5 μ m diam. Coloniae in agar Potatori carotae (PCA) chrysanthemae. Sporangia et zoosporae non observata. Oogonia terminalia, intercalaria vel pyriformia, globosa, 14–22 μ m diam. Antheridia monoclinata vel diclinata vel hypogynata. Cellulae antheridiales longus, curvus, spira vel inflatae. Oosporae singulae vel duas, pleroticae 12–20.5 μ m diam, paries 0.4–1.3 μ m crassus. Augmentum chrysanthemae quotidianum 6 mm ad 25 °C in PCA.

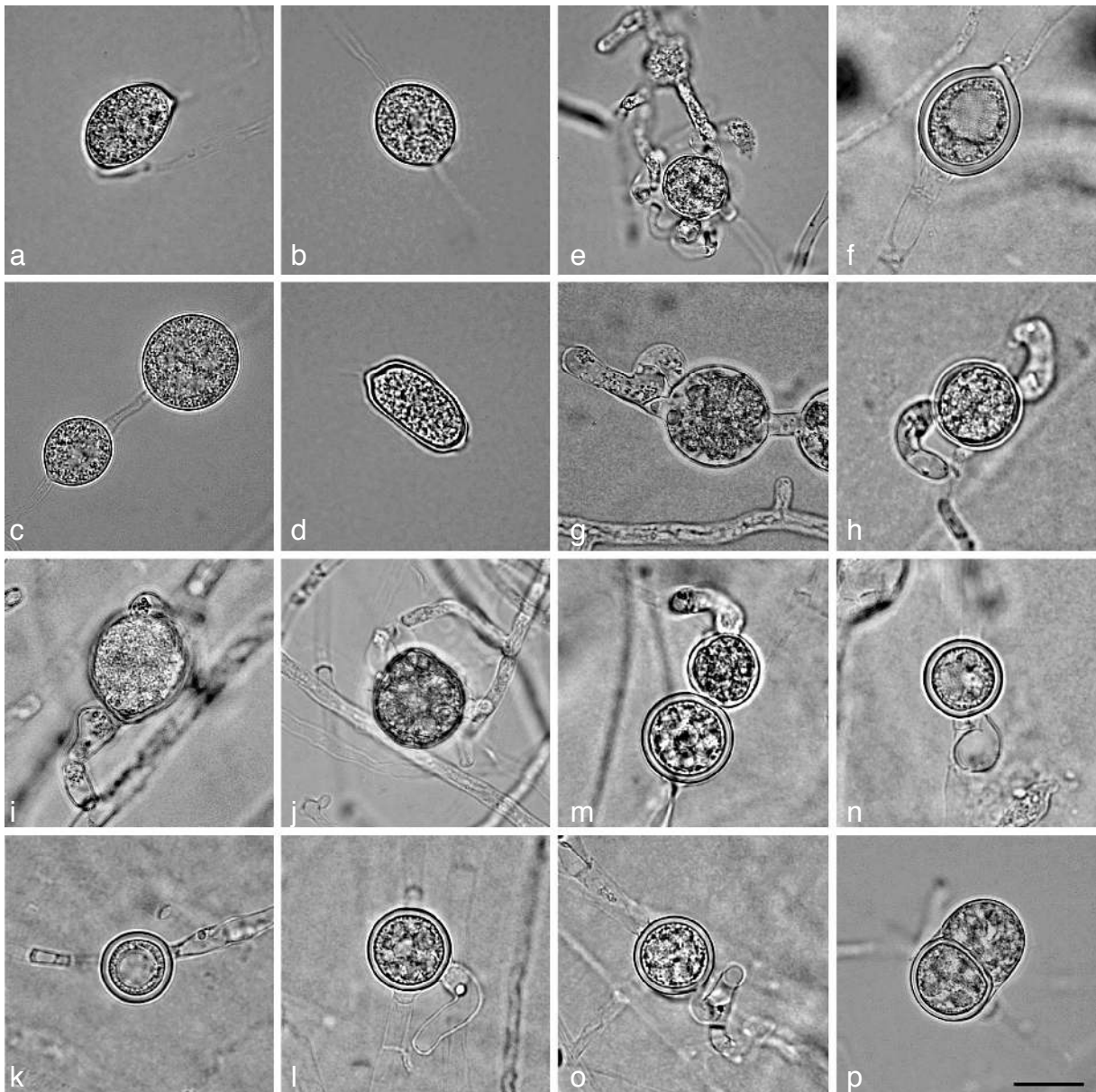
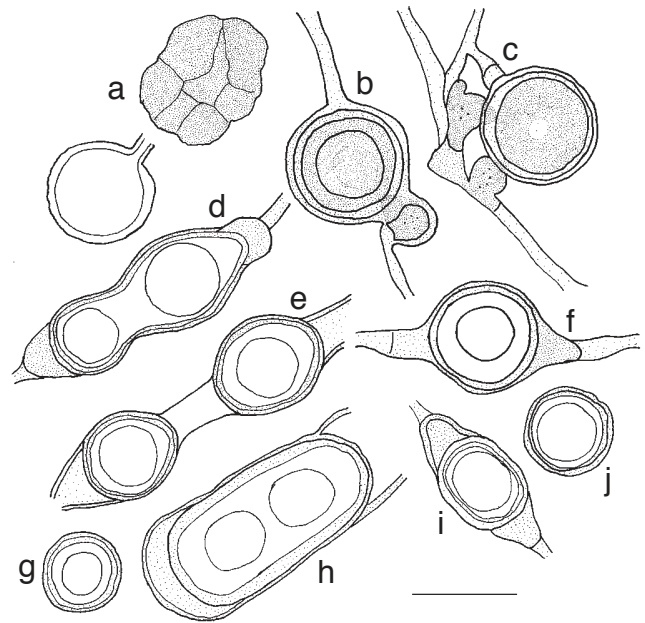


Fig. 5 Asexual and sexual reproduction in *P. camurandrum*; a–d. lemon-shaped, elongated, terminal and intercalary hyphal bodies; e. coiled antheridial stalk surrounding oogonium; f. ellipsoid oogonium with an oospore; g. monoclinous antheridia with peculiar wavy form attached to an intercalary oogonium; h. sausage-shaped antheridia making apical contact with the oogonium; i–l. antheridia and oogonial contacts; j. curved hooked, bifurcated antheridial stalk providing two antheridia to the oogonium; m–o. various contacts of antheridia with oogonia; n. inflated antheridial cell attaching to oogonium; o. antheridia intertwining on its own axis; p. double oospore. — Scale bars = 10 μ m.

Etymology: Name refers to the presence of crooked, curved, bent and hook-shaped antheridia.

Main *hyphae* up to 5 µm diam. *Colonies* on PCA show a narrow chrysanthemum pattern with an average growth rate of 6 mm/d at 25 °C. *Sporangia* and *zoospores* were not produced. Lemon-shaped and irregular hyphal bodies measuring 13–28 µm (av.) in length and 11–21 µm (av.) in width were formed abundantly in water and solid media. *Oogonia* mostly terminal, intercalary, globose and sometimes pyriform, 14–22 µm diam. *Antheridia* 1–2 per oogonium, monoclinal, declinal and hypogynous. Antheridial cells are peculiar on bifurcated stalks, very long, variously shaped typically curved, hooked, wavy, antheridial stalks coiling on their own axis and multiple antheridia surrounding the oogonia and at times inflated. *Oospores* 1–2 per oogonium, peritric, usually 12–20.5 µm diam. Oospore wall 0.4–1.3 µm thick. Double oospores are very common. The unique features of this new species are long antheridial cell, various shapes of antheridia, double oospores that differentiate it from the other closely related species.

Specimens examined. CANADA, Manitoba, Niverville, barley (*Hordeum vulgare*) seedling infected with Flame Chlorosis virus and grown in soil, 1993, D.J.S. Barr, holotype culture in liquid nitrogen DAOM BR876, culture ex-type = CBS 124059. — THE NETHERLANDS, Kennemerland, soil of bulb field, G. van Os strain 11.3, culture CBS 124096.

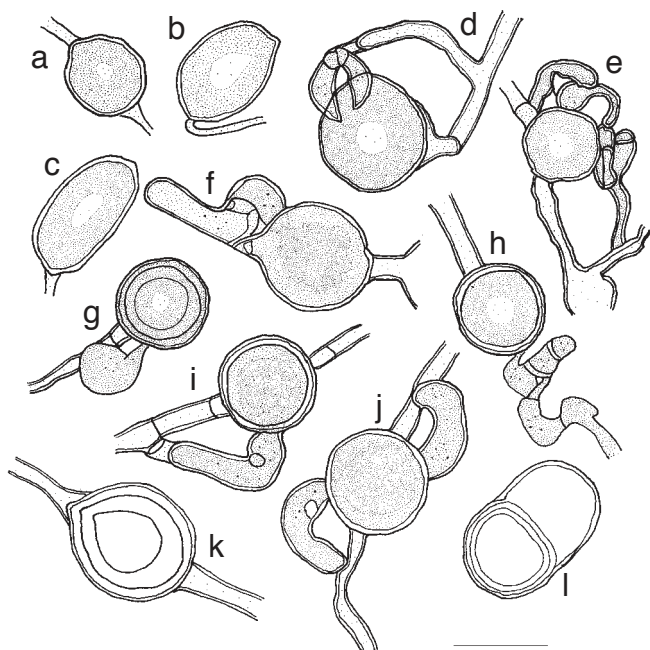


Fig. 6 *Pythium camurandrum*. a–c. Hyphal bodies; d–j. antheridia and oogonia; k. oogonia containing oospores; l. double oospores. — Scale bar = 10 µm.

The comparisons of the morphological features of new species with the related species are provided in the Tables 2–4, respectively.

Phylogenetic analyses

The ITS (779–941 bp), COI (610–727 bp) and LSU rDNA region (1389–1405 bp) sequences were searched by BLAST against all the *Pythium* sequences available in the GenBank and DAOM/CBS collections (Table 1). Neighbour-joining clustering methods were first used to construct phylogenetic trees of nuclear and mitochondrial (COI) barcodes using the 1 000 DAOM and CBS *Pythium* strains that led to the clustering of *P. oopapillum*, *P. emineosum* and *P. camurandrum* into clades B, F and E, respectively (data not shown). Representatives of neighbouring clades were used as outgroups for these three clades and the consensus of the most parsimonious trees produced by heuristic searches confirmed the position of *P. oopapillum*, *P. emineosum* and *P. camurandrum* within clades B, F and E, respectively. High bootstrap values with both maximum parsimony and maximum likelihood strongly supported grouping of the new species and gave consistent results with all three molecular markers (Fig. 7–9). Within each of the new species the isolates had identical COI and LSU sequences with minor differences of 1–2 bases in their ITS.

In maximum parsimony analyses of any of the three molecular markers, all the new species formed monophyletic groups which were supported by high bootstrap values. *Pythium oopapillum* is phylogenetically close to *P. pachycaule*, and *P. coloratum*. *Pythium emineosum* is most closely related to *P. macrosporum* and *P. intermedium*. *Pythium camurandrum* is phylogenetically close to *P. rostratum* and *P. rostratifingens*. An extension of the analysis with representatives from more clades using the LSU rRNA with maximum likelihood and Bayesian inferences further supported the emergence of these three new species of *Pythium* from clades B, F and E (Fig. 10).

DISCUSSION

The three new species reported here, originated from diverse ecological regions of Canada. The different isolates of the same species are isomorphic at different incubation temperatures ranging from 5–30 °C. However, the growth arrested on PCA after 2 d of incubation at 40 °C, but resumed when returned to room temperature. The *P. oopapillum* strain BR180 from Ottawa lost the capability to produce oogonia and oospores during subsequent subculturing. The capability of sexual reproduction could not be regained even by continuous applications of reproduction stimulators such as sterols (Hendrix 1964).

The unique morphological feature of *P. oopapillum* is the presence of papilla on oospores. The other morphological features

Table 2 Morphological differences between *P. oopapillum* and closely related *P. pachycaule* and *P. coloratum* from clade B.

Diagnostic features	<i>P. oopapillum</i>	<i>P. pachycaule</i>	<i>P. coloratum</i>
Growth and pattern on PCA at 25 °C	17 mm/d, vague chrysanthemum	15 mm/d, no particular pattern	20 mm/d, radiate
Hyphae	Up to 4.5 µm	Up to 4–10 µm	Up to 10 µm
Sporangia	Filamentous inflated, lobate elements	Filamentous, slightly inflated	Filamentous, forming dendroid structures
Antheridia	Rarely hypogynous, antheridial stalks branched	Antheridial stalks rarely branched	Antheridial stalks branched
Oogonia	Sub-terminal, intercalary, globose, 17.5–24.5 (av. 21.5 µm)	Terminal, intercalary or lateral, globose, sac-shaped, 24–34 (av. 26.6 µm)	Terminal or intercalary, globose or pyriform, sometimes with a papilla, 20–26 (av. 22.7 µm)
Oospore size	14.5–18 (av. 16.4 µm)	18–25 (av. 22.2 µm)	20–26 (av. 18.9 µm)
Oospore wall	0.8–3 µm (av. 1.9), papillate	1.5–3 µm thick	2–4 µm, lilac coloured

Table 3 Morphological differences between *P. emineosum* and closely related *P. macrosporum* and *P. intermedium* from clade F.

Diagnostic features	<i>P. emineosum</i>	<i>P. macrosporum</i>	<i>P. intermedium</i>
Growth and pattern on PCA at 25 °C	15 mm/d, vague chrysanthemum	28 mm/d, radiate and chrysanthemum-like	30 mm/d, radiate
Sporangia	Globose, terminal and intercalary vesicles and zoospores normally formed	Globose, sub-globose, terminal and intercalary vesicles and zoospores normally formed	Globose, zoospores rarely produced.
Antheridia	Monoclinous, diclinous or hypogynous, lateral, intercalary 1–3 per oogonium	Diclinous, antheridial stalks simple or branched, contorted or inflated 1–4 per oogonium	Diclinous, antheridial stalks long, branched, inflated 1–7 per oogonium
Oogonia	Normally formed, intercalary and terminal, globose, cylindrical, peanut shaped, 13.6–28.3 (av. 19.9 µm)	Sometimes formed in single cultures, abundant in dual cultures of compatible isolates, terminal, occasionally intercalary or lateral, globose 21–30 (av. 24.7 µm)	Formed in dual cultures of compatible isolates, terminal or intercalary, globose, 19–22 (av. 21.5 µm) diam
Oospores	Aplerotic, plerotic 11.9–24.4 (av. 18 µm) diam	Aplerotic, 20–25 (av. 22.4 µm)	Plerotic, occasionally double, 16–20 (av. 17.5 µm)
Oospore wall	0.4–1.2 µm (av. 0.7 µm)	Up to 3 µm thick	1–2 µm thick

Table 4 Morphological differences between *P. camurandrum* and closely related *P. rostratum* and *P. rostratifgens* from clade E.

Diagnostic features	<i>P. camurandrum</i>	<i>P. rostratum</i>	<i>P. rostratifgens</i>
Growth and pattern on PCA at 25 °C	6 mm/d, narrow chrysanthemum	8 mm/d, chrysanthemum	9 mm/d, narrow chrysanthemum
Hyphae	Up to 5 µm	Up to 8 µm	Up to 7 µm
Sporangia	Sporangia and zoospores not observed	Globose, ovoid, limoniform, or ellipsoidal, terminal or intercalary. Vesicles and zoospores normally produced	Globose, intercalary, occasionally terminal. Sporangia do often not form zoospores and germinate directly through hyphae
Antheridia	Monoclinous, diclinous and hypogynous	Monoclinous, mostly sessile or hypogynous	Monoclinous, occasionally diclinous, on a short stalk or hypogynous
Oogonia	Terminal, intercalary, globose or subglobose, 14–22 (av. 16.7 µm)	Mostly intercalary, occasionally terminal, in chains, globose or subglobose, 19–24 µm (av. 21.5 µm)	Intercalary, occasionally terminal, globose, 11–22 (av. 17.4 µm) diam
Oospores	Single, double	Single	Single
Oospore wall	0.4–1.3 µm (av. 0.68)	Up to 2 µm thick	Up to 1.5 µm thick

that distinguish *P. oopapillum* from related members are different size of oogonia and thick-walled papillate, applerotic or nearly plerotic oospores where papilla remains confined within the oogonial wall. Morphologically, this species resembles *P. coloratum*, because of the presence of diclinous antheridia with branched stalks. However, it differs from *P. coloratum* because of the presence of papillate oospores and different size of oogonia and oospores. This species is distinct from other closely related species such as *P. dissotocum* by the occasional presence of hypogynous antheridia and branched antheridial stalks. *Pythium oopapillum* differs from *P. pachycaule* in the size of oogonia and oospores (Table 2) and absence of spindle-shaped double oospores (Ali-Shtayeh & Dick 1985). The isolate fails to grow at 40 °C unlike *P. afertile* (Waterhouse 1967). On the other hand, the presence of mono as well as diclinous antheridia separates it from *P. diclinum* which has typically diclinous antheridia. The presence of papillae on the oospores is a unique character of this species. Within the genus *Pythium* to date, only one species has been reported with reticulate oospores i.e., *P. pythioides* from aquatic plants in France.

The MP and ML analysis of ITS-5.8S and COI suggests that *P. oopapillum* evolved from a common ancestor with all the other members of clade B and forms a monophyletic cluster that is supported with a bootstrap value of 99, 100 % in ITS and 97, 100 % in COI. The isolates BR632 and BR 641 differ in 1 bp from BR180 and CEF 72 in the ITS region (Fig. 7a). The *P. oopapillum* isolates cluster together with bootstrap value 97, 100 % in COI phylogeny (Fig. 7b). The phylogeny with D1–D3 regions of LSU confirms the position of *P. oopapillum* within

clade B2 of Lévesque & de Cock (2004) and is supported by high posterior probabilities (0.9) inferred by Bayesian analysis (Fig. 10). All the *P. oopapillum* isolates are genetically distant in LSU from *P. pachycaule* and other related species in clade B (Fig. 10).

The *oopapillum* strains were found to be associated with crops and agriculture soil in very different climates. These were reported to occur in Barrhead (BR632), cucumber fields in Alberta (BR641, Western Canada) with dry continental climate, central experimental farm cultivated with wheat (BR180), alfalfa and corn in Ottawa (CEF 72, eastern portion of Southern Ontario) with humid continental climate and an extreme range of temperature from -38 °C to +38 °C. Hence, *P. oopapillum* is believed to have a wide host range and to be distributed throughout the geographical habitats of central North America. The phylogenetic position of *P. oopapillum* remains consistently within clade B in multi-gene analyses. None of the other described members in clade B is known to possess papillate oospores. The morphological features together with the multi-gene phylogenetic analysis clusters *P. oopapillum* within clade B. This would be the second new species of *Pythium* of Canadian origin to be included in this clade after *P. aristosporum*.

Pythium emineosum isolates, BR479 and BR836 reproduce readily and produce plentiful globose sporangia and zoospores at room temperature. The production of vesicles and zoospores is a rare character in most members of clade F. *Pythium emineosum* is homothallic and hence different from closely related *P. intermedium* and *P. macrosporum* that are heterothallic and

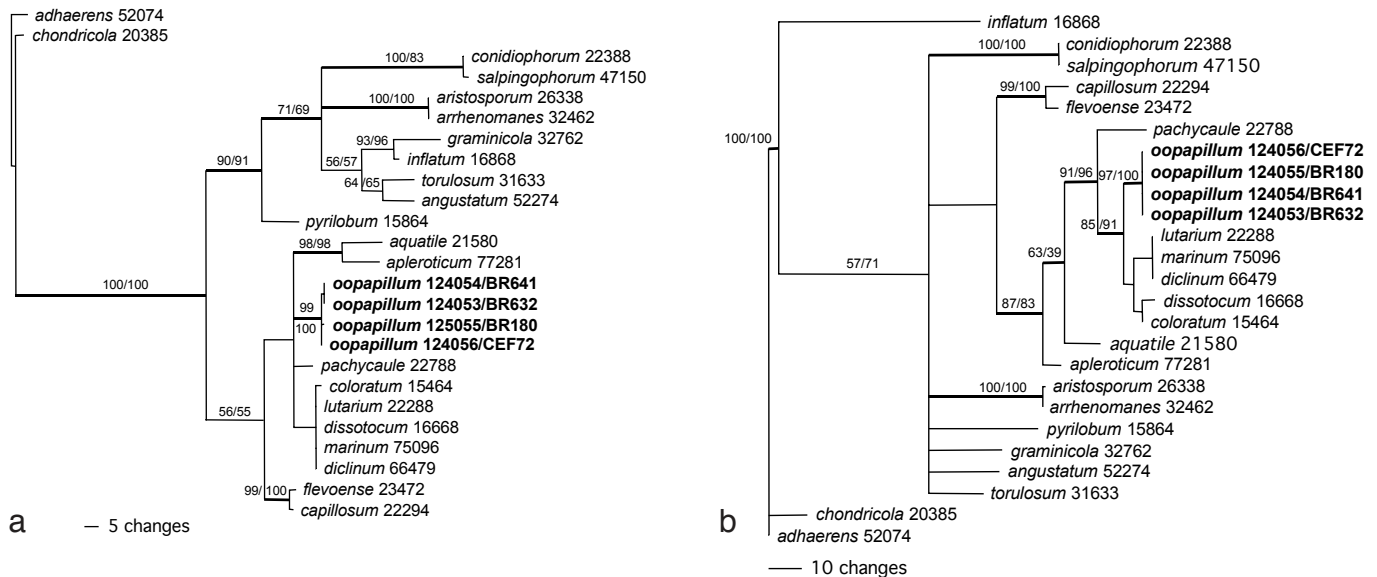


Fig. 7 a. Phylogeny in *P. oopapillum* based on ITS1, 5.8S and ITS2 of nrDNA of clade B2 *Pythium* species (see fig. 4, B2 in Lévesque & de Cock 2004). Consensus of the most parsimonious trees of a heuristic search is given. Numbers over branches represents MP (left) and ML (right) bootstrap values (1 000 replicates). *Pythium adhaerens* and *P. chondricola* from clade A were designated as outgroups. Length = 392, CI = 0.691, RCI = 0.552 and RI = 0.798; b. phylogeny in *P. oopapillum* based on COI of clade B2 *Pythium* species. Consensus of the most parsimonious trees of a heuristic search is given. Numbers over branches represents MP (left) and ML (right) bootstrap values (1 000 replicates). *Pythium adhaerens* and *P. chondricola* from clade A were designated as outgroups. Length = 379, CI = 0.509, RCI = 0.363 and RI = 0.712.

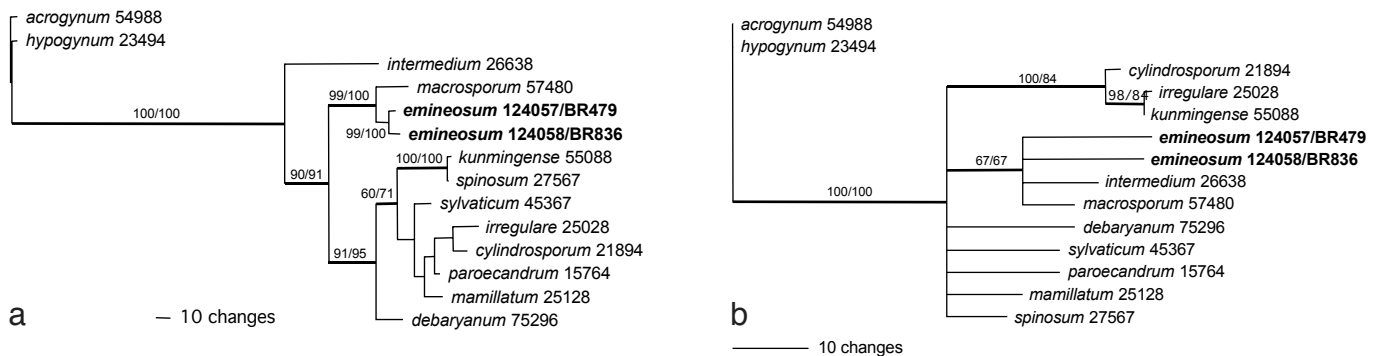


Fig. 8 a. Phylogeny in *P. emineosum* based on ITS1, 5.8S and ITS2 of nuclear rDNA of clade F *Pythium* species (see fig. 4, B2 in Lévesque & de Cock 2004). Consensus of the most parsimonious trees of a heuristic search is given. Numbers over branches represents MP (left) and ML (right) bootstrap values (1 000 replicates). *Pythium acrogynum* and *P. hypogynum* from clade E were designated as outgroups. Length = 555, CI = 0.804, RCI = 0.651 and RI = 0.810; b. phylogeny in *P. emineosum* based on COI of clade F *Pythium* species. Consensus of the most parsimonious trees of a heuristic search is given. Numbers over branches represents MP (left) and ML (right) bootstrap values (1 000 replicates). *Pythium acrogynum* and *P. hypogynum* from clade E were used as outgroups. Length = 161, CI = 0.652, RCI = 0.437 and RI = 0.671.

require two opposite strains for mating. After *P. attrantheridium* this would be another new species of Canadian origin in clade F. *Pythium emineosum* sp. nov. has some striking morphological features that are rarely observed in other members of clade F. This species is distinguished by comparatively lower growth rate of 15 mm/d than other members of clade F; presence of characteristic dumbbell or peanut-shaped and elongated oogonia containing double oospores. All of these morphological features together are unique and distinguishes *P. emineosum* from the closest relatives in clade F.

Pythium emineosum group is supported by a bootstrap value of 100 % and is resolved from *P. macrosporum* in ITS by bootstrap value of 99, 100 %. In COI, *P. emineosum* clusters together with *P. macrosporum* and *P. intermedium* supported by a poor bootstrap value of 67 % (Fig. 8b). *Pythium emineosum* is distinct in LSU from *P. macrosporum* and *P. intermedium* (Fig. 10) and exhibits posterior probability of 0.9 in LSU region (Fig. 10). The morphological features also distinguish *P. emineosum* from

P. macrosporum and *P. intermedium* (Table 3). *Pythium emineosum* has been reported to exist in the mild and wet climate of Victoria, British Columbia and mid-latitude oceanic climate of Berkshire, United Kingdom.

Pythium camurandrum has some features in common with clade E members, such as slow growth rate of 6 mm/d with a narrow chrysanthemum growth pattern on PCA and absence of sporangia and zoospores despite providing sterol supplements (Hendrix 1964). The unique feature that distinguishes *P. camurandrum* from the closest relatives, *P. rostratum* and *P. rostratifengens* are the presence of peculiar antheridia on bifurcated antheridial stalks and a very long antheridial cell that is variously shaped; at times hooked, curved, inflated, intertwining and the presence of characteristic double oopores. *Pythium camurandrum* sp. nov. exists in the continental climate of Niverville, Manitoba (BR876) and moderate maritime climate of Kennemerland, North Holland, The Netherlands (CBS 124096).

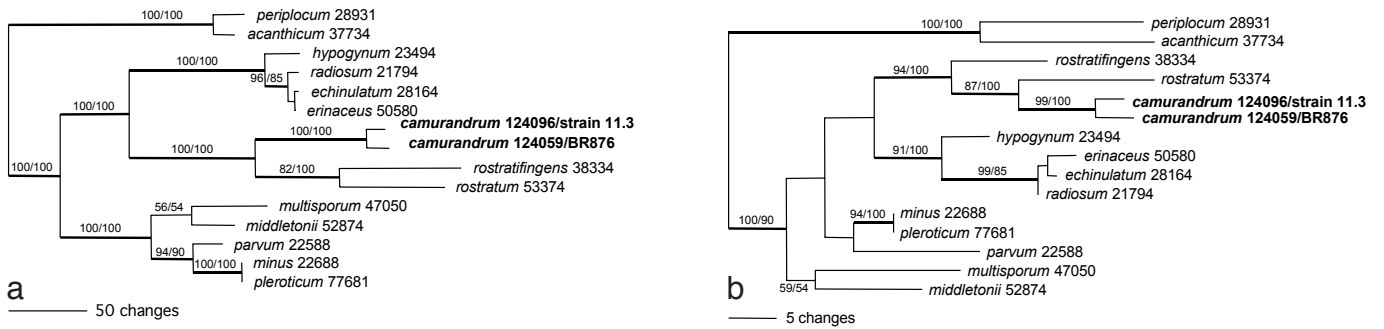


Fig. 9 a. Phylogeny in *P. camurandrum* based on ITS1, 5.8S and ITS2 of nuclear rDNA of clade E *Pythium* species (see fig. 4, B2 in Lévesque & de Cock 2004). Consensus of the most parsimonious trees of a heuristic search is given. Numbers over branches represents MP (left) and ML (right) bootstrap values (1 000 replicates). *Pythium acanthicum* and *P. periplocum* from clade D were designated outgroups. Length = 113, CI = 0.774, RCI = 0.635 and RI = 0.821; b. phylogeny in *P. camurandrum* based on COI of clade E *Pythium* species. Consensus of the most parsimonious trees of a heuristic search is given. Numbers over branches represents MP (left) and ML (right) bootstrap values (1 000 replicates). *Pythium acanthicum* and *P. periplocum* from clade D were designated outgroups. Length = 244, CI = 0.615, RCI = 0.396 and RI = 0.644.

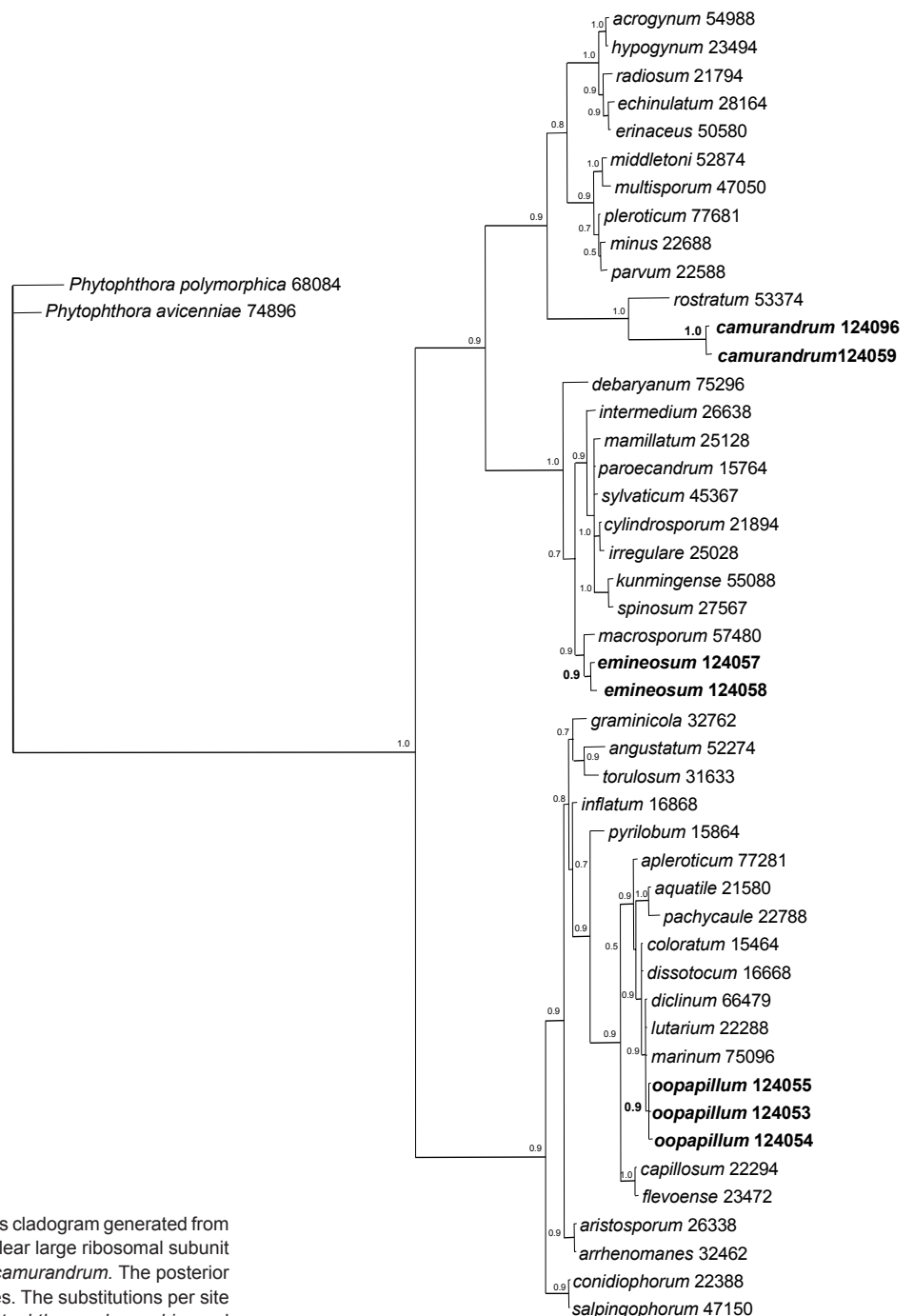


Fig. 10 Bayesian 50 % majority rule consensus cladogram generated from MCMC analysis of D1 to D3 regions of the nuclear large ribosomal subunit (LSU) of *P. oopapillum*, *P. emineosum* and *P. camurandrum*. The posterior probability values are indicated on the branches. The substitutions per site are indicated with a scale bar at the bottom. *Phytophthora polymorphica* and *Phytophthora avicenniae* were designated as outgroups. The new species are printed in **bold** type.

Pythium camurandrum isolates group together in clade E with a bootstrap support of 100 % in ITS and 99, 100 % in COI and posterior probability of 1.0 in LSU region (Fig. 10). *Pythium camurandrum* shows to originate from *P. rostratum*, however the morphological features of the former separate it from the latter (Table 4). The COI, ITS and LSU phylogeny fits *P. camurandrum* significantly into clade E.

The molecular analyses of three gene loci; ITS, COI and LSU and comparison with three computational methods; maximum parsimony, maximum likelihood and Bayesian inferences with similar model of DNA sequence evolution strongly supports a unique clade for the new species. The three molecular markers used in this study provided consistent and well-supported evidence for the three new species. These new species and their phylogenetic position within their clades are well correlated with the morphological analysis. The ITS and COI provided highly variable markers that can differentiate *Pythium* species readily and be used as DNA barcodes for their identification.

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