

Pythium subutonaiense, A New Aquatic Oomycete from Southern China Based on Morphological and Molecular Characters

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ABSTRACT

A new species, *Pythium subutonaiense*, isolated from aquatic environments (lake) in China is being described based on morphological characters and molecular evidence. The isolates grew at temperatures between 5 °C and 38 °C, and the optimum temperature was 30 °C, with a radial growth rate of 17.6 mm at 25 °C per day. It is homothallic and characterized by globose to sub-globose shaped and mostly terminal or sometimes catenulate hyphal swellings, filamentous non-inflated sporangia, and smooth oogonia with hypogynous and monoclinal antheridia that contained one plerotic oospore. In phylogenetic analysis, inferred based on the internal transcribed spacer region of the ribosomal RNA gene and mitochondrial cytochrome c oxidase subunit 1 gene, the new species formed a distinct lineage in *Pythium* clade B. Differences between the new species and phylogenetically related and morphologically similar species are discussed.

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1. Introduction

Pythium [1] (Pythiaceae, Pythiales), typified with *P. monospermum* Pringsh., is characterized by hyaline and coenocytic hyphae without septa, various shaped sporangia, and the development of zoospores in a vesicle which is formed at the tip of a discharge tube derived from a sporangium [2]. *Pythium* spp. are cosmopolitan and represent a range of functional groups, such as saprophytes in natural environments, plant and animal pathogens, and biological control agents protecting against pathogenic fungi [3]. Following recent taxonomic revisions [4,5] and discoveries (e.g., Refs. [6–11]), more than 140 species are currently recorded in the genus *Pythium* [11].

During studies on *Pythium* species diversity in southern China, one new species of *Pythium*, *P. utonaiense* was isolated. Phylogenetic analysis was performed using the internal transcribed spacer (ITS) regions of the ribosomal RNA and mitochondrial cytochrome c oxidase subunit 1 (COI) genes. Combined with the morphological characters, a new species is described in this study. Differences between the new species and phylogenetically related and/or morphologically similar species are also provided.

2. Materials and methods

2.1. Isolation

The samples were recovered from surface water of the lake in Nanjing, Jiangsu Province of China using flowers of *Bougainvillea glabra* Choisy as baits to isolate *Pythium* species [12]. The isolation procedure followed the method described by Benard and Punja [13]. Pieces of tissue 5–10 mm were cut from the baits, washed in tap water and superficially dried on a paper towel, and plated on V8 juice agar (V8A) containing rifampicin (50 mg L⁻¹), phenamacril (5 mg L⁻¹), ampicillin (50 mg L⁻¹), and pentachloronitrobenzene (50 mg L⁻¹) and incubated at 25 °C for 2–3 days. When mycelial growth was observed, purification was carried out twice by transferring a single hyphal tip of colonies onto V8A. Two isolates (Chen 220 and Chen 229) of undescribed *Pythium* species were recovered and deposited in the herbarium of the College of Plant Protection, Nanjing Agricultural University (NJAU).

2.2. Morphology and growth rate

Colony patterns of two *P. subutonaiense* isolates were examined after incubation for 3 days at 25 °C

in corn meal agar (CMA), potato carrot agar (PCA), and V8A media. Sporulation was induced using a modification of the method described by Chang [14]. Agar blocks (8 mm × 8 mm × 3 mm) were cut from the mycelia fronts of 3-day-old V8A cultures and placed in 10% clarified and sterile V8 juice (one block per dish) and incubated in darkness at 25 °C for 48–72 h. Once the mycelium has reached 4–5 cm in diameter, the V8 juice was pipetted out and the mycelia mats were then rinsed with sterile distilled water (SDW) three times at 20 min intervals. The rinsed mycelia mats were then submerged in SDW. The plates containing SDW were incubated at 25 °C for 24–48 h prior to examining for release of zoospores. Fifty measurements were taken for each morphological feature, such as sporangia, oogonia, and oospores. The cardinal temperatures of growth rates, on PCA media [2], were measured at 24 h of incubation for each isolate at 5–40 °C, with intervals of 5 °C. 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 growth revival.

2.3. DNA extraction and sequencing

A cetyl trimethylammonium bromide rapid plant genome extraction kit (Demeter Biotechnologies Co., Ltd., Beijing, China) was used to extract total genomic DNA from purified isolates, and performed the polymerase chain reaction (PCR) according to the study by Chen and Cui [15]. A small piece of dried fungal specimen (about 30 mg) was grounded to powder with liquid nitrogen, transferred to a 1.5-mL centrifuge tube and resuspended in 0.4 mL of lysis buffer (PL), and incubated at 65 °C water bath for 60 min. Then, 0.4 mL phenol–chloroform (24:1) was added to each tube and the suspension was shaken vigorously. After centrifugation at 13,000 rpm for 5 min, 0.3 mL supernatant was transferred to a new tube and mixed with 0.45 mL binding buffer (PQ). The mixture was later transferred to an adsorbing column (AC) for a centrifugation at 13,000 rpm for 0.5 min. Then, 0.5 mL inhibitor removal fluid (IR) was added to the AC for a centrifugation at 12,000 rpm for 0.5 min. After washing twice with 0.5 mL washing buffer (WB), removing the AC to a clean centrifuge tube, and adding 100 µL elution buffer in the middle of adsorbed film, the genome DNA was eluted in 100 µL elution buffer (EB). The ITS region was amplified with the primers: ITS4 and ITS5 [16]. The COI gene was amplified with the primers: OomCoxI-Levlo (CYTC HGGRTGWCCRAAAAACCAAA) and Oom CoxI-Levup (TCAWCWGMGATGGCTTTTTTCAAC) [17]. The PCR procedure for ITS was as follows: initial

denaturation at 95 °C for 3 min, followed by 35 cycles at 94 °C for 40 s, 54 °C for 45 s and 72 °C for 1 min, and a final extension of 72 °C for 10 min. The PCR procedure for COI was as follows: initial denaturation at 94 °C for 2–5 min, followed by 35 cycles at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1–2 min, and a final extension of 72 °C for 5–10 min [18]. The PCR products were purified and sequenced in Genscript Company (Nanjing, China) with the same primers.

2.4. Phylogenetic analysis

Sequences generated in this study were aligned with additional sequences downloaded from GenBank (Table 1) using ClustalX [19] and BioEdit [20].

Table 1. A list of species, cultures, and GenBank accession numbers of sequences used in this study.

Species name	Sample no.	Locality	GenBank accession no.	
			ITS	COI
<i>Pythium adhaerens</i>	CBS 520.74	The Netherlands	HQ643415	HQ708462
<i>P. afertile</i>	Lev 2066	Canada	HQ643416	HQ708463
<i>P. angustatum</i>	CBS 522.74	The Netherlands	HQ643437	HQ708484
<i>P. apoleroticum</i>	CBS 772.81	The Netherlands	HQ643444	HQ708491
<i>P. aquatile</i>	CBS 215.80	United Kingdom	HQ643445	HQ708492
<i>P. aristosporum</i>	CBS 263.38	Canada	HQ643447	HQ708494
<i>P. arrhenomanes</i>	CBS 324.62	USA	HQ643452	HQ708499
<i>P. bifforme</i>	UZ00796	Japan	KJ995584	KJ995590
<i>P. brachiatum</i>	UZ00736	Japan	KJ995581	KJ995593
<i>P. brachiatum</i>	UZ00746	Japan	KJ995583	KJ995594
<i>P. capillosum</i>	CBS 222.94	France	HQ643483	HQ708529
<i>P. catenulatum</i>	CBS 226.94	France	HQ643490	HQ708536
<i>P. chondricola</i>	CBS 203.85	The Netherlands	HQ643498	HQ708544
<i>P. coloratum</i>	CBS 154.64	Australia	HQ643501	HQ708547
<i>P. conidiophorum</i>	CBS 223.88	United Kingdom	HQ643509	HQ708555
<i>P. contiguanum</i>	CBS 221.94	Algeria	HQ643514	HQ708560
<i>P. declinum</i>	CBS 526.74	The Netherlands	HQ643523	HQ708569
<i>P. dissimile</i>	CBS 155.64	Australia	HQ643526	HQ708572
<i>P. dissotocum</i>	CBS 166.68	USA	HQ643528	HQ708574
<i>P. flevoense</i>	CBS 234.72	The Netherlands	HQ643538	HQ708582
<i>P. folliculosum</i>	CBS 220.94	Switzerland	HQ643540	HQ708584
<i>P. graminicola</i>	CBS 327.62	Jamaica	HQ643545	HQ708589
<i>P. inflatum</i>	CBS 168.68	USA	HQ643566	HQ708610
<i>P. kashmirensis</i>	CBS 122908	India	HQ643671	HQ708715
<i>P. lutarium</i>	CBS 222.88	United Kingdom	HQ643682	HQ708726
<i>P. myriotyllum</i>	CBS 254.70	Israel	HQ643701	HQ708745
<i>P. oopapillum</i>	BR 632	–	FJ655174	FJ655178
<i>P. pachycaule</i>	CBS22494	France	HQ643726	HQ708767
<i>P. pectinolyticum</i>	CBS 122643	France	HQ643739	HQ708780
<i>P. periillum</i>	CBS 169.68	USA	HQ643740	HQ708781
<i>P. phragmitis</i>	CBS 117104	Germany	HQ643746	HQ708787
<i>P. plurisporium</i>	CBS 100530	USA	HQ643749	HQ708790
<i>P. pyriformis</i>	CBS 158.64	Australia	HQ643755	HQ708796
<i>P. rhizo-oryzae</i>	CBS 119169	India	HQ643757	HQ708798
<i>P. salpingophorum</i>	BR 1024	United Kingdom	HQ643770	HQ708811
<i>P. scleroteichum</i>	CBS 294.37	USA	HQ643771	HQ708812
<i>P. subtonaiense</i>	Chen 220 ^a	China	MG654703	MG674169
<i>P. subtonaiense</i>	Chen 229 ^a	China	MG654704	MG674170
<i>P. sukuiense</i>	CBS 110030	Taiwan	HQ643836	HQ708877
<i>P. sulcatum</i>	CBS 603.73	USA	HQ643837	HQ708878
<i>P. tardicrescens</i>	Lev 1534	USA	HQ643855	HQ708896
<i>P. torulosum</i>	CBS 316.33	The Netherlands	HQ643859	HQ708900
<i>P. tracheiphilum</i>	CBS 323.65	Italy	HQ643862	HQ708903
<i>P. utonaiense</i>	UZ00758	Japan	KJ995586	KJ995588
<i>P. utonaiense</i>	UZ00769	Japan	KJ995587	KJ995589
<i>P. vanterpoolii</i>	CBS 295.37	United Kingdom	HQ643952	HQ708993
<i>P. volutum</i>	CBS 699.83	Japan	HQ643971	HQ709012
<i>P. zingiberis</i>	CBS 216.82	Japan	HQ643973	HQ709014

^aNew sequences determined in the present study.

Sequence alignment was deposited at TreeBase (<http://purl.org/phylo/treebase>; submission ID S22483).

Phylogenetic analysis was done as in the study by Chen and Cui [15]. Maximum parsimony (MP) analysis was applied to the combined dataset of ITS and COI sequences. *Pythium adhaerens* Sparrow and *P. chondricola* De Cock were used as outgroups [7]. The tree construction procedure was performed in PAUP* version 4.0b10 [21]. All characters were equally weighted and gaps were treated as missing data. Trees were inferred using the heuristic search option with TBR branch swapping and 1000 random sequence additions. Max-trees were set to 5000, branches of zero length were collapsed and all parsimonious trees were saved. Clade robustness was assessed using a bootstrap analysis with 1000 replicates [22]. Descriptive tree statistics tree length (TL), consistency index (CI), retention index (RI), rescaled consistency index (RC), and homoplasy index (HI) were calculated for each maximum parsimonious tree generated.

MrModeltest 2.3 [23] was used to determine the best-fit evolution model for the dataset for Bayesian inference (BI). BI was calculated with MrBayes3.1.2 [24] with a general time reversible (GTR) model of DNA substitution and an invgamma distribution rate variation across sites. Four Markov chains were run for 2 million generations until the split deviation frequency value <0.01 , and sampled every 100th generations. The burn-in was set to discard the first 25% of the trees. A majority rule consensus tree of all remaining trees was calculated. Phylogenetic trees were visualized using Treeview [25]. Branches that received bootstrap support for maximum likelihood (BS), maximum parsimony (MP), and Bayesian posterior probabilities (BPP) greater than or equal to 75% (MP) and 0.95 (BPP) were considered as significantly supported, respectively.

3. Results

3.1. Isolation

Water samples were collected from two lakes, respectively in Zixiahu Park and Soul Valley Temple in China in 2016. The two lakes are located in national park of Dr. Sun Yat-sen's mausoleum of southern China. Two surface water samples were collected at the edge of the lake. Two isolates of the new species, *P. subutonaiense*, were respectively obtained from two water samples and isolated from flowers of *Bougainvillea glabra* as bait on V8A.

3.2. Morphology and growth rate

Both two isolates of *P. subutonaiense* showed similar colony patterns and growth temperature results

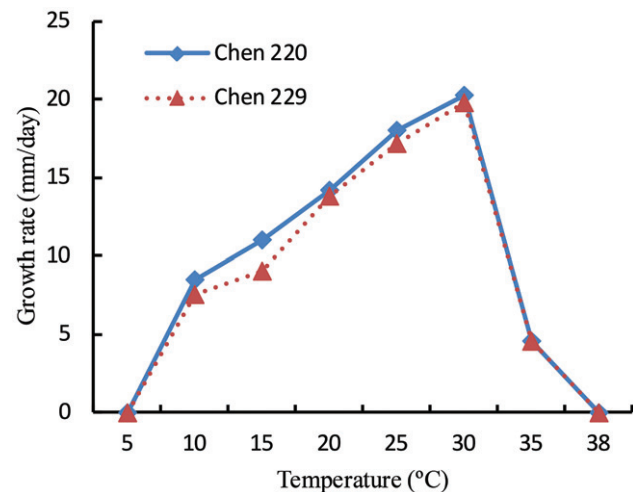


Figure 1. Mecelial growth rate of *P. subutonaiense* isolates Chen 220 and Chen 229 on potato carrot agar at different temperatures.

(Figure 1). They were rosette pattern on PCA, stellate pattern on V8A, and without a special pattern on CMA. The isolates showed maximum growth at 30°C, and no growth at 5°C and 38°C. The growth rate was 17.6 mm per day at 25°C.

The morphology of asexual and sexual structures was also similar between the two isolates. Hyphal swellings were frequently observed, and zoospores were rarely observed in both isolates. The sexual structures were abundantly produced on V8A. Oogonia were smooth-walled, but sometimes had one outstanding slender projection. Antheridia were hypogynous and monoclinal, produced one or two per oogonium. Oospores were one per oogonium.

P. subutonaiense differs other *Pythium* species from homothallic sexuality, globose to sub-globose shaped, mostly terminal or sometimes catenulate hyphal swellings, filamentous non-inflated sporangia, and smooth oogonia, hypogynous, and monoclinal antheridia with slender antheridial cells and plerotic oospores.

Further differences between the new species and other related ones are listed in Table 2.

3.3. Molecular phylogeny

Two ITS and two COI sequences were newly generated for this study and their accession numbers are available in GenBank (Table 1). BLAST analyses of ITS and COI sequences of the two isolates, described here as *P. subutonaiense*, showed the best phylogenetic matches were with species of *Pythium* clade B.

The combined ITS + COI dataset included sequences from 48 oomycetes representing 45 taxa. The dataset had an aligned length of 1563 characters, of which 1111 characters are constant, 64 are variable and parsimony-uninformative, and 388 are

Table 2. Morphological description of *Pythium subutonaiense* and the most closely related species.

	<i>P. subutonaiense</i> (Chen 218)	<i>P. brachiatum</i>	<i>P. capillosum</i>	<i>P. flevoense</i>	<i>P. utonaiense</i>
Width of hyphae (µm)	Up to 5	Up to 5.5	3–5	Up to 6	Up to 5
Hyphal swellings	Present	Absent	Absent	Absent	Absent
Sporangia or inflated structures	Filamentous non-inflated	Filamentous slightly inflated	Filamentous, non-inflated or slightly inflated	Filamentous	Filamentous non-inflated
Oogonia (µm)	17.5–22.5 (av. 20.8), intercalary or rarely terminal	13.3–34.4 (av. 22.7), intercalary or terminal, sometimes in chain	16–35.2 (av. 23.09), mostly intercalary, rarely terminal	17–20 (av. 19), mostly terminal	10–23.3 (av. 17.5), terminal, rarely intercalary
Antheridia	Hypogynous or monoclinal	Monoclinal or diclinous or rarely lacking	Mostly diclinous, rarely monoclinal	Diclinous	Lacking
Oospores	Plerotic	Plerotic, occasionally aplerotic	Plerotic or aplerotic	Aplerotic, occasionally nearly plerotic	Plerotic
Oospore wall thickness (µm)	0.5–2	0.3–3.2	2–3.5	2–3	0.3–3.1
Cardinal temperature	Min 5 °C, optimum 30 °C and max 38 °C	Min 4 °C, optimum 25 °C and max 30 °C	Min 1–2 °C, optimum 25 °C and max 40 °C	Min under 5 °C, optimum 25 °C and max over 35 °C	Min 4 °C, optimum 30 °C and max 40 °C
Daily growth rates on PCA at 25 °C (mm)	17.6/per day	14/per day	7/per day	7–10/per day	23.5/per day
Reference	This study	[7]	[27]	[28]	[7]

parsimony-informative. Maximum parsimony analysis yielded three equally parsimonious trees (TL = 1369, CI = 0.455, RI = 0.757, RC = 0.344, HI = 0.545). Best model for the combined ITS + COI sequences dataset estimated and applied in the Bayesian analysis: GTR + I + G, lset nst = 6, rates = invgamma; prset statefreqpr = dirichlet (1,1,1,1). Bayesian analysis resulted in the same topology with an average standard deviation of split frequencies = 0.006409. The sequences of *Pythium* species in clade B clustered together with strong supports (100% ML, 1 BPP, Figure 2). The phylogeny inferred from the ITS + COI dataset showed that the two newly sequenced isolates formed a lineage within *Pythium* subclade B2 with full statistical supports (100% MP, 1 BPP) and clustered with *P. utonaiense* [7].

3.4. Taxonomy

***P. subutonaiense*:** Jia J. Chen and X.B. Zheng, *sp. nov.* (Figure 3).

Mycobank no.: MB 824731.

Type.—China. Jiangsu Prov., Nanjing, Zixiahu Park, from lake water, November 1, 2016, J.J. Chen, Chen 220 (NJAU, holotype).

Etymology.—*Subutonaiense* (Lat.) referring to the species is somewhat similar to *P. utonaiense* [7].

Cardinal temperatures: minimum 5 °C, optimum 30 °C, maximum 38 °C. Main hyphae hyaline, aseptate, up to 5.0 µm wide. Hyphal swellings globose to sub-globose, mostly terminal or sometimes catenulate, 20–37.5 (mean 26.5) µm in diameter. Sporangia filamentous non-inflated, rarely giving rise to vesicles containing zoospores. Zoospores formed in SDW at room temperature, and encysted zoospores 5–7.5 µm (mean 6.5 µm) in diameter. Homothallic; oogonia globose, smooth or with one outstanding slender projection, intercalary, rarely terminal, 17.5–22.5 µm (mean 20.8 µm) in diameter. Antheridia hypogynous and monoclinal; antheridial stalks unbranched; antheridial cells slender. Oospore one per oogonium, plerotic, globose, 15.5–20.5 µm (mean 18.3 µm) in diameter, wall up to 0.5–2 (mean 1.6) µm thick.

Additional specimens examined.—CHINA. Jiangsu Prov., Nanjing, Soul Valley Temple, from lake water, November 1, 2016, J.J. Chen, Chen 229 (paratype, NJAU).

4. Discussion

P. subutonaiense is characterized by aquatic habit, homothallic sexuality, globose to sub-globose shaped, mostly terminal or sometimes catenulate hyphal swellings, filamentous non-inflated sporangia, and smooth oogonia, hypogynous, and

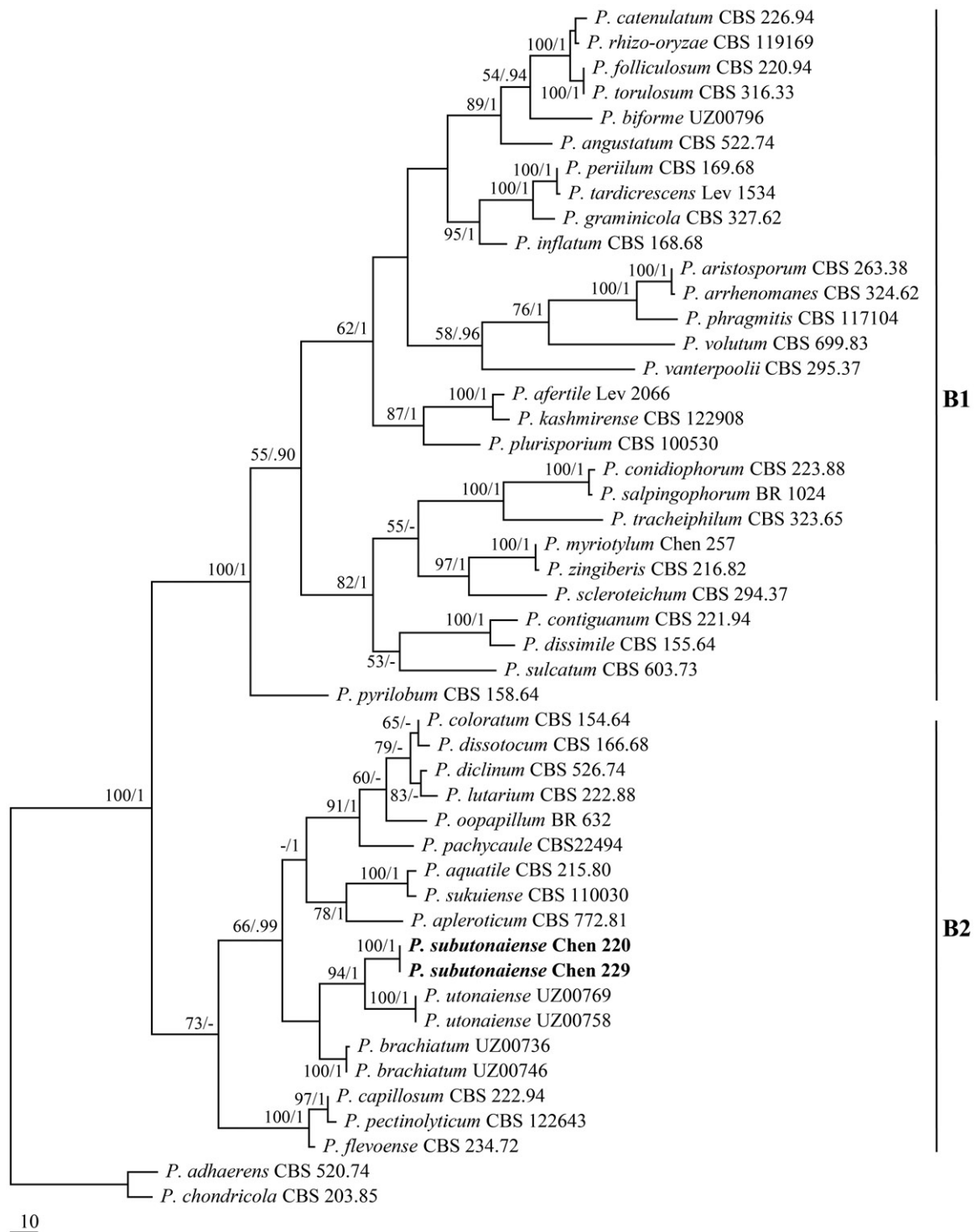


Figure 2. Phylogeny of *Pythium* clade B species inferred from ITS + COI dataset.

monoclinous antheridia with slender antheridial cells, and plerotic oospores.

According to Lévesque and de Cock [26], *Pythium* could be split into 11 clades (A–K), of which the species in subclade B2 are characterized by filamentous non-inflated to slightly inflated sporangia, smooth oogonia mostly smaller than 30 mm in diameter, and a moderate growth rate (mostly 10–20 mm per day). Phylogenetic analysis based on ITS and COI sequences indicated *P. subutonaiense* belongs to *Pythium* subclade B2 with full statistical

supports. *P. subutonaiense* shares several morphological characteristics with other *Pythium* subclade B2 species, such as filamentous sporangia, smooth oogonia, and moderate growth rate. However, *P. subutonaiense* can be readily distinguished in having globose to sub-globose shaped and mostly terminal or sometimes catenulate hyphal swellings.

P. subutonaiense has a closer relationship with *P. utonaiense* according to the ITS and COI-based phylogeny (Figure 2). *P. utonaiense* resembles *P. subutonaiense* in having aquatic habit, filamentous

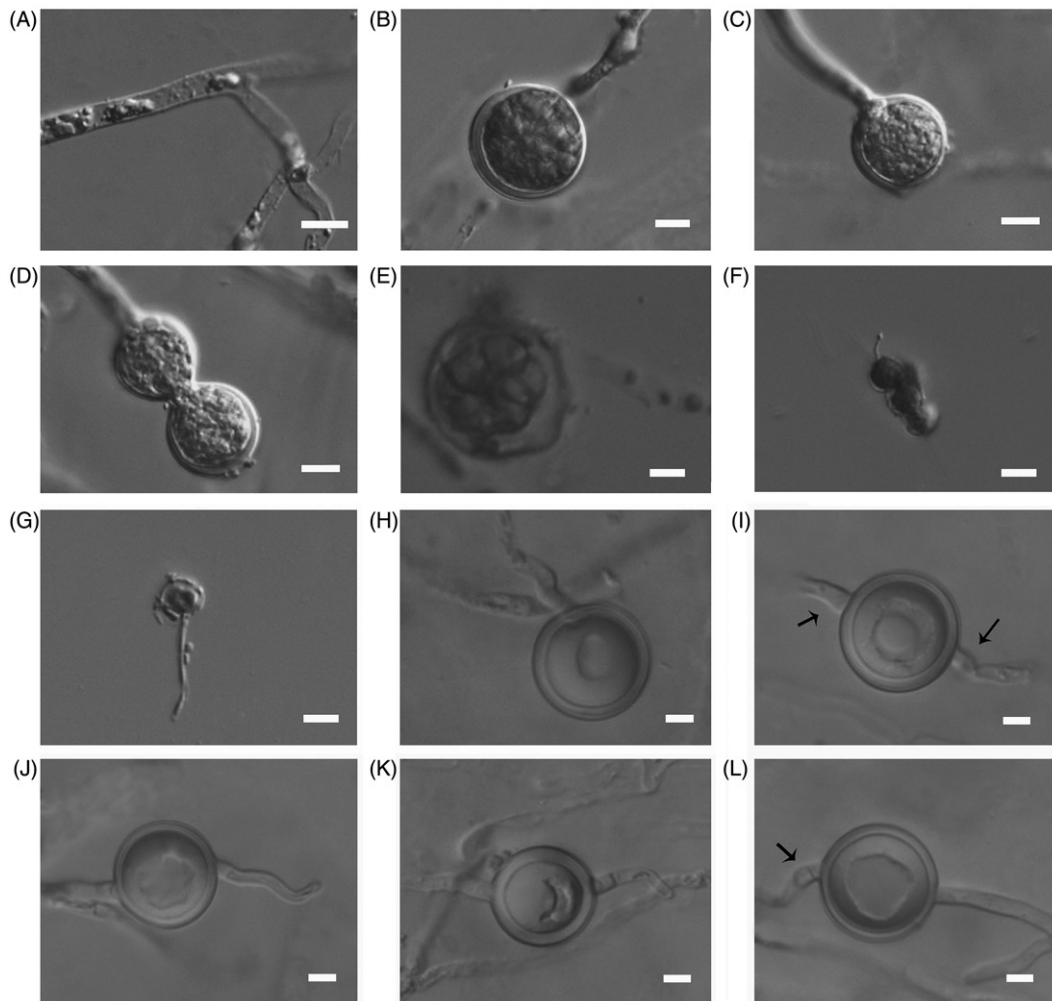


Figure 3. Asexual and sexual reproductive bodies of *P. subutonaiense* (Chen 220). (A) Mycelia; (B,C) Globose, terminal hyphal swellings; (D) Catenulate hyphal swellings; (E) A vesicle with zoospores; (F,G) Germinated encysted zoospore; (H) Terminal oogonium with plerotic oospore; (I) Intercalary oogonium with a plerotic oospore; (J,K) Terminal oogonia with a projection; (L) Plerotic oospore and a hypogynous antheridium. Arrows indicate antheridia are hypogynous. Bars A–F = 10 μm , G–L = 5 μm .

non-inflated sporangia, and plerotic oospores, but it is distinguished in its absence of hyphal swellings, faster growth rate, and the lack of antheridia [7] (Table 2).

P. brachiatum [7] is similar to *P. subutonaiense* by sharing aquatic habit and the formation of projections on oogonia, but the former species has slightly slower growth rate, lower optimum growth temperature, filamentous slightly inflated sporangia, and catenulate oogonia (Table 2). In addition, *P. brachiatum* is distant from *P. subutonaiense* in the ITS + COI sequence-based phylogeny [7] (Figure 2).

Both *P. capillosum* [27] and *P. flevoense* [28] have oogonia with a finger-like projection, and they somehow resemble *P. subutonaiense*; however, except for the lack of hyphal swellings, the two species can be distinguished from *P. subutonaiense* by slower growth rate, and thicker oospore wall [28] (Table 2). Besides, these three species clustered in different lineages in the phylogenetic analysis.

Disclosure statement

No potential conflict of interest was reported by the authors.

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