Morphological and molecular identification of newly recovered *Pythium* species, *P. sylvaticum* and *P. glomeratum* from Iran, and evaluation of their pathogenicity on cucumber seedlings

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Abstract: During a survey on the biodiversity of the genus *Pythium* in northern Iran, ten isolates of *P. sylvaticum* (clade F) and five *P. glomeratum* (clade I) were recovered from the rhizosphere of plant species. Based on combination of cultural, morphological, cardinal growth rate, sequence data from ITS-rDNA and pathogenicity assay, the isolates were identified as *P. sylvaticum* and *P. glomeratum*. Phylogenetic analyses of the ITS-rDNA sequences clustered our isolates with representative sequences for *P. sylvaticum* and *P. glomeratum* isolates from GenBank. Both fungal species represent new records for the mycobiota of Iran. Several plant species *viz*., *Taraxacum officinalis*, *Falcaria* sp., *Elaeagnus angustifolia*, *Camellia sinensis*, *Tragopogon collinus*, *Phleum iranicum*, *Lotus angustissimus* and *Ficus carica*, *Onosma* sp., *Ligustrum ovalifolium*, *Punica granatum*, and *Prunus avium* were new substrates for *P. sylvaticum* and *P. glomeratum,* respectively. With this paper, we provide full illustration for these two species and further discuss their phylogeny and morphology with closely related species and their pathogenicity on cucumber (*Cucumis sativus*) seedlings.

Zusammenfassung: Im Rahmen einer Untersuchung zur Biodiversität der Gattung *Pythium* im nördlichen Iran wurden zehn Isolate von *P. sylvaticum* (Clade F) und fünf von *P. glomeratum* (Clade I) aus der Rhizosphäre von Pflanzenarten gewonnen. Basierend auf der Kombination der Wachstumsraten, der Kulturmerkmale, ITS-rDNA Sequenzdaten und Pathogenitäts-Assays wurden die Isolate als *P. sylvaticum* und *P. glomeratum* identifiziert. Phylogenetische Analysen der ITS-rDNA Sequenzen gruppierten unsere Isolate mit repräsentativen Sequenzen für *P. sylvaticum* und *P. glomeratum* Isolate aus der GenBank. Beide Pilzarten sind neue Nachweise für die Mykobiota des Iran. Mehrere Pflanzenarten, wie *Taraxacum officinalis, Falcaria* sp., *Elaeagnus angustifolia, Camellia sinensis, Tragopogon collinus, Phleum iranicum, Lotus angustissimus* und *Ficus carica, Onosma* sp., *Ligustrum ova-*

lifolium, Punica granatum und *Prunus avium* sind neue Substrate für *P. sylvaticum* und *P. glomeratum*. In dieser Arbeit bieten wir eine vollständige Illustration für diese beiden Arten und diskutieren ihre Phylogenie und Morphologie mit eng verwandten Arten und deren Pathogenität auf Gurken(*Cucumis sativus*)-Sämlingen.

The genus *Pythium* is a large group containing more than 140 species and has wide distribution extending to terrestrial and aquatic habitats (KIRK & al. 2008). Members of *Pythium* exhibit both saprophytic and parasitic life styles (NECHWATAL & al. 2008, LARA & BELBAHRI 2011). Parasitic species of the genus may have negative effects on a wide range of hosts, including algae, mosses, humans, fish, crustaceans and mosquito larvae (MIURA & al. 2010, PHILLIPS & al. 2008, VAN DER PLAATS-NITERINK 1981). Some of phytopathogenic species have a damaging effect on crops throughout the world, showing different types of disease symptoms such as pre- and post-emergence damping-off, and seed and root rot.

Pythium glomeratum (Clade I) was isolated from soil samples in France for the first time; however, it was identified as *P. heterothallicum*. *Pythium glomeratum* does not easily form oogonia and antheridia on hemp-seed halves in water. Although, in grass-blade water cultures and on media containing agar supplemented with some sterols such as Beta-sitosterol, the oomycete produces sexual structures plentifully (PAUL 2003). *Pythium sylvaticum* (Clade F) is the first known heterothallic species of *Pythium* (CAMPBELL & HENDRIX 1967). The species was originally isolated from soil in the United States. Fresh isolates usually show no sexual organs in single culture, but after long incubation, oogonia may be produced in single cultures. Often only a part of the oogonia form mature oospores in both single and paired cultures (VAN DER PLAATS-NITERINK 1981).

Here, based on combining morphological, ecological and phylogenetic species concepts referred to as the Consolidated Species Concept (CHENARI BOUKET & al. 2015a) we describe i) new records of *Pythium glomeratum* from rhizosphere of *Ficus carica*, *Onosma* sp., *Ligustrum ovalifolium*, *Punica granatum* and *Prunus avium* and ii) *Pythium sylvaticum* from rhizosphere of *Taraxacum officinalis*, *Falcaria* sp., *Elaeagnus angustifolia*, *Camellia sinensis*, *Tragopogon collinus*, *Malus domestica*, *Phleum iranicum* and *Lotus angustissimus* in Iran. We also evaluate their pathogenicity on cucumber (*Cucumis sativus*) seedlings under laboratory conditions.

Materials and methods

Isolation: *Pythium glomeratum* isolates were recovered from rhizosphere of *Ficus carica*, *Onosma* sp., *Ligustrum ovalifolium*, *Punica granatum* and *Prunus avium* in University of Tabriz campus, Abesh-Ahmad region in East-Azarbaijan and Ardebil provinces, respectively. *Pythium sylvaticum* isolates were recovered from rhizosphere of *Taraxacum officinalis*, *Falcaria* sp., *Elaeagnus angustifolia*, *Camellia sinensis*, *Tragopogon collinus*, *Malus domestica*, *Phleum iranicum*, *Lotus angustissimus* in Kandovan, Marand, Meshkin Shahr and Rasht in East-Azarbaijan, Ardebil and Guilan provinces, respectively. Pieces of roots of plant were washed in sterile water and then dried on a paper towel, transferred onto oomycete-selective medium namely NARM (MORITA & TOJO 2007, CHENARI BOUKET & al. 2015b) and incubated at 25 °C for 1–2 days. Pure cultures were established using a hyphal tip technique. Cultures were preserved on V8A (MILLER 1955) slant vials at 10 °C in the dark until use. Detailed information of the isolates is provided in Tab. 1.

Morphology and growth temperature relationships: Mycelial patterns of all isolates were recorded three days after inoculation at 22 °C on CMA (Becton Dickinson and Company, Franklin Lakes, NJ, USA), PDA (Sigma Aldrich, St. Louis, MO, USA) and V8A (Miller 1955). Morphological obser-

vations were made on structures produced on single and paired cross cultures established on Potato Carrot Agar media and sterile grass blades floated in sterile pond water (MARTIN 1992). Twenty-five measurements were made for each microscopic structure including hyphae, sporangia, zoospores, antheridia, oogonia and oospores. Photographs were captured using an Olympus-BX43 microscope with the digital camera system (DP2-ASL) (Olympus, Tokyo, Japan). The cultures were deposited in the Culture Collection of Osaka Prefecture University (OPU), Osaka, Japan. The cardinal temperatures were determined three days after inoculation on PCA at temperature ranging from 4 to 43 °C.

DNA extraction and amplification: DNA was extracted from mycelia grown on CMA with a manual process described by MOLLER & al. (1992). The internal transcribed spacer (ITS) of ribosomal DNA was amplified by the polymerase chain reaction using primers ITS5 (GGAAGTAAAGTCGTAACAAGG) and ITS4 (TCCTCCGCTTATTGATATGC) (WHITE & al. 1990). All reactions were carried out in a total volume of 50 µl, containing 5µl 10X Taq buffer (20 mM Tris-HCl, pH 8.0, 100 mM KCl), 4µl 2.5 mM dNTP mixture, 0.5 µM of each primer, 1.25 units Taq DNA polymerase (Takara Bio, Ohtsu, Japan) and 10 ng DNA. The amplifications were performed using a PerkinElmer 9700 thermal cycler (PerkinElmer Inc. Waltham, MA, USA) with the following cycling profile: 95 °C for 5 min followed by 30 cycles including denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min, and final extension step at 72 °C for 7 min for ITS. PCR products were purified with GenElute™ PCR Clean-Up Kit (Sigma-Aldrich, St. Louis, MN, USA) based on the instructions of the manufacturer and then used for sequence analysis.

Tab. 1. Code, GenBank accession numbers, locality and references of strains used in the phylogenetic analysis.

Species	Code	Locality	Accession No.	Reference
Phytium sylvaticum	**OPU 1694**	**Meshkin Shahr, Ardebil province, (38°23′56″N 47°40′55″E)**	**KU665806**	**Present study**
P. sylvaticum	**OPU 1695**	**Kandovan, E-Azarbaijan province, (37°47′42″N 46°14′55″E)**	**KU665807**	**Present study**
P. sylvaticum	**OPU 1696**	**Marand, East-Azarbaijan province,(38°25′58″N 45°46′30″E)**	**KU665808**	**Present study**
P. sylvaticum	**OPU 1697**	**Rasht, Guilan province, (37°16′51″N 49°34′59″E)**	**KU665809**	**Present study**
P. sylvaticum	**OPU 1698**	**Meshkin Shahr, Ardebil province, (38°23′56″N 47°40′55″E)**	**KU665810**	**Present study**
P. sylvaticum	**OPU 1699**	**Moghan, Ardebil province, (39°38′54″N 47°55′03″E)**	**KU665811**	**Present study**
P. sylvaticum	**OPU 1700**	**Moghan, Ardebil province, (39°38′54″N 47°55′03″E)**	**KU665812**	**Present study**
P. sylvaticum	**OPU 1701**	**Meshkin Shahr, Ardebil province, (38°23′56″N 47°40′55″E)**	**KU665813**	**Present study**
P. sylvaticum	**OPU 1702**	**Meshkin Shahr, Ardebil province, (38°23′56″N 47°40′55″E)**	**KU665814**	**Present study**
P. sylvaticum	**OPU 1703**	**Meshkin Shahr, Ardebil province, (38°23′56″N 47°40′55″E)**	**KU665815**	**Present study**
P. glomeratum	**OPU 1781**	**Campus of University of Tabriz, (38°03′49″N 46°19′43″E)**	**KU665816**	**Present study**

Tab. 1 continued.

Species	Code	Locality	Accession No.	Reference
P. glomeratum	**OPU 1782**	**Abesh Ahmad, E-Azarbaijan Prov., (39°02′33″N 47°18′59″E)**	**KU665817**	**Present study**
P. glomeratum	**OPU 1783**	**Campus of University of Tabriz, (38°03′49″N 46°19′43″E)**	**KU665818**	**Present study**
P. glomeratum	**OPU 1784**	**Campus of University of Tabriz (38°03′49″N 46°19′43″E)**	**KU665819**	**Present study**
P. glomeratum	**OPU 1785**	**Campus of University of Tabriz (38°03′49″N 46°19′43″E)**	**KU665820**	**Present study**
P. viniferum	F-1201	France	AY455694	Direct submission by PAUL
P. viniferum	CBS119168	France	HQ643956	ROBIDEAU & al. 2011
P. lucens	CBS113342	Canada	HQ643681	ROBIDEAU & al. 2011
P. abappressorium	020162	United States	DQ091294	SCHROEDER & al. 2006
P. abappressorium	CBS110198	United States	HQ643408	ROBIDEAU & al. 2011
P. cryptoirregulare	CBS118731	United States	HQ643515	ROBIDEAU & al. 2011
P. irregulare	BR1000	South Africa	HQ643667	ROBIDEAU & al. 2011
P. irregulare	BR1001	South Africa	HQ643666	ROBIDEAU & al. 2011
P. irregulare	BR1002	South Africa	HQ643665	ROBIDEAU & al. 2011
P. irregulare	BR1003	South Africa	HQ643664	ROBIDEAU & al. 2011
P. irregulare	BR1004	South Africa	HQ643663	ROBIDEAU & al. 2011
P. irregulare	BR1005	South Africa	HQ643662	ROBIDEAU & al. 2011
P. irregulare	BR1006	South Africa	HQ643661	ROBIDEAU & al. 2011
P. irregulare	BR1008	South Africa	HQ643660	ROBIDEAU & al. 2011
P. irregulare	BR1009	South Africa	HQ643659	ROBIDEAU & al. 2011
P. irregulare	BR1013	Australia	HQ643658	ROBIDEAU & al. 2011
P. irregulare	BR1014	Australia	HQ643657	ROBIDEAU & al. 2011
P. irregulare	BR1015	Australia	HQ643656	ROBIDEAU & al. 2011
P. irregulare	BR1016	Australia	HQ643655	ROBIDEAU & al. 2011
P. irregulare	BR1017	Australia	HQ643654	ROBIDEAU & al. 2011
P. irregulare	BR1018	Australia	HQ643653	ROBIDEAU & al. 2011
P. irregulare	BR1019	Australia	HQ643652	ROBIDEAU & al. 2011
P. irregulare	BR1021	Australia	HQ643651	ROBIDEAU & al. 2011
P. irregulare	BR1022	Australia	HQ643650	ROBIDEAU & al. 2011
P. irregulare	BR1039	Canada	HQ643649	ROBIDEAU & al. 2011
P. irregulare	BR1040	Canada	HQ643648	ROBIDEAU & al. 2011
P. irregulare	BR1051	Canada	HQ643647	ROBIDEAU & al. 2011
P. irregulare	BR1052	Canada	HQ643646	ROBIDEAU & al. 2011
P. irregulare	BR1068	Canada	HQ643645	ROBIDEAU & al. 2011
P. irregulare	BR387	Canada	HQ643644	ROBIDEAU & al. 2011
P. irregulare	BR598	Canada	HQ643642	ROBIDEAU & al. 2011
P. irregulare	BR629	Canada	HQ643641	ROBIDEAU & al. 2011
P. cylindrosporum	DAOM232335	Canada	HQ643517	ROBIDEAU & al. 2011
P. cylindrosporum	CBS21894	Germany	HQ643516	ROBIDEAU & al. 2011
P. paroecandrum	ADC9909	Netherlands	HQ643737	ROBIDEAU & al. 2011
P. paroecandrum	ADC9910	Netherlands	HQ643736	ROBIDEAU & al. 2011
P. paroecandrum	BR601	Australia	HQ643735	ROBIDEAU & al. 2011
P. paroecandrum	BR773	South Africa	HQ643734	ROBIDEAU & al. 2011
P. paroecandrum	BR774	South Africa	HQ643733	ROBIDEAU & al. 2011

Tab. 1 continued.

Species	Code	Locality	Accession No.	Reference
P. paroecandrum	BR807	South Africa	HQ643732	ROBIDEAU & al. 2011
P. paroecandrum	BR929	Spain	HQ643730	ROBIDEAU & al. 2011
P. paroecandrum	CBS15764	Australia	HQ643731	ROBIDEAU & al. 2011
P. sylvaticum	BR1045	Canada	HQ643852	ROBIDEAU & al. 2011
P. sylvaticum	BR1069	Canada	HQ643851	ROBIDEAU & al. 2011
P. sylvaticum	BR171	Canada	HQ643850	ROBIDEAU & al. 2011
P. sylvaticum	BR179	Canada	HQ643849	ROBIDEAU & al. 2011
P. sylvaticum	BR599	Canada	HQ643848	ROBIDEAU & al. 2011
P. sylvaticum	BR647	Netherlands	HQ643847	ROBIDEAU & al. 2011
P. sylvaticum	Lev1544	United States	HQ643846	ROBIDEAU & al. 2011
P. sylvaticum	P15580	Canada	HQ261741	ROBIDEAU & al. 2011
P. sylvaticum	CBS45367	United States	HQ643845	ROBIDEAU & al. 2011
P. terrestris	ADC9906	Netherlands	HQ643858	ROBIDEAU & al. 2011
P. terrestris	BR922	United States	HQ643856	ROBIDEAU & al. 2011
P. terrestris	CBS112352	France	HQ643857	ROBIDEAU & al. 2011
P. mamillatum	BR648	Netherlands	HQ643689	ROBIDEAU & al. 2011
P. mamillatum	BR765	Canada	HQ643688	ROBIDEAU & al. 2011
P. mamillatum	CBS25128	Netherlands	HQ643687	ROBIDEAU & al. 2011
P. spiculum	CBS122645	France	HQ643790	ROBIDEAU & al. 2011
P. kunmingense	CBS55088	China	HQ643672	ROBIDEAU & al. 2011
P. spinosum	CBS27667	Netherlands	HQ643792	ROBIDEAU & al. 2011
P. spinosum	Lev1526	United States	HQ643794	ROBIDEAU & al. 2011
P. spinosum	CBS122663	India	HQ643791	ROBIDEAU & al. 2011
P. spinosum	CBS27567	Netherlands	HQ643793	ROBIDEAU & al. 2011
P. macrosporum	BR1029	Canada	HQ643685	ROBIDEAU & al. 2011
P. macrosporum	ADC0029	Norway	HQ643686	ROBIDEAU & al. 2011
P. macrosporum	CBS57480	Netherlands	HQ643684	ROBIDEAU & al. 2011
P. emineosum	BR836	Canada	GQ244428	BALA & al. 2010
P. emineosum	BR479	Canada	GQ244427	BALA & al. 2010
P. attrantheridium	Lev3004	United States	HQ643473	ROBIDEAU & al. 2011
P. attrantheridium	DAOM230387	United States	HQ643475	ROBIDEAU & al. 2011
P. attrantheridium	DAOM230383	Canada	HQ643477	ROBIDEAU & al. 2011
P. intermedium	BR1042	Canada	HQ643579	ROBIDEAU & al. 2011
P. intermedium	BR128	Canada	HQ643578	ROBIDEAU & al. 2011
P. intermedium	BR339	Canada	HQ643577	ROBIDEAU & al. 2011
P. intermedium	BR485	Netherlands	HQ643576	ROBIDEAU & al. 2011
P. intermedium	BR707	Canada	HQ643575	ROBIDEAU & al. 2011
P. intermedium	BR734	Poland	HQ643574	ROBIDEAU & al. 2011
P. intermedium	BR869	Canada	HQ643573	ROBIDEAU & al. 2011
P. intermedium	BR924	Spain	HQ643571	ROBIDEAU & al. 2011
P. intermedium	CBS26638	Netherlands	HQ643572	ROBIDEAU & al. 2011
P. ultimum var. *sporangiiferum*	BR651	Spain	HQ643880	ROBIDEAU & al. 2011
P. ultimum var. *sporangiiferum*	CBS21965	United States	HQ643879	ROBIDEAU & al. 2011
P. ultimum	ADC9967	Netherlands	HQ643878	ROBIDEAU & al. 2011
P. ultimum	BR1036	Canada	HQ643877	ROBIDEAU & al. 2011
P. ultimum	BR1037	Canada	HQ643876	ROBIDEAU & al. 2011
P. ultimum	BR1038	Canada	HQ643875	ROBIDEAU & al. 2011
P. ultimum	BR1060	South Africa	HQ643873	ROBIDEAU & al. 2011
P. ultimum	BR1064	South Africa	HQ643872	ROBIDEAU & al. 2011
P. ultimum	CBS72994	Canada	HQ643869	ROBIDEAU & al. 2011
P. ultimum	BR1065	South Africa	HQ643871	ROBIDEAU & al. 2011
P. ultimum	BR1089	Canada	HQ643870	ROBIDEAU & al. 2011
P. ultimum	DAOM232337	Canada	HQ643868	ROBIDEAU & al. 2011
P. ultimum	Lev1441	United Kingdom	HQ643867	ROBIDEAU & al. 2011
P. ultimum	Lev1442	Poland	HQ643866	ROBIDEAU & al. 2011
P. ultimum	CBS122650	France	HQ643864	ROBIDEAU & al. 2011

Tab. 1 continued.

Species	Code	Locality	Accession No.	Reference
P. ultimum	CBS39851	Netherlands	HQ643865	ROBIDEAU & al. 2011
P. ultimum	BR861	United Kingdom	HQ643888	ROBIDEAU & al. 2011
P. ultimum	BR858	United States	HQ643889	ROBIDEAU & al. 2011
P. splendens	BR788	South Africa	HQ643797	ROBIDEAU & al. 2011
P. splendens	Lev1497	-	HQ643796	ROBIDEAU & al. 2011
P. splendens	CBS46248	United States	HQ643795	ROBIDEAU & al. 2011
P. glomeratum	CBS119165	Bulgaria	HQ643544	ROBIDEAU & al. 2011
P. glomeratum	CBS120914	India	HQ643543	ROBIDEAU & al. 2011
P. glomeratum	CBS122644	France	HQ643542	ROBIDEAU & al. 2011
P. heterothallicum	ADC9868	Italy	HQ643563	ROBIDEAU & al. 2011
P. heterothallicum	BR749	Canada	HQ643561	ROBIDEAU & al. 2011
P. heterothallicum	BR806	South Africa	HQ643560	ROBIDEAU & al. 2011
P. heterothallicum	BR828	-	HQ643559	ROBIDEAU & al. 2011
P. heterothallicum	DAOM229136	United States	HQ643558	ROBIDEAU & al. 2011
P. heterothallicum	DAOM229137	United States	HQ643557	ROBIDEAU & al. 2011
P. heterothallicum	DAOM229138	United States	HQ643556	ROBIDEAU & al. 2011
P. heterothallicum	DAOM229140	United States	HQ643555	ROBIDEAU & al. 2011
P. heterothallicum	Lev1590	Canada	HQ643554	ROBIDEAU & al. 2011
P. heterothallicum	CBS122655	France	HQ643552	ROBIDEAU & al. 2011
P. heterothallicum	CBS122656	France	HQ643551	ROBIDEAU & al. 2011
P. heterothallicum	CBS45067	Canada	HQ643553	ROBIDEAU & al. 2011
Phythopth. capsici	CBS111333	South Korea	HQ643188	ROBIDEAU & al. 2011

DNA sequencing and phylogenetic analysis: The amplicons were sequenced in both directions using the same PCR primers and the BigDye® Terminator v. 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) on the basis of the manufacturer's manual and then analyzed on 3130x Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Raw sequence files were manually edited using SeqMan™II (DNASTAR, Madison, WI, USA) and a consensus sequence was generated for each sequence. All P. sequences obtained in this study were deposited in GenBank. The consensus sequence for each sequence was compared against the NCBI's GenBank sequence database using Megablast to identify their closest species. The sequences retrieved from GenBank together with sequences generated in this study, were aligned using MEGA v. 6 (TAMURA & al. 2013). For phylogenetic comparison, Bayesian inference analysis was performed with MrBayes v. 3.2.1 (RONQUIST & HUELSENBECK 2003). The best evolutionary model for data partition was obtained using MrModelTest v. 2.3 (NYLANDER 2004). The heating parameter was set at 0.15 and four Markov chains were run for 10000000 generations, sampling every 1000 steps, and with a burn in at 25% sampled trees until the average standard deviation of split frequencies was below 0.01.

The resulting phylogenetic tree was exported with Geneious v. 5.6.7 (DRUMMOND & al. 2012). Sequences derived from this study were released at NCBI's GenBank nucleotide database (http://www. ncbi.nlm.nih.gov; Tab. 1).

Pathogenicity assays: Pathogenicity assays were implemented using cucumber (*Cucumis sativus* L. cv. 'Aonagakei-Jibai', Takii & Co. Ltd., Kyoto, Japan) plants. One-week-old seedlings were used that had been grown on moistened filter paper in a growth chamber at 25 °C with continuous light (80 µmol $m^{-2}s^{-1}$ measured) in 9 cm diam. petri plates. 5 mm diam. agar (CMA) plugs with growing mycelia of *P. glomeratum* (OPU 1782) and plugs from the contact zone of dual culture of *P. sylvaticum* (OPU 1695 and OPU 1702) were taken from the culture, placed on the plants, and incubated in the growth chamber at 25 °C. CMA was used as a control. Mortality of plants was recorded 10 days after incubation. The presence of sexual structures such as oospores in the plant tissues was the criterion of *P. sylvaticum* and *P. glomeratum* infection. Each experiment included 20 plants and was repeated 3 times. Analysis of variance was conducted on the rates of mortality and infection using IBM SPSS Statistics software (version 22; IBM Corporation, USA). Means of the data were compared by the least significant difference based on a Tukey-Kramer honestly significant different (HSD) test ($P < 0.01$).

Tab. 2. Morphological comparison between *Pythium sylvaticum* (OPU 1695 and OPU 1702) and its morphologically and phylogenetically related species.

Characteristics	***Pythium sylvaticum*, OPU 1695 & OPU 1702**	***Pythium sylvaticum*** (VAN DER PLAATS-NITERINK 1981)	***Pythium terrestris*** (PAUL 2002)
Main hypha	>7 µm	>11 µm	>7 µm
Sporangia	Absent	Absent	Globose to ovate, intercalary or terminal, 10–35 µm in diam.
Hyphal swellings	Globose, mostly terminal, 20.5–23 µm in diam.	Globose or lemon-shaped, mostly terminal, 32 µm in diam.	Absent
Appressoria	Clavate or sickle-shaped	Present (not well described)	Absent
Oogonia	Produced on PCA paired cultures of compatible isolates; smooth, globose, terminal or intercalary, 19.5–24.5 µm in diam.	Produced on single and dual cultures, smooth; globose, terminal or intercalary, 16–19.3 µm in diam.	Produced on single cultures; smooth, globose, terminal or intercalary, 18–30 µm in diam.
Antheridia	1–2 per oogonium, monoclinous and diclinous, antheridial stalks branched, often bifurcate near the oogonium	2–4 per oogonium, diclinous, antheridial stalks branched, often bifurcate near the oogonium, vanished immediately after fertilization	Monoclinous, hypogynous, surrounding the oogonium as a complicated knob
Oospore	Aplerotic, 18–23 µm in diam.	Aplerotic, 13–19 µm in diam.	Aplerotic, 10–25 µm in diam., 1–3 oospores produced in to one oogonium
Daily growth rate at 25 °C	35 mm/day	30 mm/day	9 mm/day

Results

Morphological description

Pythium glomeratum PAUL, FEMS Microbiol. Lett. 225(1): 49 (2003). – Fig. 1. (isolate OPU 1782):

M y c e l i a l p a t t e r n : *Chrysanthemum*-like on CMA, V8A and PDA. Optimum growth at 25 °C on PCA, with an average daily growth rate of 10 mm. Minimum, optimum and maximum growth temperature on PCA 7, 25 and 30 °C, respectively.

M a i n h y p h a e : hyaline, up to 5 µm wide.

S p o r a n g i a a n d z o o s p o r e s : not formed, even after prolonged incubation at different temperatures and being flooded with sterile distilled tap and pond water.

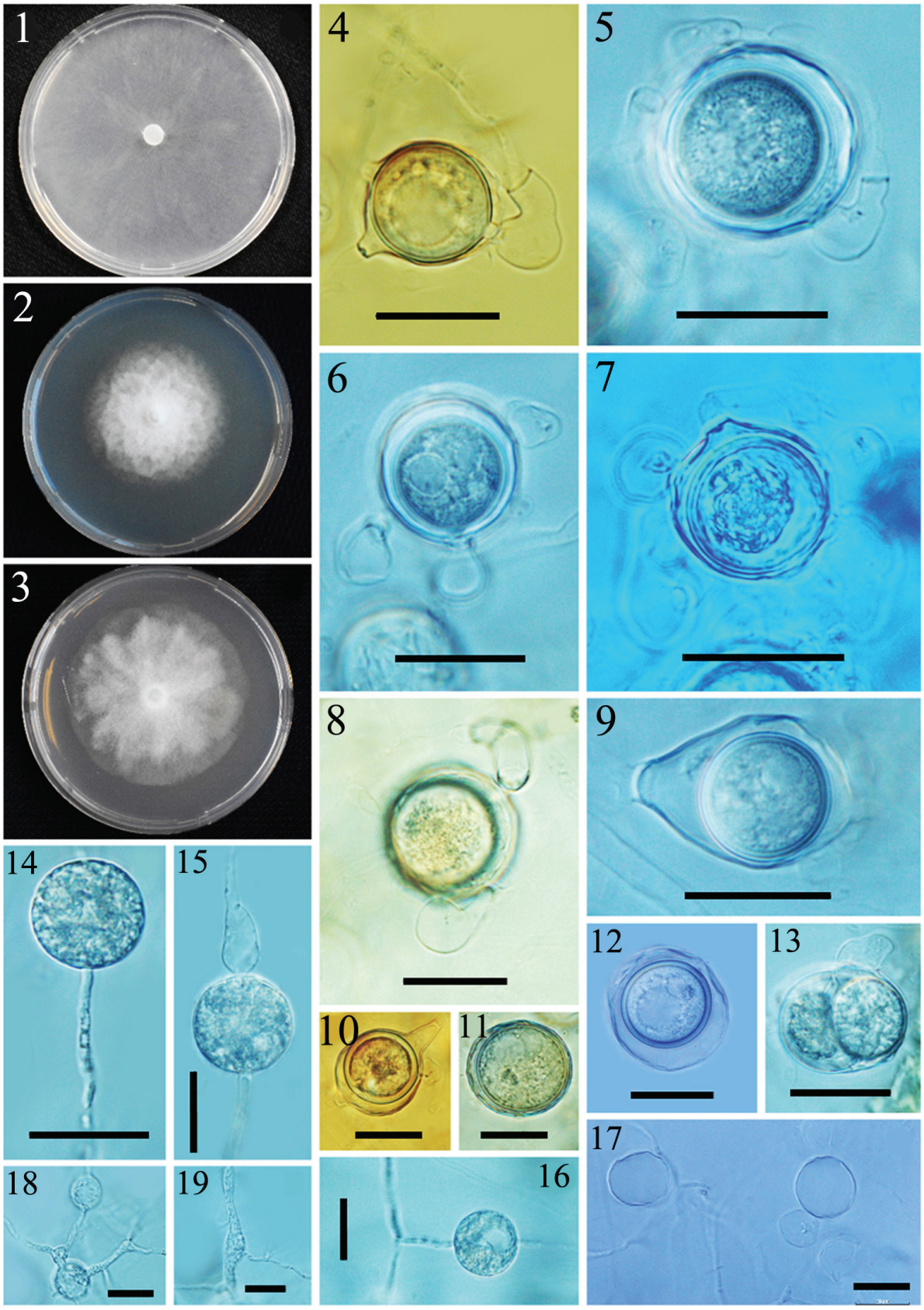

Fig. 1. *Pythium glomeratum*. *1* Colony morphology of the isolate OPU 1782 on CMA, *2* PDA, *3* V8A, *4–8* Oogonium with 1–5 diclinous antheridia, *9–12* Aplerotic oospore, *13* Two oospores into one oogonium, *14–17* Hyphal swellings, *18–19* Appresoria. Bars: 20 µm.

Tab. 3. Morphological comparison between *Pythium glomeratum* (OPU 1782) and its morphologically and phylogenetically related species.

Characteristics	**OPU 1782**	***Pythium glomeratum*** (PAUL 2003)	***Pythium heterothallicum*** (VAN DER PLAATS-NITERINK 1981)
Main hypha	>5 µm	>5 µm	>7 µm
Hyphal swellings	Globose, 19–26 µm in diam.	Globose, ovate or cylindrical, 5–21 µm in diam.	Globose or lemon-shape, terminal or intercalary, 25 µm in diam.
Appressoria	Simple or clustered	Present	Absent
Oogonia	Smooth, globose, terminal or intercalary, 20–27 µm in diam.	Smooth, globose or ovate, produced in chains or simple, terminal or intercalary, 16–43 µm in diam.	Produced on paired cultures, Smooth, globose, terminal and/or intercalary, 20–32 µm in diam.
Antheridia	1–5 per oogonium, diclinous, curved elongate, often wavy in contour	1–6 per oogonium, diclinous, curved elongate, often wavy in contour, applied lengthwise to the oogonium over their entire length	>8 per oogonium, diclinous, often forming a complicated knot around the oogonium
Oospore	Mostly aplerotic and rarely plerotic, 17–31 µm in diam. Rarely 2 oospores produced in to one oogonium	Mostly aplerotic and rarely plerotic, 15–32 µm in diam. Rarely 2 oospores produced in to one oogonium	Aplerotic, 18–28 µm in diam.
Daily growth rate at 25°C	10 mm/day	13 mm/day	20 mm/day

Tab. 4. Pathogenicity of *Pythium sylvaticum* and *P. glomeratum* isolates to seedlings of cucumber.

Pythium species inoculated	Mortality (%)	Infection (%)
P. sylvaticum	81.7 ± 2.8 **A**	100
P. glomeratum	73.3 ± 2.8 **A**	100
Control	0 **B**	0

Data given as mean ± standard errors (N = 3). Values followed by the same letters in a column do not differ significantly according to a Tukey-Kramer honestly significant different (HSD) test ($P < 0.01$).

H y p h a l s w e l l i n g s : mostly globose, 19–26 µm in diam.

O o g o n i a : smooth, globose, terminal or intercalary, 20–27 µm in diam.

A n t h e r i d i a : 1–5 per oogonium, diclinous, curved elongate, often wavy in contour, applied lengthwise to the oogonium over their entire length. Clustering of antheridia around the oogonia giving a woolly ball-like appearance and making it extremely difficult to observe the number and the type of contact of the antheridia.

O o s p o r e s : mostly aplerotic and rarely plerotic, 17–31 µm in diam. Rarely 2 oospores produced in one oogonium.

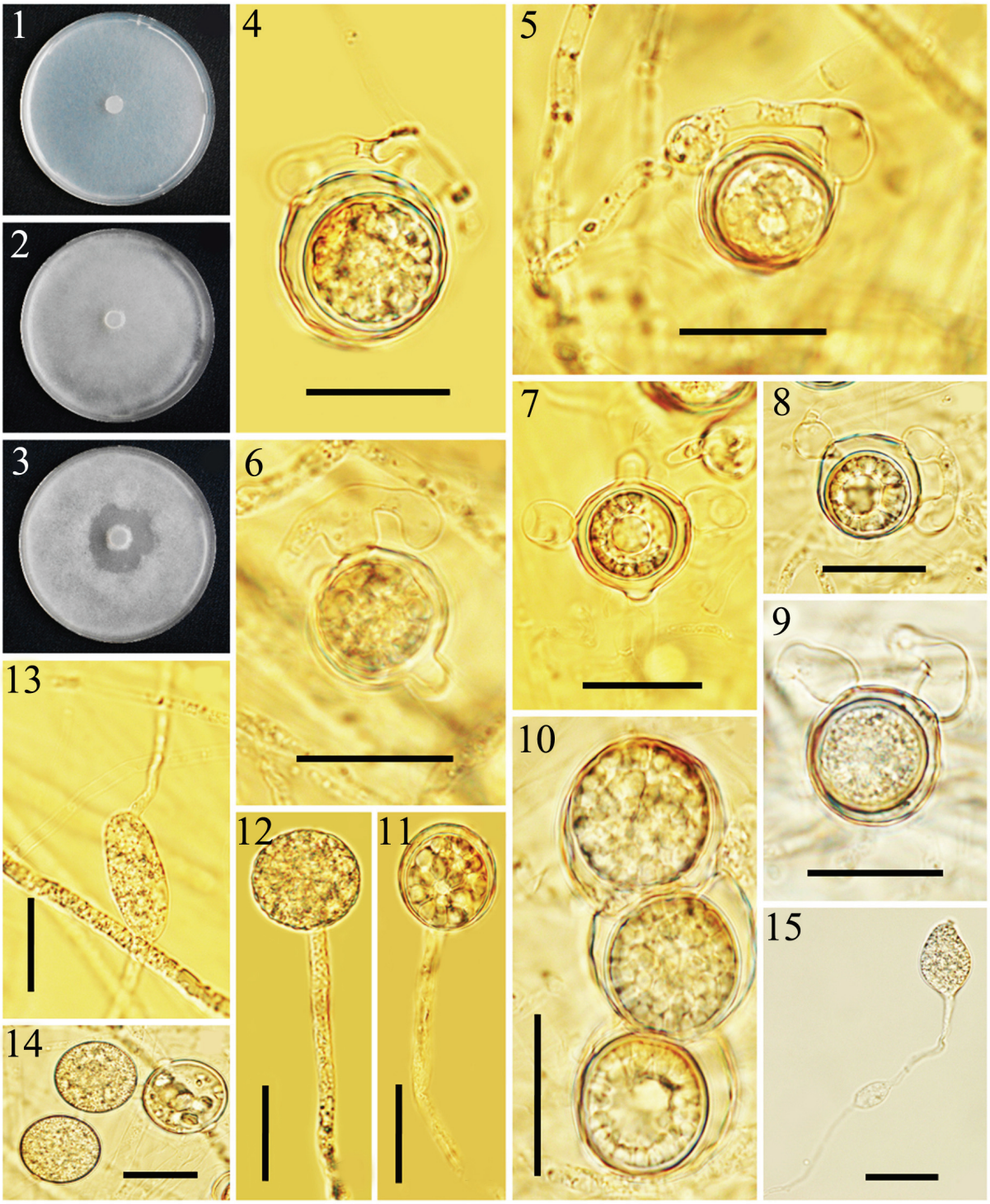

Fig. 2. *Pythium sylvaticum*. *1* Colony morphology of the isolate OPU 1702 on CMA, *2* PDA, *3* V8A, *4–9* Oogonium with 1–3 monoclinous and diclinous antheridia, *10–11* Aplerotic oospore, *12, 14* Hyphal swellings, *13, 15* Appressoria. Bars: 20 µm.

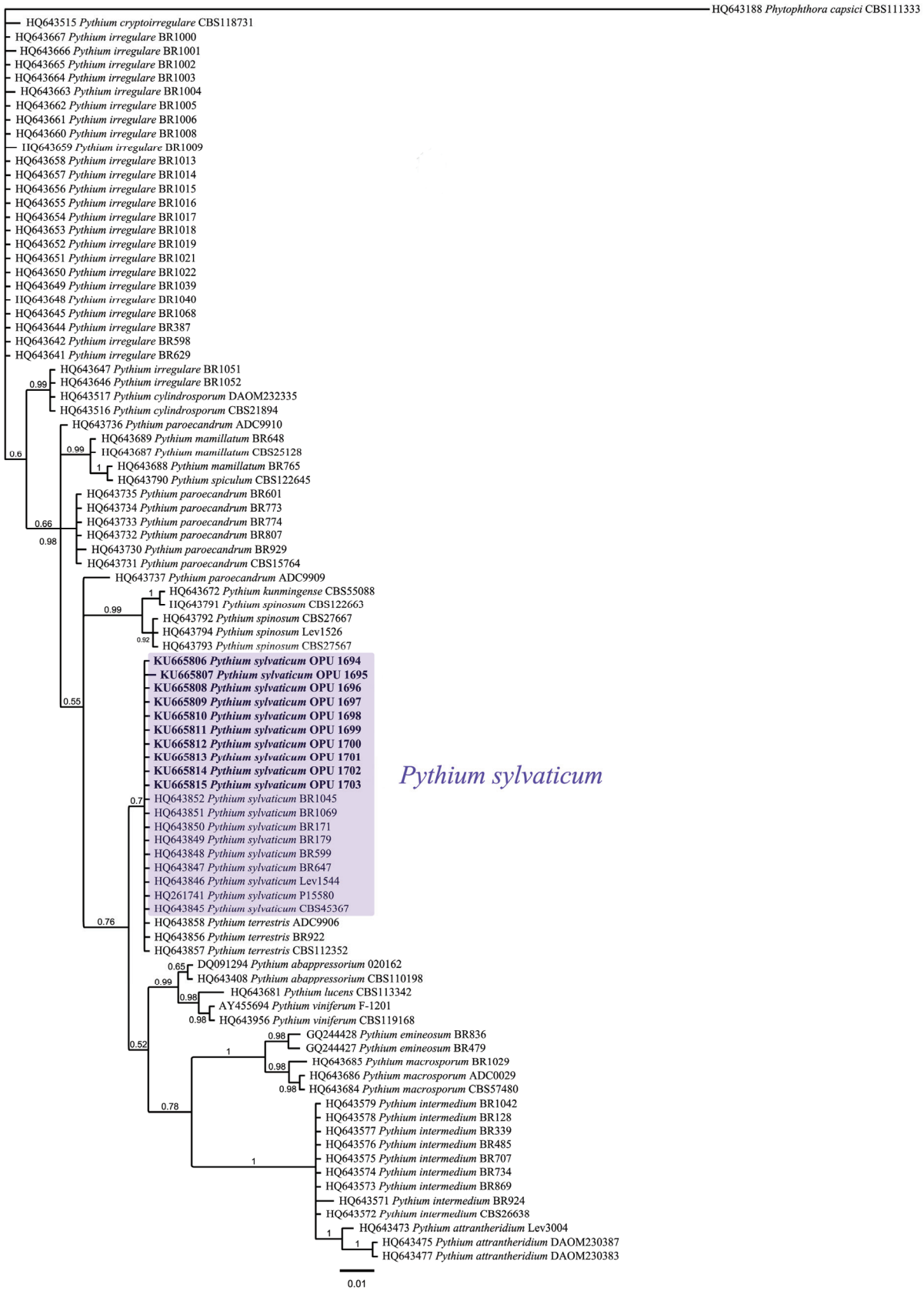

Fig. 3. Consensus phylogram (50% majority rule) of 5560 trees resulting from a Bayesian analysis of ITS sequence alignment using MrBayes v.3.2.1. The scale bar indicates 0.01 expected changes per site. Numbers above the branches represent posterior probabilities based on Bayesian analysis. The tree was rooted to *Phytophthora capsici* (GenBank accession HQ643188).

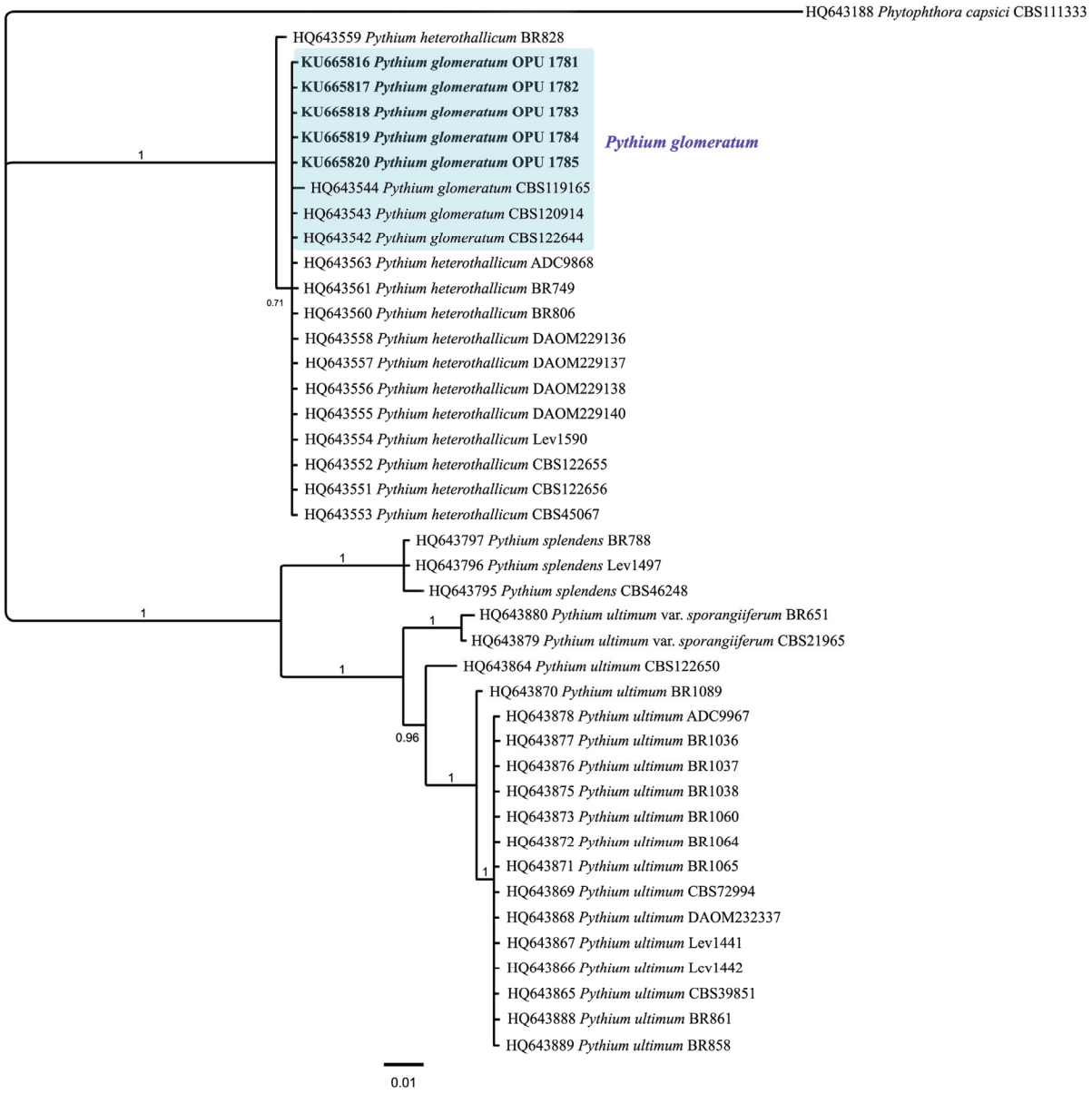

Fig. 4. Consensus phylogram (50% majority rule) of 324 trees resulting from a Bayesian analysis of ITS sequence alignment using MrBayes v.3.2.1. The scale bar indicates 0.01 expected changes per site. Numbers above the branches represent posterior probabilities based on Bayesian analysis. The tree was rooted to *Phytophthora capsici* (GenBank accession HQ643188).

Pythium sylvaticum CAMPBELL & HENDRIX, Mycologia 59(2): 274 (1967). – Fig. 2. (isolates OPU 1695 and OPU 1702):

M y c e l i a l p a t t e r n : submerged with weak growth of aerial mycelium on CMA, V8A and PDA. Optimum growth at 25 °C on PCA, with an average daily growth rate of 35 mm. Minimum, optimum and maximum growth temperature on PCA 5, 25 and 35 °C, respectively.

M a i n h y p h a e : hyaline, up to 7 µm wide.

S p o r a n g i a a n d z o o s p o r e s : not formed.

A p p r e s s o r i a : simple, clavate or sickle-shaped.

H y p h a l s w e l l i n g s : mostly terminal, globose, 20.5–23 µm in diam.

Sexual structures: formed in dual culture.

Oogonia: smooth, globose, terminal or intercalary, 19.5–24.5 µm in diam.

Antheridia: 1–2 per oogonium, monoclinous and diclinous. Antheridial stalks branched, often bifurcate near the oogonium.

Oospores: aplerotic, 18–23 µm.

DNA sequencing and phylogenetic analysis

Five isolates of *P. glomeratum* and ten isolates of *P. sylvaticum* were subjected to DNA sequence analysis. For *P. glomeratum* and *P. sylvaticum*, the final aligned ITS dataset contained 41 and 90 ingroup taxa with a total of 615 and 563 characters, containing 121 and 134 unique site patterns, respectively. *Phytophthora capsici* (GenBank accession HQ643188) served as the outgroup taxon. The heating parameter was set to 0.15. The results of MrModeltest recommended HKY+G and HKY+I+G models with a gamma distributed rate variation and dirichlet base frequencies for *P. glomeratum* and *P. sylvaticum*. During the generation of the ITS tree, a total of 432 and 7412 trees were saved, and consensus trees and posterior probabilities were calculated from the remaining 324 and 5560 (75%) trees. Based on the result of the ITS sequence data, isolates of *P. glomeratum* and *P. sylvaticum* obtained in this study resided in the well-supported clade together with the representative sequences for *P. glomeratum* and *P. sylvaticum* isolates from GenBank in the clade I and clade F, respectively (Figs. 3, 4).

Pathogenicity assays

Both *P. glomeratum* isolate and *P. sylvaticum* mated isolates were pathogenic on cucumber seedlings. Disease symptoms initially appeared as small water-soaked lesions on stem near to the root system. The lesions then coalesced and gave the stems a weak and rotten appearance. Infection rates for both *P. glomeratum* isolate and *P. sylvaticum* mated isolates in inoculated plants were 100%. No significant differences were observed in the mortality induced by different isolates according to a Tukey-Kramer honestly significant difference (HSD) test ($P > 0.01$) (Tab. 4). Oospores of *P. glomeratum* and *P. sylvaticum* were frequently found within tissues of inoculated plants (Fig. 5). These fungal isolates were consistently reisolated from inoculated plants (100 %) but not from the control plants (0 %).

Discussion

Clade F consists of important plant pathogens with a worldwide distribution. Most species do not or rarely produce zoospores. They produce either globose, non-proliferating sporangia or globose hyphal swellings and have fast growth (more than 25 mm/d) and moderate cardinal temperatures, mostly 5–25(–30–35) °C (LEVESQUE & DE COCK 2004). Based on the results of the ITS-rDNA sequence data, all *P. sylvaticum* isolate sequences obtained in this study nested within *Pythium* clade F species. All *P. sylvaticum* isolates described in this study were heterothallic. In the crossings of the type isolates on PCA at 10 °C the oogonia and oospores were formed in the contact

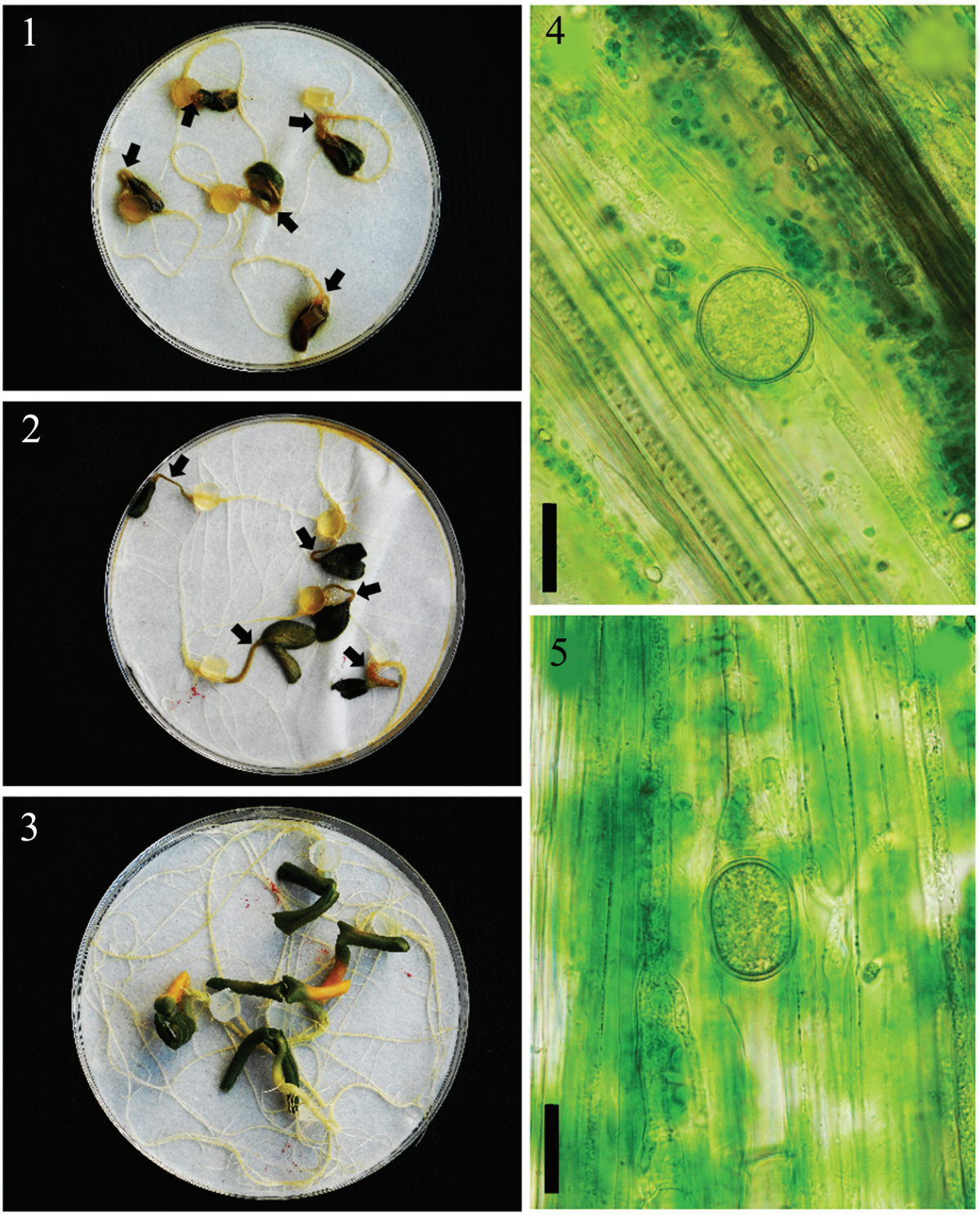

Fig. 5. In vitro pathogenicity assay of *P. sylvaticum* (A) and *P. glomeratum* (B) on cucumber seedlings. Arrows indicate the rotting symptoms after inoculation. Control plants (C). Oospore of *P. sylvaticum* (D) and *P. glomeratum* (E) formed in leaf cells of *Cucumis sativus*. Bars: 20 µm.

zone. The line of contact in dual cultures was characteristically formed, rather sharp to the side of the antheridial partner. Primarily, *P. sylvaticum* has often been mistakenly identified as *P. debaryanum* (VAN DER PLAATS-NITERINK 1981). The species was

originally isolated from soil in the United States (CAMPBELL & HENDRIX 1967). Morphologically and phylogenetically *P. sylvaticum* resembled *P. terrestris* PAUL. *Pythium terrestris* is homothallic and can grow well in hemp seed-water cultures and produce asexual and sexual structures on both solid and water media in water at room temperatures (Tab. 2).

In this study, we recovered *P. sylvaticum* as a new record for mycobiota of Iran. The species produces toxins and growth factors. Therefore, it can become pathogenic to seedlings of some host plants (VAN DER PLAATS-NITERINK 1981). We found that Iranian isolates (mated isolates) of the species were pathogenic on cucumber seedlings and formed oospores into plant tissues. Regarding previous studies, plants including *Taraxacum officinalis*, *Falcaria* sp., *Elaeagnus angustifolia*, *Camellia sinensis*, *Tragopogon collinus*, *Phleum iranicum*, and *Lotus angustissimus* are new substrates associated with *P. sylvaticum*

Based on the results of the ITS-rDNA sequence data, all *P. glomeratum* isolates obtained in this study reside in the same clade with isolates of the species deposited in GenBank in the *Pythium* clade I. Most of the members of clade I are heterothallic but *P. glomeratum* and *P. ultimum* are homothallic. First, it was isolated from soil samples taken in France in 1992, and was erroneously identified as *P. heterothallicum* for a long time. Its antheridial branches surrounding the oogonia and its failure to produce sexual structures on hemp seed-water cultures supported this conclusion (PAUL 2003). All *P. glomeratum* isolates described in this study were homothallic. Oogonia and antheridia were produced in grass blade-water culture, after two weeks at 10 °C. Morphologically and phylogenetically *P. glomeratum* resembled *P. heterothallicum* CAMPBELL & HENDRIX. *Pythium glomeratum* is homothallic. The clustering of antheridia around the oogonia gives a woolly ball-like appearance and makes it slightly difficult to detect the exact number and the type of contact of the antheridium to the oogonium (Tab. 3). Little is known about the pathogenicity of *P. glomeratum*, but the other species in clade I are mainly pathogenic to dicotyledons. In this study we found that *Ficus carica*, *Onosma* sp., *Ligustrum ovalifolium*, *Punica granatum*, and *Prunus avium* are new substrates associated with *P. glomeratum*.

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