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Morphological and molecular characterization of Oomycetes associated with root and crown rot of cucurbits in Kermanshah province, Iran

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Abstract: *Pythium* and *Phytophthora* are among the most well-known plant pathogens around the world that cause rotting of seeds, root, and crown, seedling death, and soft rot of fruits in contact with the soil. In this research, 347 isolates of these two genera and their close genus, *Phytopythium* were isolated from the cucurbits fields in Kermanshah province, Iran and examined in terms of morphological and physiological characteristics. ITS-rDNA region and the partial cytochrome oxidase II (*cox* II) gene from the selected isolates were amplified and sequenced to confirm the morphological identification. Based on the morphological, morphometrical, physiological, and phylogenetic examinations, nine species of *Pythium* including *P. aphanidermatum*, *P. dissotocum*, *P. catenulatum*, *P. kashmirense*, *P. middletonii*, *P. nodosum*, *P. oligandrum*, *P. torulosum*, and *P. ultimum*; two species of *Phytopythium* including *Pp. mercuriale* and *Pp. litorale*, and three species of *Phytophthora* including *Ph. melonis*, *Ph. nicotianae*, and *Ph. parasitica* were detected. Among the species identified in this study, *Pp. mercuriale* was a new record for mycobiota of Iran and two species, *P. aphanidermatum* and *P. ultimum* were isolated more frequently.

Key words: *Pythium*, *Phytophthora*, *Phytopythium*, damping-off, *Cucurbitaceae*

INTRODUCTION

Oomycetes such as *Pythium* and *Phytophthora* are among the most well-known plant pathogens around the world that cause rotting of seeds, root, and crown, damping and decay of the lower parts of the stem, tubers, and corms, and soft rot of fruits in contact with soil (Erwin & Ribeiro 1996, Kucharek & Mitchell 2000).

The genus *Pythium* and *Phytophthora* are taxonomically classified in the Kingdom *Stramenopila*, phylum *Oomycota*, class *Oomycetes* (Ainsworth 2008, Dick 1990). The traditional classification of genus *Phytophthora* is mainly based on the morphological characteristics of sporangia, gametangia, and oospores (Newhook et al. 1978, Stamps et al. 1990, Tucker 1931, Waterhouse 1963). Waterhouse (1963) divided the genus into six distinct groups based on morphological characteristics. She published the key for identifying isolates based on the characteristics of sporangium, antheridium shape, and homothallic or heterothallic tendency. *Pythium* spp. are traditionally classified according to sexual and non-sexual structures, in which the forms of sporangium and oogonium ornamentations are the main traits (Schroeder et al. 2013). The main constraints for the identification and classification of these species are: the lack of clear and distinct morphological characteristics, the high number of species, low number of traits, difficulty and inefficiency in culturing isolates and, comparison of their morphological characteristics with each other by microscope (Bala et al. 2010, Robideau et al. 2011, Wang et al. 2003). If there is an adequate database of reference strains, DNA-based identification can be done quickly and easily by a non-specialist and precise results can be achieved in the shortest time (Robideau et al. 2011).

Cooke et al. (2000) published the first datasets of ITS region sequences that included all known and available *Phytophthora* species. They introduced sequences in this region as a barcode for

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identification of species of this genus. In the following, Levesque & de Cock (2004) provided similar comprehensive datasets for the identification of *Pythium* species. They subdivided the genus into 11 clades (A to K), using the ITS sequences and the large subunit ribosomal DNA (28S rDNA). Villa et al. (2006) analyzed ITSI-5.8S-ITSII rDNA regions, cytochrome oxidase II gene (*cox II*), and the *β-tubulin* gene. The *β-tubulin* gene was analyzed in 58 isolates representing 39 species of *Pythium* and 17 isolates representing nine species of *Phytophthora* to examine the phylogenetic relationships between the isolates and these two genera. The results of the parsimony analysis of these three regions were four monophyletic groups. Those were completely inconsistent with the classification of isolates based on the morphology of sporangium. Further research revealed that the species belonging to the clade K were correctly intermediate between *Pythium* and *Phytophthora*, in terms of morphological and phylogenetic properties. Therefore the new genus *Phytopythium* was proposed for members of this clade (Bala et al. 2010, de Cock et al. 2015).

Iran is one of the top four countries in the world in cucurbits production and has a long history in cucurbit cultivation (Pitrat et al. 1997). Thereby, we aimed the current study to evaluate the diversity and distribution of plant-associated oomycetes. It was found that cucurbit fields in Kermanshah Province were the habitat of diverse species of oomycetes phytopathogens.

MATERIALS AND METHODS

Sampling, isolation and maintaining of isolates

Diseased samples were collected randomly from different cucurbits fields (including cucumber, watermelon, melon, and squash) in Kermanshah province, western Iran. During late May to late September 2014, cucurbit fields were visited. Crown and roots of plants showing symptoms of foliar blight were examined carefully. Samples with characteristic symptoms of oomycetes blight or seedling damping-off were collected, kept in paper bags, and transferred to the laboratory. To isolate oomycetes, 2-5mm pieces were prepared from the border of healthy and infected tissues of crown, root or stem, surface sterilized with 70% ethanol for 10 seconds, air dried on sterile filter paper, and transferred to cornmeal agar-PARP (CMA-PARP) (Jeffers & Martin 1986). The Petri dishes were kept at 25°C and the purification was carried out using the hyphal-tip method (Tuite 1969). The purified isolates were transferred to tubes containing CMA medium and kept at 15°C.

Identification of isolates

Preliminary identification of the oomycetes isolates was based on morphological and physiological examination and compared with available pieces of literature (Dick 1990, Van der Plaats-Niterink 1981). The morphological and physiological characteristics that were examined and recorded are as follows: morphology of sporangium (elliptical, egg-shaped, inverted pear-shaped, lime-shaped, spheroid, filamentous), oogonium surface decorations (flat or decorated), the amount of space that has been captured by oospore in oogonium (plerotic or aplerotic), the origin (diclinous and monoclinous), the connection type of antheridium to oogonium (paragynous or hypogynous), the diameter of the mycelium, formation of hyphal swelling, physiological characteristics including colony morphology on a variety of media such as Corn Meal Agar (CMA), Malt Extract Agar (MEA), Potato Carrot Agar (PCA), Potato Dextrose Agar (PDA) and Hemp Seed Agar (HSA), growth rate on different culture media, and growth temperatures. To ensure long-term preservation of isolates, pure cultures of all identified species were deposited at Iranian Fungal Culture Collection (IRAN …C) at the Iranian Research Institute of Plant Protection, Tehran, Iran.

DNA extraction and PCR amplification

Genomic DNA of selected isolates grown in PDB medium and extracted using the DNG^{TM}-PLUS kit (CinnaGen, Iran). ITS-rDNA region and mitochondrial cytochrome oxidase gene of sub-unit II (*cox* II) were amplified using the primer pairs ITS6/ ITS4 (White et al., 1990) and FM66/ FM58 (Martin, 2000), respectively. The PCR mixture was prepared by mixing the following: 50 ng of template DNA, one micromole of each primer, 100µM dNTPs, 0.4 µmol *Taq* DNA polymerase (Sinagen, Iran), 1.5 µmol of $MgCl_2$, 2.5 µl polymerase chain reaction buffer (200 µm Tris-HCl with pH 8 and 500 mM KCl), and 100 µM BSA for 25 µl reactions. Cycling conditions consisted of an initial denaturation at 95 °C for 2 minutes, 30 PCR cycles of denaturation at 95 °C for 20 seconds, annealing at 55 °C for 25 seconds, and extension at 72 °C for 50 seconds. These were followed by a final extension at 72 °C for 10 minutes using a Biometra thermo-cycler (Tpersonal, Germany). The PCR products were purified and sequenced from both direct and reverse directions by Macrogen, Inc. (South Korea). The sequences were manually edited using the Bioedit software (Hall, 1999). Edited sequences were submitted to the GenBank (http: //www.ncbi.nlm. nih.gov/genbank) (Table 1 and 2).

Multiple sequence alignments of the newly generated sequences and sequences of the valid species, derived from the GenBank (Tables 2 and 3), were performed with Clustal X software version 2.0.11 (Thompson et al. 1997), checked and improved manually where necessary. The neighbor-joining algorithm was used to generate the initial tree with bootstrap analysis with 500 replicates, using MEGA5 software (Tamura et al. 2011).

Table 1. Isolates of *Pythium*, *Phytopythium* and *Phytophthora* were used for phylogenetic analyses based on ITS-rDNA sequence in this study. Newly generated sequences are in bold.

Species	Isolate	Host/Substrate	ITS	Reference
P. angustatum	CBS 522.74	soil	AY598623	Levesque & de Cock 2004
P. anandrum	CBS 285.31	*Rheum rhaponticum*	AY598650	Levesque & de Cock 2004
P. amasculinum	CBS 552.88	soil vegetable garden	AY598671	Levesque & de Cock 2004
P. adhaerens	CBS 520.74	Soil	AY598619	Levesque & de Cock 2004
P. aphanidermatum	P36-3	*Agrostis* sp.	AB095052	Kageyama et al. 2005
P. aphanidermatum	**Pa1-1C**	***Cucumis sativus***	**KY785377**	**This study**
P. aristosporum	ATCC11101	*Triticum aestivum*	AB095042	Kageyama et al. 2005
P. arrhenomanes	ATCC96525	Cynodon dactylon	AB095041	Kageyama et al. 2005
P. aquatile	CBS 215.80	unknown	AY598632	Levesque & de Cock 2004
P. catenulatum	Oom089	Turf	GU233294	Barboza 2014
P. catenulatum	**Pc70-1W**	***Citrullus lanatus***	**KY785393**	**This study**
P. catenulatum	**Pc36-1C**	***Cucumis sativus***	**KY785405**	**This study**
P. carolinianum	ATCC 3643	Soil	AY987038	Robideau et al. 2011
P. chondricola	CBS 203.85	*Chondrus crispus*	AY598620	Levesque & de Cock 2004
P. coloratum	CBS 154.64	Soil	AY598633	Levesque & de Cock 2004
P. cystogenes	CBS 675.85	*Vicia faba*	AY707985	Levesque & de Cock 2004
P. deliense	MAFF305568	*Cucurbita pepo*	AJ233442	Matsumoto et al. 1999
P. diclinum	CBS 664.79	*Beta vulgaris*	AY598690	Levesque & de Cock 2004
P. dimorphum	CBS 406.72	*Pinus taeda*	AY598651	Levesque & de Cock 2004
P. debaryanum.	ATCC 48115	*Tulipa* sp.	AY598704	Levesque & de Cock 2004
P. dissotocum	KC3	Corn field	KP063129	Bolboli & Mostowfizadeh-Ghalamfarsa 2015
P. dissotocum	**Pd32-1C**	***Cucumis sativus***	**KY785397**	**This study**
P. dissimile	CBS 155.64	*Pinus radiata*	AY598681	Villa et al. 2006
P. echinulatum	CBS 281.64	soil forest nursery	AY598639	Levesque & de Cock 2004
P.erinaceum	CBS 505.80	*Triticum aestivum*	AY598694	Levesque & de Cock 2004
P.folliculosum	CBS 220.94	Soil	AY598676	Levesque & de Cock 2004
P.flevoense	CBS 234.72	Soil	AY598691	Levesque & de Cock 2004
P.glomeratum	F-304	Soil	AY263339	Paul 2003
P. graminicola	IFO31998	*Hordeum vulgare*	AB217664	Villa et al. 2006
P. grandisporangium	CBS 286.79	*Distichilis spicata*	AY598692	Levesque & de Cock 2004
Ph. helicoides	CBS286.31	*Phaseolus vulgaris*	AB108026	Villa et al. 2006
P. heterothallicum	CBS 450.67	soil	AY598654	Levesque & de Cock 2004
P. hydnosporum	MAFF305861	soil	AJ233445	Matsumoto et al. 1999
P. hypogynum	CBS 234.94	soil	AY598693	Levesque & de Cock 2004
P. inflatum	MAFF305863	soil	AJ233446	Matsumoto et al. 1999
P. insidiosum	CBS 574.85	*Equus ferus caballus*	AY598637	Levesque & de Cock 2004
P. intermedium	MAFF305570	soil	AJ233447	Matsumoto et al. 1999
P. irregulare	NBRC 10011	*Phaseolus vulgaris*	AB107995	Matsumoto et al. 1999
P. iwayamai	CBS 156.64	soil	AY598648	Levesque & de Cock 2004
P. kashmirense	LB3	Barley field	KP063131	Bolboli & Mostowfizadeh-Ghalamfarsa 2015
P. kashmirense	**Pk83-1C**	***Cucumis sativus***	**KY785396**	**This study**
P. kunmingense	CBS 550.88	soil	AY598700	Levesque & de Cock 2004
P. lutarium	CBS 222.88	soil	AY598688	Levesque & de Cock 2004
P. mamillatum	CBS 251.28	*Beta vulgaris*	AY598703	Levesque & de Cock 2004
P. marsipium	CBS 773.81	*Nymphoes peltata*	AY598699	Levesque & de Cock 2004
P. marinum	CBS 750.96	soil	AY598689	Levesque & de Cock 2004
P. macrosporum	CBS 574.80	flower bulb	AY598646	Levesque & de Cock 2004
Pp. mercuriale	V61	soybean	AB627346	Kato et al. 2013
Pp. mercuriale	**Pm23-1C**	***Cucumis sativus***	**KY785379**	**This study**
Pp. mercuriale	**Pm23-2C**	***Cucumis sativus***	**KY785381**	**This study**
Pp. mercuriale	**Pm40-1S**	***Cucurbita maxima***	**KY785380**	**This study**
P. middletonii	CBS 528.74	soil	AY598640	Levesque & de Cock 2004
P. middletonii	**Pmi77-1C**	***Cucumis sativus***	**KY785395**	**This study**
P. middletonii	**Pmi82-1C**	***Cucumis sativus***	**KY785404**	**This study**
P. monospermum	CBS 158.73	unknown	AY598621	Levesque & de Cock 2004
P. myriotylum	ATCC26082	*Spinacia oleracea*	AB095047	Kageyama et al. 2005
P. nagaii	CBS 779.96	soil	AY598705	Levesque & de Cock 2004
P. nodosum	CBS102274	soil	HQ643709	Robideau et al. 2011
P. nodosum	**Pn86-1C**	***Cucumis sativus***	**KY785399**	**This study**
P. nodosum	**Pn45-1W**	***Citrullus lanatus***	**KY785400**	**This study**
P. nunn	ATCC20693	soil	AJ233451	Matsumoto et al. 1999
Ph. oedochilum	CBS292.37	*Phlox panicula*	AB108020	Kageyama et al. 2005
P. okanoganense	CBS 315.81	*Triticum aestivum*	AY598649	Levesque & de Cock 2004
P. orthogonon	DS2-6-9D	*Zoysia japonica*	AJ233452	Matsumoto et al. 1999
P. oligandrum	CBS 382.34	*Viola* sp.	AY598618	Levesque & de Cock 2004
P. oligandrum	**Po4-2W**	***Citrullus lanatus***	**KY785383**	**This study**
P. oligandrum	**Po3-2W**	***Citrullus lanatus***	**KY785386**	**This study**
Ph. ostracodes	CBS768.73	soil	AB108022	Kageyama et al. 2007
P. paddicum	IFO31993	*Hordeum vulgare*	AB217667	Villa et al. 2006
P. parvum	CBS 225.88	soil	AY598697	Levesque & de Cock 2004
P. paroecandrum	CBS157.64	soil	AJ233453	Matsumoto et al. 1999
P. periplocum	NBRC100114	*Zoysia japonica*	AJ233455	Matsumoto et al. 1999

Table 1. Continued

Species	Isolate	Host/Substrate	ITS	Reference
P. periilum	CBS 169.68	soil	AY598683	Levesque & de Cock 2004
P. perplexum	CBS 674.85	*Vicia faba*	AY598658	Levesque & de Cock 2004
P. pleroticum	CBS 776.81	*Nymphoides peltata*	AY598642	Levesque & de Cock 2004
P. porphyrae	IFO 30347	*Porphyra yezoen*	AY598673	Matsumoto et al. 1999
P. pyrilobum	1–R–44	soil	JQ898473	Jiang et al. 2012
P. radiosum	CBS 217.94	soil	AY598695	Levesque & de Cock 2004
P. rhizooryzae	CBS119169	soil	HQ643757	Robideau et al. 2011
P. rostratum	DS5-7-1S	*Agrostis* spp.	AJ233456	Villa et al. 2006
P. rostratifingens	CBS 115464	soil	AY707986	Levesque & de Cock 2004
P. spinosum	OD231	*Daucus carota*	AJ233457	Villa et al. 2006
P. salpingophorum	CBS 471.50	*Lupinus angustif*	AY598630	Levesque & de Cock 2004
P. scleroteichum	CBS 294.37	*Ipomoea batatas*	AY598680	Levesque & de Cock 2004
P. splendens	BS 462.48	unknown	AY598655	Levesque & de Cock 2004
P. sulcatum	CTMa7	*Daucus carota*	AJ233458	Villa et al. 2006
P. sylvaticum	OM121	*Daucus carota*	AJ233459	Villa et al. 2006
P. torulosum	6–25–3	soil	JQ898476	Jiang et al. 2012
P. torulosum	**Pt35-7W**	***Citrullus lanatus***	**KY785391**	**This study**
P. torulosum	**Pt35-3W**	***Citrullus lanatus***	**KY785390**	**This study**
P. torulosum	**Pt37-1C**	***Cucumis sativus***	**KY785389**	**This study**
P. torulosum	**Pt36-5C**	***Cucumis sativus***	**KY785388**	**This study**
P. torulosum	**Pt37-2C**	***Cucumis sativus***	**KY785387**	**This study**
P. torulosum	**Pt35-6W**	***Citrullus lanatus***	**KY785403**	**This study**
P. torulosum	**Pt35-1W**	***Citrullus lanatus***	**KY785378**	**This study**
P. torulosum	**Pt35-5W**	***Citrullus lanatus***	**KY785392**	**This study**
P. torulosum	**Pt37-3C**	***Cucumis sativus***	**KY785384**	**This study**
P. tracheiphilum	CBS 323.65	*Lactuca sativa*	AY598677	Levesque & de Cock 2004
P. undulatum	CBS 157.69	soil under *Pinus* sp.	AY598708	Levesque & de Cock 2004
P. ultimom	NBRC 10012	*Beta vulgaris*	D86515	Villa et al. 2006
P. ultimom	**Pu38-1C**	***Cucumis sativus***	**KY785385**	**This study**
P. vanterpoolii	P39-1	*Agrostis* spp.	AB160847	Villa et al. 2006
Ph. Vexans	NBRC100112	*Zoysia japonica*	AJ233449	Villa et al. 2006
P. violae	OPy4	*Violax wittrockiana*	AB217669	Levesque & de Cock 2004
P. volutum	IFO31926	*Triticum aestivum*	AJ233464	Villa et al. 2006
P. zingiberum	UOP389	*Zingiber officinale*	AJ233465	Villa et al. 2006

RESULTS AND DISCUSSION

Identification of oomycetes isolates

During the field surveys, a total of 313 samples of diseased plants were collected and 347 isolates of oomycetes were isolated. As many as nine species of *Pythium* (including *P. aphanidermatum*, *P. dissotocum*, *P. catenulatum*, *P. kashmirense*, *P. middletonii*, *P. nodosum*, *P. oligandrum*, *P. torulosum,* and *P. ultimum),* two *Phytopythium* species (*Pp. mercuriale* and *Pp. litorale),* and three *phytophthora* species (including *Ph. melonis*, *Ph. nicotianae,* and *Ph. parasitica)* were identified. Those were identified on the basis of the morphological and physiological characteristics and sequence data obtained from ITS–rDNA region and *cox* II locus. Based on the available literature, *Pp. mercuriale* (among the species identified in this study) is a new record for the Iranian mycobiota. Moreover, *Pp. mercuriale*, *P. torulosum*, *P. kashmirense*, and *P. nodosum* are reported for the first time as oomycetes associated with root and crown rot of cucurbits. Furthermore, *P. dissotocum*, *Pp. litorale*, and *P. catenulatum* are reported for the first time from diseased cucurbits in Iran. Morphological description of this seven newly-recorded species in this study is given in alphabetical order as follows:

Pythium catenulatum, V.D. Matthews (1931)

The colonies had a rose-shaped pattern on CMA, PDA, and MEA, chrysanthemum colony pattern on HSA, and intermediate growth pattern on PCA. Hypha were up to 4µm wide. Hyphal swelling, 10 to 20µm in diameter and usually found in chains of three to eight (Fig. 1, a1), each producing one to three germination tubes. No chlamydospore and appressorium were observed. Sporangia were composed of jagged and flaccid mycelia, 17 to 20µm in diameter with either regular or irregular splitting (Fig. 1, a2). They produced zoospore at 20 to 25 °C. The cysts were about 8 to 9 µm in diameter. The oogonia were spherical in shape, 19 to 25 µm in diameter, with smooth walls without decorations, formed terminally or intercalary. Antheridia were commonly seen in diclinous and paragynous forms and there were more than one (often five) antheridium per oogonium (Fig. 1, a3). The oospores were spherical in shape, smooth, often aplerotic, rarely plerotic, with a wall thickness of 1.5µm on an average. The minimum, optimum, and maximum growth temperatures were 7, 30 and 37 °C respectively. The average daily growth rate was 15 mm at 25 °C on CMA. The species was placed in clade B of ITS and cytochrome oxidase II phylogenetic trees (Fig. 2 and Fig. 3).

Table 2. The list of species and isolates of *Pythium* and *Phytopythium* were used for phylogenetic analyses based on *cox* II sequence. Newly generated sequences are in bold.

Species	Isolate	Host/Substrate	Accession No	Reference
P. torulosum	1994-18	Turf	AF196628	Martin 2000
P. torulosum	**Pt37-1C**	***Cucumis sativus***	**MG813937**	**This study**
P. torulosum	**Pt35-5W**	***Citrullus lanatus***	**MG813940**	**This study**
P. torulosum	**Pt35-7W**	***Citrullus lanatus***	**MG813939**	**This study**
P. torulosum	**Pt37-3C**	***Cucumis sativus***	**MG813933**	**This study**
P. torulosum	**Pt36-5C**	***Cucumis sativus***	**MG813936**	**This study**
P. torulosum	**Pt37-2C**	***Cucumis sativus***	**MG813935**	**This study**
P. torulosum	**Pt35-3W**	***Citrullus lanatus***	**MG813938**	**This study**
P. torulosum	**Pt35-1W**	***Citrullus lanatus***	**MG813931**	**This study**
P. graminicola	ATCC96234	Corn field soil	AB160849	Kageyama et al. 2005
P. catenulatum	NBRC 100104	*Zoysia grass*	DQ071372	Villa et al. 2006
P. catenulatum	**Pc36-1C**	***Cucumis sativus***	**MG813947**	**This study**
P. catenulatum	**Pc70-1W**	***Citrullus lanatus***	**MG813941**	**This study**
P. aristosporum	UOP394	Wheat	AB095060	Kageyama et al. 2005
P. arrhenomanes	G-1	Sugar beet	AB095058	Kageyama et al. 2005
P. coloratum	CBS 154.64	Soil (tree nursery)	KJ595346	Hyde et al. 2014
P. dissotocum	UZ159	Field soil	AB468893	(Uzuhashi et al. 2010
P. dissotocum	**Pd32-1C**	***Cucumis sativus***	**MG813944**	**This study**
P. diclinum	CBS 664.79	*Beta vulgaris*	KJ595394	Hyde et al. 2014
P. lutarium	CBS 222.88	soil	KJ595359	Hyde et al. 2014
P. marinum	CBS 750.96	soil	KJ595398	Hyde et al. 2014
P. aphanidermatum	P36-3c	Bentgrass	AB095073	Kageyama et al. 2005
P. hydnosporum	MAFF305861	soil	DQ071378	Villa et al. 2006
P. periplocum	NBRC 100114	*Zoysia grass*	DQ071392	Villa et al. 2006
P. oligandrum	81-10	soil	AF196610	(Martin 2000)
P. oligandrum	**Po2-2W**	***Citrullus lanatus***	**MG813942**	**This study**
P. oligandrum	**Po4-2W**	***Citrullus lanatus***	**MG813932**	**This study**
P. oligandrum	**Po3-2W**	***Citrullus lanatus***	**MG813934**	**This study**
P. ultimum	NBRC 100122	Sugar beet	DQ071398	Villa et al. 2006
P. nodosum	MAFF305905	soil	DQ071399	Villa et al. 2006
P. nodosum	**Pn86-1C**	***Cucumis sativus***	**MG813945**	**This study**
P. middletonii	CBS528.74	soil	AB362318	Senda *et al.* 2009
P. middletonii	**Pmi77-1C**	***Cucumis sativus***	**MG813943**	**This study**
P. middletonii	**Pmi82-1C**	***Cucumis sativus***	**MG813946**	**This study**
Pp. litorale	GUCC1132	soil	AB920501	Baten *et al.* 2014
Pp. litorale	**Phl11-1W**	***Citrullus lanatus***	**MG813930**	**This study**

Pythium dissotocum Drechsler (1930)

The colonies were submerged on CMA and had no colony pattern. However, radiate growth pattern was observed on PDA and an intermediate state of chrysanthemum, rose-shape, and radiate colony patterns were observed on MEA, PCA, and HSA. The hypha were up to 7µm wide, the sporangia were filamentous, slightly swollen, branched, and tree-like (Fig. 1, b1), and the discharge tube was long (up to 11µm) (Fig. 1, b3). The encysted zoospores were 8–9µm in diameter. The oogonia were approximately spherical 20 to 24µm formed terminally, intercalary or laterally (Fig. 1, b2).

The antheridia was commonly monoclinous (Fig. 1, b2) with a stalk accurately below oogonium (paragynous) or without a stalk (hypogynous) or diclinous. For every oogonium, there were more than one to three antheridia. The oospore were spherical, ranging from 17 to 21µm (avg. 19µm) in diameter, smooth, aplerotic (Fig. 1, b2) or nearly plerotic. The minimum, optimum, and maximum growth temperatures were 5, 20-28 and 36°C, respectively. The average daily growth rate was 18mm at 25°C on CMA. This species was placed in clade B and subclade B2 of ITS and cytochrome oxidase II phylogenetic trees.

Pythium kashmirense B. Paul (2008)

No colony pattern on CMA, chrysanthemum colony pattern on MEA, and Rose-shaped colony pattern with large sections were observed on HSA, PDA, and PCA. The mycelium was highly branched, up to 6µm wide. There was no chlamydospore, hyphal swelling, and appressorium in this species. The sporangium was filamentous, tumescent, with complex and contiguous tumescence (Fig. 1, c1). Vesicle and zoospores formed after 24 hours incubation at room temperature (20 to 25 °C). The oogonia were spherical, often intercalary, 11 to 22 µm in diameter (avg. 16.4 µm). The oospores were spherical and plerotic, 10 to 21 µm in diameter (avg. 16.1 µm), with a wall thickness of 1-2µm. The antheridia were diclinous, wrapped around oogonia and formed a ring (Fig. 1, c2). The minimum, optimum, and maximum growth temperatures were 5, 25-30 and 38 °C respectively. The average daily growth rate was 15mm at 25 °C on CMA. This species was placed in clade B of ITS phylogenetic tree.

Pythium nodosum B. Paul, D. Galland, T. Bhatn & Dulieu (1998)

The colonies had radiate growth pattern on CMA, PDA, and PCA. However, there was no pattern on HSA and MEA. The hypha were 5-7 µm wide and the sporangia were varying in shape spherical, subglobose, pear-shaped or egg-like, mostly intercalary and sometimes terminally (Fig. 1, d2), 10-25 µm in diameter. The oogonia were smooth-walled, spherical, 12 to 27 µm. Antheridia, one or more, surrounding oogonium and forming node around it (Fig. 1, d1). After fertilization, the node disappeared and only one antheridium remained, which had the appearance of a bell-like cell (Fig. 1, d3). The oospores were spherical and smooth-walled, single,

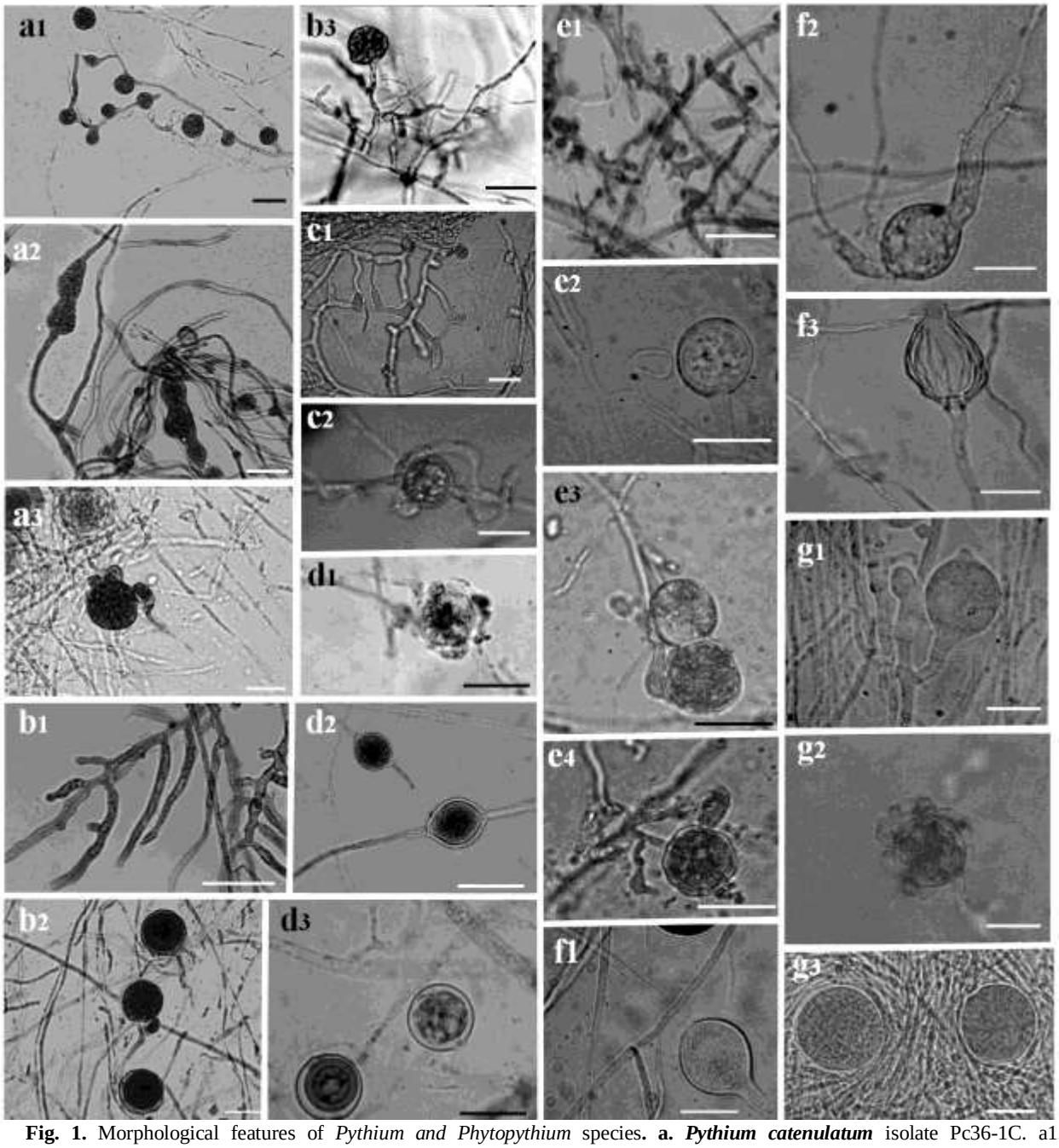

Fig. 1. Morphological features of *Pythium and Phytopythium* species**. a.** ***Pythium catenulatum*** isolate Pc36-1C. a1. Catenulate globose hyphal swelling, a2. Irregular inflated sporangia, a3. Diclinous antheridia and oogonium; **b.** ***Pythium dissotocum*** isolate Pd32-1C. b1. Filamentous dendroid sporangia, b2. Oogonium, monoclinous antheridium, aplerotic oospore, b3. Zoospores and vesicle; **c.** ***Pythium kashmirense*** isolate Pk83-1C. c1. Filamentous-inflated and continuous type of sporangia, c2. Diclinous antheridia wrapping around the oogonium; **d.** ***Pythium nodosum*** isolate Pn86-1C. d1. Oogonium surrounded by antheridia forming nodes, d2. Inercalary sporangium, d3. Oogonium with a bell-like antheridial cell; **e.** ***Pythium torulosum*** isolate Pt35-1W. e1. Flamentous inflated sporangia, e2, e3, e4. Oogonium and monoclinous antheridium; **f.** ***Phytopythium litorale*** isolate Phl11-1W. f1. Sporangium with papilla, f2. Internal extended proliferation, f3. Internally nested proliferation; **g.** ***Phytopythium mercuriale*** isolate Pm23-1C. g1. Papillate sporangium, g2. Oogonium surrounded by diclinous antheridia forming nodes, g3. Chlamydospores. — Scale bars = 20 µm.

aplerotic (Fig. 1, d3), 10 to 22 µm in diameter, and a wall thickness of about 1 µm. The minimum, optimum, and maximum growth temperatures were 10, 20-25 and 35 °C, respectively. The average daily growth rate was 17 mm at 25 °C on CMA. This species was placed in clade J of ITS and cytochrome oxidase II phylogenetic tree.

Pythium torulosum Coker & P. Patt (1927)

The colonies had subsurface growth on CMA, rose-shaped colony pattern on PCA, and uniform colony pattern on MEA, PDA, and HSA. The hypha were 5µm wide and there was no chlamydospore, hyphal swelling or appressorium. The sporangia were tumescent branches, which ran out of the main mycelium and made up the various bead-like elements in different sizes (Fig. 1, e1). The encysted zoospores were 7-8 µm in diameter. The oogonia were smooth, 15 to 23 µm (avg. 20.5) spherical, produced laterally, and intercalary or on short lateral appendages (Fig. 1, e2, e3). The antheridia were sausage-shaped and curved to club-shaped, mostly monoclinous, 5-10×3-6 µm and attached to the oogonium from their tip. One, two or sometimes three antheridia are attached to each oogonium. The stalk of oogonium or the main mycelium was the origin of monoclinous antheridium (Fig. 1, e4). The oospores were plerotic, 13 to 19 µm in diameter, and the wall thickness was up to 2µm. The minimum, optimum, and maximum growth temperatures were 5, 25-30 and 35 °C, respectively. The average daily growth rate was 14mm at 25 °C on CMA. This species was placed in clade B of ITS and cytochrome oxidase II phylogenetic tree.

Phytopythium litorale (Nechw.) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque (2014)

The colonies had satellite growth pattern on CMA, rose-shape on PDA and PCA, and radiate on HSA and EMA. The hypha were 5 µm wide and the sporangium was spherical or egg-like, 20-31×17-28 µm (avg. 25.5×22.5), with the papilla up to 70 µm (Fig. 1, f1). This papilla could form a discharge tuber or germinate directly and become branched. Sporangia were proliferating (Fig. 1, f2 and f3). The encysted zoospores were about 8- 10µm. The minimum, optimum, and maximum of growth temperatures were 5, 30 and 35 °C, respectively. The average daily growth rate was 10mm at 25 °C on CMA. The oogonium and oospore did not produce, and therefore, it was a heterothallic organism. This species was placed in clade K of cytochrome oxidase II phylogenetic tree.

Phytopythium mercuriale (Belbahri, B. Paul & Lefort) Abad, de Cock, Bala, Robideau, Lodhi & Lévesque (2014)

Colonies had subsurface growth on CMA, with a slight satellite colony pattern. The colony growth pattern was chrysanthemum, with aerial mycelia and bulk cotton form in the center on PDA and HSA. However, it was rose-shaped on MEA and cottony colony pattern on PCA. The main hyphae was up to 5µm wide. The sporangia, rarely produced in water, were mostly spherical, with papilla measuring up to 23-27 µm (Fig. 1, g1). The zoospores were produced at 17-27 °C and the discharge tube was short and about 4µm. Old sporangia often germinate from their papilla. The oogonia were spherical, measuring up to 22-28 µm, smooth-walled mostly produced terminally or laterally on the short branches. The antheridia were often diclinous, numerous, wrapped around oogonium and created a node (Fig. 1, g2). However, the oospores were not observed. The chlamydospores were mainly spherical, measuring up to 25-44 µm, thin-walled, terminally or intercalary (Fig. 1, g3). The minimum, optimum, and maximum growth temperatures were 8, 25-30 and 35 °C, respectively. The average daily growth rate was 8 mm at 25 °C on CMA. This species was placed in clade K of ITS phylogenetic tree. This species was reported for the first time in Iran.

Phylogenetic analysis

The results of the phylogenetic analysis based on ITS region of rDNA (ITS) and cytochrome oxidase II region are presented in fig. 2 and 3.

In the ITS phylogenetic tree, the species are divided into four main branches. The first branch (included clades A, B, C and D) consists of the *Pythium* species with inflated and non-inflated filamentous sporangia. The second branch (included clades E, F, G, H and I) consists of the *Pythium* species with spherical or spherical-like sporangia. All the *Phytopythium* species which are morphologically intermediate between *Pythium* and *Phytophthora* are placed in the third branch, clade K and the *Phytophthora* species as an out-group formthe fourth branch.

Clade A of Pythium ITS phylogenetic tree

This clade is heterogeneous and consists of two small and completely different clusters. *Pythium deliense* Meurs and *P. aphanidermatum* species were in the second cluster. These species, in contrast to the first cluster, have inflated filamentous sporangia, high growth rate (30 mm/day) and for each oogonium, there are one to two monoclinous and often intercalary antheridia (Levesque & de Cock 2004).

In this research, the highest number of isolates belonged to *P. aphanidermatum.* According to the results of morphological examination 166 isolates were identified as *P. aphanidermatum* and phylogenetic data (ITS analysis) confirmed the morphological identification. Diagnostic features including inflated filamentous and highly complex sporangia, intercalary and diclinous antheridia, high

and easy production of oospores and sporangia in culture, aplerotic oospores, high optimum temperature, and terminal discharge tube distinguish this species from the other species of *Pythium* and close species, such as *P. deliense* and *P. indigoferae*. Although the *P. aphanidermatum* and *P. deliense* show high similarity in their ITS regions, the sequence analysis of this region separated these two species. Lévesque & de Cock (2004) believed that the RAPD test would distinguish these two species better and more efficiently than all the other existing tools.

Clade B of the *Pythium* ITS phylogenetic tree

This cluster included *Pythium angustatum*, *P. catenulatum*, *P. torulosum*, *P. folliculosum*, and *P. kashmirense*. All of these species, except *P. angustasum*, had filamentous inflated sporangia, with an average daily growth rate of 9 to 15mm. *Pythium catenulatum* was first isolated in 1931 by Matthews from plant remains in water, soil, and grass in the United States (Van der Plaats-*Niterink*, 1981). The ITS region of *P. catenulatum* isolates were very similar to ITS region of *P. torulosum* isolates. Therefore the sequence of this region could not separate these two species. This observation confirmed the results of Lévesque & De Cock (2004). Therefore, for more accurate identification of these isolates, cytochrome oxidase II region was also sequenced. The analysis of this region was better in separation and identification of the mentioned isolates.

Pythium torulosum was first isolated from the nematodes of the genus *Teleranea* and a species of fern called *Thuidium delicatulum* in the United States (Van der Plaats-Niterink, 1981). Diagnostic features of the species are as follow. *Pythium torulosum* is reported for the first time as oomycetes associated with root and crown rot of cucurbits.

Another species in the B1a cluster was *P. kashmirense*. This species is also reported for the first time as oomycetes associated with root and crown rot of cucurbits. A significant feature of this species included a unique sequence of ITS region. Morphological characteristics, the daily growth rate at optimum temperature, and the growth pattern of isolates in this study were completely consistent with the characteristics of the type species as described by Paul and Bala (2008).

B2 Subclade

This subclade included *P. aquatile*, *P. dissotocum*, *P. diclinum*, *P. coloratum*, *P. flavoens*, *P. lutarium*, and *P. marinum*. These species had non-inflated filamentous or slightly inflated sporangia, smooth oogonia, often smaller than 30µm, with a daily growth rate of 10 to 20mm (Levesque & de Cock 2004). The species in B2 subclade show high similarity in ITS regions.

Levesque & de Cock (2004) stated that the analysis of other genes, including mitochondrial genes, would have more efficiency in differentiating the species present in this group. In this study, it was found that even the analysis of the cytochrome oxidase II gene was not sufficient for accurate identification. However, the combination of morphological, physiological, and sequencing data will facilitate the accurate identification of these species. *Pythium dissotocum* was first isolated in 1938 from sugarcane (Stevenson & Rands,1938).

Clade E of *Pythium* ITS phylogenetic tree

This clade consisted of two subclade. *Pythium middletonii*, *P. multisporum*, *P. parvum*, *P. pleroticum*, and *P. minus* are cited under subclade E2. All the members of this subclade were homothallic and had smooth-walled oogonia without decoration (Levesque & de Cock, 2004). *Pythium middletonii* was first isolated by Debary in 1881 from insect cadavers in water (van Der Plaats-Niterink 1981).

Although there is no hyphal swelling in *P. middletonii* and *P. multisporum*, the rest of the members had hyphal swellings. In addition, unlike the other species, *P. middletonii* and *P. multisporum* had spherical or lemon-shaped sporangia with internal proliferation. In *P. middletonii*, oospores are aplerotic and the discharge tube is very short. However, in *P. multisporum*, the oospores are plerotic and have longer discharge tube. Although *P. middletonii* has frequently isolated all over the world, other species of this subclade are rarely isolated (Levesque & de Cock, 2004).

Clade J from *Pythium* ITS phylogenetic tree

Based on phylogenetic evidence, *P. nodosum* was placed in clade J. This species was first isolated in 1998 by Paul et al. (1998) from a soil sample taken in the Burgundy region in France. In Iran, only one isolate from the soil of an apricot garden in Maku, East Azerbaijan, Iran, had been reported by Badali et al. (2016). Moreover, it seemed that there was no other report from other parts of the world.

Clade K of *Pythium* ITS phylogenetic tree

Species in this clade are intermediate both of *Pythium* and *Phytophthora*, in terms of the morphological and molecular characteristics.

Bala et al. (2010) classified the genus *Phytopythium* as a new genus (with *Pp. sindhum* as type species) in the *Pythiaceae* family. *Phytopythium mercuriale*, isolated from the Kermanshah Province were consistent with the isolates of Belbahri et al. (2008), in terms of morphological characteristics. The characteristics are as follows: proliferating egg-like papillate sporangia; production of zoospore in 17-27 °C; germination of old sporangium with production of germination tube derived from papilla extension, production of the rounded terminal or lateral thin-walled chlamydospore; and abundant diclinous antheridia, which produce node around oogonia.

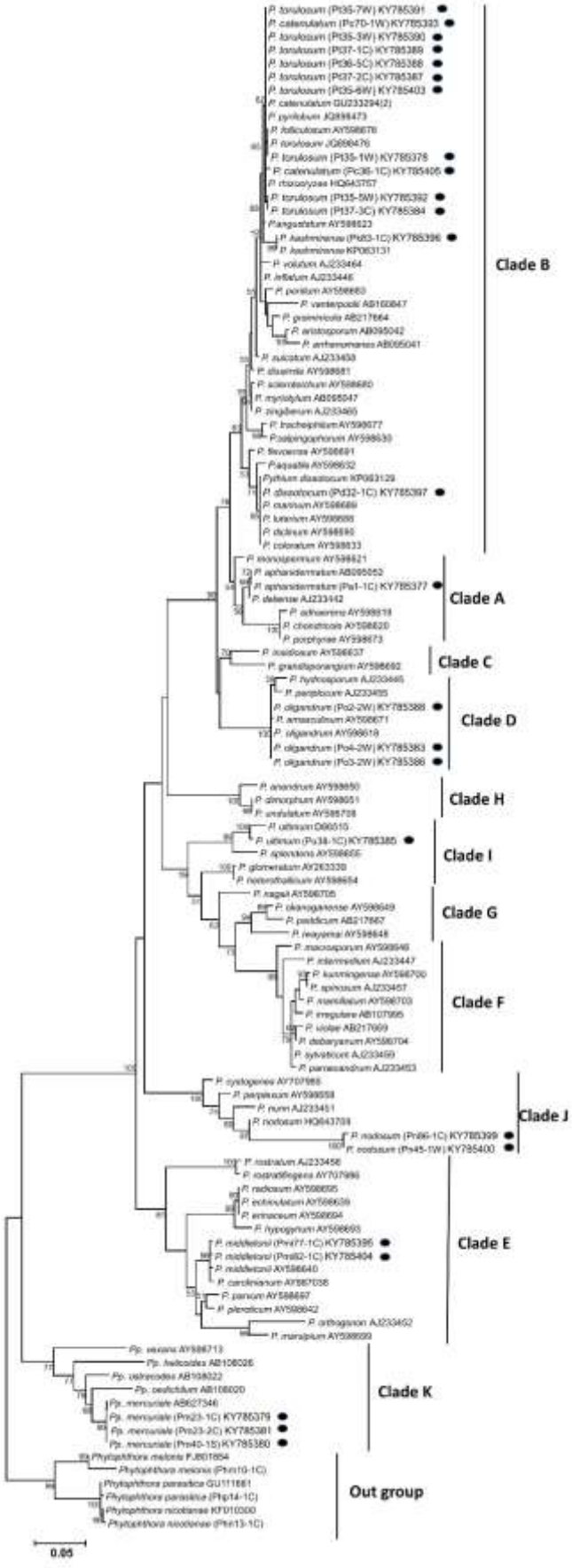

Fig. 2. Phylogenetic tree constructed from the ITS sequence alignment of *Pythium* spp. and *Phytopythium* spp. based on neighbor-joining (NJ) approach, with 500 bootstrap replicates. The Iranian specimens are shown with bold circle labels.

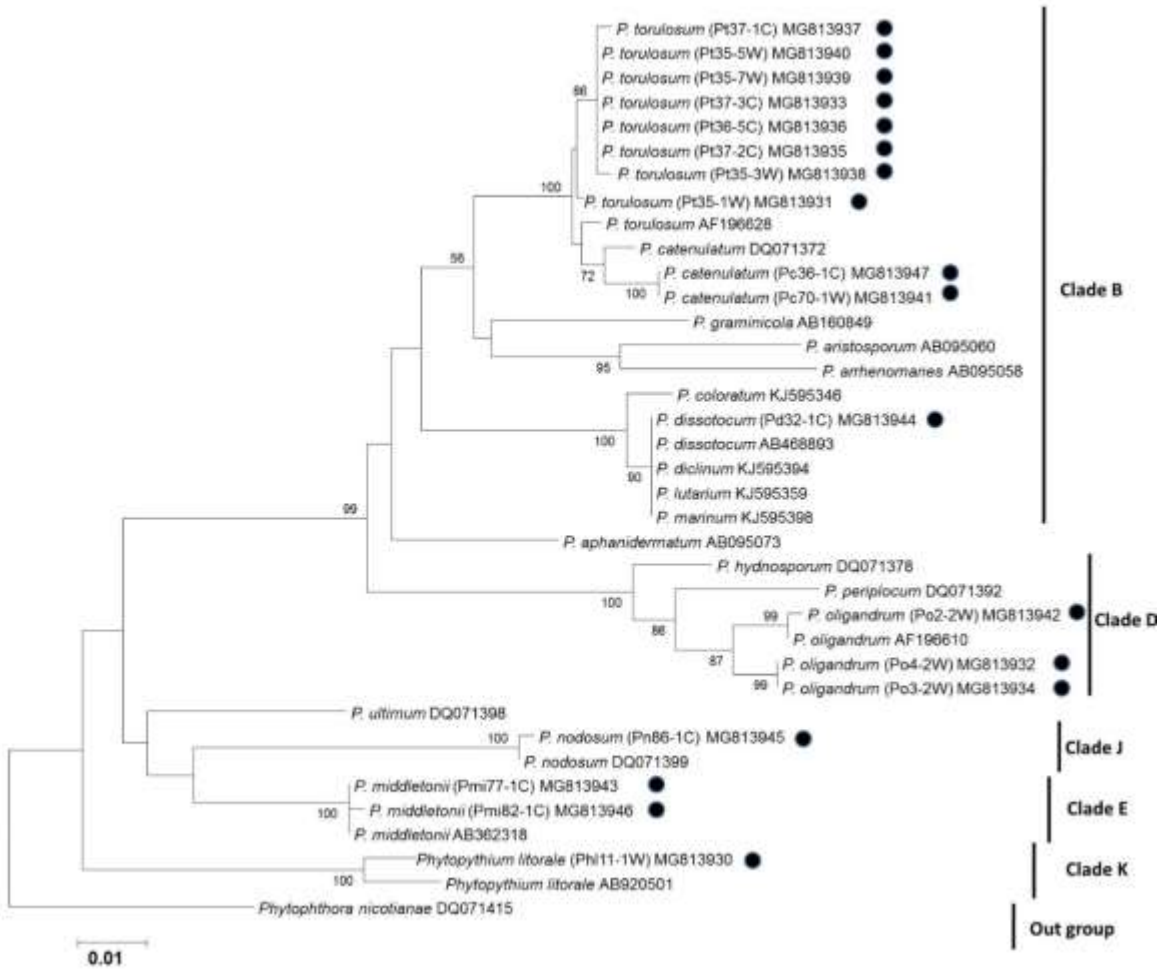

Fig. 3. Phylogenetic tree constructed from the *cox II* sequence alignment of *Pythium* spp. and *Phytopythium* spp. based on neighbor-joining (NJ) approach, with 500 bootstrap replicates. The Iranian specimens are shown with bold circle labels.

Phytopythium litorale was another species which was placed in clade K. This species was first isolated from littoral soils of Lake Constance in Germany (Nechwatal & Mendgen 2006). Parkunan and Ji (2013) reported that the species caused fruit rot and seedling damping-off of yellow squash. In Iran, *Pp. litorale* was isolated from the rhizosphere of *Juncus* sp. and *Circium* sp. (Chenari Bouket et al. 2016). The morphological and physiological characteristics of isolates of Kermanshah province were consistent with the characteristics of the previously described isolate (Chenari Bouket et al. 2016, Nechwatal & Mendgen 2006, Parkunan & Ji 2013). However, they had a lower average of daily growth rate (10 mm).

Clade I of *Pythium* ITS phylogenetic tree

This clade included *P. heterothallicum*, *P. splendens*, *P. ultimum* var. *ultimum*, and *P. ultimum* var. *sporangiiferum*.

Among the identified species, *P. ultimum* was the second most frequent species after *P. aphanidermatum*. The morphological characteristics of *P. ultimum* in this study were consistent with the characteristics of the previously described isolate (Askari Farsangi et al. 2011, Baptista et al. 2004, Rocha et al. 2001, Van der Plaats-Niterink 1981).

According to the findings of this study, cucurbit fields contained abundant and novel oomycetes flora. The reason for this might be the presence of proper environmental conditions, including high humidity condition and proper temperature in field soil. Among the identified species, *P. aphanidermatum* and *P. ultimum* were isolated more frequently than the other species. Considering the wide host range of this species and stronger virulence, it was not surprising that they had high frequency and wide distribution.

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ویژگی‌های ریخت‌شناختی و مولکولی اواومیست‌های همراه با پوسیدگی ریشه و طوقهٔ کدوییان در استان کرمانشاه

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چکیده: شبه‌قارچ‌های جنس *Pythium* و *Phytophthora* از شناخته‌شده‌ترین عوامل بیماری‌زای گیاهان در سراسر دنیا هستند که باعث پوسیدگی بذر، ریشه و طوقه، مرگ گیاهچه و پوسیدگی نرم میوه‌های در تماس با خاک می‌شوند. در این پژوهش ۳۴۷ جدایه از این دو جنس و جنس خویشاوند آن‌ها، *Phytopythium*، از مزارع جالیز استان کرمانشاه جداسازی شده و از لحاظ ویژگی های ریخت‌شناختی و فیزیولوژیک مورد ارزیابی قرار گرفتند. به منظور تأیید شناسایی ریخت شناختی، نواحی ITS-rDNA و همچنین ناحیه‌ی سیتوکروم اکسیداز II جدایه‌های منتخب تعیین توالی شد. بر اساس مطالعات ریخت‌شناختی، ریخت‌سنجی، فیزیولوژیکی و فیلوژنتیکی نُه گونه‌ی *Pythium* شامل: *P. aphanidermatum*، *P. dissotocum*، *P. catenulatum*، *P. kashmirense*، *P. middletonii*، *P. nodosum*، *P. oligandrum*، *P. torulosum* و *P. ultimum*، دو گونه *Phytopythium* به نام‌های *Pp.mercuriale* و *Pp. litorale* و سه گونه *Phytophthora* به نام‌های *Ph. melonis*، *Ph. nicotianae* و *Ph. parasitica* شناسایی شد. در میان گونه‌های شناسایی شده در این پژوهش، گونه‌ی *Pp. mercuriale* برای ایران جدید می‌باشد. در این مطالعه، بیشترین فراوانی جدایه‌ها مربوط به گونه‌های *P. aphanidermatum* و*P. ultimum* بود.

واژه‌های کلیدی: *Pythium*، *Phytophthora*، *Phytopythium*، مرگ گیاهچه، کدوئیان

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