Characterization and formal description of a new *Phytophthora* species

Report
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Introduction

The fungal-like *Phytophthora* species are closely linked to algae and cause severe economical and environmental losses throughout the world (Erwin & Ribeiro 1996). In addition to the list of the previously known 60, more than a dozen formally or informally described novel *Phytophthora* taxa have been recorded during the past decade, mainly in European forests and natural ecosystems (Brasier *et al*. 2003). Of these, several species play a role in the complex ecological phenomenon known as forest decline.

The subject of this work is an informal *Phytophthora* designated as *P.* taxon Salixsoil, of which first two isolates collected around *Salix* trees were identified in 2003. The taxon was originally assigned to the known morphospecies *P. gonapodyides* (PG), but preliminary physiological and molecular data supported the view that it was a different phylogenetic species unit. Its several putative isolates have been found by us in Hungarian alder forests too. The host range and ecological role of *P.* taxon Salixsoil is not known. Thus, the major goals of this project were (i) to clarify the taxonomical/phylogenetic status of *P*. taxon Salixsoil, and (ii) to study its pathogenicity on alder and other possible hosts.

Material and methods

Isolation

Rhizosphere soil samples from the upper 5-25 cm soil layer around alder (*Alnus glutinosa*) trees were collected in 5 alder stands of 5 locations in Hungary. Isolations were carried out either by direct plating of necrotic alder roots, following washing them in tap water, onto selective PARPBH medium (Erwin & Ribeiro, 1996), or by baiting using a modified method of Werres *et al*. (1997); leaves of common cherry-laurel (*Prunus laurocerasus*) were used instead of rhododendron. Pieces of infested leaves were then plated on the surface of the selective medium. Hyphal tip subcultures, of which several were deposited at CBS (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands) were made from every *Phytophthora* culture growing on selective medium and used for further studies. Stock cultures on CA (CA; Brasier, 1972) plates were grown at 25 °C and subcultured at 3–4 week intervals during the testing period. Additional isolates of *P*. taxon Salixsoil and *P. gonapodyides* from several other countries were received from diverse institutions or culture collections. The isolated cultures, the reference isolates used for comparisons and their sources are listed in Table 1.

Morphology and growth-temperature relationship

Colony morphology, radial growth-rate and optimal/cardinal temperatures for growth were assessed following the method of Brasier *et al*. (2003). Two 90 mm Petri dishes (20 ml agar) of CA, clarified V8 juice agar (V8) and potato dextrose agar (PDA, Oxoid) were inoculated centrally with 4 mm mycelial agar plugs taken out of the actively growing edge of a colony kept at 25 °C. Inoculated plates were incubated in the dark for 2 weeks at different temperatures. Minimum, optimum and maximum temperature values for growth were tested at 2–5, 25–33 and 33–38 °C, respectively, with 1±0.2 degree increments in at least 2 consecutive experiments. Colony morphologies were assessed and daily radial growth rates were calculated from the 7-day diameter data of cultures grown at 25±0.2 °C on all three media. In minimum and maximum temperature limit tests, three 4 mm plugs were transferred to a fresh CA plate and the peripheral growth was observed after 3 day incubation in darkness. Plates were then incubated at 25 °C for 3 additional days to check the extreme temperature-tolerance of cultures.

Production of sporangia, hyphal swellings, chlamydospores and gametangia was examined after 2 weeks in all agar media used for growth-rate tests. Additionally, mycelium mat of 3–5 day old cultures developed in liquid CA or pea-broth (Erwin & Ribeiro, 1996) was rinsed with sterile distilled water, and then overlaid with non-sterile soil-filtrate to induce sporangia formation. Morphological features of sporangia were viewed and evaluated using a light microscope at × 400–800 magnification.

Pairings

P. taxon Salixsoil and *P. gonapodyides* isolates, which do not form gametangia in single culture, were paired with a reference isolate of *P. gonapodyides* and A1 or A2 compatibility type tester strains of *P. cambivora, P. cryptogea* and *P. drechsleri*. The isolates were paired 4 cm apart on 6 cm CA plates, incubated for 21 day at 20 °C in darkness and the plates were thoroughly examined for the presence oogonia at and near the interphase.

ITS RFLP

ITS regions and the 5.8S rRNA gene of ribosomal DNA were amplified with the universal primers ITS1 and ITS4 (White *et al*., 1990). The polymerase chain reaction (PCR) was carried out with an automated thermal cycler PTC 150 Mini Cycler (MJ Research Inc, USA). The

PCR products were digested with the restriction endonuclease *Taq*I as described in Cooke *et al*. (2000). Products were run on 2.5% Agarose 1000 (Gibco).

Species specific PCR and ITS sequence analysis

DNA extracts from all isolates were also subjected to either *P.* taxon Salixsoil specific PCR, or ITS sequencing as described earlier (Nechwatal and Mendgen, 2006). All sequence analyses, alignments, and comparisons were performed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). BLAST searches were performed to compare sequences to those deposited in the GenBank database.

mtDNA RFLP analysis

Total genomic DNA (15–20 μg) was cut with 20 units of restriction endonucleases *Hae* III or *Msp* I**.** Restricted DNA was electrophoresed in agarose gel, stained with ethidium bromide and photographed. DNA fragments ≥ 1.45 kb were regarded as of mitochondrial origin.

The presence and absence of bands were scored as genetic data with 1 and 0, respectively. Data were compiled into a binary table that was used for clustering individual isolates. Unweighted pair-group method with arithmetic averages (UPGMA) hierarchical clustering (Sneath and Sokal, 1973) using the Jaccard coefficient was performed with the computer program Syn-Tax 2000 (Podani, 2001).

Pathogenicity

Pathogenicity of a selection of *P.* taxon Salixsoil isolates and of the closely related *P. gonapodyides* towards two tree species was tested in an abscised twig assay. Tree species selected were *Salix alba* and *Alnus glutinosa*, both known to inhabit temporarily flooded littoral sites. One-year-old twigs were collected in the field from single mature trees in early spring, shortly before bud burst. Twigs were cut to about 10-15 cm length, and inoculated by placing an approx. 5 x 5 mm piece from actively growing V8A cultures of the isolates tested on an equally sized area of removed bark. Five twigs per isolate were placed in glass Petri dishes containing two layers of moist filter paper. Controls received plain V8A pieces. Plates were sealed with parafilm and incubated for one week at 22°C in the dark. Random reisolations were made from necrotic tissue on PARPH after necroses size had been recorded. The tests were performed three times per isolate and tree species combination.

Results

Colony morphology

Colony morphology was tested on CA, clarified V8 and PDA (Fig. 1a–c.). Most isolates of *P.* taxon Salixsoil and all isolates of *P. gonapodyides* developed petaloid/crysanthemum-type pattern with little or without aerial mycelium. Two isolates of *P.* taxon Salixsoil (P 1/03 and H-15/02) were almost patternless and dome-shaped on CA. On CA and V8, the petals were large, whereas on PDA they were small and dense. Cultures grew fastest on CA, followed by clarified V8 and PDA at 25 ºC (Table 2). Hyphal swellings, chlamydospores and sporangia were absent on agar. However, certain isolates developed a few sporangia on CA and clarified V8, or tap water agar during the isolation and purification procedure.

Temperature–growth relationship

Mycelial growth at different temperatures was tested on CA (Table 2). There was a clear difference in the maximum temperature limit for growth between *P.* taxon Salixsoil and *P. gonapodyides*. *P.* taxon Salixsoil isolates were capable of developing new mycelia up to 36-37 °C, whereas those of *P. gonapodyides* only grew at and below 35 °C (Table 2). None of the *P.* taxon Salixsoil isolates grew at 38 °C, but many re-grew when they were returned to 25 °C.

The optimum temperature for growth ranged from 27 to 33 °C for *P*. taxon Salixsoil. The two cultures of *P. gonapodyides* tested for this feature showed the fastest growth at 25 and 26 °C. The minimum temperature at which the two species showed at least trace (> 0.5 mm) growth was generally 3 °C for both species, except for two *P.* taxon Salixsoil isolates that exhibited trace growth at 2 or 4 °C.

Sporangia

All *P.* taxon Salixsoil and *P. gonapodyides* isolates cultured in non-sterile soil-filtrate abundantly produced sporangia which were similar in shape and size. Noncaducous, nonpapillate, mostly ovoid/obpyriform sporangia with wide exit pore were developed terminally. Sporangiophores were simple, sometimes emerged just below the mature sporangia. External and internal (nested) proliferations were a common feature for both taxa.

No distinguishing could be made based on sporangial characteristics between *P.* taxon Salixsoil and *P. gonapodyides.* Dimensions of the sporangia are listed in Table 3.

Pairings

Neither *P.* taxon Salixsoil, nor *P. gonapodyides* formed sexual structures in single cultures. Pairings of each *P.* taxon Salixsoil and *P. gonapodyides* isolates with *P. cambivora* P1432 (A1), *P. cryptogea* ICMP 9673 (A1) and H-1050 (A2), *P. drechsleri* ICMP 9771 (A2) and *P. gonapodyides* (IMCP ICMP14157) did not result in oospore production. Few (up to 5) sexual structures were observed only in pairings of a *P.* taxon Salixsoil (H-10/02) and two *P. gonapodyides* (P7050 and P7186) cultures with the A2 mating type *P. cambivora* (BuKN4). These oogonia were located near the interphase, and were ornamented, suggesting that they could belong to *P. cambivora*. The oogonia either contained mature oospores (H-10/02 × BuKN4, P7050 × BuKN4) or were empty (P7186 × BuKN4). Antheridia were amphigynous.
All these support the view that both *P.* taxon Salixsoil and *P. gonapodyides* are sterile or silent A1 mating type. They are capable of incuding formation of gametangia in A2 mating type strains of certain species, but they do not form gametangia themselves.

ITS RFLP

*Taq*I restriction digest profiles of the ITS regions of the *P.* taxon Salixsoil and *P. gonapodyides* strains were different. The two taxa possessed two different patterns that could be easily distinguished by two, a 53 bp and a 90 bp, bands present only in *P.* taxon Salixsoil isolates (Fig. 2).

Species specific PCR and ITS sequence analysis

All isolates of the proposed new species, *P.* taxon Salixsoil, produced positive PCR signals when tested with the *P.* taxon Salixsoil specific primers, while isolates of *P. gonapodyides* did not. Additionally, all *P.* taxon Salixsoil isolates under investigation proved to be identical as to their ITS sequence, and where 100% identical to those GenBank database entries that have been previously designated *P.* taxon Salixsoil (AF266793, AY762973).

Mitochondrial DNA profiles

Differences in mtDNA RFLP pattern were observed between the 28 *P.* taxon Salixsoil and the 7 *P. gonapodyides* strains tested for this trait. *Hae*III generated 22 different DNA fragments within the size range of 1.54 and 9.2 kb, resulting in 8 different RFLP-types. *P.* taxon

Salixsoil isolates were sorted into 2 *Hae*III RFLP types, distinguished from each other by two, a 5.63 kb and a 4.67 kb, fragments. The Italian isolates represented one group, whereas the rests represented the other group. The 7 *P. gonapodyides* had 6 additional profiles.

*Msp*I generated 36 different DNA fragments ranging from 1.45 and 7.8 kb and forming 15 different RFLP profiles, 9 in *P.* taxon Salixsoil and 6 in *P. gonapodyides*.

By considering both *Hae*III and *Msp*I digest patterns, 15 combined mtDNA RFLP types were identified, 9 in *P.* taxon Salixsoil and 6 in *P. gonapodyides*. UPGMA clustering clearly differentiated *P. gonapodyides* from *P.* taxon Salixsoil at an average dissimilarity level of 86 %. Isolates of this latter taxon grouped in several sub-clades and were up to 30 % variable on average (Fig. 3).

Pathogenicity

Pathogenicity of *P. gonapodyides* and *P.* taxon Salixsoil towards *A. glutinosa* and *S. alba* was tested in an abscised twig assay (Table 4). Bark and cambium lesions developing on *Salix* twigs were somewhat larger than on *Alnus.* In both tree species, however, lesions caused by *P.* taxon Salixsoil were generally larger than those caused by PG. While in *Alnus* these differences were not statistically significant, in *Salix* significant differences were observed in two of the three replicate experiments. In random reisolations from necrotic tissue on selective agar media, the inoculated species were successfully recovered as indicated by colony morphology and growth trials at 37°C. No lesions developed on any of the control twigs.

Conclusion

The results obtained in this study show that *P*. taxon Salixsoil and *P. gonapodyides* are very similar to or even indistinguishable from each other based on cultural pattern, growth rate, breeding behaviour and sporangial characteristics. However, they are substantially different in their mitochondrial DNA profiles, rDNA ITS sequences and response to high temperature. It could be that *P*. taxon Salixsoil represents and independent lineage in which convergent evolution has resulted in a superficially *P. gonapodyides*-like phenotype; or, it is a product of a cross between *P. gonapodyides* and another, as yet undiscovered, taxon. All the 28 *P*. taxon Salixsoil isolates investigated had the same ITS sequence, suggesting that the speciation process has ended and the taxon has stabilised. Therefore, the taxa should be described as a new formal *Phytophthora* species.

P. taxon Salixsoil isolates were pathogenic, although not very aggressive, to two woody host plant species (*Alnus* and *Salix*) in artificial inoculation experiments. It is possible that they can infect woody hosts in nature, too. Although, the fact that they can tolerate high temperature suggests that *P*. taxon Salixsoil may also take part in the breakdown of plant litter and detritus. Such a strategy would allow maintenance of high population levels, and could account for this species' extraordinary ubiquity and abundance.

Request for re-evaluation of the report

The results of this project are intended to be published jointly with the foreign partner in an international journal (e.g. Mycological Research or Plant Pathology) with impact factor. A draft manuscript has already been prepared and sent to the foreign partner in order that he can complete it with his own data. We expect to have the paper published within two years from now. A re-evaluation of this report is requested after having the paper published.

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Table 1 Isolates, their hosts and origins

Isolate code	Host or source	Location/Date of collection
***P*. taxon Salixsoil**		
H-10/02 (CBS 117384)	*Alnus glutinosa* root	Jánossomorja, Hungary, 06/06/2002
H-11/02 (CBS 117385)	Soil with Alnus glutinosa root	Földsziget, Hungary, 06/06/2002
H-12/02	Soil with Alnus glutinosa root	Földsziget, Hungary, 06/06/2002
H-13/02 (CBS 117383)	Alnus glutinosa root	Ócsa, Hungary, 23/05/2002
H-15/02	Soil with Alnus glutinosa root	Ócsa, Hungary, 23/05/2002
H-16/02	Soil with Alnus glutinosa root	Teskánd, Hungary, 17/10/2002
P 1/03	Alnus glutinosa root	Bokod, Hungary, 18/03/2003
Ph1	reed rhizosphere soil	Konstanz, Germany, April, 2003
Ph2	reed rhizosphere soil	Konstanz, Germany
Ph3	reed rhizosphere soil	Konstanz, Germany, May, 2003
Ph4	reed rhizosphere soil	Konstanz, Germany
Ph5	reed rhizosphere soil	Konstanz, Germany
Ph6	reed rhizosphere soil	Konstanz, Germany
Ph7	reed rhizosphere soil	Konstanz, Germany
Ph8	reed rhizosphere soil	Konstanz, Germany
Ph9	reed rhizosphere soil	Konstanz, Germany
Ph14	reed rhizosphere soil	Konstanz, Germany
Ph15	reed rhizosphere soil	Konstanz, Germany
PESCO 1	peach soil	Italy
PESCO 10	peach soil	Italy
PESCO 11	peach soil	Italy
PESCO 5	peach soil	Italy
PESCO RC	peach soil	Italy
SCRP989	Unknown	unknown
SCRP990	Unknown	unknown
SCRP991	Unknown	unknown
SCRP993	Unknown	unknown
P10337 (UCA) = P245 (C.B.)	*Salix matsudana* root	Bexley Heath, Kent, UK, 1972
P. gonapodyides		
H-4/02	Unknown	Söjtör, Hungary, 17/10/2002
H-14/02	Alnus glutinosa root	Ócsa, Hungary, 23/05/2002
ICMP 14157	Castanea sativa, - sweet chesnut – roots	Geraldine, Crosby District, South Canterbury, New Zealand, 1/01/2000.
P7050 (UCA)	Vegetable debris	G.B. (England)
P7186 (UCA)	Reservoir water	G. B. (England), 1989
BuKN 1b	beech rhizosphere soil	Konstanz, Germany, May, 2003
P878	alder root debris in pond	Odense, Funen, Denmark, 1995

IMI, CABI Genetic Resource Collection; CBS, Centraalbureau voor Schimmelcultures; UCA, University of California, Riverside, ICMP, International Collection of Mirco-organisms from Plants.

Table 2 Temperature-growth relationship

Code	Average daily radial growth (mm)			Tmax (°C)	Topt (°C)	Tmin (°C)
	CA	V8	PDA			
***P.* taxon Salixsoil**						
H-13/02	5.75	3.75	2.29	37	30-33	3
H-10/02	4.86	2.68	1.93	37	31	3
H-12/02	5.39	3.18	2.29	37	nt	nt
H-16/02	4.71	2.93	2.21	36	28-29	3
H-11/02	5.18	3.00	1.82	36	nt	3
P 1/03	3.39	2.79	1.75	37	30-32	nt
H-15/02	4.04	3.18	2.54	37	30-33	4
Ph1	5.07	4.46	2.54	37	28-30	4
Ph3	4.25	2.79	2.21	37	28-29	3
Ph2	5.43	4.29	2.64	37	nt	nt
Ph4	5.43	3.93	2.71	37	29	3
Ph5	5.39	3.43	2.36	37	nt	nt
Ph6	6.14	4.25	2.71	37	28-30	3
Ph7	5.93	4.11	2.79	37	nt	nt
Ph8	4.93	2.79	2.36	37	nt	nt
Ph9	5.07	2.82	2.14	37	nt	nt
Ph14	5.25	3.39	2.04	37	30-31	3
Ph15	5.43	3.68	2.11	37	nt	nt
PESCO 1	5.32	4.29	2.43	37	28-29	4
PESCO 10	5.29	4.04	2.64	37	nt	3
PESCO 11	5.68	3.93	2.39	37	nt	3
PESCO 5	5.21	3.86	2.46	37	nt	nt
PESCO RC	5.43	4.11	2.64	37	nt	nt
SCRP989	4.4	3.9	2.0	37	29	2
SCRP990	5.5	4.6	2.9	36	28	3
SCRP991	3.7	2.9	1.1	36	28	4
SCRP993	4.0	3.0	1.8	36	28	4
P10337 (UCA) = P245	5.80	3.96	2.70	37	27	3
Average or Range	**5.07**	**3.5**	**2.3**	**36.8**	**27–33**	**3.2**
P. gonapodyides						
H-4/02	4.57	4.29	2.18	34	25	3
H-14/02	4.93	3.57	2.21	34	nt	nt
ICMP 14157	4.64	3.54	2.21	34	nt	3
P7050	4.32	3.43	1.57	nt	26	3
P7186	4.21	3.79	1.86	35	nt	nt
BuKN 1b	4.71	3.29	2.04	35	nt	3
P878	4.40	3.43	2.21	34	27-28	3
Average or Range	**4.54**	**3.62**	**2.04**	**34.3**	**25–28**	**3**

nt= not tested

Table 3 Dimensions of sporangia

Isolate	Sporangium length (n=30)[4]	Sporangium breadth (n=30)[4]	Length/Breadth
***P.* taxon Salixsoil**			
H-13/02	48.12 (38.5 - 61.6)	34.9 (23.1 - 46.2)	1.37
H-10/02	51.5 (42.3 - 65.4)	38.8 (26.9 - 65.4)	1.32
H-12/02	44.2 (31.4 - 59.6)	34.69 (22.6 – 44.7)	1.27
H-16/02	50.3 (41.8 - 66.3)	33 (21.9 - 42.3)	1.52
H-11/02	37.7 (27.9 - 57.3)	25.7 (17.0 - 43.4)	1.46
H-15/02	54.09 (38.5 - 77.0)	33.62 (23.1 - 46.2)	1.60
P. gonapodyides			
H-4/02	53.2 (42.5 - 67.7)	38 (25.6 - 69.1)	1.40

[1]Mean values and size ranges (μm).

Table 4 Lesion size (cm) caused by *Phytophthora* taxon Salixsoil and *Phytophthora gonapodyides* on *Alnus glutinosa* and *Salix alba* in an abscised twig assay

Tree species	*Alnus*				*Salix*			
***Phytophthora* sp.**	***P.* taxon Salixsoil[1]**		***P. gonapodyides*[2]**		***P.* taxon Salixsoil[1]**		***P. gonapodyides*[2]**	
	mean	SD	mean	SD	mean	SD	mean	SD
expt. 1	1.21a	0.30	0.99a	0.43	2.05a	0.81	1.12b	0.30
expt. 2	1.20a	0.51	0.89a	0.24	1.61a	0.46	0.96b	0.14
expt. 3	2.08a	0.51	2.15a	0.08	1.85a	0.50	1.67a	0.10

[1]*P.* taxon Salixsoil isolates: Germany: Ph1, Ph4; Hungary: H-16/02, H-11/02; Italy: Pesco 5, Pesco 1; UK: P245.

[2]*P. gonapodyides* isolates: Germany: BuKN1a; Hungary: H4/02.

Mean values within lines followed by the same letter are not statistically different ($p > 0.05$).

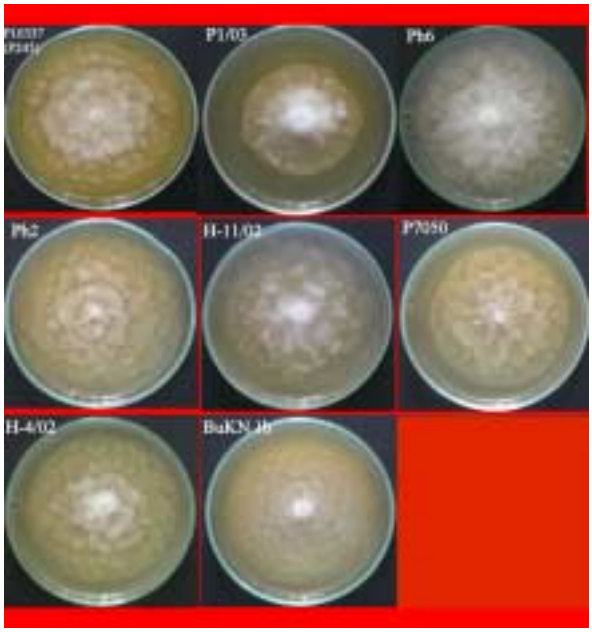

Fig. 1a. Culture morphology of 7-day old *P.* taxon Salixsoil (P10337, P1/03, Ph6, Ph2, H-11/02) and *P. gonapodyides* (P7050, H-4/02, BuKN 1b) isolates on carrot agar.

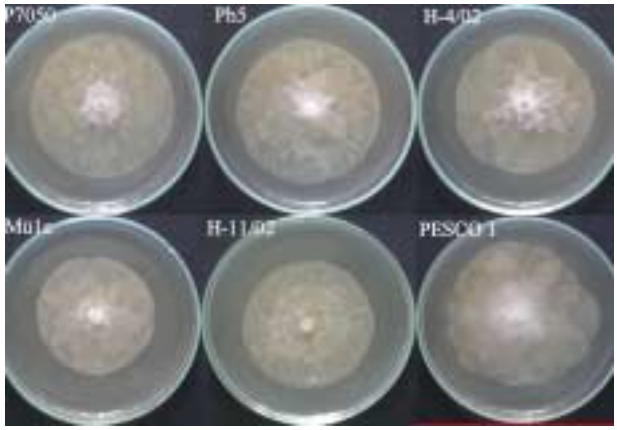

Fig. 1b. Culture morphology of 7-day old *P.* taxon Salixsoil (Ph5, Mü1c, H-11/02, PESCO 1) and *P. gonapodyides* (P7050, H-4/02) isolates on V8 agar.

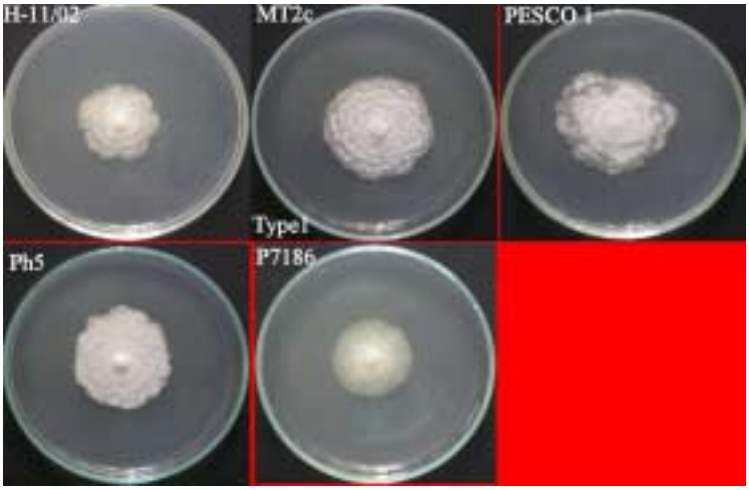

Fig. 1c. Culture morphology of 7-day old *P.* taxon Salixsoil (H-11/02, MT2c, PESCO 1, Ph5) and *P. gonapodyides* (P7186) isolates on PDA.

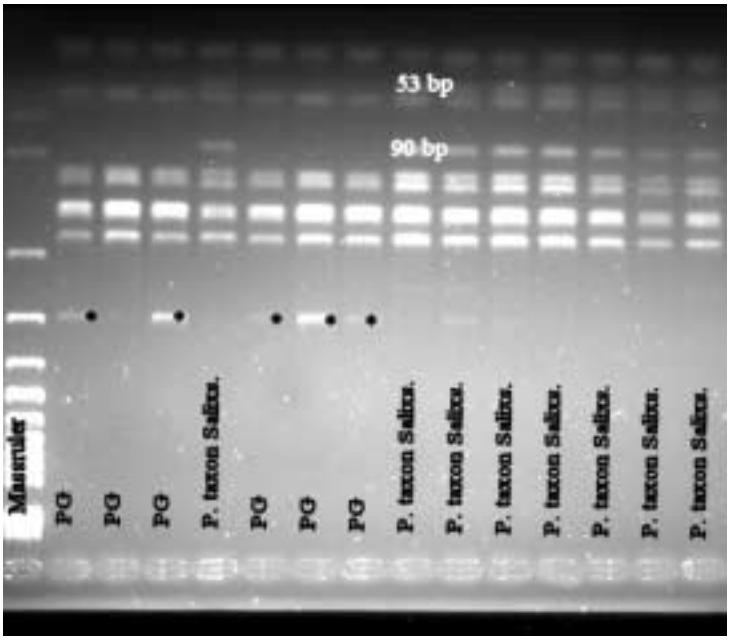

Fig. 2. Restriction fragment length polymorphisms between *P. gonapodyides* (PG) and *P*. taxon Salixsoil (PtS) after digesting rDNA ITS with *Taq*I. Note the 53 bp and the 90 bp fragments absent in PG, but present in PtS. The * marks non-digested fragments, probably due to the presence of different ITS types in some PG isolates.

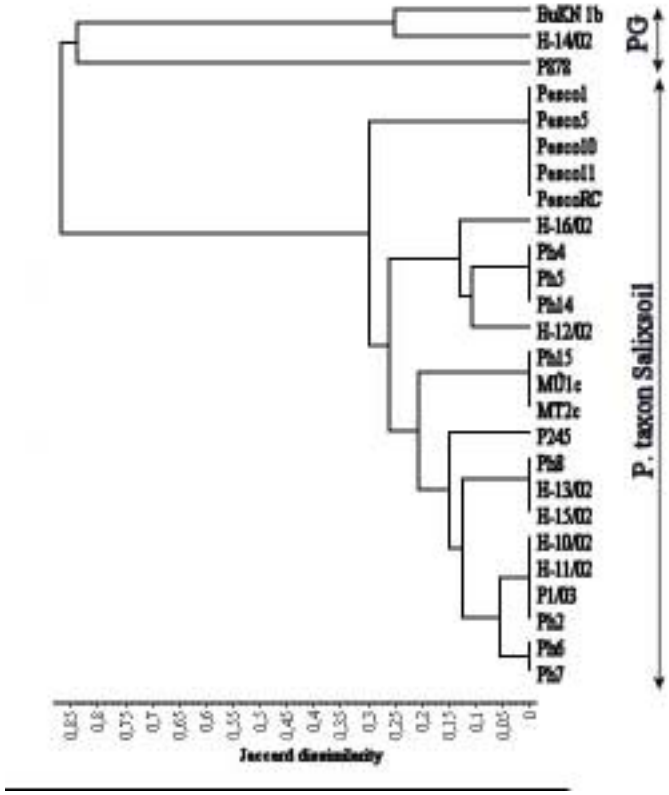

Fig. 3. UPGMA clustering of *P. gonapodyides* (PG) and *P*. taxon Salixsoil isolates based on mtDNA RFLP data. Note that PG isolates are clearly separated from *P*. taxon Salixsoil isolates.