J. Agr. Sci. Tech. (2015) Vol. 17: 1347-1363

A Revision of Iranian *Phytophthora drechsleri* Isolates from Cucurbits Based on Multiple Gene Genealogy Analysis

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ABSTRACT

The plant pathogenic *Phytophthora drechsleri* is morphologically similar to some other non-papillate *Phytophthora* spp., especially *P. melonis,* and it is difficult to discriminate these convergent taxa. It seems that the Iranian putative *P. drechsleri* isolates from different cucurbit species have been generally misidentified and their characteristics do not match with *P. drechsleri.* In order to compare these two groups, authentic *P. drechsleri* isolates and isolates from different cucurbits were assessed for morphological, physiological (cultural, temperature relations, mating type), and molecular traits. Multiple gene genealogy analysis were performed on regions of nuclear (ITS, β-tubulin, translation elongation factor 1α, elicitin) and mitochondrial (cytochrome c oxidase subunit I) gene sequences. Congruence was observed in different phylogenetic data sets. The present study demonstrated that putative *P. drechsleri* isolates from cucurbits and pistachio trees were a distinct species and belonged to *P. melonis*. Data showed that *P. melonis* was a homogenous species and there were no considerable molecular intraspecific variations between isolates from cucurbits and isolates from other hosts. Design of a molecular species-specific identification tool for *P. melonis* isolates is under investigation.

Keywords: Internal transcribed spacer of rDNA**,** Phylogeny, *Phytophthora melonis*, *Oomycota***.**

INTRODUCTION

Crown and root rot diseases of cucurbits caused by the oomycete pathogen, *Phytophthora drechsleri* Tucker, have been reported from many cucurbits in Iran and other countries (Banihashemi, 1969; Ershad and Mostowfipoor, 1969; Alavi and Strange, 1979; Mansoori and Banihashemi, 1980; Maden and Karahan, 1980; Ho *et al.*, 1984, 1995). Ershad (1971), in his original description of *P. drechsleri* from Iran, separated the putative *P. drechsleri* isolates into two cucurbit and non-cucurbit groups. Some researchers believe that this cucurbits' pathogen belongs to another non-papillate species of *Phytophthora*, namely, *P. melonis* Katsura (Wong and Jiang, 1980; Lu and Gong, 1982; Kao *et al.*, 1982; Jee *et al.*, 2001; Guharoy *et al.*, 2006). It is, however, difficult to discriminate these two taxa due to their convergent characteristics.

The status of *P. melonis* as a distinct operational taxonomic unit was subjected to doubt since it was described by Katsura in 1976 (Erwin and Ribeiro 1996). *Phytophthora melonis* is morphologically similar to *P. drechsleri* and it could grow well at 35°C. Therefore, it was considered to be conspecific by Ho *et al.* (1984), Ho (1986) and Ho and Jong (1991). Based on molecular grouping by mtDNA RFLP and isozyme patterns Mills *et al*. (1991) revealed that isolates assigned to *P. melonis* grouped with one *P. sinensis* Y.N. Yu and W.Y. Zhuang and eight *P. drechsleri* isolates from cucurbit plants (group F). Internal transcribed spacers (ITS) analysis (Cooke *et al.*, 2000) showed that *P. melonis* and *P. sinensis* share identical ITS sequences

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[DOR: 20.1001.1.16807073.2015.17.5.10.9]

consistent with their previously proposed conspecificity (Ho, 1986; Mills *et al.,* 1991). However, further proposal that they could be synonymized with *P. drechsleri* on morphological and physiological grounds was not supported by this analysis. Mirabolfathy *et al.* (2001) showed that *P. drechsleri*-like isolates from pistachio (*Pistacia vera* L.) had identical ITS sequences to those of *P. melonis*, *P. sinensis* and *P. drechsleri*-like isolates from cucurbits in Iran. Although Kroon *et al.* (2004) did not include *P. melonis* in their multiple gene analysis, they showed that *P. sinensis* was a distinct taxon in their Clade 7b and appeared as a sister group for *P. vignae* far from *P. drechsleri*, a member of their Clade 8a.

Ho *et al.* (2007) re-examined the type culture and other isolates from cucurbits and re-described *P. melonis* based on morphological and molecular traits. They conducted a single-strand conformation polymorphism (SSCP) analysis of ribosomal DNA for five isolates of *P. melonis* along with three of *P. drechsleri*. *Phytophthora melonis* produced a SSCP pattern distinct from that of *P. drechsleri,* while isolates of the same species had identical patterns. More recently, Blair *et al.* (2008) in a multi-locus phylogeny for *Phytophthora* species have illustrated that isolate of *P. melonis* is closely related to *P. sinensis* laid in Clade 7b of their phylogenetic tree.

Although some minor techniques such as induction of potato (*Solanum tuberosum* L.) pink rot by *P. drechsleri* at 20°C (Mostowfizadeh-Ghalamfarsa *et al.,* 2006) was introduced for discrimination of *P. melonis* from *P. drechsleri*, it seems that more morphological, physiological and/or molecular characteristics is needed for laboratory identification of these two convergent taxa. The objective of this study was to revise the status of *P. drechsleri* isolates from cucurbits in Iran based on multiple gene genealogy of nuclear and cytoplasmic genes and compare their morphological and physiological features with authentic *P. drechsleri* isolates.

MATERIALS AND METHODS

Organisms and Cultural Conditions

Details of the *Phytophthora* isolates examined in this study are listed in Table 1. The isolates were obtained from the culture collections of the authors or directly isolated from the host tissue on PARPH media (CMA, amendedwith 10 µg mL^{-1} pimaricin, 200 µg mL^{-1} ampicillin, 10 µg mL^{-1} rifampicin, 25 µg mL^{-1} PCNB, and 50 µg L^{-1} hymexazol) (Jeffers and Martin, 1986). Isolates were stored on cornmeal agar (CMA: Ground corn extract 40 g L^{-1}, agar 15 g L^{-1}) slopes at 15°C. Routine stock cultures for research studies were grown on French bean agar (FBA; ground French beans extract 30 g L^{-1}, agar 15 g L^{-1}) at 20°C.

Colony Morphology and Growth Rate

The isolates were grown at 20°C on CMA, clarified V8-juice agar (CV8: 100 ml V8 juice, Campbell's, UK), 900 mL distilled water, 15 g agar), malt extract agar (MEA; Sigma, UK), potato-dextrose agar (PDA: Potato extract 300 g L^{-1}, dextrose 20 g L^{-1}, agar 15 g L^{-1}), and hemp seed agar (HSA: Ground hemp seed extract 60 g L^{-1}, agar 15 g L^{-1}). Petri dishes (9 cm diameter) containing 20 mL of the test media were inoculated with 5 mm diam. discs cut from the edge of a 5-10 day-old-culture. The discs were placed upside down in the centre of each plate, and incubated in the dark. Colony morphology was examined after 8 days. For temperature-growth relationships, CMA plates were inoculated using three replicate plates per isolate and incubated at 5, 10, 15, 20, 25, 30, 35, 37 and 40°C. Growth rate (mm d^{-1}) was recorded 5 days after the onset of linear growth. Tests were repeated twice for the range of 30-37°C.

Sporangial Morphology

One disc (10 mm diam.), cut from the growing edge of a 7 day-old-culture grown

[DOR: 20.1001.1.16807073.2015.17.5.10.9]

Table 1. Origins of isolates studied and their GenBank sequence accession numbers.

Species	Isolate code		Host	Location	Year isolated	GenBank accession number				
	local	International				ITS [c]	TUB [d]	ELO [e]	COX [f]	ELI [g]
P. drechsleri [a]	SCRP222		*Solanum tuberosum*	Wales	??????	AY659435	AY659481	AY659528	AY659575	AY659622
P. drechsleri (T) [a]	SCRP232	ATCC46724, CBS292.35	*Beta vulgaris* var. *altissima*	USA	1935	AY659442	AY659488	AY659535	AY659582	AY659629
P. drechsleri [a]	SCRP236	IMI040500	*Solanum tuberosum*	Argentina	1949	AY659444	AY659490	AY659537	AY659584	AY659631
P. drechsleri [a]	SUAh4		*Beta vulgaris*	Iran, Khz. [i]	2002	AY659452	AY659498	AY659545	AY659592	AY659639
P. drechsleri [a]	SUAk2		*Beta vulgaris*	Iran, Khz.	2002	AY659453	AY659499	AY659546	AY659593	AY659640
P. drechsleri [a]	SUC5		??????	USA	1992	AY659456	AY659502	AY659549	AY659596	AY659643
P. drechsleri [a]	SUC18		*Beta vulgaris*	Iran, Far. [j]	1992	AY659457	AY659503	AY659550	AY659597	AY659644
P. drechsleri [a]	SUC20		*Helianthus annus*	Iran, Far.	1993	AY659458	AY659504	AY659551	AY659598	AY659645
P. drechsleri [a]	SUKv3		*Beta vulgaris*	Iran, Far.	2002	AY659459	AY659505	AY659552	AY659599	AY659646
P. drechsleri [a]	SUSa1		*Beta vulgaris*	Iran, Khz .	2002	AY659461	AY659507	AY659554	AY659601	AY659648
P. drechsleri [a]	SUSa2		*Beta vulgaris*	Iran, Khz.	2002	AY659462	AY659508	AY659555	AY659602	AY659649
P. drechsleri [a]	SUSd3		*Beta vulgaris*	Iran, Khz.	2002	AY659463	AY659509	AY659556	AY659603	AY659650
P. drechsleri [a]	SUSr1		*Beta vulgaris*	Iran, Khz.	2002	AY659464	AY659510	AY659557	AY659604	AY659651
P. drechsleri	SUAh2		*Beta vulgaris*	Iran, Khz.	2002	AY661865				
P. drechsleri	SUAh3		*Beta vulgaris*	Iran, Khz.	2002	AY661866				
P. drechsleri	SUAh5		*Beta vulgaris*	Iran, Khz.	2002	AY661867				
P. drechsleri	SUAh6		*Beta vulgaris*	Iran, Khz.	2002	AY661868				
P. drechsleri	SUKv4		*Beta vulgaris*	Iran, Far.	2002	AY661873				
P. drechsleri	SUKv10		*Beta vulgaris*	Iran, Far.	2002	AY661874				
P. drechsleri	SUKv14		*Beta vulgaris*	Iran, Far.	2002	AY661875				
P. drechsleri	SUSa3		*Beta vulgaris*	Iran, Khz.	2002	AY661876				
P. drechsleri	SUSd1		*Beta vulgaris*	Iran, Khz.	2002	AY661877				
P. drechsleri	SUSd2		*Beta vulgaris*	Iran, Khz.	2002	AY661878				
P. melonis [b]	SCRP455	IMI325917	*Cucumis sativus*	China	1988	AF266767 [h]	AY659687	AY661881	AY659698	AY659704
P. melonis	SUAh1		*Cucumis sativus*	Iran, Khz.	1994	AY659655				
P. melonis	SUAk1		*Beta vulgaris*	Iran, Khz.	2002	AY659656				
P. melonis	SUD2		*Cucumis melo* var. *reticulatus*	Iran, Far.	1977	AY659657				
P. melonis	SUD8		*Cucumis melo* var. *reticulatus*	Iran, Far.	1981	AY659658				
P. melonis	SUD17		*Cucumis melo*	Iran, Far.	1982	AY659659				
P. melonis	SUD26		*Cucumis melo*	Iran, Hor. [k]	1983	AY659660	AY659688	AY659693	AY659699	AY659705
P. melonis	SUD29		*Cucumis melo* var. *reticulatus*	Iran, Hor.	1985	AY659661				
P. melonis	SUD30		*Cucumis melo* var. *reticulatus*	Iran, Hor.	1985	AY659662				
P. melonis	SUD31		*Cucumis melo* var. *reticulatus*	Iran, Sis. [l]	1986	AY659663				

[a] Molecular data from Mostowfizadeh-Ghalamfarsa *et al.* 2010; [b] All *P. melonis* (except SCRP455) and *P. pistaciae* isolates formerly identified as *P. drechsleri* based on morphological and physiological characteristics. [c] Internal transcribed spacers, [d] *β-tubulin*, [e] Translation elongation factor 1α; [f] *Cytochrome c oxidase subunit I*; [g] *Elicitin,* [h] Cooke *et al.* 2000; [i] Khoozestan, [j] Fars, [k] Hormozgan, [l] Sistan and Balochestan, [m] Semnan, [n] Khorasan, [o] Kerman Provinces, T= Type isolate. continued ...

Continued of Table 1.

Species	Isolate code		Host	Location	Year isolated	GenBank accession number				
	local	International				ITS [c]	TUB [d]	ELO [e]	COX [f]	ELI [g]
P. melonis	SUD32		*Cucumis melo* var. *reticulatus*	Iran, Far.	1986	AY659664				
P. melonis	SUD33		*Cucumis melo*	Iran, Sem. [m]	1986	AY659665				
P. melonis	SUD35		*Chorozophora tinctoria*	Iran, Far.	1986	AY659666				
P. melonis	SUD36		*Cucumis melo*	Iran, Kho [n]	1986	AY659667				
P. melonis	SUD38		*Cucumis melo*	Iran, Kho	1989	AY659668				
P. melonis	SUD40		*Cucumis sativus*	Iran, Hor.	1992	AY659669				
P. melonis	SUD41		*Cucumis sativus*	Iran, Hor.	1992	AY659670				
P. melonis	SUD42		*Cucumis sativus*	Iran, Hor.	1992	AY659671				
P. melonis	SUD43		*Pistacia vera*	Iran, Ker. [o]	1993	AY659672	AY659689	AY659694	AY659700	AY659706
P. melonis	SUD45		*Cucumis sativus*	Iran, Hor.	1993	AY659673				
P. melonis	SUD48		*Citrullus lanatus*	Iran, Khz.	1994	AY659674				
P. melonis	SUD49		*Cucumis sativus*	Iran, Khz.	1994	AY659675				
P. melonis	SUHa1		*Citrullus lanatus*	Iran, Khz.	1994	AY659676				
P. melonis	SUMc1		*Cucumis sativus*	Iran, Far.	2002	AY659684	AY659690	AY659695	AY659701	AY659707
P. melonis	SUMc2		*Cucumis sativus*	Iran, Far.	2002	AY659685				
P. melonis	SUMc3		*Cucumis sativus*	Iran, Far.	2002	AY659686				
P. melonis	SURf5		*Pistacia vera*	Iran, Ker.	1993	AY659677				
P. melonis	SURf8		*Pistacia vera*	Iran, Ker.	1993	AY659678				
P. melonis	SURf9		*Pistacia vera*	Iran, Ker.	1993	AY659679				
P. melonis	SURf10		*Pistacia vera*	Iran, Ker.	1993	AY659680				
P. melonis	SURf13		*Pistacia vera*	Iran, Ker.	1993	AY659681				
P. melonis	SUSa4		*Beta vulgaris*	Iran, Khz.	2002	AY659682	AY659691	AY659696	AY659702	AY659708
P. melonis	SUSh1		*Cucumis melo*	Iran, Khz.	1994	AY659683	AY659692	AY659697	AY659703	AY659709
P. pistaciae	SUD44		*Pistacia vera*	Iran, Ker.	1993	AY659414				
P. pistaciae	SURf14		*Pistacia vera*	Iran, Ker.	1993	AY659415				
P. lateralis (T)	SCRP390	IMI040503, CBS168.42	*Chamaecyparis lawsoniana*	Cypress	1942	AF266804 [h]	AY659513	AY659560	AY659607	AY659654

[a] Molecular data from Mostowfizadeh-Ghalamfarsa *et al.* 2010; [b] All *P. melonis* (except SCRP455) and *P. pistaciae* isolates formerly identified as *P. drechsleri* based on morphological and physiological characteristics. [c] Internal transcribed spacers, [d] *β-tubulin*, [e] Translation elongation factor 1α; [f] *Cytochrome c oxidase subunit I,* [g] *Elicitin*; [h] Cooke *et al.* 2000; [i] Khoozestan, [j] Fars, [k] Hormozgan, [l] Sistan and Balochestan, [m] Semnan, [n] Khorasan, [o] Kerman Provinces, T= Type isolate.

on CV8 at 20°C in the dark, was placed in a 9 cm Petri dish and flooded, just over its surface, with non-sterile soil extract (100 g soil flooded with 1 L of distilled water for 24 hours at room temperature and then filtered). After incubation at 20°C in the dark for 48-72 hours, dimensions and characteristic features of 50 fully mature sporangia, chosen at random, were determined at X400 magnification for each isolate.

Breeding System and Morphology of Oogonia, Oospores and Antheridia

Oospores were produced in dual culture with either A1 (IMI268688) or A2 (IMI207770) mating types of *P. nicotianae* on HSA (amended with 30 mg β-sitosterol L^{-1}) plates using a one cm diam. (Pore size: 0.2 µm) polycarbonate membrane to prevent gametangia of the different species from mixing. For isolates that did not produce oospores, the test was repeated using A1 (02B-05) and A2 (02-B10) mating types of *P. infestans* on amended HSA plates. For each isolate, 50 oogonia, oospores and antheridia, chosen at random, were measured from 4-6 week old cultures grown at 20°C in the dark on amended HSA. Measurements were made at X400 magnification using a calibrated eye-piece.

Pathogenicity

As part of the procedure to discriminate *P. drechsleri* from *P. melonis*, all isolates were evaluated for their ability to cause pink-rot symptoms on potato tubers based on the method described by Mostowfizadeh-Ghalamfarsa *et al.* (2006).

DNA Extraction

Isolates were grown in 20 mL still culture of pea broth (boiled extract of 125 g frozen green peas in 1,000 ml distilled water pH= 6.2) at 20°C. After vacuum filtration, the mycelium was freeze-dried for extended storage at -20°C. DNA was extracted from mycelium using a Puregene DNA extraction kit, Flowgen (Lichfield, England).

DNA Amplification and Sequencing

DNA of the internal transcribed spacer regions (ITS) was amplified using the universal primers ITS6 and ITS4 (Cooke *et al*. 2000, White *et al.* 1990). Fragments of the *translation elongation factor 1 alpha* gene (ELO) and the *β-tubulin* (TUB) gene were amplified using, ELONGF1 and ELONGR1, TUBUF2, and TUBUR1 (Kroon *et al*. 2004) primers, respectively. The region containing the mitochondrial *cytochrome c oxidase subunit I* (COX) gene fragment was amplified using, COXF4N and COXR4N primers (Kroon *et al.* 2004).

Amplifications were performed in a Primus 96 plus thermocycler (MWG-BIOTEC, Germany). The PCR mixture contained 10 to 20 ng of template DNA, 1 µM of each primer, 100 µM of dNTPs, 0.4 U *Taq* DNA polymerase (Promega, USA), 1.5 mM of $MgCl_2$, 2.5 µL of 10X PCR buffer, and 100 mM BSA, in a reaction volume of 25 µL. For *mtDNA* gene amplification, the $MgCl_2$ concentration was raised to 3.5 mM. Successful amplification was confirmed by gel electrophoresis (1 hour at 70 Volts) on 1.0% agarose gels in 1X TBE buffer. Gels were stained using ethidium bromide and DNA fragments were visualised under UV light. PCR was originally carried out with a program of 94°C for 2 minutes (initial denaturation) followed by 30 cycles of 94°C for 20 seconds, annealing temperature (Table 2) for 25 seconds, 72°C for 50 seconds, and a final extension of 72°C for 10 minutes.

Phytophthora Extracellular Protein Genes

In order to find a novel candidate gene for multiple gene genealogy studies of selected

[DOR: 20.1001.1.16807073.2015.17.5.10.9]

Table 2. The primers designed for *Phytophthora* extra cellular proteins genes detection.

Target DNA	Primer	Primer sequence	Accession num [a]	Primer loc [b]	Ann [c] (°C)	Size [d] (bp)
Elicitin INF1	PEX-F[e]	*5´ GAT GAA CTT YCG YGC TCT G 3´*	BE776632	31-49		
	PEX-R[f]	*5´ GCG TAC GAG TAS ACG TTG AG 3´*		335-354	57	324
Cyst germination specific acidic repeat protein	PEX2-F	*5´ ACG GAA AGT ATG AAG ACS TTC GCC 3´*	BE776684	87-110		
	PEX2-R	*5´ GGC AGC GAA GTC ACT GGG CA 3´*		483-502	62	416
Acidic chitinase	PEX3-F	*5´ ACC AAG CAA GCA ACC AAA TC 3´*	BE776562	.8-27.		
	PEX3-R	*5´ ACT CCA GAC CAC CGT TGA TG 3´*		622-641	55	634
Endo-β-1,3-glucanase	PEX4-F	*5´ AGC CCC TAC AAC CCG ATC T 3´*	BE776882	29-47		
	PEX4-R	*5´GGT ACG CCG AGT ACG TGT CT 3´*		586-605	60	577
Exo-β-1,3-glucanase	PEX5-F	*5´ GTA TCC AGT TGC CAG CTT CG 3´*	BE775865	24-43		
	PEX5-R	*5´ CAG TTA CGG TTG GCT TCC TC 3´*		604-623	59	600
Necrosis inducing peptide Pi-NIP	PEX6-F	*5´ YTC RCA CGA TGC MGT KRT CC 3´*	BE776681	97-116		
	PEX6-R	*5´ GTC CAR CGC GTG RTT GAT 3´*		571-588	57	492

[a] Reference to the GenBank accession containing the DNA sequence, on which the primer is based; [b] Reference to the location of the primer within the original DNA sequence; [c] Annealing temperature of the PCR; [d] Average amplicon length; [e] Forward, [f] Reverse.

isolates, *Phytophthora* extracellular protein (Pex) cDNA information was applied. This information was based on data mining of expressed sequence tags (ESTs) of *P. infestans* database that was produced by Torto *et al.* (2003). They used an algorithm for automated identification of extracellular protein from EST data sets, called PexFinder (Torto *et al.*, 2003).

Since *P. infestans* is evolutionally divergent from *P. sojae* (Cooke *et al.*, 2000; Martin and Tooley, 2003) and also a comprehensive cDNA sequence collection available for both species, selected *Pex* genes from *P. infestans* were BLASTed against *P. sojae* gene bank (http://genome.jgi-psf.org/sojae/). The best matches selected for primer designing. These primers theoretically were supposed to amplify taxa such as *P. melonis* and *P. drechsleri*, which were located between or close to *P. infestans* and *P. sojae* in phylogenetic reconstruction trees. In order to avoid selection errors, a subset of not-matched *Pex* sequences were randomly selected to design primers as well.

Primers were designed either manually or by Primer3 Java applet (Rozen and Skaletsky, 2000) (Table 2) and tested with a group of isolates as well as *P. infestans* isolate as control. The amplified gene with proper length which showed polymorphism after sequencing in different isolates was selected for further studies.

Sequencing of Amplified Product

The amplification products of all isolates were purified through Wizard Prep columns (Promega, USA) to remove excess primers and nucleotides. PCR products were sequenced in forward and reverse orientation using the primers used for amplification and a dye terminator cycle sequencing kit (BigDye sequencing kit, Applied Biosystems, USA) on an ABI377-96 automated sequencer (Applied Biosystems, USA) according to the manufacturer's instruction.

Phylogenetic Analysis

A multiple gene genealogy approach as well as single gene comparisons was applied in the study of the phylogenetic relationships of *P. melonis* and *P. drechsleri* as described by Mostowfizadeh-Ghalamfarsa *et al.* (2010) . Sequences generated in this study were compared to those of other taxa obtained from GenBank (Sup. Table 1).

RESULTS

Preliminary Verification of Isolate Identification

Of all putative *P. drechsleri* isolates that were pre-screened by ITS analysis from various hosts, 23 were confirmed as *P. drechsleri*, 33 as *P. melonis* and 2 as *P. pistaciae* (Table 1, Figure 1). Six *P. melonis* isolates were selected from various hosts to represent the full range of genetic diversity of this taxon. For these isolates, fragments of three additional nuclear genes and one mitochondrial gene were sequenced, including *β-tubulin* (TUB), translation elongation *factor 1α* (ELO), a putative *Elicitin* (ELI, see below), and *cytochrome c oxidase subunit I* (COX). These data were compared with those in previously studied *P. drechsleri* isolates (Mostowfizadeh-Ghalamfarsa *et al.,* 2010). We applied both distance-based and maximum likelihood methods. Although the comparison of two types of trees illustrated concordance between them and results were broadly similar, neighbour-joining trees showed better separation and more intuitive grouping of taxa. *Phytophthora lateralis* Tucker and Milbrath, was selected as an outgroup.

The combined nuclear and mitochondrial DNA data set comprised *ca* 3904 characters for 19 taxa which contained 329 (8.43%) potentially phylogenetic informative sites.

Neighbour-joining as well as maximum likelihood (data not shown) analysis of the

[DOR: 20.1001.1.16807073.2015.17.5.10.9]

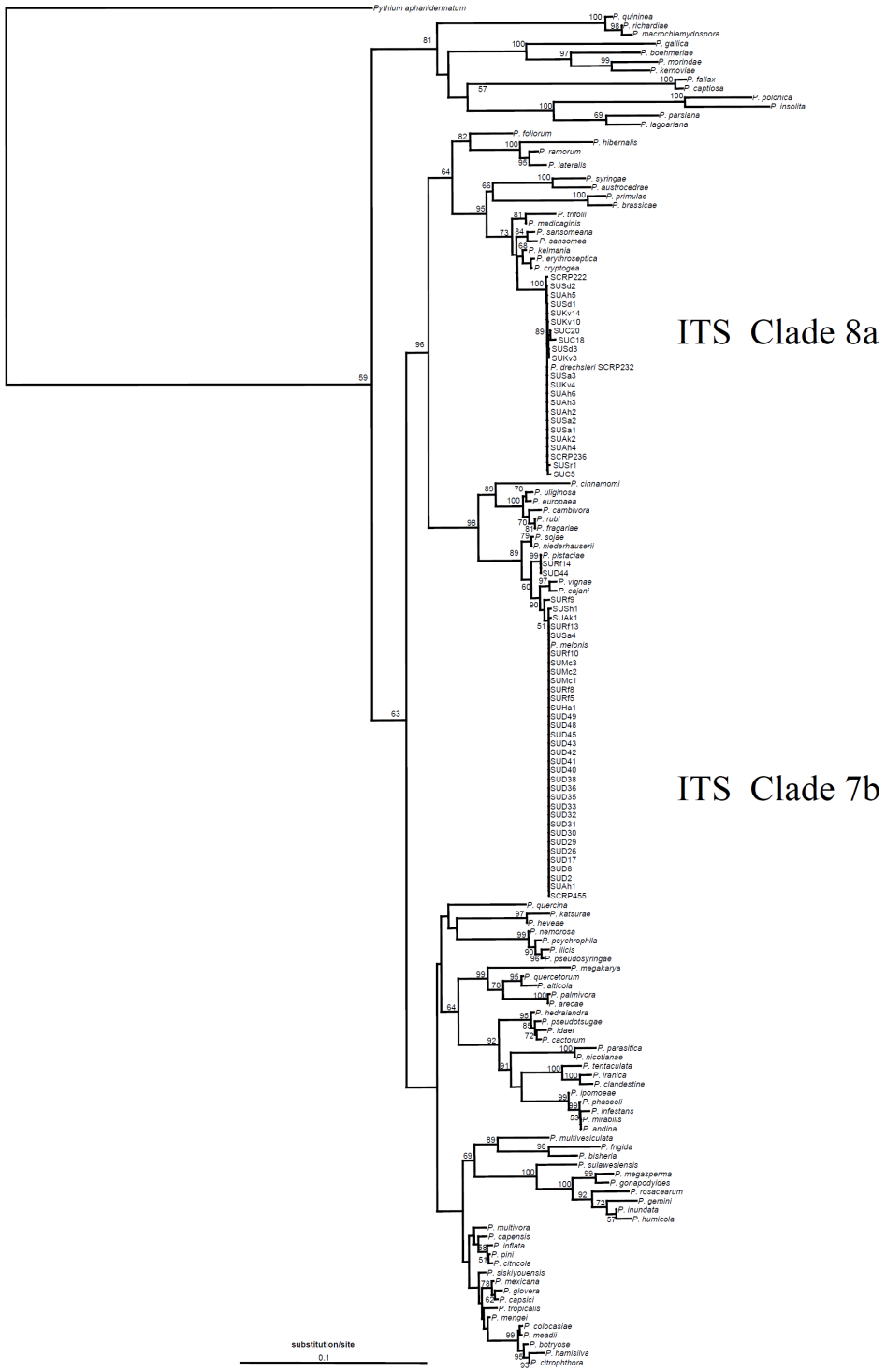

Figure 1. Detailed phylogram of 92 *Phytophthora* species and 33 *Phytophthora melonis*, 2 *P. pistaciae* and 23 *P. drechsleri* isolates. The numbers within parentheses indicate the isolate numbers. The phylogram was constructed by DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA. The numbers at the branch points indicate the percentages of bootstrap values ≥ 50%.

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five individual loci showed gene–gene concordance in the two observed lineages within the isolates.

P. melonis clade was resolved as monophyletic in the five individual neighbour-joining gene trees with bootstrap support of 100% (Figure 2). These isolates included an authentic isolate of *P. melonis* which consistently grouped in a clade with all other isolates which could be considered as *P. melonis sensu stricto*. The position of isolates in *P. melonis* clade among a wider selection of *Phytophthora* species also confirmed their identity (Figure 3).

Temperature Relations

The mean growth rate of *P. melonis*, *P. drechsleri* and *P. pistaciae* differed markedly (Table 3). In general, *P. melonis* was a slower grower than *P. drechsleri*, however, within each taxon, the range was large (Table 3). All isolates had an optimum temperature of 30°C and could grow well (more than 3.5 mm d^{-1}) at 35°C, except for the isolates of *P. pistaciae* which could grow only 1.7 and 2 mm d^{-1}.

Colony Growth Pattern

All isolates produced uniform colony pattern almost on all of the media, with few exceptions. The dominant colony pattern on PDA was rose-shaped in *P. drechsleri* and *P. pistaciae* (Table 3). *Phytophthora drechsleri* isolates also showed a relatively higher growth rate on HSA (Table 3).

Sporangium Morphology

Sporangia of *P. melonis*, *P. drechsleri* and *P. pistaciae* were non-papillate and ranged in shape from obpyriform, ellipsoid to ovoid, with or without tapered base (Table 3). One isolate of *P. drechsleri* produced both sporangia with both tapered and non-tapered bases under the same environmental conditions. Distorted shapes were also observed within *P. melonis* isolates. All isolates produced external proliferation in their sporangia. In general, the sporangia of *P. pistaciae* isolates were more elongated with a higher length/breadth ratio (2.1:1) compared with other isolates (Table 3).

Mating Behaviour and Sexual Organs' Morphology

Isolates of *P. melonis* were either homothallic or heterothallic, although some isolates did not produce oospores or any other sexual organs (antheridia and oogonia) in our experimental condition. In contrast, all isolates of *P. drechsleri* were heterothallic and *P. pistaciae* isolates showed homothallic behaviour. All of the isolates produced only amphigynous terminal antheridia (Table 3).

Pathogenicity

All *P. drechsleri* isolates produced the characteristic pink-rot symptom as described by Pethybridge (1913). The inoculated isolates were re-isolated from each of the diseased potatoes and their identity was verified. Neither *P. melonis* nor *P. pistaciae* isolates could induce potato pink-rot.

Phytophthora Extracellular Protein Genes

All designed primer pairs for extracellular protein genes were able to amplify *P. infestans* (Mont.) de Bary DNA whose expressed sequence tags (ESTs) were used for primer designing. Whereas the only amplified gene with proper length and polymorphism in the studied isolates was a putative *elicitin* (product of PEX-F and PEX-R primers), this gene could discriminate *P. melonis* from *P. drechsleri* and also was amplified in *P. lateralis* (Table 1).

[DOR: 20.1001.1.16807073.2015.17.5.10.9]

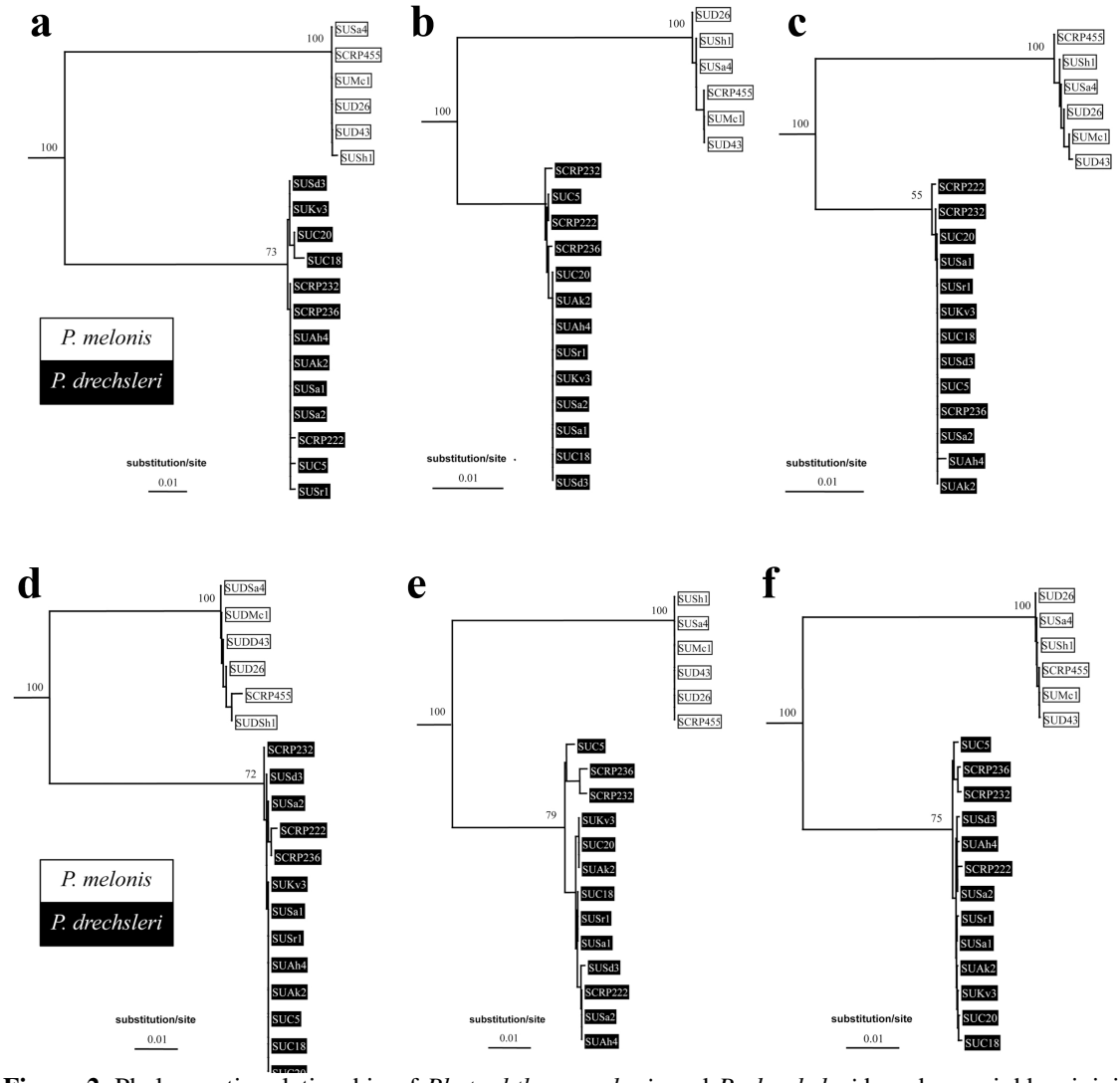

Figure 2. Phylogenetic relationship of *Phytophthora melonis* and *P. drechsleri* based on neighbor joining method. The numbers at the branch points indicate the percentages of bootstrap values ≥ 50%. (a) ITS1, 5.8S subunit, and ITS2 regions of the genomic ribosomal RNA tandem gene repeat; (b) *TUB* gene; (c) *ELO* gene; (d) *ELI* gene; (e) *COX* gene; (f) Combined genes (ITS1, 5.8S subunit, and ITS2 regions of rDNA; *TUB*; *ELO*; *ELI*; and *COX*).

DISCUSSION

Based on ITS regions of rDNA 32 isolates of *Phytophthora* from cucurbits, pistachio trees, giradol (*Chorozophora tinctoria* (L.) A. Juss.), and sugar beet (*Beta vulgaris* L.) were identified as *P. melonis* (Table 1); These *P. melonis* isolates were originally misidentified as *P. drechsleri*, typically due to their unrecognizable and overlapping morphology and the ability to grow well at 35ºC. These isolates together with an authentic *P. melonis* isolate from cucumber (*Cucumis sativus* L.) clustered in ITS Clade 7b (Figure 1). Two putative *P. drechsleri* isolates from pistachio trees also appeared next to *P. pistaciae* in ITS Clade 7b. Additionally, 10 isolates of *P. drechsleri* were identified by ITS analysis. Molecular data from these isolates together with data of 13 authentic *P. drechsleri* isolates

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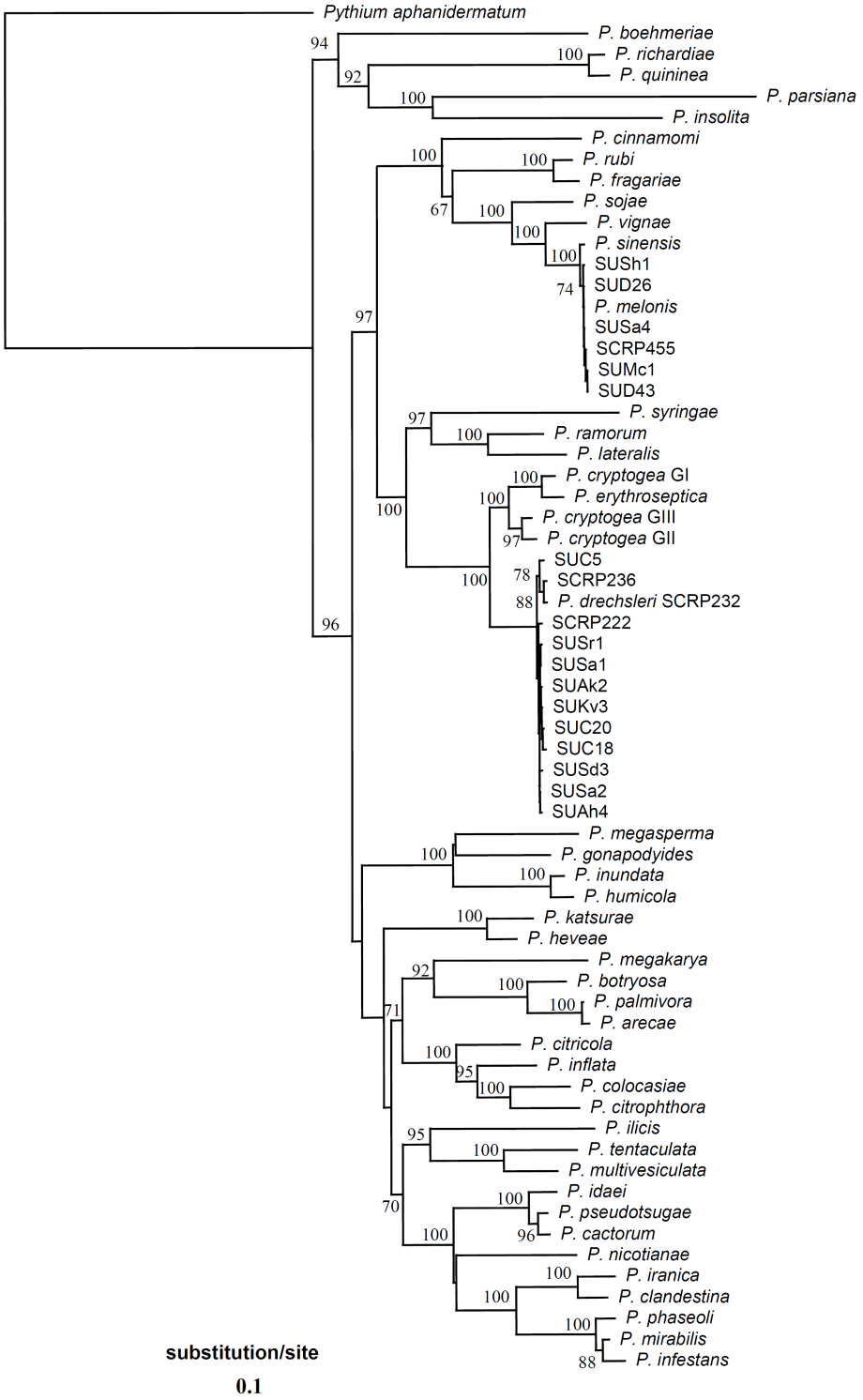

Figure 3. Combined genes phylogram of 45 *Phytophthora* taxa, 6 *Phytophthora melonis* and 13 *P. drechsleri* isolates. The phylogram was constructed after DNA distance-based analysis of the combined ITS1, 5.8S subunit, and ITS2 regions of the rDNA (Cooke *et al.*, 2000), *β-tubulin*, *translation elongation factor 1α* and *cytochrome c oxidase subunit I* (Kroon *et al.*, 2003) genes. The numbers at the branch points indicate the percentages of bootstrap values ≥ 50%.

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Table 3. Comparison of morphological characters of *Phytophthora melonis*, *P. drechsleri*, and *P. pistaciae*.

Character	*P. melonis* (33) [a]	*P. drechsleri* (23) [b]	*P. pistaciae* (2)
Sporangia			
Papilla	- [c]	-	-
Average length (µm)	53±12 [d]	32.4±8.9	73.6±8.8
Range length (µm)	18-130	17.3-88	17-119
Average breadth (µm)	28.7±6.1	19.2±4.7	35.2±4.5
Range breadth (µm)	18-67	12.5-55	15-56
Isolate averages			
Isolate length (µm)	35.2-90.2	21-57.7	67.4-79.8
Isolate breadth (µm)	18.5-45.8	12-34.6	32-38.4
Length: breadth ratio	1.9:1	1.7:1	2.1:1
Isolate averages	1.4:1-2.6:1	1.4:1-2.9:1	2.1:1
Shape(s)	El [e],Op [f]	El [e],Op [f] Ov [g]	El [e],Op [f]
Distorted shapes	(+) [h]	-	-
Tapered base	+ [i]	+	+
Caducity	-	-	-
Proliferation	+	+	+
Sympodial	(+)	(+)	(+)
Average pore diam. (µm)	7.9±1.6	5.8±1.3	10.5±0.7
Isolate averages (µm)	3-11	3-8	10-11
Homothalism	(+)	-	+
Oogonia			
Average diam. (µm)	32.8±4.5	30.1±4.5	32.3±2.1
Range (µm)	15-50	17-52	23-43
Isolate averages (µm)	24.7-40.7	22.4-36.9	30.8-33.8
Tapered base	(+)	+	(+)
Oospores			
Average diam. (µm)	30.6±3.2	27.4±3.5	32.3±2.1
Range (µm)	13-50	15-43	23-43
Isolate averages (µm)	24.7-37	20.9-33.8	30.8-33.8
Plerotic	+	+	-
Aplerotic	(+)	(+)	+
Oospore wall			
Average diam. (µm)	3.5±1.1	3.7±0.7	5±0
Isolate averages (µm)	2-5	2-5	5
Antheridia	Amphigynous	Amphigynous	Amphigynous
Average diam. (µm)	15.4±2.4	12.2±2.4	12.3±0.3
Isolate averages (µm)	9.6-18.8	9.1-15.6	12.1-12.5
Hyphae			
Average Width (µm)	5.7	5.7	5
Isolate averages (µm)	5-7.5	5-7.5	5
Hyphal swellings			
In water	(+)	(+)	-
On agar	(+)	(+)	-
CV8			
Pattern	Uniform	Uniform	Uniform
Growth rate [c]	5.1±1.8	6.7±1.3	2.9±1.5
Isolate averages	0.9-7.4	4.8-8.3	1.8-3.9

[a] Number of isolates tested; [b] A part of data from Mostowfizadeh-Ghalamfarsa *et al.* 2010**;** [c] Feature not observed.; [d] Figures are mean±standard deviation of all isolates from a particular group. [e] Ellipsoid sporangia; [f] Obpyriform sporangia, [g] Ovoid sporangia, [h] Feature occurring infrequently, [i] Feature occurring frequently.

Continued….

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Continued of Table 3**.**

Character	*P. melonis* (33)	*P. drechsleri* (23)	*P. pistaciae* (2)
MEA			
Pattern	Uniform	Uniform	Uniform
Growth rate	3.5±1.4	5±1.3	2.9±0
Isolate averages	0.7-6	2.9-5.8	2.9
HSA			
Pattern	Uniform	Uniform	Uniform
Growth rate	4.3±1.9	7.2±1.9	2.6±0
Isolate averages	1.4-8	3.6-8.7	2.6
PDA			
Pattern	Uniform	Rose-shaped	Rose-shaped
Growth rate	3.8±1.6	5.1±1.8	2.1±1.1
Isolate averages	0.7-5.8	3.2-11.4	1.3-2.8
CMA			
Pattern	Uniform	Uniform	Uniform
Average growth rate (isolate averages) at:			
5°	0±0 (0-1.2)	0.6±0.5 (0-1.5)	0±0 (0)
10°	1±0.8 (0-3)	2.5±0.6 (0.7-33)	0.8±1 (0.1-1.5)
15°	2.5±1.4 (0-5.3)	4.8±1.4 (2.1-6.2)	1.8±0.8 (1.2-2.3)
20°	4.7±1.4 (2.2-6.9)	7.1±2 (2.3-8.7)	3.2±0.8 (2.6-4)
25°	5.8±1.9 (2.5-8.9)	8.5±2.1 (3.8-11.7)	3.6±0.4 (3.3-3.8)
30°	7.6±3.1 (2.3-14.7)	12.2±3.8 (4.5-15.5)	4.6±0 (4.6)
35°	4.3±3.4 (0-9.9)	7.5±2.2 (3.2-10.1)	1.9±0.2 (1.7-2)
37°	2.1±2.4 (0-7.7)	3.5±2.6 (0-8.4)	0.7±1 (0-1.4)
40°	0±0 (0-0.1)	0.3±07 (0-3.2)	0±0 (0)
Potato pink rot	-	+	-

[a] Number of isolates tested; [b] A part of data from Mostowfizadeh-Ghalamfarsa *et al.* 2010**;** [c] Feature not observed.; [d] Figures are mean±standard deviation of all isolates from a particular group. [e] Ellipsoid sporangia; [f] Obpyriform sporangia, [g] Ovoid sporangia, [h] Feature occurring infrequently, [i] Feature occurring frequently.

(Mostowfizadeh-Ghalamfarsa *et al*., 2010) were also applied for further phylogenetic analysis.

Phylogenetic reconstruction of ITS region of rDNA revealed that *P. melonis* isolates were highly uniform (ave. 99.8% similarity). The isolates belonged to a distinct clade (ITS Clade 7b of Cook *et al.*, 2000; Figure 1) which consisted of *P. cajani* K. S. Amin, Baldev and F. J. Williams, *P. vignae* Purss, *P. pistaciae* Mirab.*, P. niederhauserii* Z.G. Abad and J.A. Abad and *P. sojae* Kaufm. and Gerd. Neighbourhood joining analysis of other genes as well as total protein electrophoresis data confirmed the homogeneity of *P. melonis* isolates. It seems that the narrow niche of this species, which was limited to cucurbits and few other species in Asia, could be responsible for the homogeneity of the isolates.

Phytophthora melonis isolates in other data sets such as *TUB, ELO*, *ELI* and *COX* demonstrated congruence in their tree topology which was confirmed in combined gene tree as well (Figure 2). This taxon always (data not shown) appeared as a sister taxon for *P. vignae,* and other members of this clade were basal to both species (Figure 3). There are no differences between relationships inferred from nuclear and

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cytoplasmic data (Figure 2). However, the rate of substitution per site was slower in *ELI* (Figure 2-d) compared to other genes of *P. melonis* isolates.

The *P. sinensis* isolate was completely identical and unrecognizable with *P. melonis* (Figure 3). Combined gene tree of *Phytophthora* species (Figure 3) confirms their "previously proposed conspecificity" (Cooke *et al.*, 2000) of this species with *P. melonis* (Ho, 1986; Mills *et al.,* 1991). There is some other published evidence which supports the premise of conspecificity. For instance, *P. melonis* and *P. sinensis* were isolated from diseased cucumber in Japan and described (Katsura, 1968) and China (Yu and Zhuang, 1982), respectively. Ho (1986) described both *P. melonis* and *P. sinensis* as *P. drechsleri* resulting in confused nomenclature of this group. However, a collection of fourteen isolates identified as *P. melonis*, *P. drechsleri* or *P. sinensis*, from *Cucumis sativus* or *Cucumis melo* from China, Iran, and Taiwan had identical isozyme patterns which discriminated them in a unique group (F) from other *P. drechsleri* isolates (Mills *et al*., 1991).

Growth pattern of *P. melonis* isolates did not reveal a very good discriminative feature between *P. melonis* and *P. drechsleri*. Most of the *P. melonis* isolates grew uniformly on PDA; on the contrary, most of the *P. drechsleri* isolates produced rose-shaped colonies on PDA, though there were some overlaps.

Phytophthora melonis isolates showed an optimal growth at 30°C and most of them could grow well at 35°C which is the main character of *P. drechsleri* (Mostowfiazdeh-Ghalamfarsa *et al.* 2010). Therefore, optimum temperature could not be a discriminative characteristic for these species. However, unlike *P. drechsleri*, some *P. melonis* isolates could not grow at 35°C at all (Table 3). *Phytophthora melonis* isolates were relatively fast growing but not as fast growing as *P. drechsleri*.

Comparison of morphological characters of *P. melonis* with other studied species showed some differences. Although the range of length/breadth ratio was almost the same, the average value for *P. melonis* isolates for this feature was more than that of *P. drechsleri*, which means they typically were more elongated (Table 3). Although the sporangia of *P. melonis* were originally described as semi-papillate by Katsura (1968), all of the isolates produced non-papillate sporangia which were consistent with Ho (1986), Ho and Jong (1991), Ho *et al.* (2007) observations of *P. melonis* and *P. sinensis.*

Phytophthora melonis was a mixture of homo and heterothallic isolates (Table 3). The evolution of this species towards heterothallism could be due to specificity on special hosts (cucurbits). Since we could not find any homothallic *P. drechsleri*, it is probable that all homothallic *P. drechsleri* originally were "misidentified *P. melonis*" isolates.

It seems that *P. melonis* isolates not only could infect cucurbit plants but also have the ability to infect other agriculturally important species such as sugar beet and pistachio trees (Table 1). It is likely that all of the *P. drechsleri* reported from cucurbits were misidentified for *P. melonis*. Some of our (Mostowfizadeh-Ghalmfarsa *et al.*, 2010) as well as others (Esmaili-Shirazi and Banihashemi, 2008; Nemati, 2010) works and the list of original hosts (Table 1) confirms this idea. Despite the fact that we identified *P. drechsleri* isolates from pistachio trees as *P. melonis*, it seems that there are some molecularly approved pathogenic *P. drechsleri* isolates on pistachio trees (A.H. Mohammadi, personal communications) as well as *P. melonis*.

Phytophthora melonis isolates did not show the ability of inciting pink rot symptoms in potato tubers (Table 3). This feature seems to be a distinctive character for discriminating *P. melonis* from *P. drechsleri* and *P. cryptogea* Pethybr. and Laff. isolates which can easily incite the pink rot symptoms in potato tubers (Mostowfizadeh-Ghalamfarsa *et al*., 2006). Observations showed that this feature was

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unique, easily scorable, not cultivar dependent, and highly conserved and could be considered as a reliable diagnostic character. However, other pathogenicity tests such as safflower (*Carthamus tinctorius* L.) seedling damping off can discriminate *P. melonis* from the pathogenic *P. drechsleri* (Banihashemi and Mirtalebi, 2007), while *P. drechsleri* cannot incite root or crown rot in melon (*Cucumis melo* L.) (Nemati, 2010).

Although *P. melonis* and *P. drechsleri* are morphologically convergent species, the multiple gene genealogy analysis and physiological studies such as host range refute the premise of their conspecificity. The results of this study revealed that the putative *P. drechsleri* isolates from cucurbits in Iran belong to *P. melonis*. There is no evidence of biogeographically or bioecologically (host range) structured lineage among isolates of the two taxa which shows the lack of intraspecific variation in *P. melonis* as well as *P. drechsleri*. Pathogenicity of *P. melonis* isolates on economically important plants emphasizes the need for a better study of their diversity and geographical distribution. Design of a molecular species-specific identification tool for *P. melonis* isolates is under investigation.

ACKNOWLEDGEMENTS

Special thanks to Dr. David E. L. Cooke (The James Hutton Institute) for his valuable ideas. This study was funded by the Iran National Science Foundation (award number 86015.03).

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[DOR: 20.1001.1.16807073.2015.17.5.10.9]

بازنگری جدایه‌های *Phytophthora drechsleri* کدوییان ایران بر اساس واکاوی دودمان‌های چند ژنی

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چکیده

گونه‌ی بیمارگر گیاهی *Phytophthora drechsleri* از نظر ریخت‌شناختی به برخی از گونه-های بدون پاپیل فیتوفتورا به خصوص *P. melonis* شبیه است و تفکیک این آرایه‌های هم‌گرا دشوار است. به نظر می‌رسد که جدایه‌هایی از *P. drechsleri* که در ایران از گونه‌های مختلف کدوییان جداسازی شده‌اند عموماً به اشتباه تشخیص داده شده، خصوصیات آن‌ها با گونه‌ی *P. drechsleri* هم‌خوانی ندارد. به منظور مقایسه‌ی این دو گروه، جدایه‌های معتبری از *P. drechsleri* به همراه جدایه‌های مربوط به کدوییان از نظر صفات ریخت شناختی، فیزیولوژیکی (خصوصیات کشتی، روابط دمایی، تیپ آمیزشی) و خصوصیات مولکولی مورد ارزیابی قرار گرفتند. واکاوی دودمان‌های چند ژنی روی توالی‌های نواحی هسته‌ای (آی‌تی‌اِس، بتاتوبولین، عامل ترجمه کشیدگی یک آلفا و الیسیتین) و توالی‌های میتوکندریایی (زیر واحد یک سیتوکروم سی اُکسیداز) انجام گرفت. در این مطالعه داده های فیلوژنتیکی به دست آمده از ژن‌های مختلف با یک‌دیگر انطباق نشان دادند. بررسی های به عمل آمده نشان داد که جدایه‌های فرضی *P. drechsleri* مربوط به کدوییان یک گونه‌ی مجزا و متعلق به گونه‌ی *P. melonis* هستند. داده‌ها نشان دادند که *P. melonis* گونه‌ای یک-نواخت است و تنوع درون گونه‌ای قابل توجهی در سطح مولکولی بین جدایه‌های کدوییان یا در مقایسه با جدایه‌هایی از سایر میزبان‌ها وجود ندارد. شناسایی مولکولی جدایه‌های *P. melonis* در دست بررسی است.

[DOR: 20.1001.1.16807073.2015.17.5.10.9]