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Two species of *Phytopythium* (Pythiaceae, Pythiales) new to China

Abstract

Two oomycetes, *Phytopythium mercuriale* and *Pp. sindhum* were found in southern China, and they are newly recorded in China. These two species were both isolated from roots of soybean. *Pp. mercuriale* is characterized by subglobose sporangia with conspicuous apical papillae, and occasionally forming oogonia. And *Pp. sindhum* is identified from other *Phytopythium* species by its globose to sub-globose sporangia with conspicuous apical papillae, large and smooth oogonia, monoclinous or diclinous antheridia, and plerotic or nearly plerotic and thick-walled oospores. Illustrations and descriptions of the two new records are provided based on the materials from China.

Keywords: *Cox1*, ITS, Oomycota, *Phytopythium mercuriale*, *Phytopythium sindhum*

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Abbreviations: BI, bayesian inference; BPP, bayesian posterior probabilities; BT, bootstrap; CMA, corn meal agar; CI, consistency index; *Cox1*, cytochrome c oxidase subunit 1; GTR, general time reversible; HI, homoplasy index; ITS, the internal transcribed spacer; MP, maximum parsimony; MPT, maximum parsimonious tree; NJAU, the College of Plant Protection, Nanjing Agricultural University; PCA, potato carrot agar; PCR, the polymerase chain reaction; RC, rescaled consistency index; RI, retention index; TL, descriptive tree statistics tree length.

Introduction

The genus *Phytopythium* Abad et al., formerly classified as *Pythium* K-clade,[1] was recently separated from *Pythium*[2] and typified by *Pp. sindhum* A.M. Lodhi, Shahzad & Lévesque. The principal characteristics of this genus include a combination of hyaline and coenocytic hyphae without septa, ovoid to globose sporangia with papillae (except for *Pp. vexans* (de Bary) Abad), common internal proliferation that is similar to that in the *Phytophthora*, zoospores that develop in a vesicle and form at the tip of a discharge tube from the sporangium like in *Pythium*, smooth oogonia, and paragynous antheridia.[3–5] *Phytopythium* spp. are cosmopolitan and represent a range of functional groups, such as saprophytes in natural environments and plant pathogens[6] and following recent taxonomic revisions[7–9] and discoveries,[5,6,10] 21 species are recognized worldwide. During studies on the occurrence and diversity of oomycetes associated with soybean in Huang-Huai area of China, two new Chinese record of *Phytopythium* were identified from our isolates based on morphological characters and molecular phylogenetic analyses of ITS regions of the ribosomal RNA and mitochondrial *Cox1* sequence data. These two species are described in this work. Moreover, comparisons of the two species and their morphological and/or phylogenetically related species are also provided.

Materials and methods

Isolates

The cultures (Chen 265 & Chen 314) of *Phytopythium* species were isolated from roots of soybean in Jiangsu and Anhui provinces in China. The isolation procedure followed the method described by Benard & Punja.[11] Pieces of tissue 5–10mm were cut from the roots, washed in tap water and superficially dried on a paper towel, and plated on CMA containing rifampicin (50mg/L), phenamacril (5mg/L), ampicillin (50mg/L), and pentachloronitrobenzene (50mg/L) and incubated at 25°C for 2–3d. When mycelial growth was observed, purification was carried out by cutting a small piece of medium with mycelia at the edge of a colony, and transferring the cutted part into the new medium plates.[12]

Morphology and growth rate

The studied cultures were deposited in the herbaria of NJAU. The purified isolates were grown on CMA for morphological studies. Isolates were transferred to sterilized distilled water for sporulation. Fifty measurements were taken for each morphological feature, such as sporangia, oogonia and oospores. The cardinal temperatures were examined on PCA according to the method of van der Plaäts-Niterink,[3] 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 the revival of the growth.

Molecular phylogeny

DNA extraction, amplification, sequencing and sequence alignment

A cetyl trimethylammonium bromide rapid plant genome extraction kit (Demeter Biotechnologies Co., Ltd, Beijing) was used to extract total genomic DNA from purified isolates, and performed PCR according to the manufacturer's instructions with some modifications.[13] The ITS region was amplified with the primers: ITS4 and ITS5.[14] The *Cox1* gene was amplified with the primers: OomCoxI-Levlo (CYTCHGGRTGWCCRAAAAACCAAA) and OomCoxI-Levup (TCAWCWMGATGGCTTTTTTCAAC).[15] The PCR procedure for ITS was as follows: initial denaturation at 95°C for 3min, followed by 35 cycles at 94°C for 40 s, 54°C for 45 s and 72°C for 1min, and a final extension of 72°C for 10min. The PCR procedure for *Cox1* 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.[16] The PCR products were purified and sequenced in Genscript company (Nanjing, China) with the same primers.

Sequences generated in this study were aligned with additional *Phytopythium* sequences downloaded from GenBank (Table 1) using ClustalX[17] and manually adjusted in BioEdit.[18] Sequence alignment was deposited at TreeBase (http://purl.org/phylo/treebase; submission ID S24904).

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

Species name	Isolate no.	Geographic origin	GenBank accession no.	
			ITS rDNA	*Cox1* mtDNA
Phytopythium aichiense	CBS137195	Japan	AB948197	AB948191
Pp. boreale	CBS551.88	China	AB725879	AB690647
Pp. carbonicum	CBS112544	France	AB725876	AB690648
Pp. chamaehyphon	CBS259.30	USA	AB690609	AB690644
Pp. citrinum	CBS119171	France	AY197328	AB690649
Pp. delawarense	382B	USA	AB725875	AB690642
Pp. fagopyri	CBS293.35	Japan	AB690617	AB690641
Pp. helicoides	CBS286.31	USA	AB725878	AB690645
Pp. iriomotense	CBS137104	Japan	AB690629	AB690659
Pp. kandeliae	CBS 113.91	China	KJ399961	KJ690245
Pp. litorale	CBS 118360	Germany	HQ643386	HQ708433
Pp. mercuriale	CBS122443	South Africa	AB725882	AB690636
Pp. mercuriale	Chen 314*	China	MN266879	MN271343
Pp. mirpurense	CBS124523	Pakistan	KJ831613	KJ831612
Pp. montanum	CBS111349	Germany	AB725883	AB690637
Pp. nanjingense	Chen 172	China	MF459634	MF459631
Pp. oedochilum	CBS292.37	USA	HQ643392	HQ708439
Pp. ostracodes	CBS 768.73	Spain	HQ643395	HQ708442
Pp. sindhum	CBS124518	Pakistan	HM244825	HQ708443
Pp. sindhum	Chen 265*	China	MF984112 MF984149	MF984149
Pp. vexans	CBS119.80	Iran	HQ643400	HQ708447
Pythium dimorphum	CBS406.72	USA	HQ643525	HQ708571
Py. ultimum	CBS398.51	Netherlands	HQ643865	HQ708906

*New sequences determined in the present study

Phylogenetic analyses

Phylogenetic analysis was done as in Chen & Cui.[19] MP analysis was applied to the combined dataset of ITS-*Cox1* sequences. *Pythium dimorphum* F.F. Hendrix & W.A. Campb. and *Py. ultimum* Trow were used as outgroups.[7] The tree construction procedure was performed in PAUP* version 4.0b10.[20] 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 BT analysis with 1000 replicates.[21] TL, CI, RI, RC, and HI were calculated for each MPT generated. Phylogenetic trees were visualized using Treeview.[22]

MrModeltest2.3[23] was used to determine the best-fit evolution model for BI. BI of the dataset was calculated with MrBayes3.1.2 (Ronquist and Huelsenbeck 2003) with a GTR model of DNA substitution and an inverse gamma distribution rate variation across sites. Four Markov chains were run for 2 runs from random starting trees for 2 million generations of the two combined datasets, and trees were sampled every 100 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. Branches that received bootstrap support for MP and BPP greater than or equal to 75% (MP) and 0.95 (BPP) respectively were considered as significantly supported.

Results and discussions

Molecular phylogeny

The combined ITS + *Cox1* dataset of *Phytopythium* species included sequences from 23 isolates representing 21 taxa. The dataset had an aligned length of 1623 characters, of which 850 characters are constant, 206 are variable and parsimony-uninformative, and 567 are parsimony-informative. Maximum parsimony analysis yielded one equally parsimonious tree (TL = 2214, CI = 0.595, RI = 0.592, RC = 0.352, HI = 0.405). Best model for the combined ITS + *Cox1*

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 = 0.003061. The Chinese isolates Chen 265 & Chen 314 were identical to the authorized sequences of *Phytopythium mercuriale* and *Pp. sindhum* available in GenBank and thus clustered within clades representing *Pp. mercuriale*, and *Pp. sindhum* with high supporting values (100% MP and 1 BPPs, Figure 1), respectively.

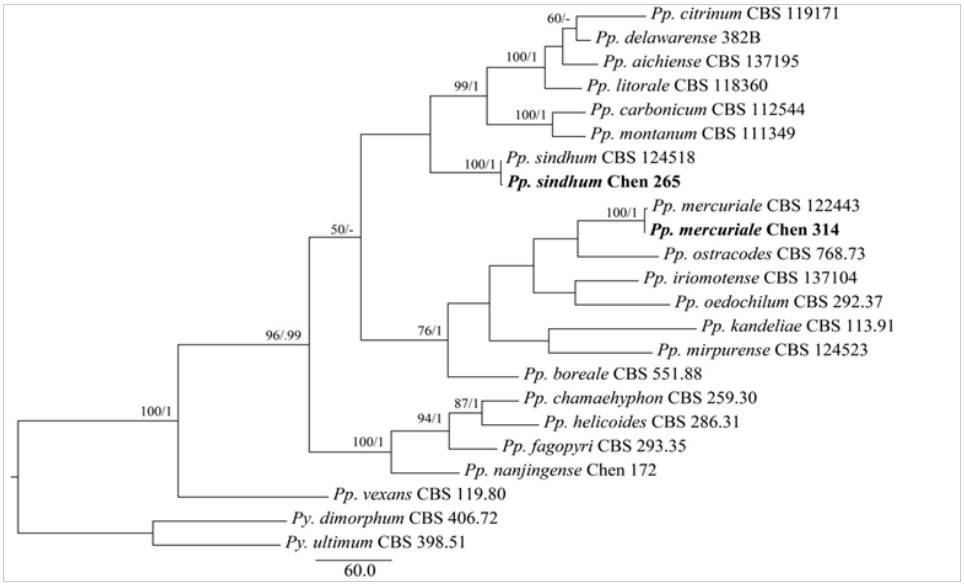

Figure 1 Phylogeny of species in *Phytopythium* and related species generated by maximum parsimony based on ITS-*Cox1* sequence data. Branches are labeled with parsimony bootstrap proportions (before slanting line) high than 50% and Bayesian posterior probabilities (after slanting line) more than 0.95. *Pp.* refers to *Phytopythium*, and *Py.* refers to *Pythium*.

Taxonomy

***Phytopythium mercuriale* (Belbahri, B. Paul & Lefort) Abad et al., Persoonia 34: 37, 2014** Figure 2

Colonies submerged, with stellate pattern on CMA. Average growth rates 3mm day^{-1} at 5°C, 7mm day^{-1} at 10°C, 10mm day^{-1} at 15°C, 12mm day^{-1} at 20°C, 15mm day^{-1} at 25°C, 16mm day^{-1} at 30°C, 10mm day^{-1} at 35°C, but when returned to room temperature both of them started to grow again. Cardinal temperatures: minimum 4°C, optimum 25–30°C, maximum 38°C. Main hyphae hyaline, aseptate, up to 6.0µm wide. No hyphal swellings. Sporangia subglobose, terminal with conspicuous apical papillae, proliferation external, internal and internally nested, 18–28 × 20–30µm (mean 25 × 26µm) in diameter. Oogonia globose, smooth, 25–40µm (mean 33µm) in diameter. Antheridia not observed. Oospores plerotic, globose, 23–38µm (mean 31µm) in diameter, hyaline. Oospore wall thin, 0.5–1.5µm (mean 1µm) thick.

Specimen examined: CHINA. Anhui Province, Bengbu, from *Glycine max*, 28 Sep 2017, *J.J. Chen*, *Chen 314* (NJAU).

Remarks: *Phytopythium mercuriale* may be confused with *Pp. boreale*[24] Abad et al. in having lacking for antheridia and plerotic oospores, but the latter species has smaller oospores (av. 22.2µm, Duan 1985, Table 2). In addition, *Pp. boreale* is distant from *Pp. mercuriale* in the phylogenetic analysis (Figure 1).

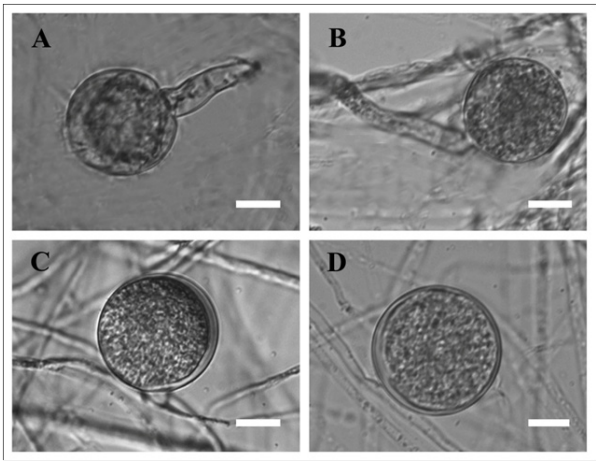

Figure 2 Asexual and sexual reproductive bodies of *Phytopythium mercuriale* (Chen 314). A-B, Subglobose sporangia with conspicuous apical papillae; C-D, Globose oogonia with plerotic oospores; Scale bars A-D=10µm.

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Table 2 Morphological description of *Phytopythium mercuriale*, *Pp. sindhum* and the most closely related species

	Pp. mercuriale (Chen 314)	*Pp. sindhum* (Chen 265)	*Pp. boreale*	*Pp. oedochilum* (Drechsler) Abad et al.	*Pp. ostracodes*
Sporangia	Subglobose	Globose to subglobose	Absent	Subglobose, lemoniform, obovoid or ovoid,	Globose to subglobose
Hyphal swelling	Absent	Absent	Present	Absent	Absent
Proliferation	External, internal and internally nested	External, internal and internally nested	Internal	External, internal and internally nested	External, internal and internally nested
Antheridia	Not observed	Monoclinous as well as diclinous	Absent	Mostly diclinous, occasionally monoclinous	Mostly monoclinous, rarely diclinous
Oospores (μm)	Plerotic av. 31	Plerotic or nearly plerotic, av. 34	Plerotic, av. 22.2	Aplerotic, av. 30.3	Plerotic or nearly plerotic, av. 32.5
Cardinal temperature	Min 4°C, optimum 25–30°C and max 38°C	Min 5°C, optimum 30°C and max 38°C	Min 4°C, optimum 25–31°C and max 43°C	Min 10°C, optimum 30°C and max 35°C	Min 10°C, optimum 30°C and max 35°C
Daily growth rates on PCA at 25°C (mm)	15/per day	14/per day	20/per day	20/per day	8/per day
Reference	This study	This study	Duan (1985)	Van der Plaats-Niterink (1981)	Van der Plaats-Niterink (1981)

***Phytopythium sindhum* A.M. Lodhi, Shahzad & Lévesque, Persoonia 24: 137, 2010** Figure 3

Colonies submerged, with rosette pattern on CMA. Average growth rates 3mm day^{-1} at 10°C, 5mm day^{-1} at 15°C, 7mm day^{-1} at 20°C, 14mm day^{-1} at 25°C, 17mm day^{-1} at 30°C, 12mm day^{-1} at 35°C, but when returned to room temperature both of them started to grow again. Cardinal temperatures: minimum 5°C, optimum 30°C, maximum 38°C. Main hyphae hyaline, aseptate, up to 6.0μm wide. No hyphal swellings. Sporangia globose to sub-globose, terminal with conspicuous apical papillae and internal nested or internal extended proliferation, 20–50 × 12.5–37.5μm (mean 32.5 × 25.5μm) in diameter. Zoospores formed in sterile water at 25°C, Encysted zoospores 7.5–12μm (mean 10μm) diam; Homothallic; oogonia globose, smooth, terminal, 30–40μm (mean 35μm) in diameter. Antheridia monoclinous or diclinous, one to two per oogonium; antheridial stalks unbranched; antheridial cells elongate, more or less lengthwise applied but crook necked, making narrow apical contact with the oogonium. Oospores plerotic or nearly plerotic, globose, 30–38μm (mean 34μm) in diameter, hyaline. Oospore wall very thick, 4.5–6.5μm (mean 5.5μm) thick.

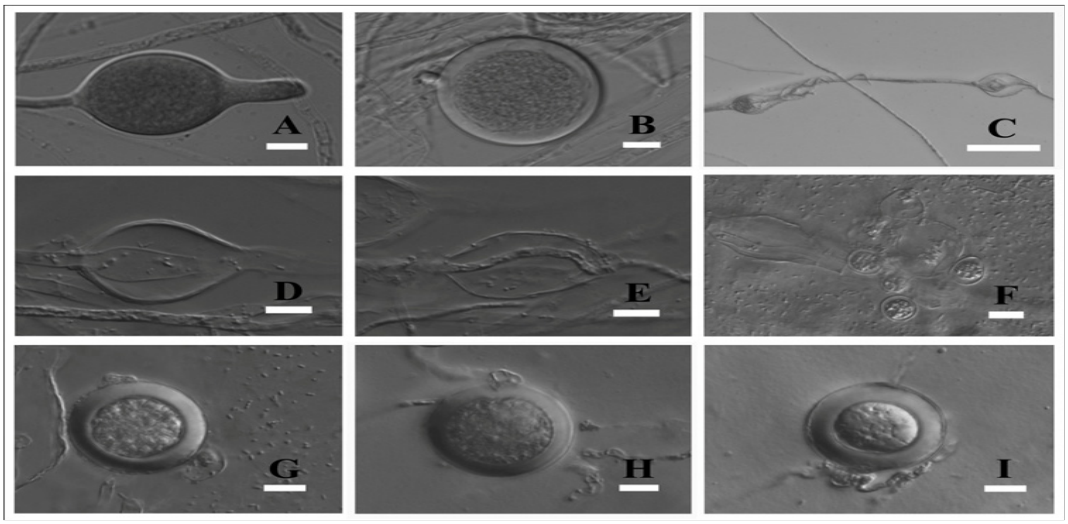

Figure 3 Asexual and sexual reproductive bodies of *Phytopythium sindhum* (Chen 265). A, Sub-globose sporangium with conspicuous apical papillae; B, Globose sporangium with short stalk; C-E, Internally nested proliferation and internal proliferation; F, Zoospore development; G, Plerotic oospore and two antheridia; H, Monoclinous antheridum; I, Diclinous antheridum; Scale bars C=5μm, A-B & D-J=10μm.

Citation: Chen JJ, Feng H, Zheng X. Two species of *Phytopythium* (Pythiaceae, Pythiales) new to China. *J Microbiol Exp.* 2019;7(5):228–233. DOI: 10.15406/jmen.2019.07.00264

Specimen examined: CHINA. Jiangsu Province, Xuzhou, from *Glycine max*, 23 Aug 2016, *J.J. Chen*, *Chen 265* (NJAU).

Remarks: *Phytopythium sindhum* is easily identified by its globose to sub-globose sporangia with conspicuous apical papillae, large and smooth oogonia, monoclinous or diclinous antheridia, and plerotic or nearly plerotic and thick-walled oospores. It is related to *Pp. ostracodes* (Drechsler) Abad et al., which also produces globose to sub-globose sporangia but differs in diclinous, occasionally monoclinous and relatively slow growth (8 mm d^{-1}).[26] Phylogenetically two cultures of *Pp. sindhum* clustered together with strong supports (100% ML, 1.0 BPPs, Figure 1) and occurred on a single branch and are distant from other species of *Phytopythium*.

Conclusion

In this study, we analyzed the phylogenetics of 19 previously accepted species of *Phytopythium*. With the aid of morphology and phylogenetic analyses of the phylogeny of ITS and *Cox1* genes, two new Chinese record, *Phytopythium mercuriale* and *Pp. sindhum* are described. Moreover, comparisons of the two new Chinese record and their morphological and/or phylogenetically related species are also provided in Table 2. Rot diseases constitute a serious challenge to soybean production. Root, seed and seedling rots in soybean in China often shows as a complex caused by several pathogenic fungi (e.g., *Fusarium graminearum* Schwabe, *F. culmorum* (Wm.G. Sm.) Sacc., and *Rhizoctonia solani* J.G. Kühn) and the oomycete *Phytophthora sojae* Kaufm. & Gerd. and *Pythium ultimum* Trow,[25] and may also be associated with plant-parasitic nematodes individually or in any possible combination.[26,27] This study significantly improved our understanding of the rare oomycetes genera *Phytopythium* associated with soybean from China. The two species obtained from this study may potentially be highly valuable. However, because little was known about the role or importance of *Phytopythium* spp. in disease on soybean in China. Thus, a further study was undertaken to determine the identity, role, pathogenicity, and virulence of *Phytopythium* spp. associated with soybean in the future.

Acknowledgments

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Conflicts of interest

Authors declare that there is no conflict of interest.

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