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Phenotypic plasticity of the isolates assigned to *Pythium plurisporium*

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Abstract: *Pythium plurisporium* was originally isolated from the roots of bentgrass (*Agrostis palustris*). It is characterized by the production of multiple oospores in oogonium, which mostly has pedicellated stalk and swollen elements below its stalk. There are not many reports of the occurrence of this species in the literature. Recently, a report of recovering *P. plurisporium* isolates from Iran has been presented. Nevertheless, the re-examination of the isolates referring to *P. plurisporium* using morphological identification as well as multiple gene genealogies, using both nuclear (ITS and *Btub*) and mitochondrial (*cox2*) loci, arises the question about the existence of intraspecific phenotypic variation within this species. A revision of morphological characteristics among isolates assigned to *P. plurisporium* is discussed in the present paper.

Key words: *Oomycota*, morphology, pathogen, phylogeny, taxonomy

INTRODUCTION

The genus *Pythium* Pringsh. is a highly diverse, cosmopolitan and heterogeneous group containing more than 230 described species (Hyde et al. 2014). Identifying the species of *Pythium* has often arisen difficulties to researchers due to various reasons such as the absence of certain structures for morphological identification, the lack of identification keys for the species, pleomorphism of the sexual and asexual structures, and the lack of molecular identification data for the species. Molecular phylogenetic studies categorized *Pythium* spp. into clades with diverse morphological characteristics (de Cock & Lévesque 2004, Villa et al. 2006, Robideau et al. 2011, Hyde et al. 2014). Uzuhashi et al. (2010) believe that *Pythium* species belonging to the clades E to J *sensu* Lévesque & de Cock (2004) should be transferred into two new genera, *Globisporangium* Uzuhashi, Tojo & Kakish (clades E to J, including a part of clade H) and *Elongisporangium* Uzuhashi, Tojo & Kakish (another part of clade H) and other species located in the clades A to D (Lévesque & de Cock 2004) should be remained as *Pythium sensu stricto*. They also described two other genera: *Ovatisporangium* Uzuhashi, Tojo & Kakish (clade K sensu Lévesque & de Cock (2004), a later synonym to *Phytopythium*) and *Pilasporangium* Uzuhashi, Tojo & Kakish (a completely new clade with only one species) among *Pythium sensu lato* species. However, these genera are still a matter of controversy.

Identification of the morphological features of various species has been a major concern of many researchers (Bala et al. 2010a). Many *Pythium* species show intraspecific variations for some specific morphological features. Although, phylogenetic analyses using DNA sequences along with other molecular techniques have significantly assisted in the identification of unknown *Pythium* species, morphological traits comprise fundamental importance to support the identifications defined by molecular techniques.

Pythium plurisporium Abad, Shew, Grand & L.T. Lucas was first isolated from bentgrass (*Agrostis palustris* Huds.) in North Carolina and formally described as a new species belong to the clade B of ITS phylogenetic tree (Abad et al. 1995). This species was characterized by possessing multiple oospores within oogonium. *Pythium plurisporium* has been reported to be morphologically close to *P. multisporum* Poitras according to the production of more than one oospore per oogonium; however, the asymmetrical shape of oogonia in *P. plurisporium*, as well as the existence of swollen elements below oogonial stalk, separate it from *P. multisporum*. Moreover, *P. multisporum* belongs to the clade E of *Pythium* ITS phylogenetic tree (Lèvesque & de Cock 2004), whereas, *P. plurisporium* is located in the clade B of *Pythium* ITS phylogenetic tress (Lèvesque & de Cock 2004; Robideau et al. 2011). *Pythium plurisporium* isolates have been recovered from diseased root and crown of bentgrass. However, it was reported as a secondary colonizer of infected roots. Since its first isolation, no other records have been presented until

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2017 (Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2017). During the investigation of rice paddy fields of Fars province, Iran, 1129 *Pythium* isolates were recovered among which seven isolates were morphologically and phylogenetically similar to *P. plurisporium* (Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2017). Although, five other isolates were morphologically identified as a new species, phylogenetic analyses revealed that they belong to *P. plurisporium.* These isolates' morphological features were completely in the conflict of the original description. These controversial findings led to the conclusion that there could be some intraspecies pleomorphism in the morphological features of the isolates assigned to *P. plurisporium*, which is discussed in this paper.

MATERIALS AND METHODS

Sampling and isolation

Sampling was randomly conducted from rhizosphere soil, water ponds and rice seedlings from different rice paddy fields and ornamental trees of Fars province, Iran from 2013 to 2018. Coordinates were recorded for each field by Global Positioning System (GPS) (Table 1). Samples were transported to the Mycology Laboratory of the Department of Plant Protection, Shiraz University. Being washed with distilled water, the rice seedlings were placed on oomycetes semi-selective medium CMA-PARP (Ground corn extract 40 g/L; agar 15 g/L; amended with 10 µg/mL pimaricin, 200 µg/mL ampicillin, 10 µg/mL rifampicin and 25 µg/mL PCNB) (Jeffers & Martin 1986). One hundred grams of each soil sample was placed in a plastic container and flooded with tap water to 1 cm above the soil surface (Tan 1996). Isolates were recovered from either soil or water samples by baiting with 5-mm surface-sterilized bitter orange (*Citrus aurantium* L.) leaf disks or 5 mm pieces of sterile meadow grass (*Poa annua* L.) at 25 °C every 8 h for 40 h in total, and plating on CMA-PARP. Isolates were purified by hyphal tip method on water agar (WA, Agar 10 g/L) and stored on CMA (Ground corn extract 40 g/L; agar 15 g/L) slopes at 15 °C.

Morphological characterization

To observe asexual organs (sporangia, vesicle, and zoospores), isolates were transferred to CMA containing sterile hemp (*Cannabis sativa* L.) seeds or turfgrass (*Poa* sp.) on agar (Mostowfizadeh-Ghalamfarsa & Banihashemi 2005) for 24 h. Hemp seeds or turfgrass were then transferred to Petri dishes containing distilled water (Ho et al. 2012), sterile soil extract (McLeod et al. 2009) or Schmitthenner solution (Schmitthenner 1973) under fluorescent light for 24 h and were checked every 8 h for six times. Sporangial formation was examined using French bean agar (FBA, French bean extract 30 g/L; agar 15 g/L) (Jeffers & Martin 1968) and sterile soil extract (Mostowfizadeh-Ghalamfarsa et al. 2008). Sexual organs were obtained with hemp seed agar (HSA, ground hemp seed extract 60 g/L; agar 15 g/L) and carrot agar (CA, carrot extract 250 g/L; agar 15 g/L) incubated in darkness (Mostowfizadeh-Ghalamfarsa & Banihashemi 2005). To study colony morphology, isolates were grown on CMA, HSA, CA, potato-dextrose agar (PDA, potato extract 300 g/L; dextrose 20 g/L; agar 15 g/L) and malt extract agar (MEA, Malt extract 25 g/L; agar 15 g/L) (Mostowfizadeh-Ghalamfarsa & Banihashemi 2005). Five mm diameter plugs from the edge of a 3 d old culture were placed on Petri dishes containing 20 mm of a particular test media. The plates were incubated at 25 °C for 48 h. Temperature-growth relationships were tested on PDA with three replicate plates per isolate and incubated at 0, 5, 10, 15, 20, 25, 30, 35, and 40 °C. The growth rate was recorded 2–12 d after the onset of linear growth.

Sequencing and phylogenetic analyses

The method described by Mirsoleimani & Mostowfizadeh-Ghalamfarsa (2013) was employed for DNA extraction. Potato extract broth (extract of 300 g/L boiled potato in distilled water) was used for the growth of isolates. Mycelia were harvested, freeze-dried, and DNA extracts were obtained using a DNG™-PLUS extraction kit (Sinagene, Iran) according to the manufacture's instruction. The DNA quality was examined with an MD-1000 Nanodrop machine (NanoDrop Technologies, USA). The primers used for amplification and sequencing of nuclear (Internal transcribed spacers 1, 2 and 5.8S region of rDNA (ITS-rDNA) and *β-tubulin* gene (*Btub*)) as well as mitochondrial (cytochrome c oxidase subunit II (*cox*2)) loci are listed in Table 3. The PCR conditions for these loci are listed in Table 4. PCR products were purified and sequenced with the primers used for amplification by a dye terminator cycle (Bioneer, South Korea). Sequenced data were deposited into GeneBank and accession numbers were obtained.

Resulting sequences were edited by Bioedit (Hall 1999). The sequence alignment of the amplicons together with data extracted from GenBank (Table 2) was conducted by ClustalX (Thompson et al. 1997) with subsequent visual adjustment. To reconstruct the phylogenetic trees, Bayesian inference analyses on individual and concatenated ITS, *Btub* and *cox2* loci were carried out with MrBayes v. 3.1 (Rounquist & Huelsenbeck 2003), imposing a general time-reversible (GTR) substitution model with gamma (G) and proportion of invariable site (I) parameters to accommodate variable rates across sites. Bayesian analyses were conducted with the same data set according to Safaeifarahani et al. (2015). The best nucleotide substitution model was determined by MrModelTest v. 2.3 (Nylander 2004). Two independent runs of Markov Chain Monte Carlo (MCMC) using four chains were run over 1,000,000 generations. Trees were saved each 1000 generations, resulting in 10001

trees. Burn-in was set at 5% generations. *Pythium nagaii* S. Ito & Tokun. was chosen as an outgroup. Phylogenetic trees were edited and displayed with TREEGRAPH (Stöver & Müller 2010). Partition homogeneity tests were conducted on combined nuclear and mitochondrial gene alignments by PAUP* 4.0a136 (Swofford 2002) using 100 replicates and heuristic general search option. Alignments and trees were submitted to TreeBASE (http://www.treebase.org).

Pathogenicity

Resulting isolates were evaluated for their ability to cause stunting, post- and pre-emergence damping-off and seed rot, and their pathogenicity on rice plants. Inoculum preparation was conducted by the method described by Salmaninezhad & Mostowfizadeh-Ghalamfarsa (2019) with mycelium inoculated vermiculite amended with 120 mL/L hempseed extract (extract of 60 g boiled hemp seed).

Table 1. List of *Pythium plurisporium* isolates recovered from rice paddy fields of Fars Province of Iran with their GenBank accession numbers.

Isolate Code	Date	Location	Substrate	Coordinates		Accession No.		
				Latitude	Longitude	ITS	Tub	Cox2
***Pythium plurisporium* Group I**								
Kb440	16-Aug-14	Kamfiruz	*Oryzae sativa* Pond water	30°20.426'N	052°16.460'E	N/A	N/A	N/A
KC12	20-May-14	Ramjard	*Oryzae sativa* root	30°05.671'N	052°35.522'E	KX228082	N/A	N/A
Kh419	16-Aug-14	Kamfiruz	*Oryzae sativa* Nursery soil	30°17.131'N	052°19.039'E	N/A	N/A	N/A
Kh423	16-Aug-14	Kamfiruz	*Oryzae sativa* Nursery soil	30°17.412'N	052°18.682'E	N/A	N/A	N/A
Kh424	16-Aug-14	Kamfiruz	*Oryzae sativa* Nursery soil	30°17.396'N	052°18.698'E	N/A	N/A	N/A
Kh425	16-Aug-14	Kamfiruz	*Oryzae sativa* Pond water	30°17.395'N	052°18.696'E	N/A	N/A	N/A
Kh426	16-Aug-14	Kamfiruz	*Oryzae sativa* Pond water	30°17.392'N	052°18.693'E	N/A	N/A	N/A
***Pythium plurisporium* Group II**								
045-1	1-Aug-14	Kamfiruz	*Oryzae sativa* root	30°16.545'N	052°19.659'E	KX228085	KX228110	KX228123.2
SS	1-Nov-15	Ramjard	*Oryzae sativa* crown	30°06.568'N	052°34.164'E	KX228084	KX228111	KX228122
PS	1-Aug-14	Kamfiruz	*Oryzae sativa* root	30°17.421'N	052°18.692'E	KX228086	KX228112	KX228121
HS	1-Aug-14	Kamfiruz	*Oryzae sativa* Soil	30°18.199'N	052°17.635'E	N/A	N/A	N/A

Table 2. GenBank accession numbers of *Pythium* species used for phylogenetic reconstructions.

Species	Isolate Code	Substrate/Host	GenBank Accession no.		
			ITS	*cox2*	*Btub*
P. afertile	LEV 2066	Turf grass	HQ643416	KJ595440	KJ595563
P. angustatum	CBS 522.74	Soil	AY598623	KJ595387	KJ595511
P. apleroticum	CBS 772.81	*Nymphyoides petata*	AY598631	KJ595400	KJ595524
P. aquatile	CBS 215.80	Soil	AY598632	KJ595355	KJ595481
P. aristosporum	ATCC 11101	*Triticum aestivum*	AY598627	AB095060	DQ071297
P. arrhenomanes	1994-15	Unknown	AY598628	AF196587	KJ595451
P. capillosum	CBS 222.94	Soil	AY598635	KJ595360	KJ595485
P. catenulatum	CBS 842.68	Turf grass	AY598675	KJ595404	KJ595528
P. coloratum	CBS 154.64	Soil (tree nursery)	AY598633	KJ595346	KJ595474
P. conidiophorum	CBS 223.88	Soil	AY598629	KJ595361	KJ595486
P. diclinum	CBS 664.79	*Beta vulgaris*	AY598690	KJ595394	KJ595518
P. dissimile	CBS 155.64	*Pinus radiata*	AY598681	KJ595347	KJ595475
P. dissotocum	CBS 166.68	*Triticum aestivum*	AY598634	KJ595351	KJ595479
P. flevoense	CBS 234.72	Soil	AY598691	KJ595363	KJ595488
P. folliculosum	CBS 220.94	Unknown	HQ643540	N/A	MK752994
P. graminicola	CBS 327.62	*Saccharum officinarum*	AY598625	AF196593	KJ595452
P. inflatum	CBS 168.68	Unknown	AY598626	KJ595352	DQ071313
P. kashmirense	CBS 122908	Soil	HQ643671	KJ595429	KJ595553
P. longipapillum	CBS 141231	*Oryzae sativa*	KX228104	KX228128	KX228116
P. lutarium	CBS 222.88	Soil	HQ643682	KJ595359	KJ595484
P. marinum	CBS 750.96	Soil	AY598689	KJ595398	KJ595522
P. myriotylum	CBS 254.70	*Arachis hypogaea*	AY598678	KJ595365	KJ595490
P. nagaii	CBS 779.96	Soil	AY598705	KJ595402	JX397970
P. oopapillum	CBS 124053	*Cucumis sativus*	FJ655174	KJ595431	KJ595556
P. pachycaule	CBS 227.88	Soil	AY598687	KJ595362	KJ595487
P. pectinolyticum	CBS 122643	Unknown	MK015671	N/A	KJ595469
P. periilum	CBS 169.68	Unknown	AY598683	N/A	N/A
P. phragmitis	CBS 117104	Soil (*Phragmites australis*)	HQ643746	AJ890351	EU152854
P. plurisporium	CBS 100530	*Agrostis*	AY598684	KJ595405	KJ595529
P. pyrilobum	CBS 158.64	*Pinus radiata*	AY598636	KJ595349	KJ595477
P. rhizo-oryzae	CBS 119169	Soil	HQ643757	KJ595420	KJ595545
P. rishiriense	CBS 139278	Water	AB998878	N/A	N/A
P. salpingophorum	CBS 471.50	*Lupinus angustifolius*	AY598630	KJ595384	KJ595508
P. scleroteichum	CBS 294.37	*Ipomoea batatas*	AY598680	KJ595370	KJ595495
P. sukuiense	CBS 110030	Soil	HQ643836	KJ595408	KJ595532
P. sulcatum	CBS 603.73	*Daucus carota*	AY598682	KJ595393	KJ595517
P. tardicrescens	LEV 1534	Turf grass	HQ643855	KJ595439	KJ595562
P. torulosum	CBS 316.33	Grass	AY598624	KJ595374	KJ595499
P. tracheiphilum	CBS 323.65	Unknown	AY598677	KJ595375	N/A
P. vanterpoolii	CBS 295.37	*Triticum aestivum*	AY598685	KJ595371	KJ595496
P. volutum	CBS 699.83	*Triticum* and *Hordeum*	AY598686	KJ595397	KJ595521
P. zingiberis	CBS 21682	Unknown	HQ643973	DQ071402	DQ071349

Table 3. List of primers used in this study.

Target DNA	Primer name	Primer sequence (5′→3′)	Reference
ITS[a]	ITS4	TCCTCCGCTTATTGATATGC	White et al. 1990
	ITS6	GAAGGTGAAGTCGTAACAAGG	Cooke et al. 2000
Btub[b]	BT5	GTATCATGTGCACGTACTCGG	Villa et al. 2006
	BT6	CAAGAAAGCCTTACGACGGA	Villa et al. 2006
cox2[c]	FM66	TAGGATTTCAAGATCCTGC	Villa et al. 2006
	FM58	CCACAAATTTCACTACATTGA	Villa et al. 2006

[a]Internal transcribed spacers 1, 2 and 5.8S gene of rDNA. [b] β-tubulin. [c] cytochrome c oxidase subunit II.

Table 4. PCR conditions for primers used in this study.

Gene	Initial desaturation	Number of cycles	Desaturation	Annealing	Expansion	Final expansion
ITS[a]	95 (120) [d]	30	95 (20)	55 (25)	72 (50)	72 (600)
Btub[b]	95 (120)	30	95 (20)	63 (25)	72 (50)	72 (600)
cox2[c]	95 (120)	30	95 (20)	52 (25)	72 (50)	72 (600)

[a]Internal transcribed spacers 1, 2 and 5.8S region of rDNA. [b] β-tubulin. [c] cytochrome c oxidase subunit II. [d] Temperature '°C' (time s')

For pre-emergence damping-off tests, rice seeds were washed and planted in sandy loam (1:1) soil (500 mL) amended with 10 mL inoculum. Post-emergence damping-off was examined on 20 d old seedlings inoculated with 10 mL inoculum per pot (500 mL soil). Control pots contained only hempseed extract amended vermiculite. Symptoms were monitored two weeks after inoculation. To evaluate the ability of various isolates in the colonization of root and crown tissues of rice, after being cut into 0.5 mm pieces, roots and crown were washed, blotted and placed on the CMA-PARP medium at 25 °C. The growth rate was checked every 12 h during a week (Afeck et al. 1990).

RESULTS

Pythium plurisporium isolates from Iran

A total of 12 isolates of *Pythium* spp. With filamentous sporangia were recovered from rice paddy fields of Fars province. These isolates formed two distinct morphological groups: the first group consists of seven isolates which were morphologically similar to the original description of *P. plurisporium* (Abad et al. 1996). Morphometric results can be seen in Table 5. The representative sequenced isolate of the group was located in the vicinity of *P. plurisporium* in Clade B of ITS phylogenetic tree (Lévesque & de Cock 2004) (Fig. 1).

Some other recovered isolates from Iran were morphologically different from the others as well as the original description of *P. plurisporium*. The representative sequenced isolates of this group also appeared as *P. plurisporium* both in ITS and combined gene trees (Fig. 1 and 2). Variations have been observed in these isolates (Table 5). The morphological features of both groups (i.e. Group I and Group II) are described below.

Morphological Group I

Colonies on PDA, MEA, HSA, and CA show rosette pattern, on CMA with uniform pattern (Fig. 3a). Main hyphae up to 6 µm wide. Sporangia are filamentous, slightly inflated with long discharge tube (Fig. 4a). Sporangia were abundantly formed in liquid media after 10 h (Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2017). Vesicles and zoospores are produced on sterile hempseed in water cultures after 12 h at 25 °C. Oogonia are obpyriform, smooth, terminal, aplerotic, consisting of at most two oospores, (Fig. 4b–f), and variable in size (Table 5).

None of the isolates produced more than two oospores in a single oogonium. Antheridia are 6–12 per oogonium, crook-necked, mostly monoclinous, and sometimes diclinous, with a terminal contact, paragynous (Fig. 4b–f). Antheridium origination in monoclinous oospores is near oogonium stalk. Although no swollen elements were observed in the oogonial stalk, the stalk was swollen itself (Fig. 4). None of these isolates produced any papillae on oogonium. Oospores are mostly globose, aplerotic, up to 30 µm in diam, with a wall which is up to 3 µm thick. Morphometric results are shown in Table 5. Colonies on PDA have an average radial growth rate of 5.5 mm/d at 15 °C, 10 mm/d at 20 °C, 25 mm/d at 25 °C, 27 mm/d at 30 °C and 35 °C, 7 mm/d at 40 °C and no growth at 5 °C and 10 °C (Fig. 7). *Cardinal temperatures:* optimum 35 °C, minimum 15 °C, and maximum 40 °C.

Specimens examined. IRAN, Fars Province: Kamfiruz, (30°20.426'N−052°16.460'E), from pond water of *Oryzae sativa*, 16 Aug. 2014, *F. Salmaninezhad Kb440;* Kamfiruz, (30°17.131'N−052° 19.039'E), from nursery soil of *Oryzae sativa*, 16 Aug. 2014, *F. Salmaninezhad Kh416;* Kamfiruz, (30°17. 412'N −052°18.682'E), from nursery soil of *Oryzae sativa*, 16 Aug. 2014, *F. Salmaninezhad Kh423;* Kamfiruz, (30°17.396'N−052°18.698'E), from nursery soil of *Oryzae sativa*, 16 Aug. 2014, *F. Salmaninezhad Kh424;* Kamfiruz, (30°17.395'N−052°18.696'E), from pond water of *Oryzae sativa*, 16 Aug. 2014, *F. Salmaninezhad Kh425;* Kamfiruz, (30°17.392'N−052°18.693'E), from pond water of *Oryzae sativa*, 16 Aug. 2014, *F. Salmaninezhad Kh426;* Ramjard, (30°05.671'N−052°35.522'E), from root of *Oryzae sativa*, 20 May 2014, *F. Salmaninezhad KC12*. GenBank: ITS = KX228082.

All isolates of the Group I was extremely pathogenic on rice seedlings causing post-emergence

damping-off (Fig. 5a; Table 6). The representative isolate of this grouped was located in the vicinity of *P. plurisporium* in the Clade B of ITS phylogenetic tree (Lèvesque & de Cock 2004).

Morphological Group II

Colonies on MEA show no specific pattern, on PDA, HSA and CA show an intermediate pattern between radial to rosette and on CMA show an approximately radial pattern (Fig. 3b). Main hyphae have 2.9–4.3 (av. 3.2) µm width. Sporangia are filamentous, slightly inflated with a rather long discharge tube (Fig. 6a). Vesicles and zoospores are formed plentifully on sterile hempseed in water cultures after 12–24 hours at 20–25 °C. Oogonia are globose, smooth, terminal and intercalary, 25.9–27.2 (av. 26.2) µm. More than 50 % of the oogonia have one or two papillae which are 1.5–6.7 (av. 2.2) µm long (Fig. 6b).

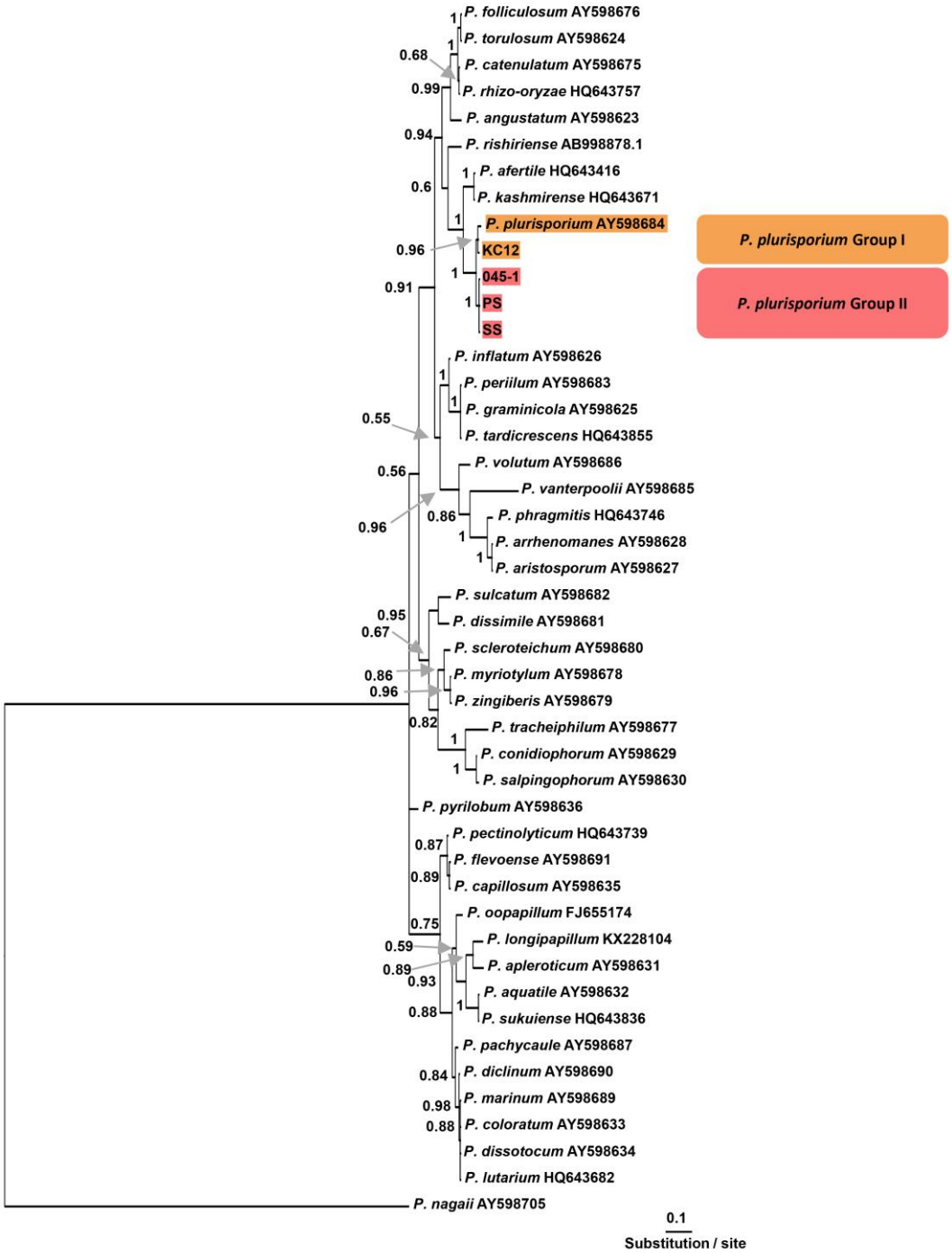

Fig. 1. Phylogenetic relationships of *Pythium plurisporium* from paddy fields of Fars province among 42 *Pythium* species based on the comparison of internal transcribed spacers 1, 2 and 5.8S region of rDNA sequences in a Bayesian probability tree. Numbers above the branches represent posterior probability based on Bayesian analysis. *Pythium nagaii* is used as an outgroup taxon.

Antheridia are 2–5 per oogonium, crook-necked, mostly diclinous, and sometimes monoclinous, with a terminal contact, paragynous (Fig. 6c). Antheridium origination in monoclinous oospores is near oogonium stalk. Oospores are globose, aplerotic, 22.3–23.2 (av. 23.0) µm with a wall which is 1.3–2.1 (av. 1.5) µm thick. Morphometric results are shown in Table 5. Colonies on PDA have an average radial growth rate of 3.5 mm/d at 15 °C, 5 mm/d at 20 °C, 10 mm/d at 25 °C, 11 mm/d at 30 °C and 35 °C, 2 mm/d at 40 °C and no growth at 5 °C and 10 °C (Fig. 7). *Cardinal temperatures:* optimum 35 °C, minimum 15 °C, and maximum 40 °C.

Phylogenetic analyses using both nuclear (ITS, *Btub*) and mitochondrial (*cox2*) loci revealed that this taxon is located in Clade B in the vicinity of *P. plurisporium* in a separate monophyletic group (Fig. 1 and 2).

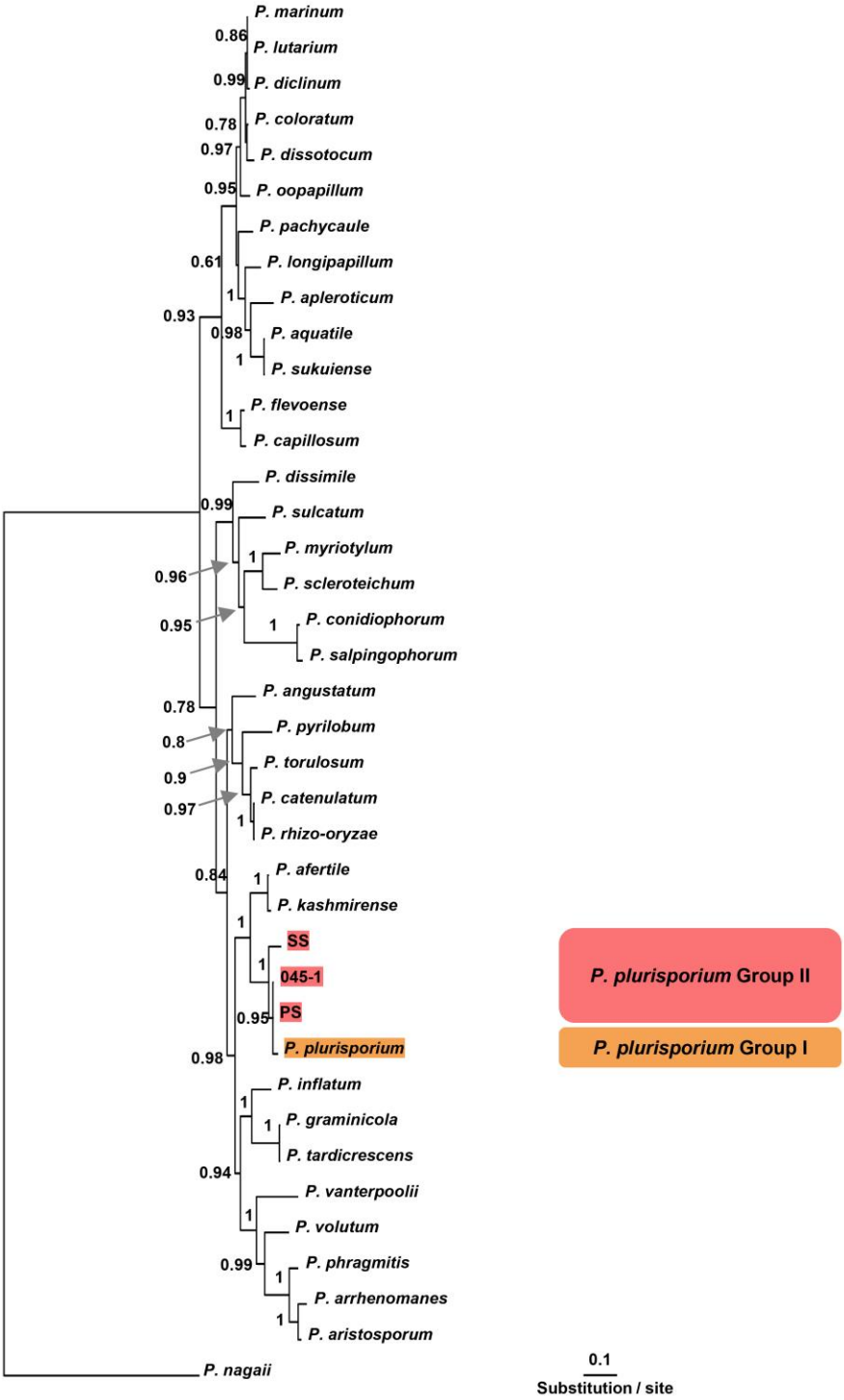

Fig. 2. Phylogenetic relationships of *Pythium plurisporium* from paddy fields of Fars Province among 36 *Pythium* species based on the analysis of multigene genealogies of nuclear (ITS and *Btub*) and mitochondrial (*cox2*) sequences. Values on branches are posterior probability based on Bayesian analysis greater or equal to 0.5. *Pythium nagaii* is used as an outgroup taxon.

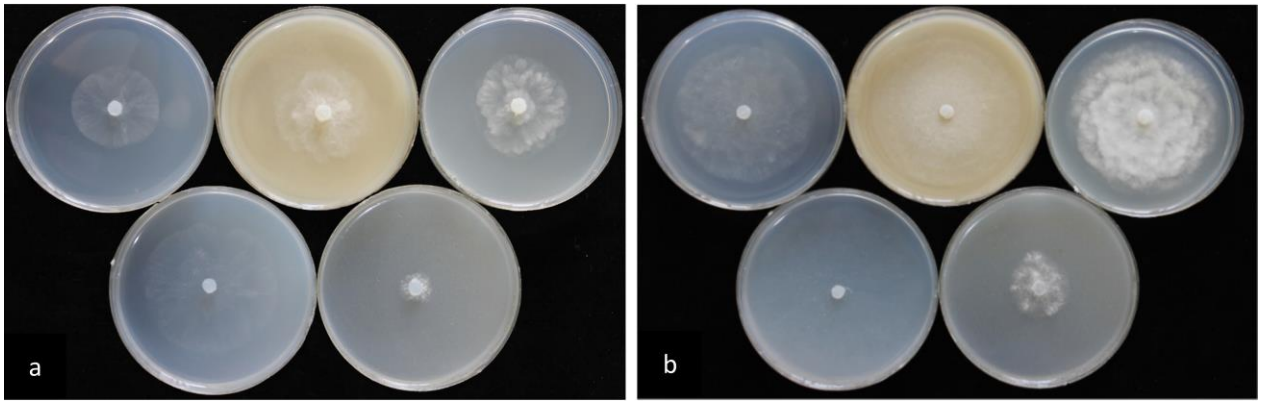

Fig. 3. Colony morphology of examined groups of *Pythium plurisporium* in this study: a. Group I; b. Group II after 24 h on various media at 25 °C; top (from left to right): carrot agar, malt extract agar and potato-dextrose agar; bottom (from left to right): cornmeal agar and hempseed agar.

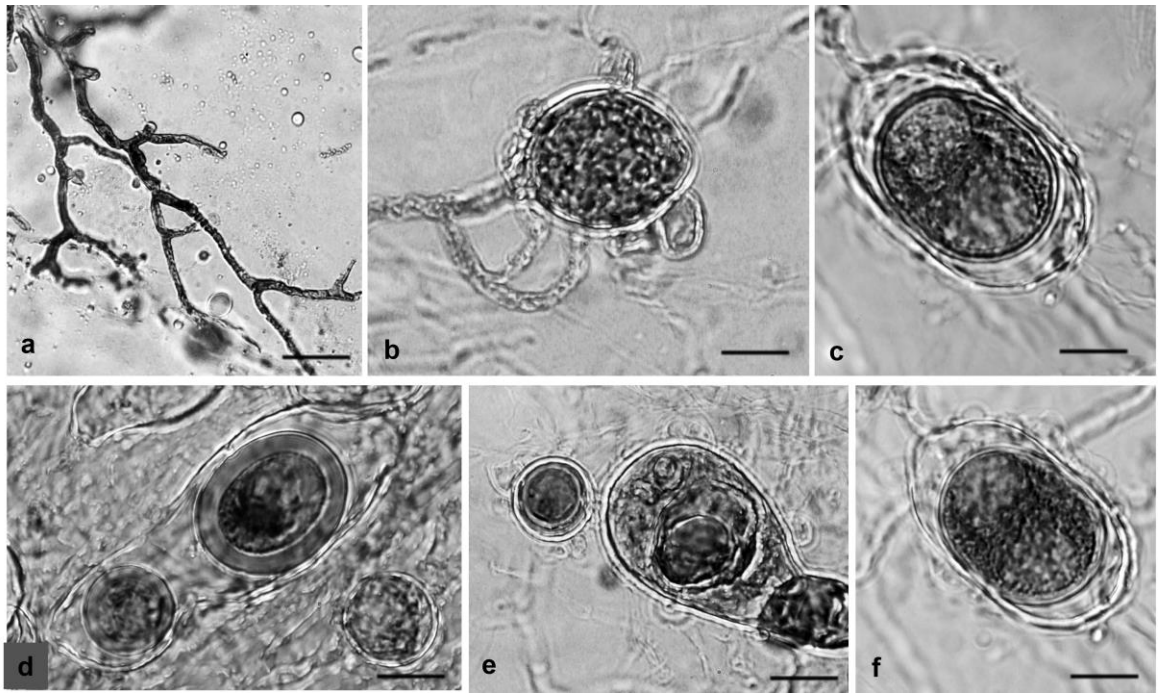

Fig. 4. Morphology of *Pythium plurisporium* (Group I). a. filamentous, slightly inflated sporangium; b. oospore with monoclinous and diclinous antheridia; c. ellipsoid aplerotic oogonium; d. aplerotic oogonium with two oospores; e. oogonium with swollen stalk; f. Oogonium with 10 to 12 paragynous antheridia. — Scale bars = b-f: 10 µm, a: 20 µm .

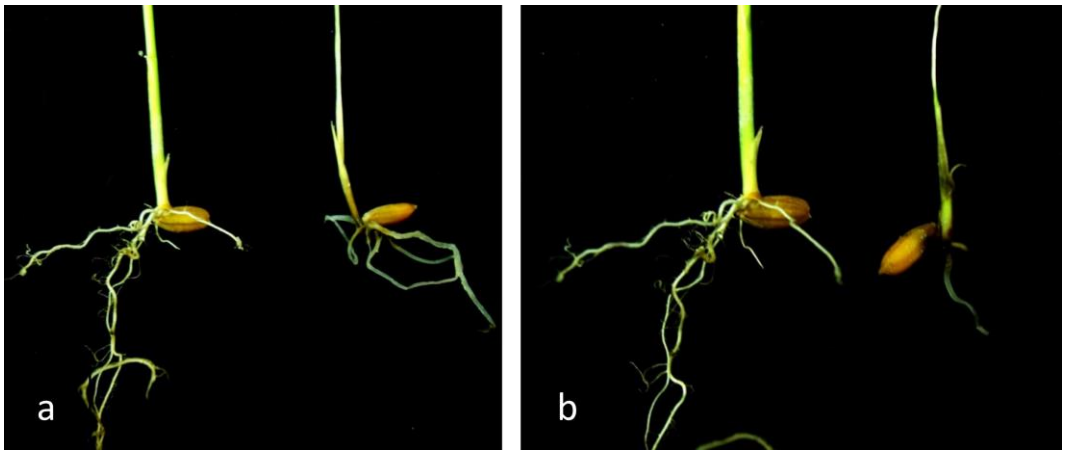

Fig. 5. Pathogenicity tests on roots and crown of rice (*Oryzae sativa*) by representatives of *Pythium plurisporium* groups. a. Group I (KC12) which causes post-emergence damping-off and root rot (left: control; right: infected crown and roots). b. Group II (045-1) which causes severe root and crown rot; pre- and post-emergence damping-off (left: control; right: infected crown and root).

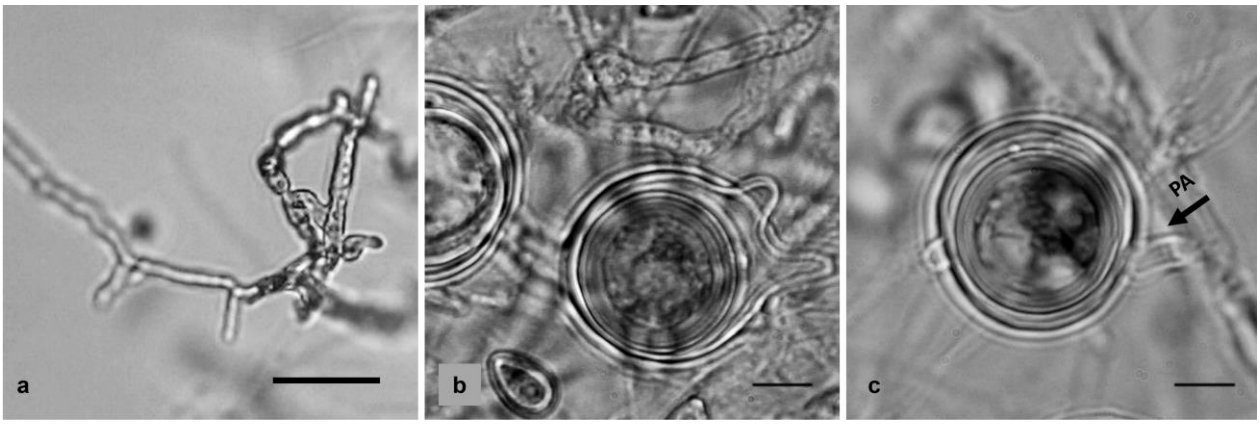

Fig. 6. Morphological structures of *Pythium plurisporium* (Group II). A. filamentous slightly inflated sporangium; b. aplerotic oospore with two long papillae; c. aplerotic oospore with a papilla and paragynous antheridium. — Scale bars = a: 20 µm, b-c: 10 µm.

Specimens examined. IRAN, FARS PROVINCE: Kamfiruz (30°16.545'N−052°19.659'E), from the roots of *Oryzae sativa*, 16 Aug 2014, *F. Salmaninezhad 045-1* (CBS 140940). GenBank: ITS = KX228085; *Btub* = KX228110; *cox*2 = KX228123.2. IRAN, FARS PROVINCE: Ramjard (30°06.568'N−052°34.164'E), from *Oryzae sativa* crown, 9 Nov 2015, *F. Salmaninezhad SS*. GenBank: ITS = KX228074; *Btub* = KX228111; *cox*2 = KX228122. IRAN, FARS PROVINCE: Kamfiruz (30°17.421'N−052°18.692'E), from root of *Oryzae sativa*, 16 Aug 2014, *F. Salmaninezhad PS*. GenBank: ITS = KX228086; *Btub* = KX228112; *cox*2 = KX228121. IRAN, FARS PROVINCE, Kamfiruz (30°18.199'N−052°17.635'E), from rhizosphere soil of *Oryzae sativa* paddy fields, Aug 2014, *F. Salmaninezhad*, HS.

All isolates of the Group II were severe pathogens of rice seedlings, causing pre- and post-emergence damping-off, as well as root, crown, and seed rot (Table 6).

Note. The length of papilla was mainly more than 3 µm in the isolate 045-1. Generally, oogonia in this isolate (i.e. 045-1) contained more than one papilla. However, more than 60% of oogonia had only one papilla in other isolates.

DISCUSSION

Among 1129 *Pythium* isolates recovered from rice paddy fields of Fars Province, Iran, 12 isolates were assigned to *P. plurisporium* (Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2017). These isolates formed two distinct morphological groups:

Group I consist of isolates KC12, Kb440, Kh419, Kh423, Kh424, Kh425, and Kh426. These isolates were recovered from rice root, pond water, and nursery soil (Table 1). All the isolates produced 1 to 2 oospores per oogonium. Colony morphology on all examined media was rosette form, except for CMA, which was uniform. This was in contrast with the original description of *P. plurisporium* which was reported to be chrysanthemum on CA and CMA (Abad et al. 1996).

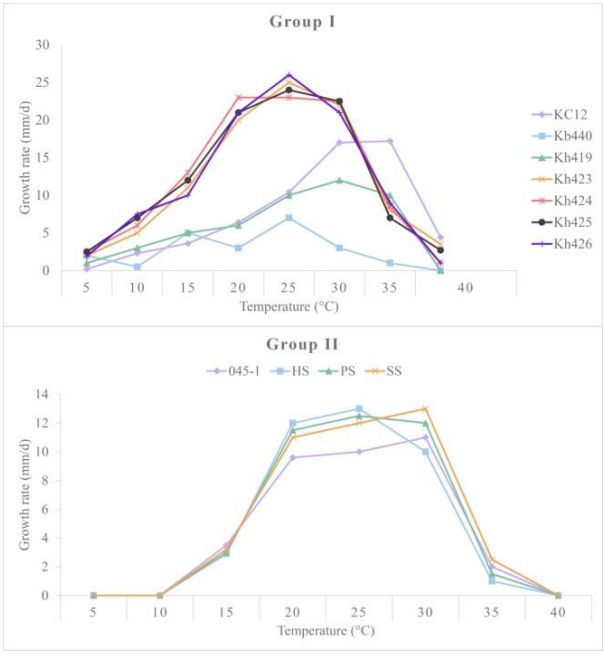

Fig. 7. Average radial growth rate of *Pythium plurisoprium* isolates from Iran on potato-dextrose agar at different temperatures; Group I (upper diagram) Group II (lower diagram).

In our study of this group, total oogonial size was larger than the first description (Table 5). No hyphal rings or appressoria was observed in our isolates. This was in contrast with the isolates described by Abad et al. (1996) where hyphal rings were observed. Iran's isolates' total oospore, oogonium, and antheridium size were larger than North Carolina's (Table 5). Furthermore, the first report described *P. plurisporium* as a species with subglobose oogonia; however, our isolates produced obpyriform oogonia. The original description of *P. plurisporium* reported that 4 to 8 antheridia exist per oogonium; whereas, we've observed more antheridia (6 to 12 per oogonium).

Table 5. Comparison of *Pythium plurisporium* isolates from the original description (North Carolina) and Iran isolates.

Characters	Isolates													
	Abad *et al.* 1996			*Pythium plurisporium* Group I							*Pythium plurisporium* Group II			
	L39	L143	L147	Kh426	Kh425	Kh424	Kh423	Kh419	Kb440	KC12	045-1	HS	PS	SS
Colony morphology on PDA	No data	No data	No data	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Int.	Rad.	Int.	Int.
Colony morphology on CA	Chry.	Chry.	Chry.	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Int.	Int.	Ros.	Int.
Colony morphology on HSA	No data	No data	No data	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Int.	Int.	Int.	Chry.
Colony morphology on MEA	No data	No data	No data	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	Ros.	N/P.	N/P.	N/P.	N/P.
Colony morphology on CMA	Chry.	Chry.	Chry.	Uni.	Uni.	Uni.	Uni.	Uni.	Uni.	Uni.	App. Rad.	Rad.	Rad.	Uni.
Growth on PCA (mm/d)	25	25	25	25	25	27	25	24	27	25	10	10	10	10
Sporangia	Lobate	Lobate	Lobate	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.
Hyphae (µm)	5-6.25	5-6.25	5-6.25	5.168-6.992	5.535-7.004	4.783-6.524	4.003-6.052	4.993-7.024	4.914-6.012	5.003-6.312	2.9–4.3	2.7–4.0	2.8–4.4	2.9–4.2
Hyphal rings (µm)	50	50	50	A	A	A	A	A	A	A	A	A	A	A
Appressoria	Rare	Rare	Rare	A	A	A	A	A	A	A	A	A	A	A
Sporangia	Lobate	Lobate	Lobate	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.
Hyphae (µm)	5-6.25	5-6.25	5-6.25	5.168-6.992	5.535-7.004	4.783-6.524	4.003-6.052	4.993-7.024	4.914-6.012	5.003-6.312	2.9–4.3	2.7–4.0	2.8–4.4	2.9–4.2
Hyphal rings (µm)	50	50	50	A	A	A	A	A	A	A	A	A	A	A
Appressoria	Rare	Rare	Rare	A	A	A	A	A	A	A	A	A	A	A
Sporangia	Lobate	Lobate	Lobate	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.
Hyphae (µm)	5-6.25	5-6.25	5-6.25	5.168-6.992	5.535-7.004	4.783-6.524	4.003-6.052	4.993-7.024	4.914-6.012	5.003-6.312	2.9–4.3	2.7–4.0	2.8–4.4	2.9–4.2
Hyphal rings (µm)	50	50	50	A	A	A	A	A	A	A	A	A	A	A
Appressoria	Rare	Rare	Rare	A	A	A	A	A	A	A	A	A	A	A
Sporangia	Lobate	Lobate	Lobate	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightly Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.	Fila. Slightl y Infla.
Antheridia type	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mono-Dic	Mostly Dic.	Mostly Dic.	Mostly Dic.	Mostly Dic.
Antheridia per oogonium	5–8	5–8	5–8	6–12	6–12	6–12	6–12	6–12	6–12	6–12	2–5	2–5	2–5	2–5
Antheridia attachment	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.	Para.
Antheridia size (µm**)**	6-8 × 12-17	6-8 × 12-17	6-8 × 12-17	5.993*13.902-8.831*18.083	6.071*13.135-8.913*18.325	6.083*14.910-8.120*18.544	7.503*15.993-10.030*21.231	6.989*14.068-9.025*20.001	6.921*13.015-8.531*18.603	6.042*13.031-8.515*18.407	6.0 * 14.0–8.4 * 16.7	5.8 * 12.7–8.0 * 15.2	6.0 * 14.2–8.0 * 16.0	6.0 * 13.9–8.3 * 15.7
Oospore shape	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Mostly Glo.	Glo.	Glo.	Glo.	Glo.
Oospore size (µm)	12.1-25	12.1-25	12.1-25	23.067-37.242	19.063-27.246	20.357-38.962	11.351-28.585	14.368-30.005	14.782-28.359	12.143-24.805	22.3–23.2	21.9–23.0	22.5–23.2	22.5–23.5
Oospore wall (µm)	1.25-2.50	1.25-2.50	1.25-2.50	2.411	3.146	2.098	2.984	3.014	3.062	2.143	1.5±0.5	1.3±1.0	1.5±0.5	1.5±0.5

Table 6. Pathogenicity results of the *Pythium plurisporium* isolates examined in this study.

Species	Isolate	Pathogenicity on rice	Symptom					Host tissue colonization*
			Post-emergence damping-off (%)	Pre-emergence damping-off (%)	Seed rot (%)	Stunting (%)	No growth (%)	
***Pythium plurisporium* Group I**								
	Kb440	+	55	0	0	0	0	+
	KC12	+	60	0	0	0	0	+
	Kh424	+	55	0	0	0	0	+
	Kh425	+	60	0	0	0	0	+
	Kh426	+	50	0	0	0	0	+
	Kh423	+	65	0	0	0	0	+
	Kh419	+	65	0	0	0	0	+
***Pythium plurisporium* Group II**								
	HS	+	80	60	50	50	60	+
	045-1	+	90	70	60	50	40	+
	PS	+	80	50	40	60	50	+
	SS	+	70	50	50	40	40	+

On the other hand, variation between these isolates has also been observed (i.e. antheridial and oogonial size as well as growth rate on PCA) (Table 5). The sequenced isolate appeared in Clade B of ITS phylogenetic tree (Lèvesque & de Cock 2004), as *P. plurisporium.*

Group II included the isolates 045-1, SS, PS, and HS. All isolates produced filamentous, slightly inflated to dendroid sporangia, aplerotic oospores with 2–5 paragynous, crook-necked antheridia per oogonium. In contrast to *P. plurisporium* original description, these isolates never produced more than one oospore in a single oogonium (FIG. 2). Moreover, the existence of one to two papillae on oogonial surface was only observed in this group of isolates. Globose oogonia and smaller size of oogonium and antheridium were other distinct characteristics from the original description of *P. plurisporium.* Furthermore, this group's growth rate on PCA was relatively slower than group I and the first description of *P. plurisporium.* Besides, this group had variable colony morphology on different media (Table 5). However, the phylogenetic analyses of this group showed a very close relationship with original *P. plurisporium* (see below).

Pathogenicity test results also confirmed the existence of two groups within *P. plurisporium* examined isolates. Despite being able to colonize root and crown tissues, Group I isolates could only cause post-emergence damping-off; whereas Group II isolates could severely cause pre- and post-emergence damping-off, stunting, seed rot, and prohibit seedlings' growth (Table 6).

These isolates are located in the clade B of ITS phylogenetic tree (Lévesque & de Cock 2004), in the *P. kashmirense* B. Paul, *P. afertile* Kanouse & T. Humphery, and *P. plurisporium* group. The isolates were morphologically close to *P. kashmirense* and ex-type of *P. plurisporium* from Abad *et al.* (1995) study. Formation of loose loops of antheridia filaments around oogonium and coiling around the oogonial stalk separate *P. kashmirense* (Paul & Bala 2008) from these isolates. Production of strictly filamentous sporangia and globose to irregular hyphal swellings separates *P. afertile* (Van der Pläats-Niterink 1981) from our isolates. The production of papilla on the oogonial surface has been reported for *P. oopapillum* Bala & Lèvesque (Bala et al. 2010b) and another recently described species, *P. longipapillum* Mostowfizadeh-Ghalamfarsa & Salmaninezhad (Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2019). However, *P. oopapillum* has only one papilla on each oogonium (Bala et al. 2010b), whereas these isolates produced two papillae per oogonium. Moreover, *P. longipapillum* produces strictly filamentous sporangia, indistinguishable from the vegetative hyphae, and rarely up to three antheridia per oogonium, while these isolates have filamentous slightly inflated sporangia and a greater number of antheridia per oogonium. Another important feature of the isolates is the production of two adjacent papillae per oogonium, however, *P. longipapillum* only produces one papilla per oogonium (Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2019).

Group I isolates were morphologically relatively close to *P. plurisporium*. Although it has been reported that *P. plurisporium* produces lobate sporangia with complex structures, all our isolates (i.e. Group I and II) produced only filamentous slightly inflated sporangia. Group II and *P. plurisporium* main isolates are thoroughly different from each other based on sexual structures, colony morphology, and cardinal temperatures. The absence of more than one oospore per oogonium, fewer antheridia, and specific colony morphology of these isolates, differentiated them from *P. plurisporium* original description. Besides, most of our Group II isolates produced two long papillae on oogonium, which has never been reported in *P. plurisporium.* Hence, it could be hypothesized that intraspecific variation exists within *P. plurisporium* at least in the matter of morphology.

The isolates of Group II appeared as a member of *P. plurisporium* clade in ITS, *Btub*, and combined gene trees in phylogenetic analyses. It was not true for *cox2*, where the isolate SS was separated from *P. plurisporium* (Data not shown), however, other isolates of this group located in the vicinity of *P. plurisporium* in *cox2* tree. This might be due to the existence of different haplotypes in the cytoplasmic genome of SS, which has a maternal inheritance. Our further investigation on isolate SS did not confirm the hybrid origin of this isolate. Generally, phylogenetic studies on multiple genealogies of nuclear (ITS and *Btub*) sequences, consistently showed that *P. plurisporium* lineage formed a robust monophyletic group which shared a common ancestor with all the *Pythium* species within clade B.

Morphological plasticity is a common issue in *Pythium* species (Mostowfizadeh-Ghalamfarsa & Salmaninezhad 2020). Many *Pythium* species can have multiple variations of a specific morphological feature within a single species (Van der Pläats-Niterink 1981, Zitnick-Anderson 2013). For instance, *P. deliense* antheridia can be in the monoclinous, diclinous, intercalary, or terminal positions (Van der Pläats-Niterink 1981). Other examples of this phenomenon are *P. adhaerens* Sparrow with both terminal and intercalary oogonia, *P. anadrum* Drechsler with both monoclinous and diclinous antheridia as well as unisporous and multisporous oogonia, *P. catenulatum* V. D. Matthews with both terminal and intercalary oogonia as well as monoclinous, diclinous, clavate and crook-necked antheridia, *P. hydnosporum* (Mont.) J. Schröt. and *P. mastophorum* Drechsler with both plerotic and aplerotic oospore, *P. hypogynum* Middleton with both terminal and intercalary sporangia, and *P. multisporum* with subglobose, globose, oblong and limoniform

sporangia as well as both monoclinous and diclinous antheridia (Van der Pläats-Niterink 1981). In most of these examples, the intraspecific variation in morphological features show overlapping ranges, however, in the case of *P. plurisporium* groups these features hardly overlap. Although rare, the shape of the oogonium can be smooth or ornamented in some *Pythium* species such as *P. heteroogonium* Mostowfizadeh-Ghalamfarsa & Salmaninezhad, *P. irregulare* Buisman, and *P. carbonicum* B. Paul (Middleton 1943, Van der Pläats-Niterink 1981; Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2019). The same phenomenon has been observed in our isolates, forming two morphological groups, in this study.

Several challenges have been reported in the taxonomy of the genus *Pythium*, such as overlapping of morphological features, difficulties in isolation of certain species, lack of definite morphological structures, pleomorphism, uncertainty in GenBank database, and conflicts between morphological identification and phylogenetic analyses. Considering both morphological and molecular identification methods and their advantages and defects, it is extremely recommended to use both morphological and molecular methods to an accurate identification of *Pythium* species (Mostowfizadeh-Ghalamfarsa & Salmaninezhad 2020). Our results suggested that there might be intraspecific variation within *P. plurisporium* isolates. However, only 12 isolates from Iran and three isolates from North Carolina have been thoroughly examined for *P. plurisporium*. Although, there are few world records of *P. plurisporium* isolates (Abad et al. 1996, Salmaninezhad & Mostowfizadeh-Ghalamfarsa 2017), finding and examining a larger number of isolates could better impose the existence of plasticity in *P. plurisporium.* Moreover, only three loci have been examined in this study. Therefore, conducting a comprehensive phylogeny based on more nuclear and mitochondrial loci could also reveal that whether these isolates belong to the same phylogenetic species or the variation within morphological characters would also appear in molecular taxonomy. Furthermore, the pathogenicity of *P. plurisporium* has been tested only on rice and bentgrass. Even though, it has been originally described as a second colonizer of bentgrass roots, our results revealed that two distinct morphological groups of *P. plurisporium* are able to cause different symptoms at different rates. As a consequence, studying the host range of isolates assigned to *P. plurisporium* and conducting a comparison between their abilities to colonize various hosts would clarify the biological borders of this taxon.

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انعطاف پذیری پدیدگانی جدایه‌های منسوب به *Pythium plurisporium*

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چکیده:

گونه *Pythium plurisporium* در اصل از ریشه‌های چمن (*Agrostis palustris*) جداسازی شده است. این گونه با تولید چندین اُاسپور در یک اُاگونیوم شناخته می‌شود که اُاگونیوم اغلب دارای دم‌پایک و عناصر متورم در زیر پایه است. گزارش‌های زیادی از وجود این جنس در مقالات نیست. اخیراً گزارشی از جداسازی *P. plurisporium* از ایران ارائه شده است. اما ارزیابی مجدد جدایه-های منصوب به *P. plurisporium* با استفاده از شناسایی ریخت‌شناختی و دودمان‌های چند ژنی، با به کارگیری هر دو ژن‌گاه‌های هسته‌ای (ITS و *Btub*) و میتوکندریایی (*cox2*)، پرسش‌هایی را در مورد وجود تنوع پدیدگانی درون‌گونه‌ای در این گونه مطرح کرده است. بازبینی خصوصیات ریخت‌شناختی در جدایه‌های منصوب به *P. plurisporium* در این مقاله بحث شده است.

کلمات کلیدی: اُاُمیکوتا، ریخت‌شناسی، بیمارگر، فیلوژنی، تاکسونومی

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