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 Plas-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), Conmeel 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...
aguar. 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'-CGTAACAAGGTTCGGTAGGTGAC-3') (Lévesque & de Cock 2004) and Un-Lo28S1220 (5'-GTTGTTACACACTCTTAGCGGAT-3') designed for a high annealing temperature. COI amplification was performed using the forward primer Oom-COI-Lev-up 5'-TCAWCWMGATGGCTTTTTTCAAC-3' and the reverse primer FM85-mod 5'- RRHWACKTGACTDATRATACAAAC-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 (GCATACAAATAAGCGAGGAAAG) and Un-lo28S576B (CTCTTTGCTGCGTTTCCAAGAGC) and Un-Up28S577 (CGTCTTGAACAGCGACAGAAGG) and Un-lo28S522 (GTGTTCTTCTCCGGTTATGATG) were used as internal sequencing primers (Schurko et al. 2003). Sequencing reactions were performed 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 calculated 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 praecipae 4.5 µm diam. Coloniae in agaro Potatori carotae (PCA) chrysanthemale. Sporangia filamentosa. Oogonia intercalaria, globosa, subterminalia 17.5–24.5 µm diam. Antheridia monoclinata vel diclinata raro hypogynata. Oosporae singulae, apleroticae vel pleroticae, globosae, subglobosae 14.5–18 µm diam, paries 0.8–3 µm crassus, papillis. Augmentum chrysanthemale quotiadianum 17 mm ad 25 °C in agaro Potatori carotae (PCA). Temperatura minima crescentis 5 °C, optima 30 °C maxima 30 °C.

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

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

| Features                      | P. oopapillum                                      | P. emineosum                                      | P. camurandrum                                    |
|-------------------------------|---------------------------------------------------|--------------------------------------------------|--------------------------------------------------|
| ITS COI                       | 779–780 bp 727 bp                                  | 851–921 bp 717–727 bp                             | 941bp 610 bp                                      |
| GenBank accession number      | FJ655174 FJ655178                                  | GQ244427 GQ244423                                | GQ244426 GQ244425                                |
| CBS accession numbers         | CBS 124053, CBS 124054                             | 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                                              |

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 plentiful 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, monoclinous or diclinous, 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.S. Barr, culture DAOM BR 180 = 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. monoclinous antheridia; g. lateral attachment of antheridium to subterminal oogonium; h. diclinous 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.](image-url)
**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 µm crassa. Coloniea in agaro Potatori carotae (PCA) chrysanthemae. Sporangia et zoosporae observata. Oogonia globosa, cylindrosa vel elongata, terminalia vel intercalaria, 13.6–28.3 µm diam. Antheridia monoclinata vel diconlata vel hypogynata. Oosporae singulae vel duas, apleroticae vel pleroticae, globosae, 11.9–24.4 µm diam, paries 0.4–1.2 µm crassus. Incrementum chrysanthemae 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 µm wide. Colonies on PCA show a broad chrysanthemal 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 µm diam. Vesicles and oospores produced plentifully at room temperature. Encysted zoospores usually 7–9 µm 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 µm diam, elongated ones 28.8 µm (av.) in length and 21.4 µm (av.) in width. Antheridia usually 1–3 per oogonium, monoclinous, sessile or hypogynous, or diconlous, occasionally intercalary. Oosporae 1–2 per oogonium, double oospores very common, elongated, cylindrical, plerotic and aplerotic, 11.9–24.4 µm diam, wall 0.4–1.2 µm. 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. 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; e. monoclinous antheridal cell arising just below oogonium and making apical contact; f. intercalary antheridium; 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 µm.](image-url)
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 µm diam. Coloniae in agaro Potatori carotae (PCA) chrysanthemale. Sporangia et zoosporae non observata. Oogonia terminalia, intercalaria vel pyriformia, globosa, 14–22 µm diam. Antheridia monoclinata vel diclinata vel hypogynata. Cellulae antheridiales longus, curvus, spira vel inflatae. Oosporae singulae vel duae, pleroticae 12–20.5 µm diam, paries 0.4–1.3 µm crassus. Augmentum chrysanthemale quotidianum 6 mm ad 25 °C in PCA.

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

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; g–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 µm.
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 oospores 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, mononclinous, diploid 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, pleonastic, 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 ITSs.

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 RNA 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 | P. oopapillum | P. pachycaule | P. coloratum |
|---------------------|---------------|---------------|--------------|
| 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 |
that distinguish *P. oopapillum* from related members are different size of oogonia and thick-walled papillate, aplerotic 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 oosposes and different size of oogonia and oospores. This species is distinct from other closely related species such as *P. dissolocum* by the occasional presence of hypogynous antheridia and branched antheridial stalks. *Pythium oopapillum* differs from *P. pachyaule* 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 diciploous antheridia separates it from *P. diclinum* which has typically diciploous 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, *P. oopapillum*.

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 B. *Pythium emineosum* is homothallic and hence different from closely related *P. intermedium* and *P. macrosporum* that are heterothallic and

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

| Diagnostic features | *P. emineosum* | *P. macrosporum* | *P. intermedium* |
|---------------------|----------------|------------------|------------------|
| 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, diciploous or hypogynous, lateral, intercalary | Diciploous, antheridial stalks simple or branched, contorted or inflated | Diciploous, antheridial stalks long, branched, inflated |
| 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, pericentral 11.9–24.4 (av. 18.1 µm) diam | Aplerotic, 20–25 (av. 22.4 µm) | Plerotic, occasionaly 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. rostratifingens* from clade E.

| Diagnostic features | *P. camurandrum* | *P. rostratum* | *P. rostratifingens* |
|---------------------|------------------|----------------|-------------------|
| 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 not form zoospores and germinate directly through hyphae |
| Antheridia | Monoclinous, diciploous and hypogynous | Monoclinous, mostly sessile or hypogynous | Monoclinous, occasionally diciploous, 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 (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 |
require two opposite strains for mating. After *P. atrantheridium* 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. rostratifinges* are the presence of peculiar antheridia on bifurcated antheridial stalks and a very long antheridial cell (Table 3).
Fig. 5a. ITS
Figure 5b. COI

outgroups. Length = 244, CI = 0.615, RCI = 0.396 and RI = 0.644.

b.

(1 000 replicates).

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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