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Gene flow analysis of *Phytophthora porri* reveals a new species: *Phytophthora brassicae* sp. nov.

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Abstract

Isozyme analysis and sequence analysis of the internal transcribed spacer regions (ITS-1 and ITS-2) and the 5.8S subunit of the ribosomal DNA gene repeat were used to examine whether isolates of *Phytophthora porri* from *Allium* and *Brassica* represent a single homogeneous species. Twenty-six strains of *P. porri*, 16 strains isolated from the genus *Allium*, and 10 strains isolated from the genus *Brassica*, were analyzed using malate dehydrogenase (MDH), isocitrate dehydrogenase (IDH) and lactate dehydrogenase (LDH), represented altogether by four putative loci (*Mdh-2*, *Idh-1*, *Idh-2*, and *Ldh-2*). Isozyme analysis revealed that strains isolated from *Allium* contained five private alleles at three isozyme loci (*Ldh-2*^{83}, *Ldh-2*^{104}, *Idh-1*^{108}, *Idh-1*^{112}, and *Idh-2*^{98}), whereas six different alleles were observed at four isozyme loci (*Ldh-2*^{85}, *Ldh-2*^{100}, *Ldh-2*^{114}, *Idh-1*^{100}, *Idh-2*^{100}, and *Mdh-2*^{111}) in strains obtained from *Brassica*. The heterozygosity at the *Ldh-2* locus, differing in allele composition, however, between strains from *Allium* and *Brassica,* was present in all strains, indicating that it is probably fixed. Sequence analysis of the ITS regions and the 5.8S subunit showed consistent differences between isolates from *Allium* and isolates from *Brassica*. Based on isozyme data, ITS sequence analysis and formerly published differences in restriction enzyme patterns of mitochondrial DNA, morphology and pathogenicity, it was concluded that the isolates of *P. porri* Foister did not represent a homogeneous species. Isolates from *Brassica* constitute a distinct species which is described here as *P. brassicae* sp. nov. It was inferred from isozyme patterns, which were in no case intermediate between the two species, that *P. porri* and *P. brassicae* do not hybridize and are reproductively isolated by barriers to gene flow.

Introduction

Phytophthora porri Foister is a member of the genus *Phytophthora* in the phylum Oomycota which now comprises more than 50 recognized species (Erwin and Ribeiro, 1996), including some that were recently described (Ilieva et al., 1998; Jung et al., 1999; Cooke et al., 1999; Werres et al., 2001).

Phytophthora porri was originally described as a pathogen of *Allium porrum* (Foister, 1931) and was subsequently reported to be a pathogen of other hosts, namely, other *Allium* species, and species of the genera *Brassica*, *Campanula*, *Daucus*, *Dianthus*, *Gladiolus*, *Hyacinthus*, and *Tulipa* (Erwin and Ribeiro, 1996). So far, *P. porri* seems to be confined to Canada, Western Europe, and Japan, though there is one report on its occurrence in South Africa (von Maltitz and von Broembsen, 1984).

Phytophthora porri is characterized by non-papillate to semi-papillate sporangia, both amphigynous and paragynous antheridia, slow growth, low maximum growth temperatures, and coiling growth of hyphae. *P. porri* is a homothallic species. However, oogonia are often abortive and mature oospores are rarely produced (de Cock et al., 1992). Isolates from different hosts, may show some differences. According

to de Cock et al. (1992), isolates from *Allium* have mainly paragynous antheridia, whereas antheridia in isolates from *Brassica* were predominantly amphigynous. Some isolates, however, deviated in this respect. All isolates obtained from *Allium* produced oogonia consistently, whereas oogonial production by isolates from *Brassica* rapidly declined after isolation. Only the isolates from *Brassica* produced sporangia in large numbers on agar. Infection experiments with *P. porri* isolates indicated different host specificity. Isolates obtained from *Allium* were only capable of infecting *Allium* plants, whereas isolates obtained from *Brassica* exclusively infected *Brassica* plants (de Cock et al., 1992). This strongly suggests that these two groups of host-specific pathogens may in reality represent two different taxa. Restriction enzyme analysis of mitochondrial DNA using six restriction enzymes revealed that the patterns in all isolates from *Brassica* were consistent, and different from the patterns of all isolates from *Allium*. The Japanese isolates from *Allium* showed a different pattern, but were more similar to the other isolates from *Allium* (de Cock et al., 1992).

A considerable number of molecular techniques are now available for taxonomic studies of *Phytophthora* species. Sequences of internal transcribed spacer region of the ribosomal DNA gene repeat (ITS-1 and ITS-2) have been used to distinguish many *Phytophthora* species and particularly to establish their phylogenetic relationships (Cooke et al., 1997, 2000). Intraspecific variation is low and sequence variation at the interspecific level is generally large enough to delineate species. However, sometimes differences in ITS sequences between species are absent (e.g. *P. infestans* and *P. mirabilis*). Apparently, ITS sequence homology between these two clearly distinct species does not necessarily imply that these two species are conspecific (Goodwin et al., 1999).

Isozyme analysis has been successfully employed to solve taxonomical problems in *Phytophthora* (Oudemans and Coffey, 1991). Thus, conspecificity between *P. parasitica* and *P. nicotianae* (Oudemans and Coffey, 1991) and between *P. arecae* and *P. palmivora* (Mchau and Coffey, 1994) was demonstrated. Moreover, isozyme analysis has the advantage over other biochemical techniques that, when dimeric enzymes are employed, crosses can be detected due to the presence of heterodimeric bands. Isozyme analysis revealed the existence of natural hybrids of *P. nicotianae* and *P. cactorum* (Man in 't Veld et al., 1998).

The aim of this study was to test the hypothesis that *Allium* and *Brassica* isolates of *P. porri* may represent two separate species. Isozyme genotyping and ITS sequence analysis were used to assess gene flow between the two groups of isolates.

Materials and methods

Isolates and cultivation

Isolates used in this study, their origin, year of isolation, and reference numbers are listed in Table 1. Isolates which were also used by de Cock et al. (1992) were studied as described in the cited paper. In addition, morphology and colony patterns of all isolates were examined on V8 agar (Gams et al., 1998) at 20 °C.

For DNA isolation, strains were grown in pea broth medium for up to two weeks (de Cock et al. 1992). Mycelium was harvested by vacuum filtration and stored at −20 °C.

Isozyme analysis

Three enzymes were employed to monitor the possible genetic variation of *P. porri*: isocitrate dehydrogenase (IDH, EC 1.1.1.42), lactate dehydrogenase (LDH, EC 1.1.1.27), and malate dehydrogenase (MDH, EC 1.1.1.37). Isozyme analysis was essentially performed as described by Man in 't Veld et al. (1998) with the following additions: the reaction components for LDH were: 23 ml of 0.1 M tris–HCl pH 7.5, 2 ml of lactate solution (5.3 ml lactic acid (98%) + 24.5 ml 1 M Na_2CO_3 + 10.2 ml distilled water), 12.5 mg of nicotinamide adenine dinucleotide (NAD), 7.5 mg of nitroblue tetrazolium (NBT), 1 mg phenazine methyl sulfate (PMS); for IDH: 25 ml of 0.1 M tris–HCl, pH 8.0, 12.5 mg of isocitrate (trisodium salt), 100 mg of $MgCl_2$; 10 mg of nicotinamide adenine dinucleotide phosphate (NADP), 7.5 mg of NBT, 1 mg of PMS; for IDH glycine was added to the samples to a final concentration of 0.22 M.

Alleles were scored according to the method of Spielman et al. (1990). Arbitrarily the mobility of one allele was designated 100, and other alleles were numbered according to their mobilities relative to this allele. Since *Phytophthora* spp. are diploid, two identical numbers were assigned to one band. A three-banded pattern was interpreted as the product of two different alleles encoding for a dimeric enzyme (Richardson et al., 1986). Both MDH and IDH are known to be dimeric enzymes (Richardson et al., 1986). When two zones of activity were present on the gel, the slowest

Table 1. Isolates of *Phytophthora* used in this study, their reference numbers, their origins (host plant and location), the year of isolation and their isozyme electrophoretic type

Isolates	Host	Location	Year	ET[1]	Genbank
Phytophthora porri					
CBS 138.87	*Allium cepa*	Japan	1987	1	AF380150
CBS 139.87	*Allium grayi*	Japan	1987	1	n.s.[2]
CBS 140.87	*Allium cepa*	Japan	1987	1	AF380151
CBS 142.87	*Allium porrum*	Belgium	1987	1	AF380152
CBS 567.86	*Allium porrum*	The Netherlands	1986	1	AF380153
CBS 802.95 (PD92/214)	*Allium porrum*	The Netherlands	1992	1	n.s.
PD 98/8/2700	*Allium porrum*	The Netherlands	1998	1	n.s.
PD 98/8/2701	*Allium porrum*	The Netherlands	1998	1	n.s.
PD 98/8/2705	*Allium porrum*	The Netherlands	1998	1	n.s.
SMILDE EE	*Allium porrum*	The Netherlands	1994	1	n.s.
SMILDE E4	*Allium porrum*	The Netherlands	1994	1	n.s.
SMILDE O (CBS 783.97)	*Allium porrum*	The Netherlands	1994	1	n.s.
SMILDE R	*Allium porrum*	The Netherlands	1994	1	n.s.
SMILDE W	*Allium porrum*	The Netherlands	1994	1	n.s.
SMILDE X	*Allium porrum*	The Netherlands	1994	1	n.s.
SMILDE GG	*Allium porrum*	United Kingdom	1994	2	n.s.
CBS 180.87	*Brassica oleracea*	The Netherlands	1987	3	n.s.
PD 94/166	*Brassica oleracea*	The Netherlands	1994	3	n.s.
CBS 178.87	*Brassica oleracea*	Germany	1987	4	AF380147
CBS 212.82	*Brassica oleracea*	The Netherlands	1982	4	n.s.
PD 94/64	*Brassica chinensis*	The Netherlands	1994	4	n.s.
PD 95/1954	*Brassica oleracea*	The Netherlands	1995	4	n.s.
SMILDE HH (CBS 782.97)	*Brassica chinensis*	The Netherlands	1994	4	AF266801
SMILDE II	*Brassica oleracea*	The Netherlands	1994	4	n.s.
CBS 179.87	*Brassica oleracea*	The Netherlands	1987	5	AF380148
CBS 686.95	*Brassica oleracea*	The Netherlands	1995	5	AF380149
Phytophthora erythroseptica					
ATCC 36302	*Solanum tuberosum*	USA	—	—	AF266797
Phytophthora primulae					
CBS 620.97	*Primula acaulis*	Germany	1997	—	AF266802
Phytophthora syringae					
CBS 132.23	Unknown	UK	1923	—	AF380146
IMI 296829	*Rubus idaeus*	Scotland	1985	—	AF266803

[1]Isozyme electrophoretic type. [2]Not sequenced.

zone was assigned the first locus and the fastest zone the second locus.

DNA isolation

Mycelium was freeze-dried and DNA was isolated by CsCl ultracentrifugation (de Cock et al., 1992) or using the miniprep method described by Möller et al. (1992).

ITS sequencing and analysis

Representative isolates from the two groups of *P. porri* as well as *P. syringae* (CBS 132.23), a species closely related to *P. porri* according to Cooke et al. (2000), were selected for sequencing (see Table 1 for details). Universal eukaryotic primers UN-UP18S42 (5′-CGTAACAAGGTTTCCGTAGGTGAAC-3′) with UN-LO28S576B (5′-CTCCTTGGTCCGTGTTTCA-AGACG-3′) or UNLO28S22 (5′-GTTTCTTTT-CCTCCGCTTATTGATATG-3′) were used to prepare the sequencing template by amplifying the internal transcribed spacers, the 5.8S gene of nuclear ribosomal DNA, and part of the large ribosomal subunit. DNA was amplified by polymerase chain reaction (PCR), purified and sequenced using the procedure from Bakkeren et al. (2000) and BigDye Terminator chemistry (Applied Biosystems,

Forster City, CA). The ITS 1 was sequenced using UN-UP18S42 and a reverse primer (OOM-LO5.8S47 = 5′-GACGCTTGACGCTATGCATTA-3′), while the ITS 2 was sequenced with a forward primer (OOM-UP5.8S01 = 5′-CAACTTTCAGCAGTGGATGTCT-3′ or OOM-UP5.8S55 = 5′-TTAAGCGTAATGCATAGCGT-3′), and UNLO28S22 as reverse primer. Sequences were edited using Sequence Navigator version 1.01 (Applied Biosystems, Forster City, CA) or Seqman 4.05 (DNASTAR, Madison, WI). MegAlign (DNASTAR, Madison, WI) was used to perform the final alignment. Parsimony phylogenetic analysis of the ITS 1, 5.8S, and ITS 2 region was performed with phylogenetic analysis using parsimony (PAUP) version 4.0b6 software (Swofford, 1999) using the Heuristic approach. The following species were included as outgroups: *P. erythroseptica* ATCC 36302 (Genbank accession number AF266797), *P. primulae* CBS 620.97 (Genbank AF266802) and *P. syringae* IMI 296829 (Genbank AF266803; in addition to strain CBS 132.23) (Cooke et al., 2000). Gaps were treated as missing data. Bootstrap values were generated using 1000 repetitions.

Results

Morphology

All isolates were identified as *P. porri* on the basis of their low optimum growth temperature (∼20 °C), slow growth, non-papillate to semi-papillate sporangia, hyphal swellings, and coiling growth of hyphae. All isolates from *Allium* showed fluffy aerial mycelium on V8, whereas all isolates from *Brassica* exhibited a powdery appearence (Figure 5). The antheridia of isolates from *Allium* were mainly paragynous, except SMILDE X, which had predominantly amphigynous antheridia, and SMILDE O and GG which had paragynous and amphigynous antheridia in equal frequency. SMILDE R and SMILDE E4 did not form oogonia at all. All isolates from *Brassica* had been maintained in culture for a few years and no longer formed sexual structures. This is typical for isolates obtained from *Brassica* (de Cock et al., 1992).

Isozyme analysis

Up to three zones of activity were observed on the gel using LDH (Table 2). These zones, assumed to represent three different loci, were assigned *Ldh-1*, *Ldh-2*, and *Ldh-3*. At the first locus, *Ldh-1*, the bands were usually very faint or not visible at all and this locus was not further analyzed. The second locus, *Ldh-2*, consisted of a conspicuous three-banded pattern. All isolates from *Allium* had the same three bands at *Ldh-2*. Likewise, isolates from *Brassica*, except CBS 179.87 and CBS 686.95, had also three bands at *Ldh-2*, all differing, however, from those of *Allium* isolates (Figure 1A). In *Brassica* isolates CBS 179.87 and CBS 686.95, a five-banded pattern was generated at *Ldh-2* (Figure 2), of which three bands comigrated with three bands consistently present in other isolates from *Brassica*. The third locus, *Ldh-3*, mostly exhibited high activity but was sometimes missing and was therefore not analyzed further.

In order to test whether bands of *Ldh-2* contain dimeric enzymes, the enzyme assay was performed in the presence of 0.5% w/v octyl-β-D-glucopyranoside, a non-ionic detergent (Roux et al., 1991). This substance is capable of dissociating the subunits of dimeric enzymes, resulting in the loss of all enzymatic activity. Indeed, when the enzyme stain was developed in the presence of octyl-β-D-glucopyranoside, the three-banded pattern was markedly reduced, whereas the activity of bands of the other loci was unaffected (Figure 1A,B). We conclude that the isozymes at *Ldh-2* are dimeric (Table 2) and that the isozymes at *Ldh-3* are monomeric.

Isozyme analysis with IDH showed two zones of activity, assigned to two putative loci, *Idh-1*, and *Idh-2*. Three unique bands were found in isolates from *Allium*, and two unique bands were present in all isolates from *Brassica* (Table 2, Figure 3). Isozyme analysis of MDH also showed two zones of activity, which were assigned to two putative loci, *Mdh-1* and *Mdh-2*. The first locus was not analyzed due to smearing; the bands at the second locus gave clearly interpretable bands. All isolates had one band in common at *Mdh-2*. Eight isolates from *Brassica* generated a three-banded pattern (Table 2).

ITS sequence analysis

Sequences were easy to align, showing consistency within *P. porri* from *Allium* and *P. porri* from *Brassica*. The ITS 1 sequences of both *P. porri* from *Allium* and *P. porri* from *Brassica* were 202 base pairs in length, with two separate single base pair deletions in *P. porri* from *Allium* and one double base pair deletion in *P. porri* from *Brassica*. The ITS 2 sequence of

Table 2. Isozyme alleles of *P. porri* scored at the *Ldh-2*, *Idh-1*, *Idh-2*, and *Mdh-2* loci, their mitochondrial DNA types and their internal transcribed spacer sequences type (ITS)

Isolates	Isozyme genotype				mtDNA[1]	ITS[2]
	Ldh-2	*Idh-1*	*Idh-2*	*Mdh-2*		
Phytophthora ex *Allium*						
CBS 138.87	83/104	108/108	98/98	100/100	A′	α
CBS 139.87	83/104	108/108	98/98	100/100	A′	
CBS 140.87	83/104	108/108	98/98	100/100	A″	α
CBS 142.87	83/104	108/108	98/98	100/100	A	α
CBS 567.86	83/104	108/108	98/98	100/100	A	α
CBS 802.95	83/104	108/108	98/98	100/100		
PD 98/8/2700	83/104	108/108	98/98	100/100		
PD 98/8/2701	83/104	108/108	98/98	100/100		
PD 98/8/2705	83/104	108/108	98/98	100/100		
SMILDE EE	83/104	108/108	98/98	100/100		
SMILDE E4	83/104	108/108	98/98	100/100		
SMILDE O	83/104	108/108	98/98	100/100		
SMILDE R	83/104	108/108	98/98	100/100		
SMILDE W	83/104	108/108	98/98	100/100		
SMILDE X	83/104	108/108	98/98	100/100		
SMILDE GG	83/104	112/112	98/98	100/100		
Phytophthora ex *Brassica*						
CBS 180.87	100/114	100/100	100/100	100/100	B	
PD 94/166	100/114	100/100	100/100	100/100		
CBS 178.87	100/114	100/100	100/100	100/111	B	β
CBS 212.82	100/114	100/100	100/100	100/111	B	
PD 94/64	100/114	100/100	100/100	100/111		
PD 95/1954	100/114	100/100	100/100	100/111		
SMILDE HH	100/114	100/100	100/100	100/111		β
SMILDE II	100/114	100/100	100/100	100/111		
CBS 179.87[3]	85/100/114	100/100	100/100	100/111	B	β
CBS 686.95	85/100/114	100/100	100/100	100/111		β

[1]Mitochondrial DNA types as revealed by restriction enzyme analysis with six enzymes; A′ and A″ have 70% similarity with A; A′ has 90% similarity with A″ (after de Cock et al., 1992). [2]ITS sequence types: α differs at 19 nucleotide positions from β. [3]Typestrain of *P. brassicae*.

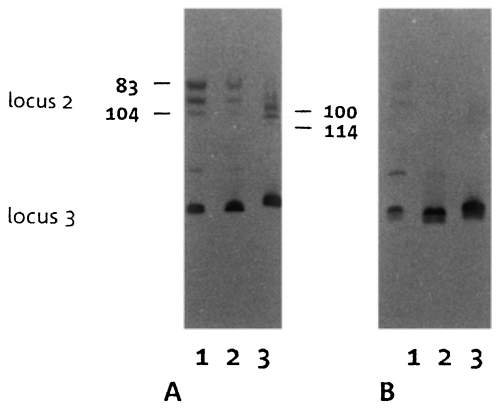

Figure 1. Isozyme patterns generated by LDH in the absence (A) and in the presence (B) of octyl-β-D-glucopyranoside. Lane 1: CBS 802.95 ex *Allium porrum*; Lane 2: SMILDE O ex *Allium porrum*, and Lane 3: SMILDE II ex *B. oleracea*.

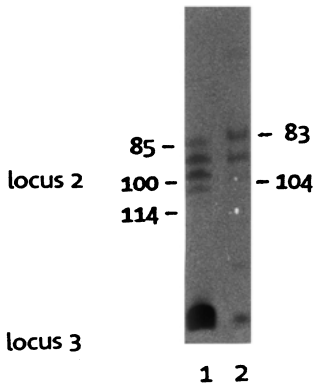

Figure 2. Isozyme patterns generated by LDH. Lane 1: CBS 686.95 ex *B. oleracea* and Lane 2: CBS 802.95 ex *Allium porrum*.

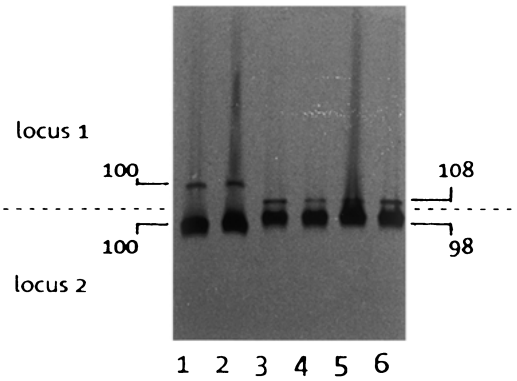

Figure 3. Isozyme patterns generated by IDH. Lane 1: CBS 179.87 ex *B. oleracea*; Lane 2: SMILDE II ex *B. oleracea*; Lane 3: SMILDE O ex *Allium porrum*; Lane 4: CBS 802.95 ex *Allium porrum*; Lane 5: CBS 142.87 ex *Allium porrum*; and Lane 6: CBS 567.86 ex *Allium porrum*.

P. porri from *Allium* was 445 base pairs, whereas that of *P. porri* from *Brassica* amounted 446 base pairs, with two separate single base pair deletions in *P. porri* from *Allium* and one in *P. porri* from *Brassica.* When comparing the two groups, there were five single and one double base pair substitutions in the ITS 1, and 12 single base pair substitutions in the ITS 2. Fourteen of these substitutions were transitions and five were transversions. *P. porri* from *Allium* and *P. porri* from *Brassica* are clearly separated, as indicated by the high bootstrap values in the phylogenetic analysis (Figure 4). Most sequences obtained were of excellent quality showing no signs of heterozygosity. The exceptions were the Japanese isolates of *P. porri* from *Allium* which showed seven possible dimorphic sites. The other *Allium* isolates showed seven single base-pair substitutions compared to *P. primulae* (CBS 620.97, AF266802); exactly at those sites where the sequence differed from *P. primulae*, the Japanese isolates were possibly dimorphic.

Discussion

De Cock et al. (1992) suggested that isolates of *P. porri* from *Brassica* represent a species distinct from *P. porri* from *Allium*. This suggestion was based on differences they found in morphology, pathogenicity and restriction patterns of mtDNA. Our observations on the morphology of isolates used in this study are in agreement with the former study. In addition, significant differences in colony morphology on V8 agar between isolates of the two hosts were established. Results of ITS sequencing were concurrent with morphological differences: isolates from the same host showed identical ITS sequences and they differed from those of the other host. This was reflected in the phylogenetic tree in which the two groups of *P. porri* isolates formed discrete clades, more closely related to each other than to *P. syringae* or *P. erythroseptica*. Surprisingly, one of the intended outgroups, *P. primulae*, represented by an isolate sequenced by Cooke et al. (2000), clustered within the group of *P. porri* isolates, close to the isolates from *Allium*. *P. primulae* resembles *P. porri* in some cardinal characters (e.g. coiling hyphae, low optimum temperature for growth, semi-papillate sporangia). The main difference seems to be the relatively high length/width ratio of the sporangia in *P. primulae*, which, however, may be related to their often distorted shapes (Stamps et al., 1990; Erwin and Ribeiro, 1996). This species is probably therefore in need of revision. In the ITS-based phylogenetic tree by Cooke et al. (2000), *P. porri* was represented by a strain obtained from *Brassica*, the sequence of which was identical to those of our isolates from *Brassica* (Figure 4). A strain from *Allium* was not included in their tree. However, *P. primulae* is at the same distance from the *Brassica* isolates as are the *Allium* isolates, viz. 18 and 19 nucleotides, respectively. This allows us to compare the distance between the host related groups of *P. porri* with the distances between other *Phytophthora* species in the tree of Cooke et al. (2000). The comparison shows that many species are more closely related to each other than are the two *P. porri* groups. In other words, the ITS sequence difference between the *Allium* and *Brassica* isolates is larger than that found between several other species.

The ITS sequences of the two Japanese isolates CBS 138.87 and CBS 140.87 are identical to those found in the other isolates obtained from *Allium*, but at seven sites evidence of dimorphism was apparent. These dimorphic sites were exactly those sites where the sequences of the *Allium* isolates differed from *P. primulae*. This is remarkable since within a species these tandemly repeated ITS regions are generally homogenized via concerted evolution. This suggests that these isolates have been involved in reticulation events in the course of their evolution. Dimorphic sites were also discovered in *P. cambivora* and in the alder hybrid *Phytophthora* population (Brasier et al., 1999). Interestingly, the mitochondrial DNA profiles of the Japanese isolates also differ slightly from other *P. porri* isolates obtained from *Allium* (de Cock

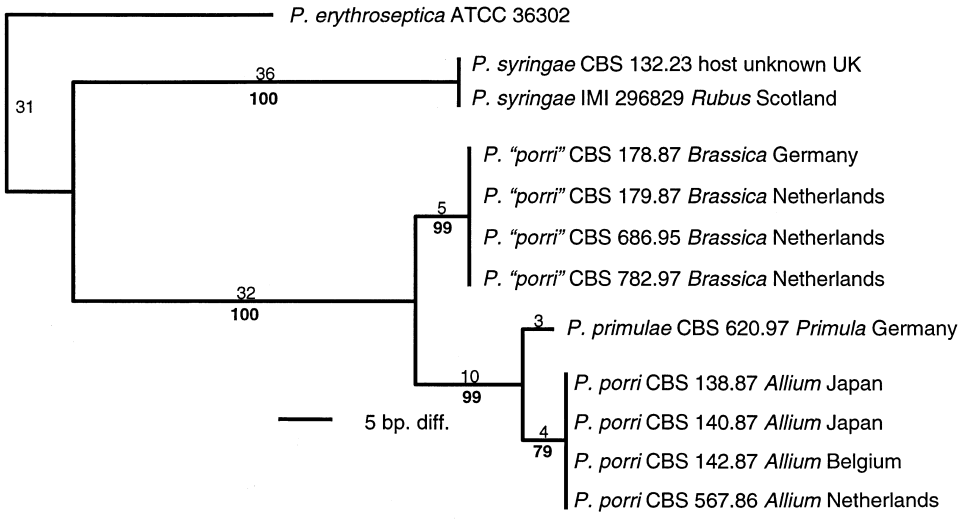

Figure 4. Phylogenetic relationship of isolates of *P. porri* and related species, deduced from maximum parsimony analysis of sequences of the internal transcribed spacer region (ITS 1, 5.8S, and ITS 2). Branch length and percentage bootstrap values (in bold) based on 1000 replications are indicated.

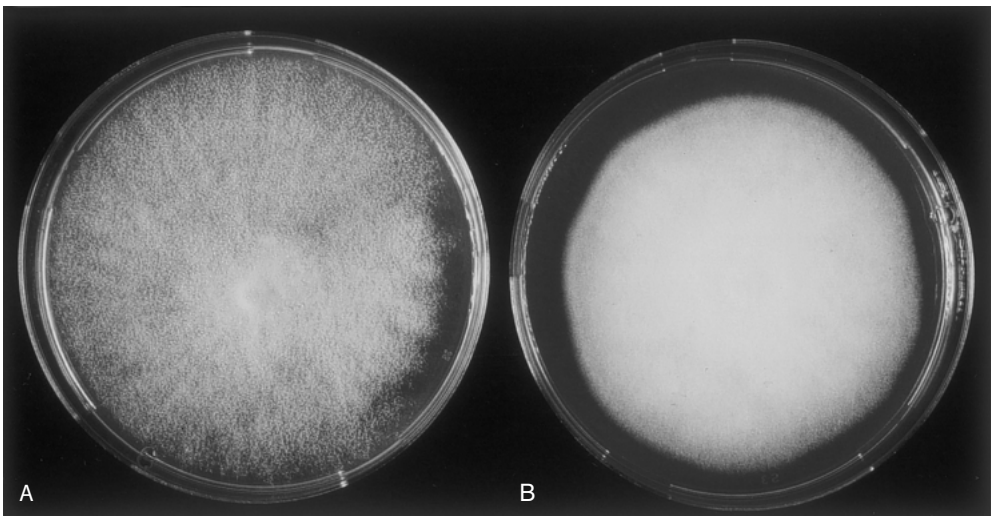

Figure 5. Colony appearance of (A) *P. brassicae* (CBS 212.82) and (B) *P. porri* (SMILDE O) on V8 agar.

et al., 1992). Currently, these isolates are under investigation.

In the case of dimeric isozymes, the common explanation for a three-banded pattern is that gene products of two different alleles combine in three different ways, resulting in two different homodimeric isozymes and one heterodimeric isozyme with intermediate physicochemical properties (Goodwin et al., 1994). Hence, the three-banded patterns at *Ldh-2* and *Mdh-2* are assumed to be due to the presence of two different alleles at their respective loci. Two isolates, CBS 179.87 and CBS 686.95, showed a five-banded pattern at *Ldh-2*, three of which comigrated with the standard three-banded pattern at *Ldh-2* in other isolates obtained from *Brassica* (Figure 5). Since the isozymes at *Ldh-2* have been shown to be dimeric, the most obvious explanation is that three different alleles are responsible for this pattern, generating six bands, one of which is

superimposed on another band on the gel. The only logical explanation is that the homodimeric isozyme encoded for by the *Ldh-2*^{100} allele comigrates with the heterodimeric isozyme encoded for by the *Ldh-2*^{85} and the *Ldh-2*^{114} alleles. A similar case has been described by Goodwin et al. (1992), who found a five-banded pattern at the *Gpi* locus in *P. infestans*. By making crosses, he showed that the middle band was indeed a superposition of a homodimeric and a heterodimeric isozyme.

The co-migration of a heterodimeric isozyme and a homodimeric isozyme, creating the middle band may not be coincidental. A possible explanation would be that during the pairing of the homologuous chromosomes in meiotic prometaphase, unequal crossing-over has taken place in the course of evolution at the *Ldh-2* locus. A deletion in the *Ldh-2*^{100}, thus creating the *Ldh-2*^{114} allele, matches exactly in size an insertion in the *Ldh-2*^{100} allele, thus creating the *Ldh-2*^{85} allele. As a result, the heterodimeric isozyme, constituted by the gene products of the *Ldh-2*^{85} allele and the *Ldh-2*^{114} allele, has similar physico-chemical features as the homodimeric isozyme encoded by the *Ldh-2*^{100} allele. Although the three-dimensional structure of the resulting proteins constituting the isozymes is very complex, it is clear that the amino-acid sequences near to and in the active centre of the enzyme, and those in the outer domains, involved in the dimerization proces, hardly tolerate any replacements and must be highly conserved. It can be inferred from this that crossing-over events will generate differences between the various isozymes which probably only consist in the presence or absence of loops, which do not influence the three-dimensional structure of the enzymes as a whole, but only result in changes in net charge of the isozymes. It is likely therefore that the identical physico-chemical features of the homodimeric isozymes and the heterodimeric isozymes, creating the middle band, can be accounted for by this mechanism.

Isozyme analysis revealed a total of 12 different alleles at four loci among 26 *P. porri* isolates. Five alleles, *Ldh-2*^{83}, *Ldh-2*^{104}, *Idh-1*^{108}, *Idh-1*^{112}, and *Idh-2*^{98} were unique to isolates from *Allium* and six alleles, *Ldh-2*^{85}, *Ldh-2*^{100}, *Ldh-2*^{114}, *Idh-1*^{100}, *Idh-2*^{100}, and *Mdh-2*^{111} were unique to isolates from *Brassica*. Only a single allele, *Mdh-2*^{100} was shared by both groups. According to the definition by Hansen (1991), species are populations of organisms that represent a common gene pool, reproductively isolated from other such populations by genetic barriers to gene flow. If *P. porri* sensu lato were a homogeneous species, alleles should be distributed at random in the population. This, however, is not the case. The apparent lack of gene flow between isolates of *P. porri* from *Allium* and *Brassica*, as indicated by the presence of unique alleles in the two groups, implies that they are reproductively isolated from each other. Probably, a strong isolating mechanism between the two species results in the inability to infect each other's host.

Regarding the presence of unique alleles in both host groups of *P. porri*, it is important to note that neither the isolates from *Allium* nor those from *Brassica* studied here represent a clonal lineage. Three *P. porri* isolates, CBS 138.87, CBS 139.87, and CBS 140.87, originating from Japan, contained the alleles at *Ldh-2*, *Idh-1*, and *IDH-2* typical for isolates obtained from *Allium*, yet their mitochondrial DNA restriction patterns differed from those found in some other isolates from the same host (similarity 70%) (de Cock et al., 1992). Moreover one isolate (SMILDE GG), originating from the United Kingdom, differed at the *Idh-2* locus from the other isolates obtained from *Allium*. Although *Ldh-3* was not analyzed in detail, variation was observed at this locus; especially isolate CBS 142.87 is worth mentioning, because it contains a unique band, not present in the other isolates (data not shown).

Isozyme analysis of the isolates from *Brassica* revealed three electrophoretic types (ET) differing at the *Mdh-2* locus and at the *Ldh-2* locus, also indicating that the these isolates do not represent a clonal lineage. Two *P. brassicae* isolates (CBS 180.87 and PD 94/166) are homozygous at *Mdh-2*, whereas all other isolates of this species are clearly heterozygous indicating that outcrossing occurs within this group. It has been assumed for a long time that outbreeding occurred only in heterothallic species. Recently, however, it has been demonstrated by Whisson et al. (1994) that homothallic isolates of *P. sojae* are capable of outbreeding. Moreover, it has been found by Man in 't Veld et al. (1998) that even outbreeding (hybridization) can occur between the heterothallic *P. nicotianae* and the homothallic *P. cactorum*. Apparently, outbreeding by homothallic species is more common than previously assumed.

None of the isolates showed the homozygous state with regard to any of the alleles of *Ldh*-2, though outbreeding apparently occurs, as discussed above. Though *Ldh*-2 patterns in isolates from *Allium* differed from those in isolates from *Brassica*, only heterozygous patterns were observed.

It is very unlikely that the heterozygosity at *Ldh-2* remains in all isolates after outcrossing, suggesting that the heterozygosity at *Ldh-2* is probably fixed. This

hypothesis is supported by the observation of three alleles in CBS 179.87 and 686.95: if the alleles *Ldh*-2^{100} and *Ldh*-2^{114} were not fixed, only one of the two would have been present together with *Ldh*-2^{85}, instead of both.

Taking into account the differences in host specificity, the (slight) differences in morphology, the ITS sequence polymorphisms, the apparent lack of gene flow as shown by isozyme genotyping and the differences in mtDNA profiles observed previously (de Cock et al., 1992), we conclude that isolates of *P. porri* from *Brassica* are different from isolates from *Allium* and represent a distinct species, which we describe below.

Phytophthora brassicae *De Cock and Man in 't Veld, sp. nov. (Figures 5–7)*

Coloniae in agaro farina maydis confecto submersae vel parco mycelio aerio obtectae et pulverulentae. Hyphae primariae ad 7 μm latae, saepe convolutae. Intumescentiae hypharum in seminibus cannabis aqua submersis formatae, intercalares, saepe catenatae, nonnumquam hyphas radiantes proferentes, raro terminales, (sub-)globosae, 10–40(–53) μm diam.

Sporangia copiosa in agaro farina maydis confecto, submersa vel superficialia, singula vel proliferatione sympodiali aggregata, terminalia, raro lateralia vel intercalaria, globosa vel ovoidea, nonnumquam distorta, plerumque papilla lata et indistincta praedita, 25–48 × 40–74 μm, longitudo : latitudo = 1.4. Nonnulla sporangia sine negotio liberata, pedicello brevi praedita. Zoosporae 20 °C vel temperatura minore formatae.

Oogonia in agaro farina maydis vel suco V8 confecto solum paulo post isolationem formata, terminalia, plerumque ramulis lateralibus supportata, globosa, partem hyphae supportantis includentia, 30–41(–65) μm diam; paries extus levis sed intus undulatus, hyalinus vel dilute flavus, ad 2(–6) μm crassus. Antheridia singula vel bina, terminalia, diclina, plerumque amphigyna, raro paragyna, forma variabilia, 16–20 μm diam. Oosporae apleroticae, paries ad 2 μm crassus.

Temperatura minima crescentiae minus quam 3 °C, optima 21 °C, maxima 27 °C. Incrementum diurnum radiale in agaro farina maydis confecto 21 °C 6.4 mm, 24 °C 2.3 mm. Putredinem brunneam in *Brassica* praeservata provocans.

Typus CBS 179.87, in herb. CBS (ex vivux ex-type).

Colonies on cornmeal agar submerged or with some low aerial mycelium, no distinct growth pattern; on V8 with low aerial mycelium and powdery appearance. Main hyphae up to 7 μm wide, often coiled. Hyphal swellings present in hemp-seed/water cultures, intercalary, often in chains and/or provided with radiating hyphae, occasionally terminal, (sub-)globose, 10–40(–53) μm diam.

Sporangia abundantly produced in and on cornmeal agar as well as on hemp seeds in soil extract, developing singly or in sympodia, terminally, occasionally laterally attached or intercalary, globose to ovoid, sometimes distorted, with broad and indistinct papilla (in some other isolates none), without or with weak apical thickening, 25–48 × 40–74 μm, length to width ratio 1.4 (in other isolates 1.3–1.5). Part of the sporangia caducous, easily seceding upon preparation, with a short pedicel, consisting of a plug in the subtending hyphae. Zoospores developed below 20 °C.

Oogonia developed on cornmeal agar and V8 agar, only during a short period after isolation, only occasionally later, terminal, mostly on short side branches, globose including a part of the subtending hypha, 30–41 μm diam. (in some other isolates up to 65 μm); wall smooth, inner side of the wall undulate, colourless to pale yellow, up to 2 μm thick (in some other isolates also dark yellow and up to 6 μm thick). Antheridia 1–2 per oogonium, terminal, diclinous, mostly amphigynous, occasionally paragynous, variable in shape, 16–20 μm diam. Oospores aplerotic, wall up to 2 μm thick. Cardinal temperatures for growth: minimum below 3 °C, optimum 21 °C, maximum 27 °C. Daily radial growth on cornmeal agar at optimum temperature 6.4 mm, at 24 °C 2.3 mm. Pathogenicity: causing brown rot in stored cabbages.

Holotype: CBS 179.87, dried culture. Oogonia documented by this description and Figure 7. Type culture from which the holotype was derived: CBS 179.87 from *Brassica oleracea*, The Netherlands. Paratype cultures: CBS 212.82, CBS 180.87 (both from *B. oleracea*, The Netherlands) and CBS 178.87 (from *B. oleracea*, Germany).

Phytophthora brassicae is characterized by low cardinal temperatures for growth, coiled hyphae, and semi- to non-papillate sporangia. It is morphologically similar to and closely related with *P. porri*, but can be distinguished by the abundant sporangium production on agar, the appressed aerial mycelium on V8, and rapid decrease of oogonium production after isolation. More distinct and consistent differences, however, are found in ITS sequences and isozyme patterns (present study) or

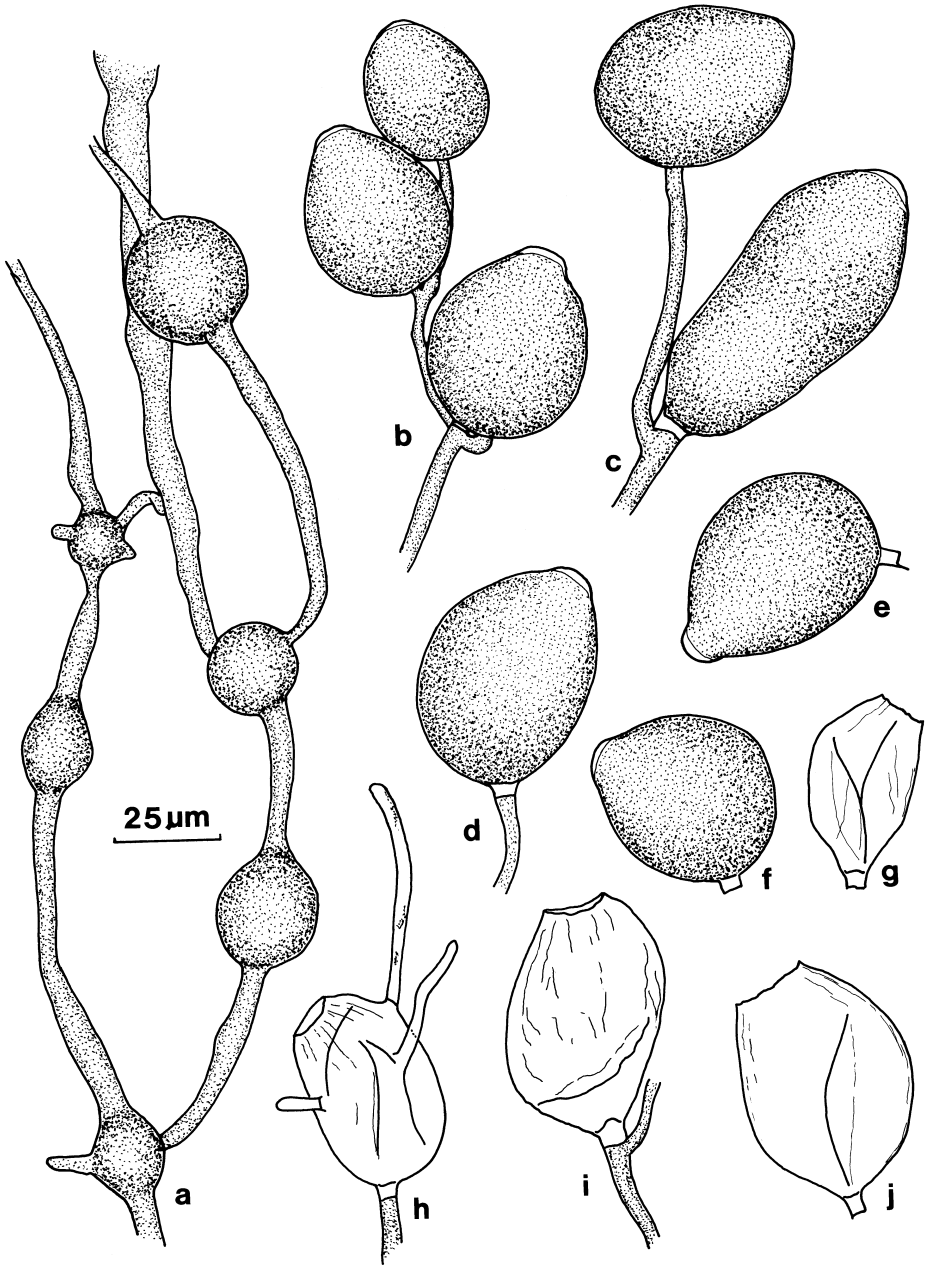

Figure 6. P. brassicae. a. Hyphal swellings. b–j. Sporangia: (b, c), sympodially arranged sporangia; (e–g, j), caducous sporangia with short pedicel; (g–j), empty sporangia after release of zoospores, showing wide opening.

mitochochondrial DNA profiles (de Cock et al., 1992).

Other *Phytophthora* species with semi-papillate sporangia and low cardinal temperatures are *P. infestans*, *P. mirabilis*, *P. phaseoli*, *P. hibernalis*, *P. ilicis*, *P. primulae*, and *P. syringae*. The former three, *P. infestans*, *P. mirabilis*, and *P. phaseoli* differ from *P. brassicae* by producing compound sympodial sporangiophores with basal swellings and no hyphal swellings or chlamydospores; in addition to

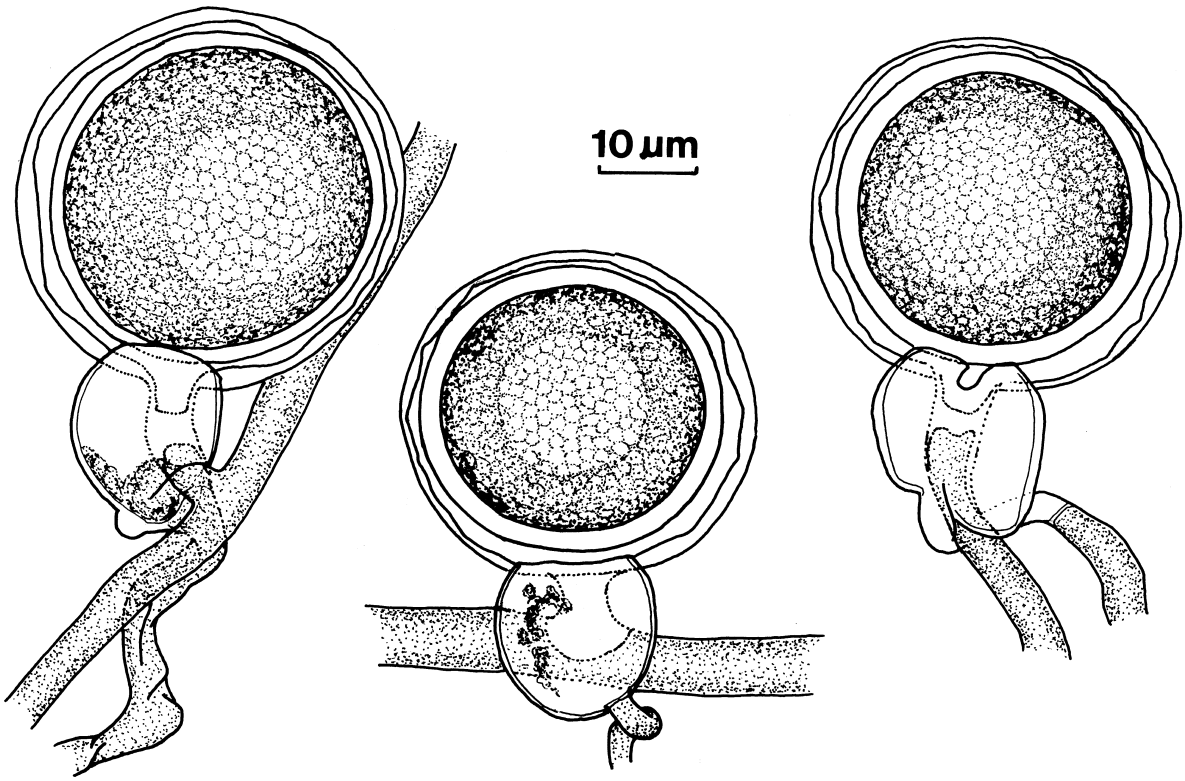

Figure 7. P. brassicae. Oogonia with amphigynous antheridia.

the morphological differences, restriction patterns of mitochondrial DNA of these species are quite different from those of *P. brassicae* (De Cock et al., 1992; Möller et al., 1993). *P. hibernalis* differs by its characteristic obovoid sporangia, which are caducous with a long pedicel; *P. ilicis* by its small, caducous sporangia with medium length pedicel, abundant oogonia, absence of hyphal swellings and chlamydospores and very slow growth. *P. primulae* differs by often distorted sporangia, frequent oogonium production and paragynous antheridia; *P. syringae* by small chlamydospores and small, paragynous antheridia with tangled hyphae below.

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References

Bakkeren G, Kronstad JW and Lévesque CA (2000) Comparison of AFLP fingerprints and ITS sequences as phylogenetic markers in Ustilaginomycetes. Mycologia 92: 510–521

Brasier CM, Cooke DEL and Duncan JM (1999) Origin of a new *Phytophthora* pathogen through interspecific hybridization. Proceedings of the National Academy of Sciences USA 96: 5878–5883

De Cock WAM, Neuvel A, Bahnweg G, de Cock JCJM and Prell HH (1992) A comparison of morphology, pathogenicity and restriction fragment patterns of mitochondrial DNA among isolates of *Phytophthora porri* Foister. Netherlands Journal of Plant Pathathology 98: 277–289

Cooke DEL and Duncan JM (1997) Phylogenetic analysis of *Phytophthora* species based on ITS 1 and ITS 2 sequences of the ribosomal RNA gene repeat. Mycological Research 101: 667–677

Cooke DEL, Jung T, Williams NA, Schubert R, Bahnweg G, Osswald W and Duncan JM (1999) Molecular evidence supports *Phytophthora quercina* as a distinct species. Mycological Research 103: 799–804

Cooke DEL, Drenth A, Duncan JM, Wagels G and Brasier CM (2000) A molecular phylogeny of *Phytophthora* and related Oomycetes. Fungal Genetics and Biology 30: 17–32

Erwin DC and Ribeiro OK 1996. *Phytophthora* Diseases Worldwide. APS Press: Minnesota

Foister CE (1931) The white tip disease of leeks and its causal fungus, *Phytophthora porri* n.sp. Transactions of the Botanical Society of Edinburgh 30: 257–281

Gams W, Hoekstra ES and Aptroot A (eds) (1998) CBS Course of Mycology, 4th edn. Centraalbureau voor Schimmelcultures, Baarn/Delft

Goodwin SB, Spielman LJ, Matuszak JM, Bergeron SN and Fry WE (1992) Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in Northern and Central Mexico. Phytopathology 82: 955–961

Goodwin SB, Legard DE, Smart CD, Levy M and Fry WE (1999) Gene flow analysis of molecular markers confirms that *Phytophthora mirabilis* and *Phytophthora infestans* are separate species. Mycologia 91: 796–810

Goodwin SB, Webster RK and Allard RW (1994) Evidence for mutation and migration as sources of genetic variation in populations of *Rhynchosporium secalis*. Phytopathology 84: 1047–1053

Hansen EM (1991) Variation in the species of the *Phytophthora megasperma* complex. In: Lucas JA, Shattock RC, Shaw DS, Cooke LR (eds) Phytophthora (p 157) Cambridge University Press, Cambridge

Ilieva E, Man in 't Veld WA, Veenbaas-Rijks W and Pieters R (1998) *Phytophthora multivesiculata*, a new species causing rot in *Cymbidium*. European Journal of Plant Pathology 104: 677–684

Jung T, Cooke DEL, Blaschke H, Duncan JM, Osswald W (1999) *Phytophthora quercina* sp. nov., causing rot of European oaks. Mycological Research 103: 785–798

Von Maltitz PA and von Broembsen SL (1984) *Phytophthora porri* on onions in South Africa. Plant Disease 68: 732

Man in 't Veld WA, Veenbaas-Rijks WJ, Ilieva E, de Cock AWAM, Bonants PJM and Pieters R (1998) Natural hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* demonstrated by isozyme analysis and Random Amplified Polymorphic DNA. Phytopathology 88: 922–929

Mchau GRA and Coffey MD (1994) Isozyme diversity in *Phytophthora palmivora*: evidence for a southeast Asian centre of origin. Mycological Research 98: 1035–1043

Möller EM, Bahnweg G, Sandermann H and Geiger HH (1992) A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Research 20: 6115–6116

Möller EM, de Cock AWAM and Prell HH (1993) Mitochondrial and nuclear DNA restriction enzyme analysis of the closely related *Phytophthora* species *P. infestans*, *P. mirabilis* and *P. phaseoli*. Journal of Phytopathology 139: 309–321

Oudemans P and Coffey MD (1991) A revised systematics of twelve papillate *Phytophthora* species based on isozyme analysis. Mycological Research 95: 1025–1046

Richardson BJ, Baverstock PR and Adams M (1986) Electrophoresis. In: Allozyme Electrophoresis (p 23) Academic Press, New York

Roux P and Labarère J (1991) Determination of genes and subunit composition of three isozyme activities in *Agaricus bitorquis*. Mycological Research 95: 851–860

Spielman LJ, Sweigadr JA, Shattock RC and Fry WE (1990) The genetics of *Phytophthora infestans*: Segregation of allozyme markers in F2 and backcross progeny and the inheritance of virulence against potato resistance R2 and R4 in F1 progeny. Experimental Mycology 14: 57–69

Stamps DJ, Waterhouse GM, Newhook FJ and Hall GS (1990) Revised tabular key to the species of *Phytophthora*, 2nd edn. Mycological Papers 162

Werres S, Marwitz R, Man in 't Veld WA, de Cock AWAM, Bonants PJM, de Weerdt M, Themann K, Ilieva E and Baayen RP (2001) Phytophthora ramorum sp. nov., a new pathogen on *Rhododendron* and *Viburnum*. Mycological Research 105(10): 1155–1165

Whisson S, Drenth A, Maclean DJ and Irwin JAG (1994) Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. Current Genetics 27: 77–82