Eur J Plant Pathol (2010) 127:341–350
DOI 10.1007/s10658-010-9601-5

Molecular characterization of *Phytophthora porri* and closely related species and their pathogenicity on leek (*Allium porrum*)

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Accepted: 3 March 2010 / Published online: 24 March 2010

Abstract White tip, caused by *Phytophthora porri*, is a destructive disease in the cultivation of European leek (*Allium porrum*). *P. porri* and closely related species such as *P. brassicae*, *P. primulae* and *P. syringae* belong to the phylogenetic clade 8b within the genus *Phytophthora*. The objectives of this study were to establish the position of *P. porri* and closely related species within the *Phytophthora* clade 8b; to study genetic variation among *P. porri* isolates from leek and closely related species and to test the hypothesis that host-driven speciation has occurred within this clade. AFLP analysis could clearly make a distinction between isolates of *P. porri* from *Allium* species and related *Phytophthora* species such as *P. brassicae*, *P. syringae* and *P. primulae*. DNA similarity and cluster analysis based on 353 markers demonstrated little genetic diversity within the *P. porri* population from *Allium* species although Belgian and Dutch *P. porri* isolates from leek could be distinguished from Japanese *P. porri* isolates from other *Allium* species and the *P. porri* isolate from carrot. Our results point to incipient speciation within the *P. porri* isolates, which could have been driven by the host plant or by geographic isolation. ITS sequence analysis confirmed the results obtained by AFLP and showed a close relationship between *P. porri* isolates from *Allium* and *P. primulae* and between the *P. porri* isolate from carrot and *P. brassicae*. We hypothesize that interspecific hybridization has occurred within this clade.

Keywords Homothallic · ITS-rDNA · Oomycetes · Sequencing · Virulence

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Introduction

Leek (*Allium porrum*) is a worldwide grown vegetable, although its importance is most significant in Europe (De Clercq et al. 2003; Pink 1993). The largest areas of leek cultivation can be found in France, Belgium, The Netherlands, Germany, Spain, United Kingdom and

East-European countries such as Poland and Bulgaria. One of the most devastating diseases of leek during autumn and winter in Europe is white tip, caused by the soil-borne pathogen *Phytophthora porri* Foister, a homothallic oomycete (Erwin and Ribeiro 1996; Foister 1931). Typical disease symptoms are papery white local lesions on leaves, sometimes surrounded by a green water-logged zone. Sporangia can develop in these wet lesions although this is not frequently observed in the field. During unfavorable conditions, the pathogen forms sexual oospores on and in the leaves, which enter the soil when the leaves die. The oospores of *P. porri* are surrounded with a very thick wall that helps them survive during the crop-free period. In wet conditions, oospores germinate with the formation of sporangia that release 10 to 30 zoospores upon maturation. These zoospores are considered as the main infection structures and probably reach the plant by rain splash (Declercq 2009, Erwin and Ribeiro 1996; Smilde 1996; Smilde et al. 1996a; Smilde et al. 1996b).

P. porri has also been reported to be a pathogen of other hosts, including other *Allium* species, *Daucus carota* and species of the genus *Brassica* (see Erwin and Ribeiro 1996 for references). In the studies reported in Erwin and Ribeiro (1996), *P. porri* had been identified on morphological grounds. Morphological characterization of species in the genus *Phytophthora* is laborious because of difficulties in producing all morphological stages necessary for identification (Gallegly and Hong 2008) and closely related species are not easily distinguished. *P. porri* strains from the Brassicaceae have recently been reclassified as *P. brassicae* since sequencing of the ITS region and restriction fragment length polymorphism of mitochondrial DNA revealed that they are genetically different from *P. porri* strains from leek (de Cock et al. 1992, Man in 't Veld et al. 2002).

Lately, progress in the phylogeny of the genus *Phytophthora* has been made due to advancements in molecular methods (Cooke et al. 2000; Forster et al. 2000; Blair et al. 2008). Cooke et al. (2000) divided the different species of the genus *Phytophthora* into eight clades based on sequence analysis of the ITS region. *P. porri* and closely related species such as *P. primulae* and *P. syringae* are grouped in clade 8b. A recent study by Blair et al. (2008) based on multilocus sequencing, identified ten clades within the genus *Phytophthora,* but the clustering of *P. porri, P. brassicae, P. primulae* and *P. syringae* corresponded to previously performed studies. It should be noted, however, that *P. porri* isolates from *Alllium* were not included in these phylogenetic studies since an isolate from carrot (*Daucus carota*) was used in the study by Blair et al. (2008), while *P. porri* was represented by an isolate from *Brassica* in the study of Cooke et al. (2000).

The objectives of this study were: (1) to establish the position of *P. porri* and closely related species within the *Phytophthora* clade 8b; (2) to study genetic variation among *P. porri* isolates from leek and closely related species; and (3) to test the hypothesis that host-driven speciation has occurred within clade 8b.

Knowledge about the intraspecific variation of *P. porri* isolates and their interspecific relationship with closely related species can assist plant breeders in disease resistance breeding programs for leek.

Materials and methods

Collecting of *Phytophthora porri* isolates

Phytophthora porri strains were isolated from symptomatic leaves of leek plants, collected from different fields in Flanders during 2005 and 2006 (Table 1). Leaf tissue was excised from the margin of the green water-logged outer zone of foliar lesions and cut into pieces of approximately 0.5 by 0.5 cm. These pieces were disinfected for 2 min in 1% NaOCl and washed three times in sterile distilled water. Two pieces were placed in a Petri dish filled with cornmeal agar (17 g l^{-1}; Oxoid, code CM 0103) amended with 10 mg l^{-1} of amphotericin, 250 mg l^{-1} of carbenicillin, 100 mgl^{-1} of rifampicin and 100 mg l^{-1} of pentachloronitrobenzene, which makes it selective for oomycetes (Jeffers and Martin 1986). Cultures were subsequently hyphal tipped, as described by Donahoo et al. (2006) and put on V8 juice agar (Miller 1955) to ensure pure single isolates of *P. porri.* After incubation at 18°C in the dark for 7 until 10 days, all isolates were microscopically examined on the basis of morphological characteristics of the mycelium, hyphal swellings and the presence of oospores. Isolates were maintained on V8 juice agar at 18°C in the dark.

Besides the *P. porri* strains collected in the field (36 isolates), a collection of strains was retrieved from

Table 1 Isolates of *Phytophthora porri* obtained from leek plants in Belgium used in this study

Origin (Province)	Isolate[a]	Year of isolation	Genbank accession
East-Flanders	K05019, K05020(2), K05021(1), K05025(1)	2005	–
	K06006(2)	2006	FJ643567
	K06007(2)	2006	FJ643556
	K06004(1), K06004(2), K06005(1)	2006	–
West-Flanders	B05004(2)	2005	–
	B06004(3)	2006	FJ643550
	B06005(1)	2006	FJ643553
	B06009	2006	FJ643554
	B06004(1), B06004(2),B06005(2), B06008, B06010(2), B06011(1)	2006	–
Antwerp	S05001(1)	2005	–
	S05005(1),	2006	FJ643557
	S05014(1)	2006	FJ643551
	S05015(1)	2006	FJ643552
	S05029(1)	2006	FJ643558
	S05006(1), S05006(2),S05007(1), S05009, S05011, S05013(2), S05014(2), S05017(2), S05018(2), S05029(2), S05032(1), S05034(2)	2006	–

[a] Isolates with the same four-digit number were obtained from the same field. Values between brackets refer to different isolates within one field

the CBS Fungal Biodiversity Centre (Utrecht, The Netherlands). Their origin, year of isolation and reference numbers are listed in Table 2.

Morphological characterisation

Hyphal structures and oospores were obtained by growing *P. porri* isolates on V8 juice agar at 18°C during 7 days in the dark. The typical coiling of the mycelium, hyphal swellings and chlamydospores were observed with an Olympus BX51 microscope (Hamburg, Germany). The diameter of the oospores was also measured.

Sporangia were produced by placing 15 mycelial plugs (5 mm diameter) in a Petri dish containing 20 ml of V8 juice broth at 18°C in the dark. After 3 days, the V8 juice was replaced by 20 ml of Schmitthenner solution (Erwin and Ribeiro 1996). The sporangia were formed after 3–4 days and were observed using an Olympus BX51 microscope (Hamburg, Germany).

DNA extraction

Phytophthora strains were cultured in 50 ml of liquid clarified V8 medium during one week in an incubator at 18°C in the dark (Erwin and Ribeiro 1996). Mycelial mats were sieved from the medium, blotted dry between sterile filter paper (Whatman, Dassel, Germany) and frozen at −20°C. The mycelium was crushed with a ball mill (Retsch MM301, Haan, Germany), followed by DNA extraction with the help of a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany).

AFLP analysis

AFLP analysis was performed following the protocol described by Vos et al (1995) with minor modifications. Genomic DNA (500 ng) was digested with 10U μl^{-1} of *Eco*RI (Fermentas, St. Leon-Rot, Germany) and 10U μl^{-1} of *Mse*I (New England Biolabs, Ontario, Canada) restriction enzymes in NEB buffer 2 (10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM Dithiothrietol). In the same reaction, carried out for 7 h at 37°C, adapters were ligated. PCR pre-amplification was performed in a 50-µl reaction, containing 10 µl of template DNA, primers *Eco*RI and *Mse*I without selective nucleotides (1.5 µL of *Eco*RI+0 and 1.5 µL of *Mse*I + 0), 5 µl of 10×PCR buffer (100 mM Tris-HCl pH 8.3, 25 mM $MgCl_2$, 500 mM KCl), 2 µl of 5 mM dNTP, 0.1 µl of Taq polymerase and 29.9 µl of highly purified water. The PCR program consisted of 20 cycles of 30 s at 94°C, 60 s at 56°C and 60 s at

Table 2 Collection information about the isolates of *Phytophthora* used in this study

Phytophthora sp.	Isolate	Host plant	Origin	Year of isolation	Genbank accession
P. porri	CBS 138.87	*Allium cepa*	Japan	1987	AF380150
	CBS 139.87	*Allium grayi*	Japan	1987	FJ643559
	CBS 140.87	*Allium cepa*	Japan	1987	AF380151
	CBS 141.87	*Allium porrum*	Netherlands	1987	FJ654564
	CBS 142.87	*Allium porrum*	Belgium	1987	AF380152
	CBS 181.87	*Allium porrum*	Netherlands	1987	FJ643565
	CBS 576.86	*Allium porrum*	Netherlands	1986	AF380153
	CBS 673.95	*Allium porrum*	Netherlands	1995	FJ643566
	CBS 688.79	*Daucus carota*	Canada	1979	FJ643560
	CBS783.97	*Allium porrum*	Netherlands	1997	FJ643555
P. brassicae	CBS 178.87	*Brassica* sp.	Germany	1987	AF380147
	CBS 180.87	*Brassica oleraceae*	Netherlands	1987	FJ643570
	CBS 686.95	Stored cabbage	Netherlands	1995	AF380149
	CBS 782.97	*Brassica chinensis*	Netherlands	1997	AF266801
P. syringae	CBS 114.110	Almond	Australia	2004	FJ643562
	CBS 110.161	Rhododendron	Germany	1995	FJ643561
	CBS 114.108	Cherry	USA	N	FJ643568
P. primulae	CBS 114.346	*Primula polyantha*	New Zealand	2003	FJ643563
	CBS 110.167	*Primulae elatior*	Germany	1999	FJ643569
	CBS 620.97	*Primula acaulis*	Germany	1997	AF266802

72°C. The pre-amplified product was quantified in a 1% agarose gel with ethidium bromide. The selective *Eco*RI and *Mse*I primers (Table 3) used in the hot PCR were chosen based on the highest degree of polymorphism in an initial screening with a limited number of *P. porri* isolates. The *Eco*RI primer with selective nucleotides was labelled with γ-[^{33}P]-ATP (Amersham Biosciences Europe, Roosendaal, The Netherlands). The PCR program consisted of 13 cycles of 30 s at 94°C, 30 s at 65°C to 56°C, $\Delta T = 0.7$°C, 60 s at 72°C followed by 18 cycles of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C with a PCR mix including 2 µl of 10 x PCR buffer, 0.4 µl of dNTP, 0.5 µl of *Eco*RI primer, 0.5 µl of *Mse*I primer, 0.08 µl of Taq polymerase and 11.42 µl of highly purified water. After the hot PCR, equal volumes of formamide dye were added to the PCR product. The denaturated fragments were electrophoresed on a 5% denaturing polyacrylamide gel containing 1 × TBE running buffer at 100 V for 2.5 h. The gels were vacuum-dried on 3 MM Whatman paper at 80°C and were visualized by an X-ray film (Kodak Biomax MR, Rochester, New York, USA). For each primer combination, only intense and unambiguous poly-

Table 3 The different primer combinations used in the AFLP analysis

	*Eco*RI-primer	*Mse*I-primer	Number of bands	Number of polymorphic bands	Number of monomorphic bands
1	*Eco*RI+C	*Mse*I+G	73	47 (64%)	26 (36%)
2	*Eco*RI+AT	*Mse*I+CA	72	56 (78%)	16 (22%)
3	*Eco*RI+ACA	*Mse*I+GG	74	70 (95%)	4 (5%)
4	*Eco*RI+AC	*Mse*I+G	72	60 (83%)	12 (17%)
5	*Eco*RI+AA	*Mse*I+CG	62	58 (94%)	4 (6%)
Total			353	291 (82%)	62 (18%)

morphic AFLP fragments were visually scored as the presence or absence of bands. This data set resulted in a binary presence/absence matrix. The genetic similarities were graphically represented by means of a tree using the UPGMA (unweighted pair group method, arithmetic average) clustering algorithm. Genetic distances and trees were constructed with the assistance of the TREECON software package (Vandepeer and Dewachter 1994) using the method of Link et al. (1995) for genetic distance estimation. Bootstrap values were generated using 1000 repetitions.

Sequencing of the ITS Region

Target DNA was amplified using the universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990). The PCR reaction was carried out in a 25-µl reaction volume, containing 2.5 µl of Q-buffer (Qiagen, Hilden, Germany), 0.5 µl of dNTPs (10 mM, Fermentas, Ontario, Canada), 5 µl of Q-solution (Qiagen, Hilden, Germany), 1.75 µl of each primer (10 mM), 0.15 µl of Taq-polymerase (5U μl^{-1}, Fermentas, Ontario, Canada), 11.35 µl of sterile water and 2 µl of DNA template. A thermal cycler (Eppendorf Mastercycle gradient, Hamburg, Germany) was programmed as follows: 10 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and finally 10 min at 72°C. The PCR product was purified using the Qiagen PCR purification kit and afterwards, the concentration was determined by use of a spectrophotometer (UVIKON 932, Kontron Instruments, Zurich, Switzerland) and quartz cuvettes. Sequencing was performed by the VIB lab (Antwerp, Belgium) and the resulting data, consisting of the complete sequence of ITS1, the 5.8 S subunit and ITS2, were transformed into consensus sequences with BioEdit. Genbank accessions of the sequences obtained in this study are listed in Tables 1 and 2. The following species were already sequenced by Man in 't Veld et al. (2002) and Cooke et al. (2000) (Table 2): *P. porri* CBS 138.87, *P. porri* CBS 140.87, *P. porri* CBS 142.87, *P. porri* CBS 576.86, *P. brassicae* CBS 782.97, *P. brassicae* CBS 178.87, *P. brassicae* CBS 686.95 and *P. primulae* CBS 620.97. The alignment of the sequences was carried out using the computer package ClustalW (Thompson et al. 1994) and a phylogenetic study was performed using the TREECON software program (Vandepeer and Dewachter 1994). Bootstrap values were generated using 1000 repetitions.

Pathogenicity experiments in laboratory conditions

Healthy leaves of the leek cultivar Apollo F1 (Syngenta Seeds, Enkhuizen, The Netherlands), in phenological development stage BBCH 49 (Growth complete; length and stem diameter typical for variety reached), which is a sensitive cultivar for *P. porri*, were collected in research stations in Flanders. The leaves were not treated with fungicides against *P. porri*. The outer two leaves at each side of the plant were cut at the division between the green leaf and the white stalk. The lower part (15 cm) of the leaf was cut into 3 equal parts of 5 cm each. Wetted sterile cotton was put into infection trays on which Petri dishes were placed upside down. On those Petri dishes, the leaf pieces were placed with an 8-mm-diameter plug of mycelium in contact with the leaf surface. The plugs were made with a cork borer out of 14-day-old cultures grown on V8 juice agar at 18°C in the dark. Leaf pieces without mycelial plugs served as control treatments. Inoculations were repeated between 7 and 16 times for the same isolate. The infection trays were covered with transparent plastic and placed in an incubator room at 12°C (+/−1°C) with a 8/16 light regime. After 5 days the plastic was removed from the infection tray and the plugs were removed from the leaves. Disease incidence was determined 12 days after inoculation by individually scoring each leaf piece as diseased (1) or not diseased (0) based on visual symptoms. Subsequently, the percentage of diseased leaf pieces was calculated.

Pathogenicity experiments in field conditions

Leek plants of the cultivar Harston F1 (Nunhems Seeds, Haelen, The Netherlands) or Apollo F1 (Syngenta Seeds, Enkhuizen, The Netherlands), which are equally sensitive to *P. porri*, were grown in a plastic tunnel greenhouse. Earlier experiments showed that there was no infection of *P. porri* under these tunnel greenhouses, because there is no rain splash, which brings the inoculum onto the leek plants. The extremities of the tunnel were opened to have the same temperature conditions as outside. Leek plants were irrigated for 15 min and afterwards

randomly at 4 times 10 plants were inoculated with mycelial plugs. The plugs were made with a cork borer out of 14-day-old cultures grown on V8 juice agar at 18°C in the dark. Disease incidence was determined 5 weeks after inoculation by individually scoring each plant as diseased (1) or not diseased (0) based on visual symptoms.

Results

Strains isolated

All 36 strains (Table 1) isolated from leaf samples collected in Flanders during the winter of 2005 and 2006, were characterized as *Phytophthora porri* based on their slow growth, their low growth optimum and their morphological characteristics such as non-papillate to semi-papillate sporangia, hyphal swellings and coiling growth of the hyphae of the mycelium (Erwin and Ribeiro 1996; Gallegly and Hong 2008; Man in 't Veld et al. 2002; Stamps et al. 1990).

Detection of polymorphisms

The intraspecific diversity of *P. porri* and the interspecific relationship between *P. porri* and other related *Phytophthora* strains was determined by conducting an AFLP analysis on 56 isolates. Twenty isolates were obtained from the CBS culture collection (CBS Fungal Biodiversity Centre, Utrecht, The Netherlands). The CBS isolates included nine *P. porri* isolates from different *Allium* species, one *P. porri* isolate from carrot, four closely related *P. brassicae* previously called *P. porri* (Man in 't Veld et al. 2002), three isolates of *P. syringae* and also three isolates of *P. primulae*. The remaining 36 isolates were freshly isolated Flemish *P. porri* isolates from *Allium porrum*.

AFLP fingerprinting was carried out using five *Eco*RI and *Mse*I primer combinations, listed in Table 3, to quantify the similarity among all 56 *Phytophthora* isolates. The isolates generated a total of 353 unambiguous markers of which 291 were polymorphic (82%) and 62 monomorphic (18%). A similarity tree was generated in TREECON, after clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Fig. 1). The dendrogram clearly showed different clusters with very high bootstrap percentages. Particularly at the species level, the bootstrap values were high, showing percentages of 98 and 100 percent. There was a clear partitioning of genetic diversity. The isolates clearly clustered together based on species. The isolates of *P. syringae*, *P. primulae, P. brassicae* and *P. porri* all formed different clusters, the only exception being the *P. porri* isolate CBS 688.79 from carrot, which did not cluster with the *P. porri* isolates from *Allium* species. Within the cluster with all *P. porri* isolates from *Allium* species, two subclusters could be distinguished, one with all leek isolates and the second with the Japanese isolates from *Allium cepa* and *A. grayi.* The *P. porri* isolates from leek obtained from the CBS collection, which were collected 10 and more years ago, did not differ much from the isolates collected in Flanders during 2005–2006. Among all isolates of *P. porri* from leek (*A. porrum*) only eight polymorphic markers were found. There was less intraspecific genetic diversity between the *P. porri* isolates from *Allium* species than within the other *Phytophthora* species.

ITS sequences

The ITS1, 5.8 S and ITS 2 region of a subset of isolates investigated in the AFLP analysis was determined and a phylogenetic tree was generated in TREECON using ClustalW for alignment and the Neighbour Joining Algorithm for clustering. The dendrogram of the ITS sequences (Fig. 2) supported the results obtained by the AFLP analysis. Similar as in the AFLP analysis, the *P. porri* isolates from *Allium* species were a very homogenous group not showing many differences in the ITS region. Only one *P. porri* isolate, CBS 139.87 from *A. grayi*, did not cluster together with the *P. porri* isolates from *Allium* species, but clustered together with the *P. primulae* strains. All *P. brassicae* isolates formed a separate cluster together with the CBS 688.79 *P. porri* isolate from carrot (Cooke et al. 2000; Man in 't Veld et al. 2002).

Pathogenicity tests

The results of pathogenicity tests, both in field and in laboratory conditions, are shown in Table 4. In general, only the recently isolated strains of *P. porri* were able to cause serious infection on leek. *P. porri* isolates from the CBS culture collection were not or only

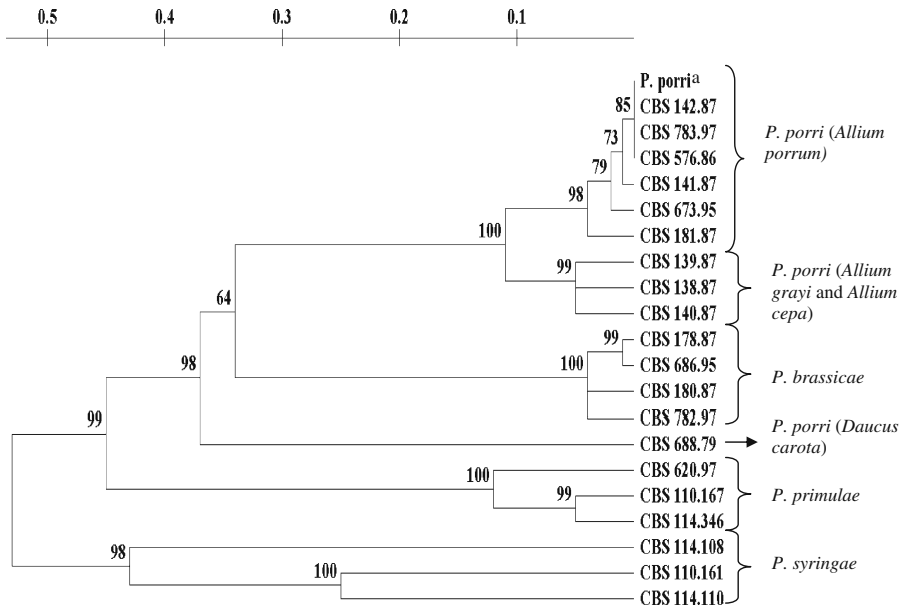

Fig. 1 Distance tree generated in TREECON, using clustering with the Unweighted Pair Group Method with Arithmetic Mean (UPGMA), illustrating the clustering between *Phytophthora porri* isolates and related *Phytophthora* isolates based on AFLP analysis. The numbers above branches indicate bootstrap percentage from 1000 replicates (above 50%). [a] *P. porri* refers to the 36 Belgian isolates obtained from leek in this study (see Table 1). They all showed a similar AFLP pattern

weakly pathogenic on leek. *P. brassicae*, *P. primulae* and *P. syringae* isolates from the CBS culture collection also seem to have the potential to infect leek, but their virulence was very low.

Discussion

AFLP analysis and ITS sequencing clearly revealed two distinct groups among the *Phytophthora* isolates from clade 8b defined by Cooke et al. (2000). One group contains *P. syringae* isolates while the second group is composed of *P. porri, P. brassicae* and *P. primulae* isolates. AFLP analysis separated the second group into subgroups according to species with bootstrap values of 100%, while these subgroups were not clearly distinguished based on ITS sequence analysis.

AFLP fingerprinting clearly showed that *P. porri* isolates clustered in two distinct groups; one cluster

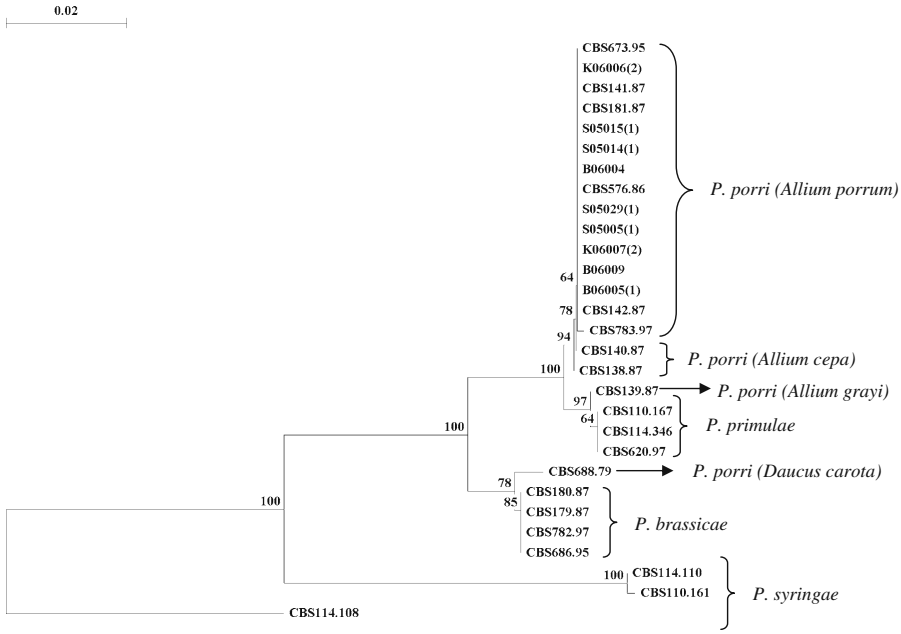

Fig. 2 A neighbour-joining phylogenetic tree, generated in TREECON using ClustalW for alignment. The tree illustrates the estimated phylogenetic relationships among *P. porri* and related strains based on ITS sequencing. The numbers above branches indicate bootstrap percentages from 1000 replicates (above 50 %)

Table 4 Pathogenicity on leek of a subset of isolates used in the AFLP analysis

Phytophthora sp.	Isolate	Disease incidence in laboratory	Disease incidence in field
P. porri	B06004 (3)	80% (8/10)	N[a]
	B06005 (1)	100% (7/7)	N
	B06009	57% (4/7)	N
	K06006 (2)	75% (9/12)	93%[b]
	K06007 (2)	60% (6/10)	27%
	S05005 (1)	90% (9/10)	78%[b]
	S05014 (1)	100% (7/7)	N
	S05015 (1)	78% (7/9)	N
	S05029 (1)	50% (5/10)	N
	CBS 138.87	25% (4/16)	0%
	CBS 139.87	0% (0/16)	0%
	CBS 140.87	0% (0/16)	N
	CBS 141.87	31% (5/16)	7%
	CBS 181.87	25% (4/16)	10%
	CBS 576.86	13% (2/16)	0%
	CBS 673.95	0% (0/16)	0%
	CBS 688.79	0% (0/16)	0%
	CBS 783.97	13% (2/16)	7%
P. brassicae	CBS 178.87	6% (1/16)	0%
	CBS 180.87	0% (0/16)	3%
	CBS 686.95	0% (0/16)	13%
	CBS 782.97	0% (0/16)	7%
P. primulae	CBS 110.167	38% (3/8)	3%
	CBS 114.346	N	13%
P. syringae	CBS 110.161	N	3%
	CBS 114.108	25% (2/8)	3%
	CBS 114.110	N	7%

[a] N: not tested, [b] Tested on cultivar Apollo

contains all *P. porri* isolates from leek, while the other cluster contains the Japanese isolates from *A. cepa* and *A. grayi*. Although the ITS sequences of two of the Japanese isolates (CBS138.87 and CBS140.87) are identical to those found in the *P. porri* isolates from *Allium porrum*, Man in 't Veld et al. (2002) already observed that at seven sites in the ITS sequence evidence of dimorphism was apparent. These dimorphic sites were exactly those sites where the sequence of the *Allium* isolates differed from *P. primulae*. In this context, it is interesting to notice that the ITS sequence of the third Japanese isolate, CBS139.87 from *Allium grayi,* did not show dimorphic sites and was identical to those found in *P. primulae* isolates except for one base-pair substitution. Dimorphic sites are indicative of heterozygosity and have been demonstrated in various *Phytophthora* hybrids (Brasier et al. 1999, Man in 't Veld et al. 2007). It can be hypothesized that the Japanese *Allium* isolates form an incipient species that has arisen by interspecific hybridization between *P. porri* and *P. primulae*. However, more genes should be sequenced to test this assumption. Whether this speciation is driven by the host plant or geographic isolation is at present unclear and Japanese *P. porri* isolates from leek should be investigated to clarify this point.

P. porri isolates from *A. porrum* form a very homogenous group. Even isolates of *P. porri* from the CBS collection, which were collected more than 10 years ago, clustered together with our recently collected isolates from Flanders. The lack of genetic variation within the *P. porri* population from leek is probably due to different reasons such as a short life cycle in the field, the lack of selection imposed by host resistance genes or limited sampling, since all leek isolates in this study originated from the same geographic region (the Netherlands and Belgium). In a homothallic species such as *P. porri* it is likely that mutation is the primary source of variation. It was stated by Goodwin (1997) that mutation rates do not have to be excessively high to create variation when the selection is strong and a huge number of propagules are produced. But *P. porri* has a very short life cycle in the field, and the formation of sporangia in field conditions is not frequently observed. *P. porri* easily forms oospores when conditions are not suitable for pathogen infection. Oospores are probably the only fungal structures that can survive in leek-free soil for years (Smilde et al. 1996a). One of the causes of selection in *Phytophthora* species is host resistance genes (Goodwin, 1997). Rapid evolution and high genetic diversity has been observed for the homothallic *P. sojae*, a pathogen that causes root rot of soybean, a disease that is typically managed by breeding resistant cultivars (Forster et al., 1994; Drenth et al., 1996; Gally et al., 2007). Breeding of leek, however, is time consuming and complicated because leek is a tetraploid and shows strong inbreeding depression (loss of vigour) after self pollination (De Clercq et al. 2003). Despite leek breeding programs aiming at

the development of improved cultivars which are less susceptible to *P. porri,* no resistant cultivars are available at the moment so there is no selection imposed by host resistance genes.

Based on AFLP analysis and ITS sequencing the *P. porri* isolate from carrot (CBS 688.79) appears to be different from the *P. porri* isolates from *Allium* species. de Cock et al. (1992) also found differences between CBS688.79 and other *P. porri* isolates from leek based on restriction patterns of mitochondrial DNA. Both the data from de Cock et al. (1992) and our AFLP analysis suggest that *P. porri* from carrot belongs to a new species. Based on its ITS sequence it appears to be more closely related to *P. brassicae* than to *P. porri* isolates from *Allium.* Interestingly, the sites where the ITS sequence differs from *P. brassicae* are either unique within clade 8b or identical to the ITS sequence of *P. syringae*. It is tempting to speculate that also this species could be the result of an interspecific hybridization. More isolates from carrot need to be studied and some additional genes should be sequenced to test this hypothesis.

The close relationship between *P. porri* and *P. primulae* (isolate CBS 620.97) based on ITS sequencing, was already shown by Man in 't Veld et al (2002) and Cooke et al. (2000). Man in 't Veld (2002) even stated that *P. primulae* is in need of revision because of this close relationship with *P. porri*. However, our AFLP data demonstrate that *P. primulae* isolate CBS 620.97 clusters with other *P. primulae* isolates and forms a separate group clearly distinct from *P. porri* and *P. brassicae*.

Pathogenicity tests with *P. porri* isolates from leek and closely related species were not very successful, since various *Phytophthora* isolates obtained from culture collections had lost their pathogenicity during storage, as had been observed before (de Cock et al. 1992). No differences in virulence could be detected among the recent *P. porri* isolates from leek. Although host specialization is involved in the grouping of *Phytophthora* isolates from clade 8b, no clear indications for strict host specificity could be found since some of the *P. brassicae*, *P. primulae* and *P. syringae* isolates were able to infect leek, although their virulence was low. It remains to be investigated to what extent *P. porri* isolates from leek are able to infect other hosts, although de Cock et al. (1992) already showed that *P. porri* isolates from *Allium* spp. were not or weakly pathogenic on white cabbage.

In conclusion it can be stated that *P. porri* isolates from leek form a homogenous group which can be distinguished from *P. porri* isolates from other *Allium* species and the *P. porri* isolate from carrot which points to incipient speciation within clade 8b. We are currently testing the hypothesis that this speciation is the result of interspecific hybridization between closely related species. Although the host plant is probably an important selective force for species formation in clade 8b, geographic isolation could also be involved.

Acknowledgements The authors want to thank Tina Kyndt for the fruitful discussions and her helpful comments during this work. This study is funded by the Institute for Promotion and Innovation through Science and Technology in Flanders (IWT-Vlaanderen).

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