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ORIGINAL ARTICLE

Two new *Pythium* species from China based on the morphology and DNA sequence data

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Abstract In an investigation of *Pythium* species in lawn grassland of south China, two new species, *Pythium breve* and *P. baisense*, were identified based on morphological characteristics and DNA sequence data. These two new species differ morphologically from the other *Pythium* species by the oogonia encompassed by many antheridia and with 1–3 antheridia on the wavy and curved stalks. Furthermore, *P. baisense* with complexly lobed antheridial stalks differs from *P. breve* with antheridial stalks entwining the hyphae nearby the oogonia with several turns. Results of phylogenetic analyses showed that these two new species were clearly separated from morphologically similar *Pythium* species based on the internal transcribed spacer region (ITS1, 5.8S, ITS2) and cytochrome c oxidase subunit I (COXI) gene sequences using maximum parsimony and Bayesian methods. These two new species are described and illustrated in detail.

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Keywords *Pythium breve* · *Pythium baisense* · Morphology · ITS · COXI

Introduction

The genus *Pythium* Pringsheim, established by Pringsheim in 1858, belongs to Pythiaceae, Pythiales, Oomycetes, Oomycota, and Straminipila (Hulvey et al. 2010). *Pythium* species are widely distributed throughout the world, and most species are saprobic in soil, water and remains of dead plants and animals. Some species are important pathogens of plants and animals, and some can be beneficial as biological control agents protecting against pathogenic fungi (van der Plaats-Niterink 1981; Ali-Shtayeh and Saleh 1999).

Van der Plaats-Niterink (1981) revised the genus *Pythium* according to morphological characteristics, occurrence and pathogenicity, and accepted 87 species in this genus. More than 60 new *Pythium* species have been described since van der Plaats-Niterink's monograph (Paul 1986, 1991, 1999, 2000, 2001, 2002, 2003a, b, 2004; Mathew et al. 2003; Paul et al. 1998, 2005; Nechwatal and Oßald 2003; Allain-Boulé et al. 2004; Nechwatal et al. 2005; Garzón et al. 2007; Belbahri et al. 2008; De Cock et al. 2008; Karaca et al. 2008, 2009; Moralejo et al. 2008; Senda et al. 2009; Uzuhashi et al. 2009; Bala et al. 2010a; Long et al. 2010).

The taxonomy of *Pythium* was mainly based on the morphological characteristics, such as the size and shape of oogonia, antheridia and sporangia. However, some important morphological structures are highly variable, overlap considerably and are absent in many species. These disadvantages, therefore, contributed to the historical lack of consensus on the most important morphological

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characteristics for species identification (Lévesque and de Cock 2004).

Molecular techniques have been employed in the species identification and systematics of *Pythium*. Internal transcribed spacer regions (ITS1, 5.8S, ITS2) of rDNA sequences have been successfully used in the identification of *Pythium* species (e.g., Paul et al. 1998; Nechwatal and Oßald 2003; Allain-Boulé et al. 2004; De Cock et al. 2008; Karaca et al. 2009), while there were identical ITS sequences in some distinctly morphological species (Lévesque and de Cock 2004; Long et al. 2010) and intra-specific heterogeneity of ITS sequences in some species (Kageyama et al. 2007; Belbahri et al. 2008; Mcleod et al. 2009; Senda et al. 2009). Bala et al. (2010a) pointed out that ITS region and cytochrome c oxidase subunit I (COXI) gene could provide good species recognition and were used as DNA barcodes for *Pythium* species identification. Therefore, *Pythium* species can be accurately identified by the combination of morphological characteristics and DNA sequence data (e.g., Nechwatal et al. 2005; Garzón et al. 2007; Moralejo et al. 2008; Uzuhashi et al. 2009). Recently, Bala et al. (2010b) established a new genus *Phytopythium* Abad, de Cock, Bala, Robideau & Lévesque comprising the *Pythium* species from clade K in Lévesque and de Cock (2004), and Hulvey et al. (2010) transferred this new genus from Pythiaceae of Pythiales to Peronosporaceae of Peronosporales based on the analyses of ITS and partial 28S rDNA gene sequences.

In an ongoing study of *Pythium* species in China, two new *Pythium* species were identified based on morphological characteristics and ITS and COXI sequence data.

Materials and methods

Isolation

Soil samples were collected from lawn grassland in Baise, Guilin, Liuzhou and Nanning of Guangxi, China. The samples were taken to the laboratory for isolation within 7 days. The particles of the soil samples were put on Petri dishes with corn meal agar (CMA) and were incubated at 25°C for 2–3 days. When mycelial growth was observed, purification was carried out by cutting a small piece of media with mycelia at the edge of a colony and then transplanted onto new medium plates.

Morphology and growth rate

The purified isolates were grown on CMA for morphological studies. *Pythium* isolates have been transferred to sterilized distilled water for sporulation. Fifty measurements were taken for each morphological characteristics, such as sporangia, oogonia and oospores.

The cardinal temperatures were examined on potato carrot agar (PCA) according to the method of van der Plaats-Niterink (1981), and growth rates were measured at 24 h incubation. Each isolate was incubated at 5–40°C with intervals of 5°C on PCA media. When no growth was observed, the intervals were reduced from 5 to 2 or 1°C and the culture was returned to room temperature to check whether or not growth resumed.

DNA extraction, PCR amplification and sequencing

Isolates of *Pythium* species were cultured on sterilized cellophane sticked on potato dextrose agar (PDA) at 25°C for 3–5 days. Fresh cultures were harvested by sterilized spatulas. Genomic DNA was extracted from fresh cultures following the protocol of Guo et al. (2000). The ITS regions were amplified with universal primer pairs ITS5 and ITS4 (White et al. 1990). COXI gene was amplified with primer pairs FM52R (5′-TTAGAATGGAATTAGCA CAAC-3′, reversed the complementation of FM52) and FM55 (5′-GGCATACCAGCTAAACCTAA-3′, Martin 2000). Amplification was performed in a 50-μL reaction volume which contained PCR buffer (20 mM KCl, 10 mM $(NH_4)_2SO4$, 2 mM $MgCl_2$, 20 mM Tris-HCl, pH8.4), 200 μM of each deoxyribonucleotide triphosphate, 15 pmols of each primer, 100 ng template DNA, and 2.5 units of Taq DNA polymerase (Biocolor BioScience and Technology, Shanghai, China). The thermal cycling program was as follows: 3 min initial denaturation at 95°C, followed by 35 cycles of 40 s denaturation at 94°C, 50 s primer annealing at 54°C, 60 s extension at 72°C for ITS, 70 s extension at 72°C for COXI, and a final 10 min extension at 72°C.

PCR products were purified using PCR Cleanup Filter Plates (MultiScreen® PCRμ96; Millipore, USA) according to the manufacturer's protocol. Purified PCR products were directly sequenced with primer pairs as mentioned above in an ABI 3730-XL DNA sequencer (Applied Biosystems, USA).

Phylogenetic analysis

The sequences were aligned with MAFFT using the G-INS-i option (Katoh et al. 2005). All sites were treated as unordered and unweighted, and gaps were treated as missing in the phylogenetic analyses. Maximum parsimony analyses of the alignment ITS and COXI datasets were conducted with the heuristic search algorithm with tree-bisection-reconnection branch swapping in PAUP 4.0b10 (Swofford 2002). For each search, 1,000 replicates of

random stepwise sequence addition were performed and all trees were saved per replicate. The strength of the internal branches of the trees was tested with bootstrap analyses using 1,000 replications with the same search settings.

Bayesian analyses of the same alignment dataset were conducted in MrBayes 3.1.2 (Huelsenbeck and Ronquist 2001). The best-fit evolutionary model was determined for each dataset by comparing different evolutionary models via MrModeltest 2.3 (Nylander 2008). The general time-reversible model of molecular evolution was used for both ITS and COXI datasets according to the MrModeltest suggestion, in which a proportion of the site was assumed to be invariable, while the rate for the remaining sites was approximated from a gamma distribution with four categories (GTR + I + G), and a random starting tree. The prior probability density is a flat Dirichlet (all values are 1.0) for both Revmatpr and Statefreqpr as default settings. Four simultaneous chains of Markov Chain Monte Carlo were run starting from random trees and sampling every 100 generations. The analyses were halted at 1,000,000 generations, when the calculation approached to stationarity. At the end of the analysis, 10,000 trees were generated and 25% of them were excluded as the "burn in" when calculating the posterior probabilities. Bayesian posterior probabilities were obtained from the 50% majority rule consensus of the trees kept. If more than 95% of the sampled trees contained a given clade, it was considered to be significantly supported by our data.

Results

Taxonomy

Pythium breve Y.Y. Long, J.G. Wei & L.D. Guo sp. nov. (Fig. 1)

MycoBank. MB 561132

Etymology The oomycete is named as *P. breve* because of its short side-branches on hyphae.

Latin description Hyphae praecipae usque ad 5 μm diam; Sporangia terminalia vel intercalaria, globosa vel subglobosa, interdum papillis, 11.3–31.3 μm diam, non prolifis; Oogonia globosa, subglobosa, intercalaria, interdum terminalia, 22.5–31.3 μm; Antheridia diclinata, interdum monoclinata, uno vel duobus per oogonium, interdum plures frequentatio inter singulum oogonium, clavatis vel elongato, interdum crispans in contactus, stipite elongato curvo saepe sinuato praeditis; Oosporae singulae, pleuroticae, interdum apleuroticae, globosa, 17.5–28.8 μm diam. Incrementum radiale quotiadianum 16 mm 25°C in agaro Solani tuberosi et Dauci carotae (PCA). Holotypus in Herbario Mycologico Academiae Sinicae in Beijing (HMAS242231) conservatur.

Holotype China, Guangxi, Nanning, from the soil of dawn, Y.Y. Long, 10.4.2009 HMAS242231. Living culture (CNN213) deposited in the Department of Plant Protection, College of Agriculture, Guangxi University.

The oomycete grows well on CMA, PCA and PDA. Its mycelium in water hyaline, well branched, the main hyphae up to 5 μm wide, and dendroid, often with short side-branches (Fig. 1a). Colonies on PCA submerged, without a special pattern. Average growth rates 3 mm day^{-1} at 15°C, 8 mm day^{-1} at 20°C, 16 mm day^{-1} at 25°C, 17.5 mm day^{-1} at 30°C, 7.5 mm day^{-1} at 35°C, no growth at 7°C and 38°C, but when returned to room temperature both of them started to grow again. Cardinal temperatures: minimum 7°C, optimum 30°C, maximum 38°C. Appressoria sausage-shaped, often catenulate, occasionally present (Fig. 1b). Zoospores formed in sterile water at 20°C (Fig. 1c). Sporangia globose or subglobose, sometimes provided with papilla, occasionally oval to elongate or gourd-shaped; mostly terminal, sometimes intercalary, 11.3–31.3 (average 21) μm in diameter (Fig. 1d–i). The female gametangia (oogonia) globose, subglobose or sac-like, smooth-walled, at times with a papilla, mostly terminal, sometimes intercalary (Fig. 1j–k, o, s), 22.5–31.3 μm in diameter (average 28.2 μm). The female gametangia filled with dense, coarsely granulated protoplasm. The male gametangia (antheridia) mostly diclinous, occasionally monoclinous, sometimes crowding around the oogonia, making it very difficult to determine their origin, type of contact and numbers (Fig. 1k–l). Antheridial stalks sometimes wavy and more or less curved, arising at various distances from the oogonium, at times entwining the hyphae nearby the oogonium with several turns (Fig. 1m, n). Antheridial cells club-shaped or elongated, sometimes wavy in contour or large, 1–8 per oogonium, making apical or lateral contact with the oogonium (Fig. 1k, p–q, t). Oospores plerotic or nearly plerotic, at times aplerotic, globose, 17.5–28.8 μm in diameter (average 24.2 μm), generally single, rarely 2 in an oogonium, colorless (Fig. 1j–k, r).

Pythium baisense Y.Y. Long, J.G. Wei & L.D. Guo sp. nov. (Fig. 2)

MycoBank. MB 561133

Etymology The oomycete is named as *P. baisense* because its holotype strain was isolated from lawn soil collected from Baise city, Guangxi Province, P.R. China.

Latin description Hyphae praecipae usque ad 6.3 μm diam; Sporangia terminalia vel intercalaria, globosa vel subglobosa, interdum papillis, 5–15 μm diam, non prolifis; Oogonia globosa, subglobosa, terminalia vel

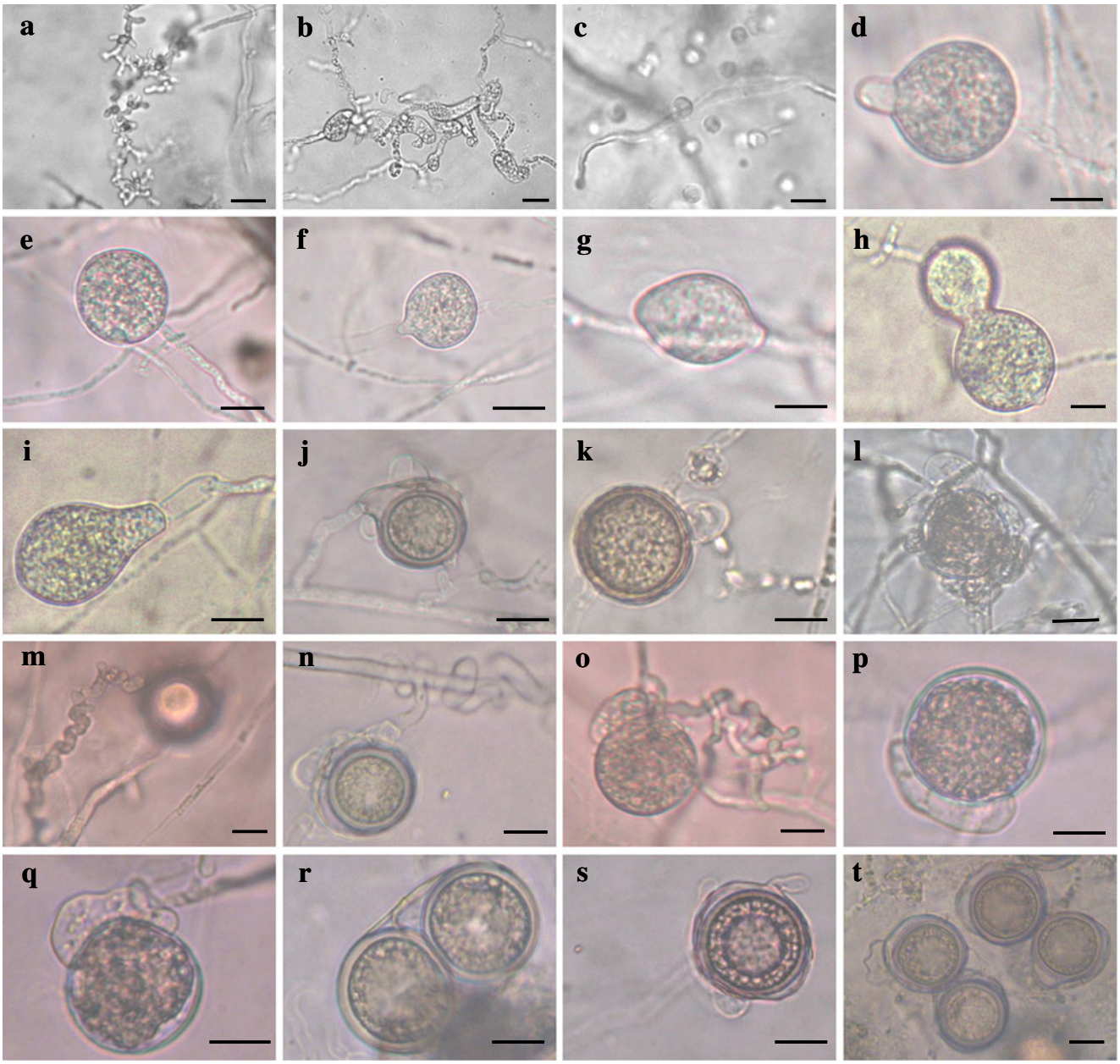

Fig. 1 Asexual and sexual reproductive bodies of *Pythium breve*. **a** Hyphal dendroid structures; **b** sausage-shaped appressoria; **c** released zoospores; **d** globose sporangium provided with papilla; **e** terminal sporangium; **f** intercalary sporangium; **g** oval sporangium; **h** gourd-shaped sporangium; **i** elongate sporangium; **j** sac-like oogonium with aplerotic oospore; **k** intercalary oogonium with plerotic oospore; **l** many antheridia crowding around oogonia; **m** wavy and curved antheridial stalk; **n** antheridial stalk entwining the hypha nearby the oogonium with several turns; **o** diclinous antheridium; **p** elongate antheridial cell; **q** large antheridial cell; **r** 2 oospores in an oogonium; **s** oogonium with a pappila; **t** antheridial cell wavy in contour. *Scale bar* 10 μm

emanio directus ex hyphae, interdum pleurogena, 13.8–28.8 μm; Antheridia diclinata vel monoclinata, interdum emanio directus ex hyphae vel plures frequentatio inter singulum oogonium, inflatae campanulatae, clavatis vel incomposite, interdum elongate et crispans in contactus antheridial coma interdum crispans quod pandus vel universa lobed; Oosporae singulae, interdum duobus, apleuroticae, interdum pleuroticae, globosa, 11.3–23.8 μm diam. Incrementum radiale quotiadianum 30 mm 25°C in agaro Solani tuberosi et Dauci carotae (PCA). Holotypus in Herbario Mycologico Academiae Sinicae in Beijing (HMAS242232) conservatur.

Holotype China, Guangxi, Baise, from the soil of dawn, Y.Y. Long, 5. 10. 2009, HMAS242232. Living culture (QBS123) deposited in the Department of Plant Protection, College of Agriculture, Guangxi University.

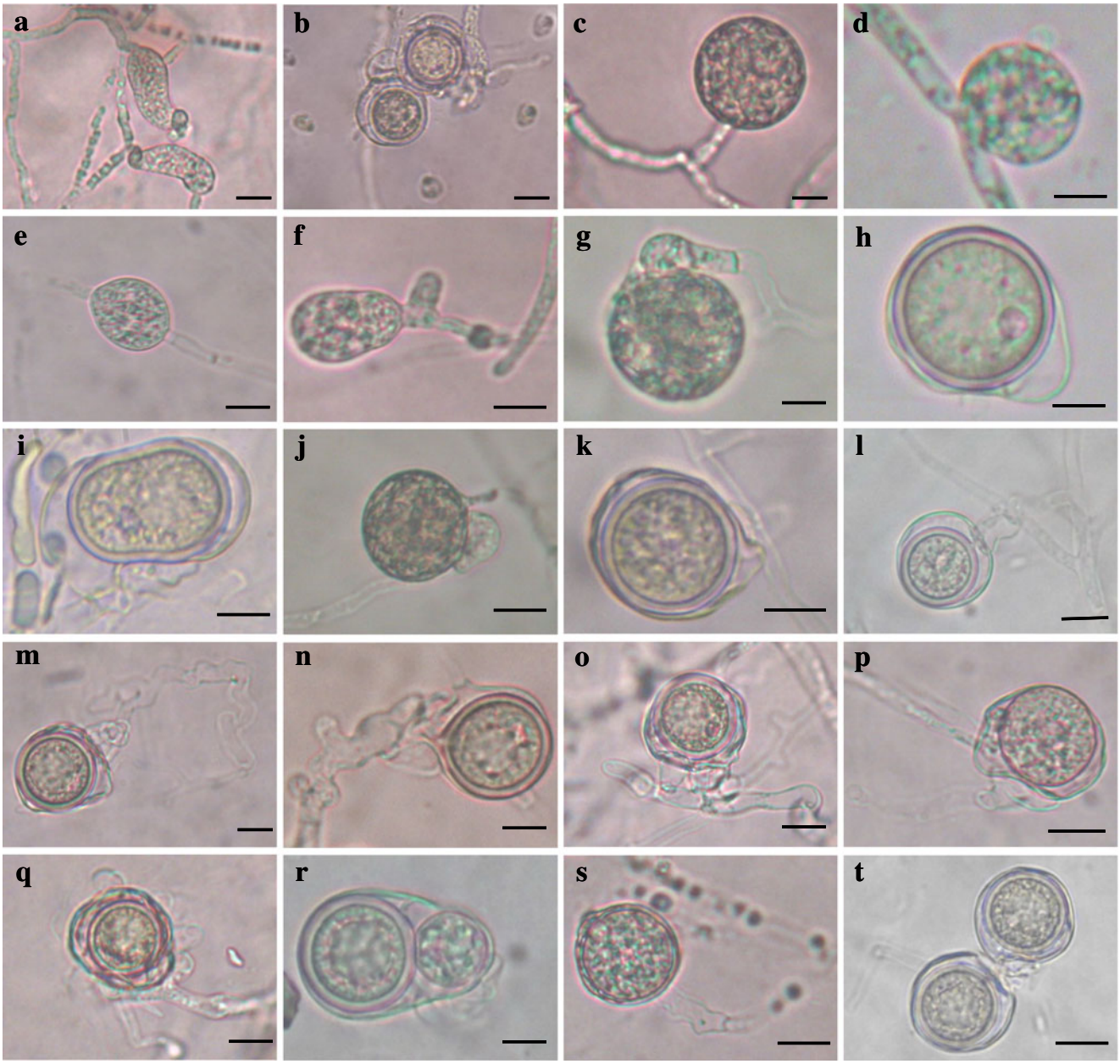

Fig. 2 Asexual and sexual reproductive bodies of *Pythium baisense*. **a** Sausage-shaped appressoria; **b** released zoospores; **c** globose sporangium; **d** lateral sporangium with a papilla; **e** intercalary sporangium; **f** elongate sporangium; **g** globose oogonium; **h**–**i**, p sac-like oogonium with aplerotic oospore; **j**, **l** antheridium contact with oogonium in the neighbourhood of the oogonial stalk; **k** oogonium originating immediately from the hyphae; **m** wavy and curved antheridial stalk; **n** intercalary oogonium with antherium on complexly lobed stalk; **o** antheridia originating immediately from the hyphae; **q** clustering of several antheridial cells around the oogonium; **r** 2 oospores in an oogonium; **s** elongated antheridial cell wavy in contour; **s** 2 oogonia clustering in a small group. *Scale bar* 10 μm

Except the type strain, isolate XGL324 collected from the soil of lawn in Guilin, Guangxi, China, by Y.Y. Long, 15.7.2009. Isolate CLZ223 collected from the soil of lawn in Liuzhou, Guangxi, China, by Y.Y. Long, 3.5.2009. Living culture all deposited in the Department of Plant Protection, College of Agriculture, Guangxi University.

The oomycete grows well on CMA, PCA and PDA. Its mycelium in water hyaline, well branched, the main hyphae up to 6.3 μm diam. Colonies on PCA showed a vague rosette pattern, average growth rates 9.5 mm day^{-1} at 15°C, 21 mm day^{-1} at 20°C, 30 mm day^{-1} at 25°C, 36 mm day^{-1} at 30°C, 20 mm day^{-1} at 35°C, no growth at 8°C and 39°C, but when returned to room temperature both of them started to grow again. Cardinal temperatures: minimum 8°C, optimum 30°C, maximum 39°C. Appressoria sausage-shaped, sometimes catenulate, often present (Fig. 2a).

Zoospores formed in sterile water at 25°C (Fig. 2b). Sporangia globose, subglobose to elongate, at times with a papilla, terminal or intercalary, 5–15 μm in diameter (average 12.3 μm) (Fig. 2c–f). The female gametangia (oogonia) globose or subglobose, sometimes sac-like, smooth-walled, originating directly from the hyphae or terminal on short side branches, occasionally lateral, 13.8–28.8 μm in diameter (average 21.2 μm), sometimes 2–4 clustering in small groups (Fig. 2g–l, s–t). The female gametangia filled with dense, coarsely granulated protoplasm. The male gametangia (antheridia) monoclinous and diclinous, sometimes terminal on wavy and curved antheridial stalks or originating directly from the hyphae or crowding around the oogonia, making it very difficult to determine their origin, type of contact and numbers (Fig. 2g, m, o, q). Antheridium occasionally with complexly lobed stalk (Fig. 2n). Antheridial cells bell-shaped, club-shaped or irregular, occasionally elongated and wavy in contour, 1–8 per oogonium, often making apical or lateral contact with the oogonium in the neighbourhood of the oogonial stalk (Fig. 2g, j, l–q, s). Oospores mostly aplerotic, sometimes nearly plerotic, globose, sometimes subglobose, rarely peanut-shape or elongate, 11.3–23.8 μm in diameter (average 18.5 μm), generally single, sometimes 2 in an oogonium, colorless (Fig. 2k–r).

Phylogenetic analyses

The ITS sequences of two new species, 32 reference taxa of *Pythium* (Pythiaceae) and *Phytopythium boreale*, *P. helicoides* and *P. vexans* (Peronosporaceae) used as the outgroup were included in the phylogenetic analyses. In the alignment of the 37 taxa, the data matrix comprised 1,279 characters, of which 432 (33.8%) characters were constant and 666 (52.1%) were parsimony informative. The maximum parsimony (MP) analysis of the alignment dataset was subjected to a heuristic search for the most parsimonious trees and a strict consensus tree was

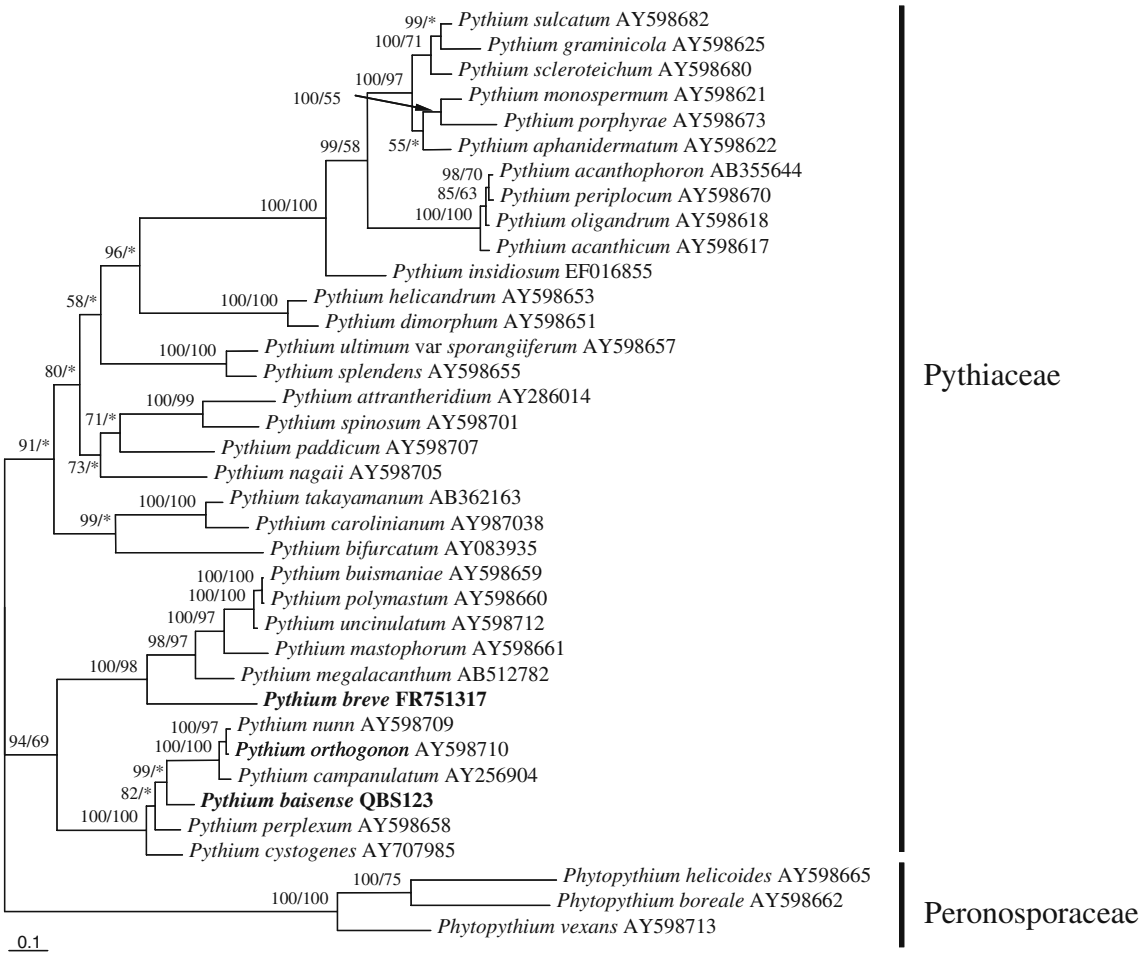

Fig. 3 Bayesian tree based on ITS (ITS1, 5.8S, ITS2) region sequences. The tree was rooted with *Phytopythium helicoids*, *P. boreale* and *P. vexans*. The numbers at each branch point represented Bayesian posterior probabilities (*left*) and percentage bootstrap support calculated from 1,000 replicates (*right*). *Indicates lack of support or support less than 50% for a particular clade. *Bar* 0.1 expected changes per site

obtained from the five equally most parsimonious trees (CI=0.4612, RI=0.6611, RC=0.3049, HI=0.5388). The same alignment dataset was also performed using MrBayes program, and a similar topology as the strict consensus tree was obtained. Therefore, a Bayesian tree with posterior probabilities (BPP) and parsimony bootstrap values at branches is shown in Fig. 3. In the phylogenetic tree, *P. breve* formed a clade with *P. buismaniae* Plaats-Niterink, *P. mastophorum* Drechsler, *P. megalacanthum* de Bary, *P. polymastum* Drechsler and *P. uncinulatum* Plaats-Niterink with strong support (BPP 100%, MP 98%). *P. baisense* formed a clade with *P. cystogenes* De Cock & Lévesque, *P. perplexum* H. Kouyeas & Theoh, *P. campanulatum* R. Mathew, K.K. Singh & B. Paul, *P. orthogonon* Ahrens and *P. nunn* Lifsh, Stangh & R.E.D. Baker with 100% support of BPP and MP. These two new species formed a single branch, respectively.

Phylogenetic study was also performed based on COXI sequences from two new species and ten reference *Pythium* species, with *Phytopythium boreale*, *P. ostracodes* and *P. vexans* as the outgroup. In the alignment of the 15 taxa, the data matrix comprised 593 characters, of which 420 (70.8%) characters were constant and 127 (21.4%) were parsimony informative. The maximum parsimony analysis was subjected to a heuristic search for the most parsimonious trees and a strict consensus tree was obtained from four equally most parsimonious trees (CI=0.6113, RI=0.6113, RC=0.3736, HI=0.3887). The same alignment dataset was also performed using MrBayes program, and a similar topology as the strict consensus tree was obtained. Therefore, a Bayesian tree with posterior probabilities and parsimony bootstrap values at branches was shown in Fig. 4. In the phylogenetic tree, *P. breve* formed a clade with *P. buismaniae*, *P. mastophorum* and *P. uncinulatum* (BBP 93%, MP 59%). *P. baisense* clustered together with *P. perplexum* with relatively low support.

Discussion

The isolate of *P. breve* was obtained from the lawn soil collected in Nanning, Guangxi Province, China. This new species is characterized by smooth oogonia crowded by many antheridia, the large antheridial cells to be sometimes wavy in contour, and the antheridial stalks entwining the hyphae nearby the oogonia occasionally with several turns. Although many *Pythium* species have some similarly morphological features with *P. breve*, the distinctly wavy antheridial stalks were reported only in *P. bifurcatum* B. Paul, *P. scleroteichum* Drechsler, and *P. takayamanum* M.

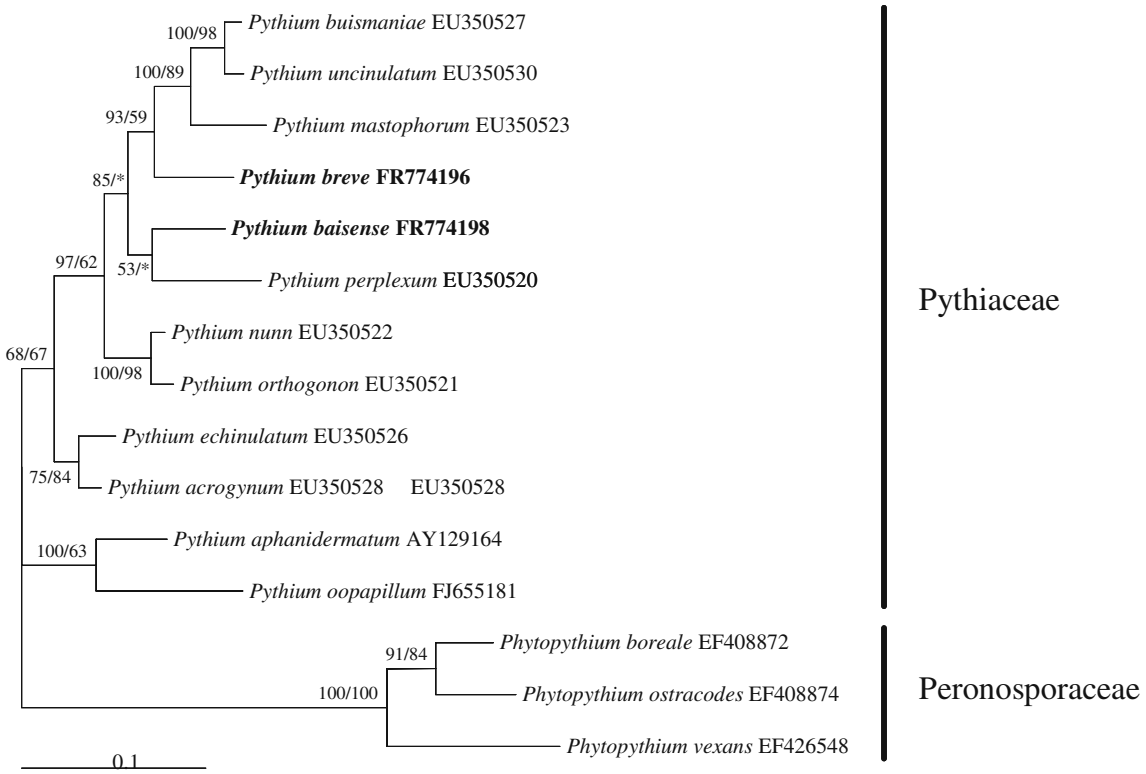

Fig. 4 Bayesian tree based on COXI sequences. The tree was rooted with *Phytopythium boreale*, *P. ostracodes* and *P. vexans*. The numbers at each branch point represented Bayesian posterior probabilities (*left*) and percentage bootstrap support calculated from 1,000 replicates (*right*). *Indicates lack of support or support less than 50% for a particular clade. *Bar* 0.1 expected changes per site

Table 1 A comparison of *Pythium breve* with morphologically similar species

Character	*P. breve*	*P. bifurcatum*	*P. scleroteichum*	*P. takayamanum*	*P. palingenes*
Growth rate on PCA	16 mm day^{-1} at 25°C	11 mm day^{-1} at 25°C	20 mm day^{-1} at 25°C	15.4 mm day^{-1} at 30°C	Unknown
Sporangia	Globose or subglobose, 11.3–31.3 μm diam (av. 21 μm), terminal, sometimes intercalary	Globose to cylindrical, 25–50 μm diam (av. 35 μm), mostly intercalary	Never observed	Subglobose, 13–28 μm diam (av. 19.4 μm), terminal or intercalary	Subglobose or ovoid, 24–42×18–33 μm diam (av. 33×29 μm), papillate, terminal
Oogonia	Globose or subglobose, sometimes sac-like, smooth, 22.5–31.3 μm diam (av. 28.2 μm), intercalary or terminal	Globose, smooth, 17–36 μm diam (av. 26.7 μm), terminal, subterminal, rarely intercalary	Globose, smooth, (17) 21–29 (34) μm diam (av. 25.3 μm), terminal or intercalary	Globose to subglobose or sac-like, ellipsoidal, smooth, 12–21 μm diam (av. 14.9 μm), terminal or intercalary	Subglobose, smooth, (19) 28–40 (41) μm diam (av. 34 μm), terminal, sessile, intercalary or unilaterally
Antheridia	1–8 per oogonium, diclinous, occasionally monoclinous	Often 2 per oogonium, hypogynous or monoclinous sessile or monoclinous	1–5 per oogonium, monoclinous, sometimes diclinous	1–2 per oogonium, monoclinous, occasionally diclinous, sessile	(1) 2 (4) per oogonium, diclinous
Oospores	Globose, 17.5–28.8 μm diam (av. 24.2 μm), plerotic or nearly plerotic, sometimes aplerotic, 1 (2) per oogonium	Globose, 16–35 μm diam (av. 24.9 μm), plerotic, sometimes aplerotic, 1 (2) per oogonium	Globose, (13) 16–25 (30) μm diam (av. 20 μm,) aplerotic, 1 per oogonium	Globose, 11–19 μm diam (av. 13.8 μm), aplerotic to nearly plerotic, 1 per oogonium	Subglobose, (18) 26–36 (37) μm diam (av. 31.3 μm), aplerotic, 1 per oogonium
Reference	This study	Paul 2003a	Van der Plaats-Niterink 1981	Senda et al. 2009	Van der Plaats-Niterink 1981

Table 2 A comparison of *Pythium baisense* with related species

Character	*P. baisense*	*P. hypoandrum*	*P. perplexum*	*P. campanulatum*
Growth rate on PCA	30 mm day^{-1} at 25°C	Unknown	15 mm day^{-1} at 25°C	15 mm day−1 at 25°C
Sporangia	Globose, subglobose to elongate, 5–15 μm diam (av. 12.3 μm) , terminal or intercalary	Globose or subglobose, 13–29 μm diam (av. 20.4 μm), mostly terminal, sometimes intercalary	Globose, elongated, 13–41 μm diam (av. 17 μm), terminal or intercalary, occasionally in series	Globose to oval, somewhat elongated, 11–25 μm diam (av. 18 μm), terminal or intercalary
Oogonia	Globose or subglobose, sometimes sac-like, smooth, 13.8–28. 8 μm diam (av. 21.2 μm), terminal or sessile, occasionally lateral	Globose, smooth, 18–28 μm diam (av. 20.5 μm), mostly terminal, sometimes intercalary	Globose, smooth, 16–27 μm diam (av 20 μm), terminal on lateral branches	Globose, smooth, 13–27 μm diam (av. 20.6 μm), mostly terminal
Antheridia	1–8 per oogonium, monoclinous and diclinous, sometimes sessile or crowding around the oogonia; Antheridial cells bell-shaped, club-shaped or irregular, occasionally elongated and wavy in contour, often contact with the oogonium in the neighbourhood of the oogonial stalk	1 (4) per oogonium, monoclinous or diclinous; Antheridial cells subelliptic or pseudofusiform, contact with the oogonium in the neighbourhood of the oogonial stalk	1–2 per oogonium, mostly monoclinous, originating at a short distance from the oogonium; Antheridial cell bell-shaped	1–5 per oogonium, monoclinous or diclinous, many wrap around the oogonia with a very complicated knot which disappears at last leaving 1–2 antheridial cell; Antheridial cells campanulate or at times elongated
Oospores	Globose or subglobose, 11.3–23. 8 μm diam (av. 18.5 μm), aplerotic or nearly plerotic	Globose, 11–25 μm diam (av. 16.7 μm), aplerotic	Globose, 12–20 μm diam (av. 16 μm), aplerotic	Globose, 11–20 μm diam (av. 16.8 μm), aplerotic, sometimes plerotic
Reference	This study	Yu 1998	Galland Paul 2001	Mathew et al. 2003

Senda (van der Plaats-Niterink 1981; Paul 2003a; Senda et al. 2009). However, *P. breve* can be differentiated from *P. bifurcatum* by the absence of hypogynous and monoclinous antheridia, from *P. takayamanum* due to the absence of the ellipsoid oogonia with constricted area, and from *P. scleroteichum* by the lack of sporangia (Table 1). In addition, *P. breve* is morphologically similar to *P. palingenes* Drechsler in the shape of wavy antheridial cells and the oogonial stalks coiled by antheridial stalks in a few turns, but *P. breve* differs from *P. palingenes* in the absence of sessile oogonia and proliferating or forming secondary sporangia on branches originating just below the septum of the primary sporangia (van der Plaats-Niterink 1981). Furthermore, *P. breve* has smaller sporangia, oogonia and oospores compared to *P. palingenes* (Table 1). The results of the phylogenetic analysis of ITS sequences indicated that *P. breve* was clearly separated from these three morphologically similar species. Due to the lack of the molecular data of *P. palingenes*, we could not analyze the phylogenetic relationship between *P. breve* and *P. palingenes*.

P. breve had a closer relationship with *P. megalacanthum* and *P. mastophorum* compared to the other species in the phylogenetic trees (Figs. 3 and 4), but *P. breve* had low DNA sequence similarities with *P. megalacanthum* (64.9% ITS identity with AB512782) and with *P. mastophorum* (65.4% ITS identity with AY598661 and 92.9% COXI identity with EU350523). Furthermore, in morphology, *P. breve*, with smooth-walled oogonia and smaller sporangia, oogonia and oospores, conspicuously differs from *P. megalacanthum* and *P. mastophorum* with ornamented oogonia and bigger sporangia, oogonia and oospores (van der Plaats-Niterink 1981).

P. baisense (strains CLZ223, XGL324 and QBS123) were collected from the lawn soils in Guilin, Liuzhong and Baise of Guangxi in China, respectively. These three isolates were isomorphic and had almost the same growth rates. The morphological combination of variously sac-like oogonia originating directly from the hyphae and diclinous sessile antheridia is unique in the genus *Pythium*. In addition, *P. baisense* is morphologically similar to *P. hypoandrum* Y.N. Yu & Y.L. Wang in the shape of sporangia and the antheridia contacting with oogonia in the neighbourhood of the oogonial stalk (Yu 1998). However, *P. baisense* has smaller sporangia (5–15 vs 13–29 μm diam.) and more antheridia (1–8 vs 1 per ooganium) compared to *P. hypoandrum*. Furthermore, antheridial cells are bell-shaped, club-shaped or irregular in *P. baisense*, but subelliptic or pseudofusiform in *P. hypoandrum*. The diclinous antheridia of *P. baisense* are sessile or born on either wavy and curved or complexly lobed stalks which are completely absent in *P. hypoandrum* (Table 2). In addition, *P. baisense* is morphologically close to *P. breve* with many antheridia crowding around the oogonium and curved and waved antheridial stalks. However, *P. baisense* with antheridial stalks occasionally complexly lobed is obviously distinguished from *P. breve* with antheridial stalks entwining the hyphae nearby the oogonia with several turns. The results of the phylogenetic analyses of ITS and COXI sequences indicated that *P. baisense* was clearly separated from *P. breve* (Figs. 3 and 4). Due to a lack of the molecular data of *P. hypoandrum*, we could not analyze the phylogenetic relationship between *P. baisense* and *P. hypoandrum*.

In addition, *P. baisense* had closer relationships with *P. perplexum* and *P. campanulatum* compared to the other species in the phylogenetic analyses of ITS and COXI sequences (Figs. 3 and 4), but *P. baisense* had low DNA sequence similarity with *P. perplexum* (84.4% ITS identity with AY598658 and 93.7% COXI identity with EU350520) and with *P. campanulatum* (79.9% ITS identity with AY256904). Furthermore, in morphology, *P. baisense* with monoclinous and diclinous antheridia and more (1–8) antheridia conspicuously differs from *P. perplexum* with mostly monoclinous antheridia and fewer (1–2) antheridia (Galland and Paul 2001) and from *P. campanulatum* with fewer (1–5) antheridia which wrap around oogonia with a very complicated knot (Mathew et al. 2003) (Table 2).

In conclusion, the distinctively morphological and molecular characteristics of *P. breve* and *P. baisense*, compared to other *Pythium* species described, certify that these two taxa are new to science.

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References

Ali-Shtayeh MS, Saleh ASF (1999) Isolation of *Pythium acanthicum*, *P. oligandrum*, and *P. periplocum* from soil and evaluation of their mycoparasitic activity and biocontrol efficacy against selected phytopathogenic *Pythium* species. Mycopathologia 145:143–153

Allain-Boulé N, Tweddell R, Mazzola M, Bélanger R, Lévesque CA (2004) *Pythium attrantheridium* sp. nov.: taxonomy and comparison with related species. Mycol Res 108(7):795–805

Bala K, Robideau GP, Désaulniers N, de Cock AWAM, Lévesque CA (2010a) Taxonomy, DNA barcoding and phylogeny of three new species of *Pythium* from Canada. Persoonia 25:22–31

Bala K, Robideau GP, Lévesque CA, de Cock AWAM, Abad ZG, Lodhi AM, Shahzad S, Ghaffar A, Coffey MD (2010b) *Phytopythium* Abad, de Cock, Bala, Robideau & Lévesque, gen. nov. and *Phytopythium sindhum* Lodhi, Shahzad & Lévesque, sp. nov. Persoonia 24:136–137

Belbahri L, McLeod A, Paul B, Calmin G, Moralejo E, Spies CFJ, Botha WJ, Clemente A, Descals E, Sánchez-Hernández E, Lefort F (2008) Intraspecific and within-isolate sequence variation in the ITS rRNA gene region of *Pythium mercuriale* sp. nov. (Pythiaceae). FEMS Microbiol Lett 284:17–27

De Cock AWAM, Lévesque CA, Melero-Vara M, Serrano Y, Guirado ML, Gómez J (2008) *Pythium solare* sp. nov., a new pathogen of green beans in Spain. Mycol Res 112:1115–1121

Galland D, Paul B (2001) *Pythium perplexum* isolated from soil in France: morphology, molecular characterisation and biological control. Mycol Res 156:185–189

Garzón CD, Yánez JM, Moorman GW (2007) *Pythium cryptoirregulare*, a new species within the *P. irregulare* complex. Mycologia 99(2):291–301

Guo LD, Hyde KD, Liew ECY (2000) Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. New Phytol 147:617–630

Huelsenbeck JP, Ronquist F (2001) MRBAYES: Bayesian inference of phylogeny trees. Bioinformatics 17:754–755

Hulvey J, Telle S, Nigrelli L, Lamour K, Thines M (2010) Salisapiliaceae - a new family of oomycetes from marsh grass litter of southeastern North America. Persoonia 25:109–116

Kageyama K, Senda M, Asano T, Suga H, Ishiguro K (2007) Intra-isolate heterogeneity of the ITS region of rDNA in *Pythium helicoides*. Mycol Res 6:416–423

Karaca G, Tepedelen G, Belghouthi A, Paul B (2008) A new mycoparasite, *Pythium lycopersicum*, isolated in Isparta, Turkey: morphology, molecular characteristics, and its antagonism with phytopathogenic fungi. FEMS Microbiol Lett 288:163–170

Karaca G, Jonathan R, Paul B (2009) *Pythium stipitatum* sp.nov. isolated from soil and plant debris taken in France, Tunisia, Turkey, and India. FEMS Microbiol Lett 295:164–169

Katoh K, Kuma K, Toh H, Miyata T (2005) MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res 33:511–518

Lévesque CA, De Cock AWAM (2004) Molecular phylogeny and taxonomy of the genus *Pythium*. Mycol Res 108:1363–1383

Long YY, Wei JG, Huang CL, He YQ, Yuan GQ, Shi Y, Xiong Y (2010) A new *Pythium* species isolated from vegetable fields and analysis by rDNA ITS sequence. Mycosystema 29(6):795–800

Martin FN (2000) Phylogenetic relationships among some *Pythium* species inferred from sequence analysis of the mitochondrially encoded cytochrome oxidase II gene. Mycologia 92(4):711–727

Mathew R, Singh KK, Paul B (2003) *P. campanulatum* sp. nov., isolated from the rhizosphere of maize, its taxonomy, ITS region of rDNA, and comparison with related species. FEMS Microbiol Lett 226:9–14

Mcleod A, Botha WJ, Meitz JC, Sples CFJ, Tewoldemedhin YT, Mostert L (2009) Morphological and phylogenetic analyses of *Pythium* species in South Africa. Mycol Res 113:933–951

Moralejo E, Clemente A, Descals E, Belbahri L, Calmin G, Lefort F, Spies CFJ, McLeod A (2008) *Pythium recalcitrans* sp. nov. revealed by multigene phylogenetic analysis. Mycologia 100 (2):310–319

Nechwatal J, Oßald WF (2003) *Pythium montanum* sp. nov., a new species from a spruce stand in the Bavarian Alps. Mycol Prog 2 (1):73–80

Nechwatal J, Wielgoss A, Mendgen K (2005) *Pythium phragmitis* sp. nov., a new species close to *P. arrhenomanes* as a pathogen of common reed (*Phragmites australis*). Mycol Res 109(12):1337–1346

Nylander JAA (2008) MrModeltest 2.3 README. http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html. Accessed 22 May 2008

Paul B (1986) A new species of *Pythium* from Algerian waters. Hydrobiologia 131(1):31–38

Paul B (1991) *Pythium folliculosum*, a new species from the bank of Lake Zürich. Mycol Helvetica 4:203–208

Paul B (1999) *Pythium ornacarpum*: a new species with ornamented oogonia isolated from soil in France. FEMS Microbiol Lett 180:337–344

Paul B (2000) *Pythium contiguanum* nomen novum (syn. *Pythium dreschleri* Paul), its antagonism to *Botrytis cinerea*, ITS1 region of its nuclear ribosomal DNA, and its comparison with related species. FEMS Microbiol Lett 183:105–110

Paul B (2001) ITS region of the rDNA of *Pythium longandrum*, a new species; its taxonomy and its comparison with related species. FEMS Microbiol Lett 202:239–242

Paul B (2002) ITS region of *Pythium canariense* sp. nov., its morphology and its interaction with *Botrytis cinereas*. FEMS Microbiol Lett 208:135–141

Paul B (2003a) Characterisation of a new species of *Pythium* isolated from a wheat field in northern France and its antagonism towards *Botrytis cinerea* causing the grey mould disease of the grapevine. FEMS Microbiol Lett 224:215–223

Paul B (2003b) *Pythium glomeratum*, a new species isolated from agricultural soil taken in north-eastern France, its ITS region and its comparison with related species. FEMS Microbiol Lett 225:47–52

Paul B (2004) A new species of *Pythium* isolated from burgundian vineyards and its antagonism towards *Botrytis cinerea*, the causative agent of the grey mould disease. FEMS Microbiol Lett 234:269–274

Paul B, Galland D, Bhatnagar T, Dulieu H (1998) A new species of *Pythium* isolated from the Burgundy region in France. FEMS Microbiol Lett 158:207–213

Paul B, Bala K, Gognies S, Belarbi A (2005) Morphological and molecular taxonomy of *Pythium longisporangium* sp. nov. isolated from the Burgundian region of France. FEMS Microbiol Lett 246:207–212

Senda M, Kageyama K, Suga H, Lévesque CA (2009) Two new species of *Pythium*, *P. senticosum* and *P. takayamanum*, isolated from cool-temperate forest soil in Japan. Mycologia 101(4):439–448

Swofford DL (2002) Phylogenetic Analysis Using Parsimony (and other methods). Version 4, Sinauer, Sunderland, Mass. http://paup.csit.fsu.edu/Cmd_ref_v2.pdf

Uzuhashi S, Tojo M, Kobayashi S, Kakishima M (2009) *Pythium apinafurcum*: its morphology, molecular phylogeny, and infectivity for plants. Mycoscience 50:281–290

Van der Plaats-Niterink AJ (1981) Monograph of the genus *Pythium*. Stud Mycol 21:1–242

White TJ, Bruns TD, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal DNA genes for phylogenetics. In: Innis MA, Sninsky DH, White TJ (eds) PCR Protocols. Academic, London, pp 315–322

Yu YN (1998) Peronosporales [Flora Fungorum Sinicorum Vol. 6]. Science Press, Beijing (in Chinese)