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Pythium glomeratum and *Py. nodosum*, two new records from China

Abstract

Two oomycetes, *Pythium glomeratum* and *Py. nodosum* were found in southern China, and they are newly recorded in China. These two species were both isolated from roots of soybean. *Py. glomeratum* is characterized by absence of zoosporangia and zoospores, and slow growth rate. And *Py. nodosum* is identified from other *Pythium* species by its smooth oogonia which are crowded with different antheridial branches making a complicated knot, and aplerotic oospores. Illustrations and descriptions of the two new records are provided based on the materials from China.

Keywords: Cox1, ITS, Oomycota, *Pythium glomeratum*, *Pythium nodosum*

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Abbreviations: BI, bayesian inference; BPP, bayesian posterior probabilities; BT, bootstrap; CI, consistency index; CMA, corn meal agar; Cox1, cytochrome c oxidase subunit 1; CTAB, cetyl trimethylammonium bromide; 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 *Pythium* Pringsheim[1] was typified by *Py. monospermum* Pringsh. The principal characteristics of this genus include a combination of 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,[6-8] more than 140 species are currently recorded in the genus *Pythium*.[8] During studies on the occurrence and diversity of *Pythium* in China, two new Chinese record of Pythium 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.

Materials and methods

Isolates

The cultures (Chen 276, 409 & 412) of *Pythium* species were isolated from roots of soybean in Beijing and Jiangsu provinces in China. The isolation procedure followed the method described by Benard & Punja.[9] Pieces of tissue 5–10 mm 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 2–3 d. 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.

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,[2] 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.[10]

Molecular phylogeny

DNA extraction, amplification, sequencing and sequence alignment

A CTAB 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.[7]

The ITS region was amplified with the primers: ITS4 and ITS5.[11] The Cox1 gene was amplified with the primers: OomCoxI-Levlo (CYTCHGGRTGWCCRAAAAACCAAA) and OomCoxI-Levup (TCAWCWMGATGGCTTTTTTCAAC).[12] 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 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.[13] 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 *Pythium* sequences downloaded from GenBank (Table 1) using ClustalX[14] and manually adjusted in BioEdit.[15] Sequence alignment was deposited at TreeBase (http://purl.org/phylo/treebase; submission ID S25483).

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 ostracodes	CBS 768.73	Spain	HQ643395	HQ708442
Pythium baisense	HMAS 242232	China	FR775440	FR774198
Py. breve	HMAS 242231	China	FR751317	FR774196
Py. buismaniae	CBS 288.31	Netherlands	HQ643479	HQ708526
Py. cystogenes	CBS 675.85	Netherlands	HQ643518	HQ708564
Py. glomeratum	CBS 122651	France	HQ643541	HQ708585
Py. glomeratum	**Chen 276**	**China**	**MN732893**	**MN756479**
Py. heterothallicum	CBS 450.67	Canada	HQ643553	HQ708597
Py. sp. jasmonium	DAOM 229150	USA	HQ643670	HQ708714
Py. mastophorum	CBS 375.72	United Kingdom	HQ643691	HQ708735
Py. megalacanthum	DAOM 229154	Germany	HQ643693	HQ708737
Py. nodosum	CBS 102274	France	HQ643709	HQ708753
Py. nodosum	**Chen 409**	**China**	**MN732894**	**MN756480**
Py. nodosum	**Chen 412**	**China**	**MN732895**	**MN756481**
Py. nunn	CBS 808.96	USA	HQ643711	HQ708755
Py. orthogonon	CBS 376.72	Lebanon	HQ643723	HQ708764
Py. perplexum	CBS 674.85	Netherlands	HQ643744	HQ708785
Py. polymastum	CBS 811.70	Netherlands	HQ643752	HQ708793
Py. splendens	CBS 462.48	USA	HQ643795	HQ708836
Py. ultimum var. sporangiiferum	CBS 219.65	USA	HQ643879	HQ708920
Py. ultimum var. ultimum	CBS398.51	Netherlands	HQ643865	HQ708906
Py. uncinulatum	CBS 518.77	Netherlands	HQ643944	HQ708985

New sequences are shown in bold

Phylogenetic analyses

Phylogenetic analysis was done as in Chen & Cui.[16] MP analysis was applied to the combined dataset of ITS-Cox1 sequences. *Phytopythium ostracodes* (Drechsler) Abad, de Cock, Bala, Robideau, A.M. Lodhi & Lévesque was used as an outgroup. The tree construction procedure was performed in PAUP* version 4.0b10.[17] 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.[18] TL, CI, RI, RC, and HI were calculated for each MPT generated. Phylogenetic trees were visualized using Treeview.[19]

MrModeltest2.3[20] 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 & discussions

Molecular phylogeny

The combined ITS + Cox1 dataset included sequences from 23 isolates representing 20 taxa. The dataset had an aligned length of 1662 characters, of which 842 characters are constant, 218 are variable and parsimony-uninformative, and 602 are parsimony-informative. MP analysis yielded one equally parsimonious tree (TL=1868, CI=0. 675, RI=0.806, RC=0.543, HI=0.325). The best model for the combined ITS + Cox1 sequences dataset estimated and applied in the BI was GTR+I+G. BI resulted in a similar topology with an average standard deviation of split frequencies = 0.001950 to MP analysis, and thus only the MP tree was provided. Both BT values (≥50%) and BPPs (≥0.95) are shown at the nodes (Figure 1). The Chinese isolates Chen 276, 409 & 412 were identical to the authorized sequences of *Pythium glomeratum* and *Py. nodosum* available in GenBank and thus clustered within clades representing *Py. glomeratum* and *Py. nodosum* with high supporting values (Figure 1), respectively.

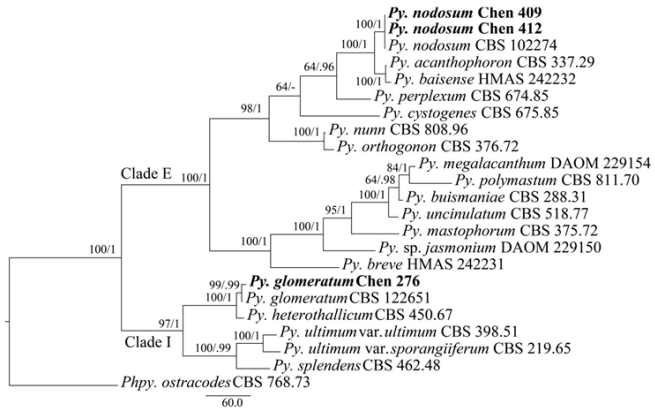

Figure 1 Phylogeny of *Pythium glomeratum* and *Py. nodosum* and related species generated by maximum parsimony based on ITS-*Cox1* sequence data. Branches are labeled with parsimony bootstrap proportions (before slanting line) high than 50% and Bayesian posterior probabilities (after slanting line)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

Taxonomy

***Pythium glomeratum* B. Paul, FEMS Microbiol. Lett. 225: 49, 2003 Figure 2**

Colonies submerged, with radiate pattern on CMA. Average growth rates 7 mm day^{-1} at 10°C, 10 mm day^{-1} at 15°C, 12 mm day^{-1} at 20°C, 14 mm day^{-1} at 25°C, 10 mm day^{-1} at 30°C, 6 mm day^{-1} at 35°C, but when returned to room temperature both of them started to grow again. Cardinal temperatures: minimum 5°C, optimum 25°C, maximum 36°C. Main hyphae hyaline, aseptate, up to 5.0µm wide. Hyphal swellings globose, sub-globose, ovoid to peanut shaped, 5–30 (mean 16.5)µm in diameter. Sporangia and zoospores not observed. Homothallic; oogonia globose, smooth, 17.5–25µm (mean 21µm) in diameter. Antheridia not observed. Oospore one per oogonium, plerotic, globose, 15.5–23.5µm (mean 18.5µm) in diameter, wall up to 0.5–2 (mean 1.6)µm thick.

Specimen examined: CHINA. Beijing, from Glycine max, 7 Sep 2017, J.J. Chen, Chen 276 (NJAU).

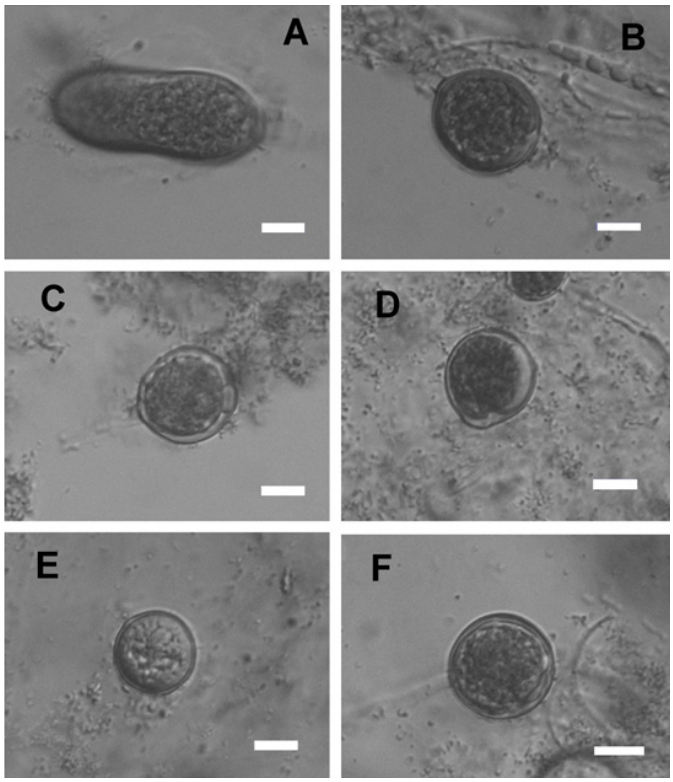

Figure 2 Asexual and sexual reproductive bodies of *Pythium glomeratum* (Chen 276). A-B. Hyphal swellings. C-F. Globose oogonia. Scale bars A-E=10µm.

Pythium nodosum (Figure 3)[21]

Colonies submerged, with radiate pattern on CMA. Average growth rates 10 mm day^{-1} at 10°C, 12 mm day^{-1} at 15°C, 15 mm day^{-1} at 20°C, 20 mm day^{-1} at 25°C, 18 mm day^{-1} at 30°C, 4 mm day^{-1} at 35°C, but when returned to room temperature both of them started to grow again. Cardinal temperatures: minimum 5°C, optimum 25°C, maximum 36°C. Main hyphae hyaline, aseptate, up to 7.0µm wide. No hyphal swellings. Sporangia globose to sub-globose, 10–27µm (mean 20µm) in diameter. Zoospores formed in sterile water at 25°C; Homothallic; oogonia globose, smooth, terminal, 10–25µm (mean 18.5µm) in diameter. Antheridia monoclinous, one to three per oogonium; antheridial stalks unbranched; antheridial cells elongate, more or less lengthwise applied but crook necked, making narrow apical contact with the oogonium. Oospores aplerotic, globose, 8–21.5 µm (mean 15.5 µm) in diameter, hyaline. Oospore wall thin, 0.5–2µm (mean 1.2µm) thick.

Specimen examined: CHINA. Jiangsu Province, Nanjing, from Glycine max, 20 June 2017, J.J. Chen, Chen 409 & 412 (NJAU).

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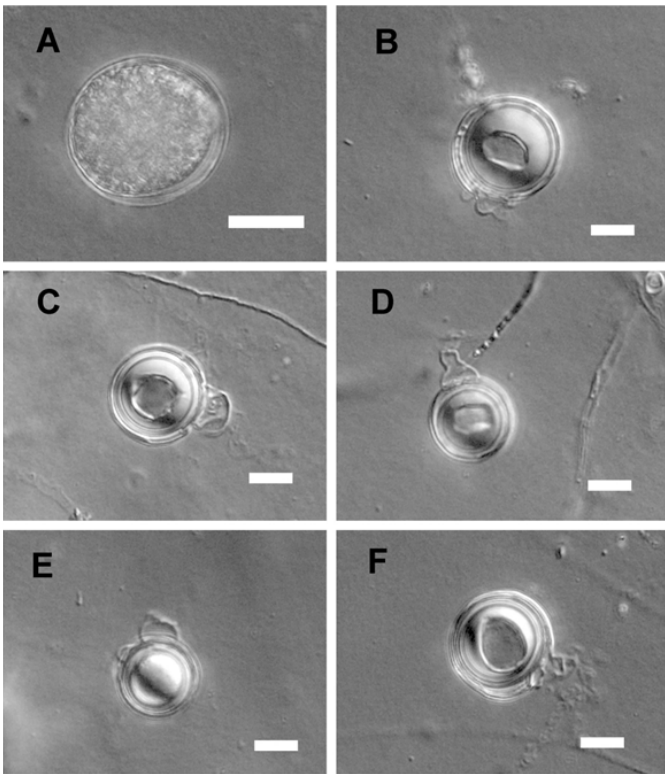

Figure 3 Asexual and sexual reproductive bodies of *Pythium nodosum* (Chen 409). A. Sub-globose sporangium. B-E. Globose oogonia, aplerotic oospores. Scale bars A-E=10μm.

Conclusion

In this study, we analyzed the phylogenetics of 14 species of *Pythium* clade E and five species of *Pythium* clade I. With the aid of morphology and phylogenetic analyses of the phylogeny of ITS and Cox1 genes, two new Chinese record, *Pythium glomeratum* and *Py. nodosum* are described. *Pythium glomeratum* has a closer relationship with *Py. heterothallicum* W.A. Campb. & F.F. Hendrix to the ITS and Cox1-based phylogeny (Figure 1). *Py. glomeratum* resembles *Py. heterothallicum* in having globose oogonia, but it is distinguished in its absence of zoosporangia and zoospores, slower growth rate and presence of sex organs.[22] *Pythium nodosum* is easily identified by its smooth oogonia which are crowded with different antheridial branches making a complicated knot, and aplerotic oospores. *Py. acanthophoron* Sideris and *Py. baisense* Y.Y. Long, J.G. Wei & L.D. Guo resemble *Py. nodosum* and the combined ITS and Cox1 sequences also suggested a close relationship between the these two species and *Py. nodosum* within *Pythium* clade E (Figure 1); however, Py. acanthophoron has ornamented oogonia,[2] while Py. baisense is distinguished from *Py. nodosum* by faster growth rate, and presence of double oospores.[10] This study significantly improved our understanding of the oomycetes genera *Pythium* 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 *Pythium* spp. in disease on soybean in China. Thus, a further study was undertaken to determine the identity, role, pathogenicity, and virulence of *Pythium* spp. associated with soybean in the future.

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Conflicts of interest

Authors declare that there is no conflict of interest.

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