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Gene flow analysis demonstrates that *Phytophthora fragariae* var. *rubi* constitutes a distinct species, *Phytophthora rubi* comb. nov.

Willem A. Man in 't Veld

Plant Protection Service, Department of Mycology, P.O. Box 9102, 6700 HC Wageningen, the Netherlands

Abstract: Isozyme analysis and cytochrome oxidase sequences were used to examine whether differentiation of *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* at the variety level is justified. In isozyme studies six strains of both *P. fragariae* varieties were analyzed with malate dehydrogenase (MDH), glucose phosphate isomerase (GPI), aconitase (ACO), isocitrate dehydrogenase (IDH) and phosphogluconate dehydrogenase (PGD), comprising altogether seven putative loci. Five unique alleles (*Mdh-1*^{A}, *Mdh-2*^{B}, *Gpi*^{A}, *Aco*^{B} and *Idh-1*^{B}) were found in strains of *P. fragariae* var. *fragariae*, whereas five unique alleles (*Mdh-1*^{B}, *Mdh-2*^{A}, *Gpi*^{B}, *Aco*^{A} and *Idh-1*^{A}) were present in strains of *P. fragariae* var. *rubi*. It was inferred from these data that there is no gene flow between the two *P. fragariae* varieties. Cytochrome oxidase I (*Cox I*) sequences showed consistent differences at 15 positions between strains of *Fragaria* and *Rubus* respectively. Based on isozyme data, cytochrome oxidase I sequences, and previously published differences in restyriction enzyme patterns of mitochondrial DNA, sequences of nuclear and mitochondrial genes, AFLP patterns and pathogenicity, it was concluded that both specific pathogenic varieties of *P. fragariae* are reproductively isolated and constitute a distinct species. Consequently strains isolated from *Rubus idaeus* are assigned to *Phytophthora rubi* comb. nov.

Key words: Cytochrome oxidase I, isozymes, *Phytophthora rubi* comb. nov.

INTRODUCTION

Phytophthora fragariae Hickman originally was described as a pathogen of strawberry (*Fragaria* x *ananassa*), causing red core root rot (Hickman 1940). Being homothallic, this species produces oospores autonomously that can rest in the soil up to 15 y without losing the ability to germinate and to infect strawberry plants. *P. fragariae* is assigned a quarantine status in EU countries on which a ''nil tolerance''is placed. Later morphologically similar isolates were obtained from *Rubus idaeus* (raspberry) and described as *P. fragariae* var. *rubi* (Wilcox et al 1993). These isolates were highly pathogenic to raspberry, but caused only small amounts of necrosis in strawberry roots. The typical symptoms of red core root rot could not be produced in strawberry by isolates obtained from raspberry in pathogenicity experiments. Several molecular studies were done to define the delimitation of both varieties. Total protein patterns obtained with SDS-PAGE of the raspberry isolates were almost identical with those of *P. fragariae* (Duncan et al 1991). Restriction enzyme analysis of mitochondrial DNA (Förster et al 1992, StammLer et al 1993) with seven restriction enzymes showed no difference between strains from strawberry and strains from raspberry, whereas seven other restriction enzymes revealed fragment length differences between the two populations. It was inferred from these data that mtDNA of the two populations have a similarity value of 55%, indicating that they probably originate from a common ancestor. It was concluded that the strains from strawberry and the strains from raspberry represent two different homogeneous groups which however are closely related. Förster et al (1992) suggested that, considering the intraspecific diversity of *P. capsici*, *P. citricola*, *P. citrophthora* and *P. megakarya*, the strains from strawberry and the strains from raspberry might represent the genetic diversity of one single morphological species. Wilcox et al (1993) therefore decided to assign the raspberry isolates to *P. fragariae*. However it was also their opinion that there were sufficient differences between the two populations to justify separation at the variety level. It was noted by Wilcox et al (1993) that ''should reproductive isolation be demonstrated between the raspberry and the strawberry forms of *P. fragariae* in future studies, it may be necessary to recognize them as separate biological species''. Based on the high sequence homology of the ITS regions of the ribosomal DNA gene repeat of the two *P. fragariae* varieties, Cooke and Duncan (1997) concluded that taxonomical separation at the variety level was justified.

The purpose of this paper was to test whether *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* indeed are reproductively isolated by genetic barriers to gene flow by subjecting strains of both varieties to isozyme analysis. In addition cytochrome oxidase I sequences of some strains were determined.

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[1] Corresponding author. E-mail: w.a.man.in.t.veld@minlnv.nl

MATERIALS AND METHODS

Isozyme analysis. Cultivation of mycelium.—Isolates of all *Phytophthora* spp. were grown in 50 mL of tryptone soy broth medium (TSB) in 250 mL Erlenmeyer flasks on a rotary shaker at 40 rpm in the dark. Each flask had been inoculated with three V8 agar disks (5 mm diam) with mycelium, taken from activily growing colony margins of cultures (3 d old). Cultures were incubated at 23 C. After 7 d the mycelium of each isolate was collected by sieving, after which the tissue was dried by pressing between filter paper. The mycelium was stored overnight at –80 C before extraction of enzymes.

Enzyme extraction.—Frozen mycelium was thawed at 4 C for 3 h before enzyme extraction. Routinely about 0.5 g of mycelium was ground in a chilled mortar with sand and 70 µL of extraction medium. The extraction medium consisted of 0.1 M Tris-HCl (pH 7.0), 1 mM dithiothreitol, 50 mM ethylene diamine tetra-acetic acid (EDTA, disodium salt, M.W.: 372.2), 10% polyvinyl pyrrolidone (PVP, M.W.: 25.000) w/v, 50 µg/mL soybean trypsin inhibitor, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) and 5% glycerol (v/v). All mycelia were ground for 3 min. The homogenate was transferred to Eppendorf tubes and centrifuged 10 min at 14 000 rpm (4 C). The supernatant (40–80 µL) was collected and stored at –80 C before use.

Electrophoresis and enzyme staining.—Isozymes were separated by electrophoresis with the automated PhastSystem of Amersham Pharmacia Biotech (Roosendaal, the Netherlands). Crude extracts, obtained as described before, were loaded on native polyacrylamide gels and subsequently electrophoresis was performed at 4 C for about 40 min. Bromphenol blue marker dye was added to the samples to monitor the progress of electrophoresis. The gels were made with 0.11 M Tris-acetate buffer (pH 6.4). The running buffer, contained in 2% agarose gel, consisted of a 0.25 M Tris and 0.88 M L-alanine buffer (pH 8.8). At completion of electrophoresis, gels were immersed in freshly prepared staining solutions in the dark at 37 C. Five enzymatic stains gave clearly interpretable bands, notably those for aconitase (ACO, EC 4.2.1.3), glucose-phosphate isomerase (GPI, EC 5.3.1.9), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37) and 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44). The reaction ingredients for each enzyme were as follows (between brackets the geltype used in the assay):

ACO contained 25 mL of 0,2 M Tris-HCl pH 8.0, 50 mg of cis-aconitic acid (Sigma A 3412), 100 mg of $MgCl_2$, 6 units of isocitrate dehydrogenase (Sigma I 2002), 12.5 mg of β-nicotinamide adenine dinucleotide phosphate (NADP), 7.5 mg of nitro blue tetrazolium (NBT) (Sigma N 6876) and 1 mg of phenazine metho sulfate (PMS) (Sigma P 9625). (10–15% gradient gel)

GPI contained 25 mL of 0.1 M Tris-HCl pH 8.0, 20 mg of fructose-6-phosphate, 50 mg of $MgCl_2$, 10 units of G6PDH (Sigma G 5760), 12.5 mg of β-nicotinamide adenine dinucleotide (NAD), 7.5 mg of NBT and 1 mg of PMS. (12.5% homogeneous gel)

IDH contained 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 NADP, 7.5 mg of NBT and 1 mg of PMS. (8–25% gradient gel)

MDH: 25 mL 0.2 M Tris-HCl pH 8.0, 440 mg L-malic acid (disodium salt), 12.5 mg NAD, 7.5 mg NBT, 1 mg PMS. (8–25% gradient gel)

PGD contained 25 mL of 0.1 M Tris-HCl pH 8.0, 10 mg of 6-phosphogluconic acid (trisodium salt) 50 mg of $MgCl_2$, 10 mg of NADP, 7.5 mg of NBT and 1 mg of PMS. (10–15% gradient gel)

Isozyme activity was recorded based on the relative mobility of the banding patterns. Each band was considered to be an allele belonging to a specific locus, and alleles were numbered alphabetically according to their relative mobilities. Because the genus *Phytophthora* is diploid, two identical letters were assigned to one band. When two zones of activity were present on the gel, the slowest zone was assigned the first locus and the fastest zone the second locus. It is known from isozyme analysis of several *Phytophthora* species that malate dehydrogenase and isocitrate dehydrogenase generate two loci (Oudemans and Coffey 1991b).

Sequence analysis of cytochrome oxidase I (Cox I).—Sequence analysis of *Cox I* was performed with COXF4N and COXR4N primers according to the protocol of Kroon et al (2004). One strain of *P. fragariae* var. *fragariae* (P965-DQ674735) and two strains of *P. fragariae* var. *rubi* (R49-DQ674736 and P1282-DQ674737) were sequenced and their sequences were aligned with sequences in GenBank . Sequences were edited with Seqman 4.05 (DNASTAR, Madison, Wisconsin). MegAlign (DNASTAR) was used to perform the final alignment.

RESULTS

Altogether five enzyme systems, which comprised seven putative loci, were used to monitor gene flow in *Phytophthora fragariae sensu lato* (TABLE I). All enzymes generated clear bands except at *Mdh-1*, which showed a smeary appearance in three strains (TABLE I). Two isozyme loci, notably *IDH-2* and *PGD*, were monomorphic for all isolates in this study. Five other loci, notably *ACO*, *GPI* (FIG. 1), *IDH-1*, *MDH-1* and *MDH-2* (FIG. 2), were polymorphic and resolved the population into two groups: one comprised the *P. fragariae* isolates from *Fragaria* and the second those from *Rubus idaeus*.

Sequences of *Cox I*, aligned with sequences in GenBank, showed differences at 15 positions between strains ex *Fragaria* and ex *Rubus idaeus*, supporting the division in two groups as determined by isozyme analysis. Phylogenetic analysis based on *Cox I* sequences revealed that, although closely related, strains ex *Fragaria* clustered together in one branch whereas strains ex *Rubus idaeus* grouped in a separate branch (FIG. 3).

TABLE I. Features of *Phytophthora* spp. used in this study, year of isolation, origin and isozyme alleles

Isolates	Year	Origin	Isozyme loci						
			Mdh-1	*Mdh-2*	*Gpi*	*Aco*	*Idh-1*	*Idh-2*	*Pgd*
ex *Fragaria* x *ananassa*									
FVF 9 [1]	1968	England	smear	BB	AA	BB	BB	AA	AA
FVF 50	1982	Scotland	smear	BB	AA	BB	BB	AA	AA
P965 [2]	1993	Scotland	AA	BB	AA	BB	BB	AA	AA
P1284	1945	England	AA	BB	AA	BB	BB	AA	AA
P1285	1979	Scotland	AA	BB	AA	BB	BB	AA	AA
P1286	1982	Scotland	AA	BB	AA	BB	BB	AA	AA
ex *Rubus idaeus*									
P823	1991	Scotland	BB	AA	BB	AA	AA	AA	AA
P1281	1985	England	BB	AA	BB	AA	AA	AA	AA
P1282	1985	Scotland	BB	AA	BB	AA	AA	AA	AA
P1283	1989	Norway	BB	AA	BB	AA	AA	AA	AA
PD 94/961 [3]	1994	Scotland	smear	AA	BB	AA	AA	AA	AA
R49 [1] (Type sp.)	1985	Scotland	BB	AA	BB	AA	AA	AA	AA

[1] Scottish Crop Research Institute, Invergowrie, Dundee, UK.
[2] Forest Research Station, Alice Holt Lodge, Farnhem, Surrey, UK.
[3] Plant protection Service, Wageningen, the Netherlands.

DISCUSSION

As noted by Duncan et al (1989) pathogenicity per se is not a valid criterion for separating taxa at the species level. Because at that time *P. fragariae* strains from *Fragaria* and *Rubus* only seemed to differ in pathogenicity, strains from *Rubus* were assigned the subordinate rank of variety *rubi* (Wilcox et al 1993). If however, in spite of almost similar morphology and identical ITS sequences, *P. fragariae* strains from strawberry and those from raspberry do not share a common gene pool and hence apparently are reproductively isolated by genetic barriers to gene flow, separating the two populations at the species level is justified (Wilcox et al 1993). It is clear from this study that *P. fragariae* strains from strawberry as well as those from raspberry each contain several unique isozyme alleles, indicating that they indeed are isolated reproductively. In Southern hybridization the probes pPS2 and pPS42 generated different hybridization patterns in both *P. fragariae* varieties, whereas probe pPS39 hybridized exclusively with total DNA from strains isolated from *Rubus* (including R49, which also is analysed in the present study) (StammLer et al 1993). Molecular analysis based on AFLP patterns of both varieties of *P. fragariae* (including P965 and P823, which also are analysed in the present study) also indicated that both varieties represent different genetic entities (Brasier et al 1999). Moreover both taxa contained different *Cox I* sequences as well (FIG. 3). In addition sequence analysis of four nuclear genes (*ASF*-like, *GPA*1, *Ras-Ypt* and *TRP*1) and two

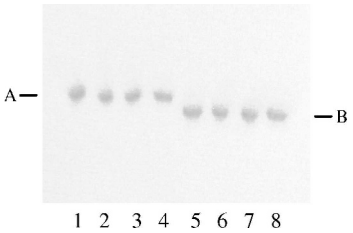

FIG. 1. Glucose phosphate isomerase isozymes generated by *Phytophthora fragariae* (lanes 1–4) and *Phytophthora rubi* (lanes 5–8):

1: P965 5: P1283
2: P1286 6: P1282
3: P1285 7: P1281
4: P1284 8: P823

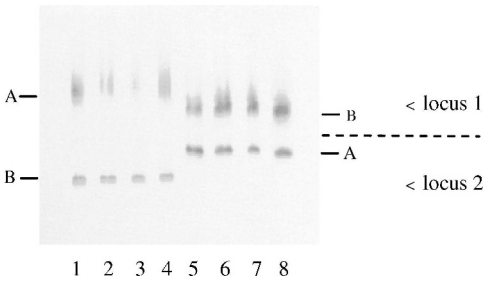

FIG. 2. Isozyme patterns of malate dehydrogenase generated by *Phytophthora fragariae* (lanes 1–4) and *Phytophthora rubi* (lanes 5–8):

1: P965 5: P1283
2: P1286 6: P1282
3: P1285 7: P1281
4: P1284 8: P823

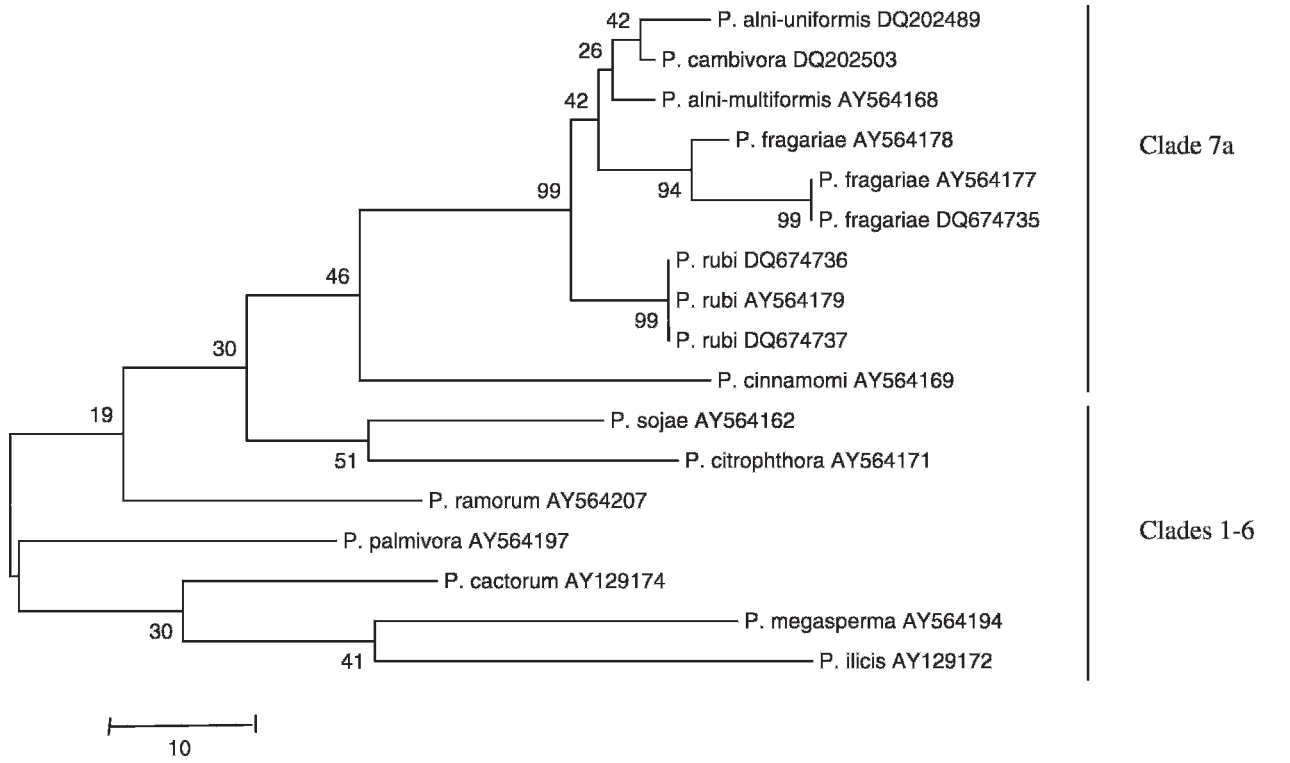

FIG. 3. Phylogenetic tree, calculated using bootstrap Maximum Parsimony, based on the cytochrome oxidase I (*Cox I*) gene of *Phytophthora rubi* and related species. Numbers at the branch points indicate the percentages of bootstrap values, based on 1000 replicates. Scale bar indicates 10 nucleotides.

mitochondrial genes (*Nadh* and *Cox II*) showed that in all cases both taxa contained different sequences (Martin and Tooley 2003, Ioos et al 2006). Hence it is concluded that it is justified to raise the rank of *P. fragariae* var. *rubi* to the species level for which the name *P. rubi* is proposed.

The question whether both populations are reproductively isolated by pre- or post mating barriers awaits an answer. Attempts to infect each other's host hitherto have been unsuccesful, and this lack of success in itself indicates a strong premating reproductive isolating mechanism. Restriction enzyme analysis of mitochondrial DNA revealed that both populations have homogeneous but different DNA profiles with a similarity level of ~55% (Förster et al 1992) indicating that it is unlikely that reticulation has taken place recently. The sequences of *Cox II* differed also between strains from *Fragaria* and *Rubus* (Martin and Tooley 2003) and restriction enzyme analysis of a region spanning the *Cox I* and *Cox II* genes could distinguish isolates from *Fragaria* and *Rubus* as well (Martin and Tooley 2004), thus supporting the results of isozyme analysis. Sequence analysis of *Cox I* showed variation in strains ex *Fragaria* as well as in strains ex *Rubus idaeus*, indicating that both taxa are not clonal.

A third variety is reported by Wang and Lu (1978), *P. fragariae* var. *oryzobladis* Wang & Lu. According to Ho and Jong (1998) the type strain no longer is available for examination. In contrast to strains isolated from *Fragaria* and *Rubus*, this variety is characterized by rapid growth and the abundance of chlamydospores. The assignment to *P. fragariae* by Wang and Lu (1978), according to Ho and Jong (1998), is not justified at all by morphological data. Therefore *P. fragariae* var. *oryzobladis* has no affinity with *P. fragariae* at all and hence varieties of *P. fragariae* automatically cease to exist.

A combination of isozyme analysis and mitochondrial DNA analysis has been used before to separate taxa with high morphological similarity. Based on host range, nutritional, biochemical and morphological criteria, it was proposed by Galindo and Hohl (1985) to name strains from *Mirabilis jalapa* as a variety of *P. infestans*. The sequences of the ITS regions of the ribosomal DNA gene repeat were identical between the two varieties, but small yet distinct differences were found in mtDNA restriction profiles. Möller et al (1993) therefore proposed assigning the isolates obtained from *Mirabilis jalapa* to *P. infestans* forma specialis *mirabilis*. Goodwin (1999) however concluded that, based on isozyme analysis and mitochondrial haplotypes, isolates obtained

from *Mirabilis jalapa* and *P. infestans sensu stricto* were reproductively isolated by genetic barriers to gene flow, and hence he proposed to assign isolates obtained from *Mirabilis jalapa* to be *P. mirabilis*. Isozyme analysis and mitochondrial DNA analysis also were used to demonstrate that *P. porri*-like strains isolated from *Brassica* constitute a distinct species: *P. brassicae* (Man in 't Veld et al 2002). Isozymes, separated by electrophoresis under native conditions, are well defined by their mobility and identity. Isozyme analysis is one of the oldest techniques used to characterize *Phytophthora* species genetically. Due to rapid developments of DNA-related techniques in the past decade, isozyme analysis is not widely used anymore. However its potential in studying the population structure (Oudemans and Coffey 1991a, b), delineating species (Goodwin et al 1999, Man in 't Veld et al 2002) and, by using dimeric enzymes, detecting crossings and hybrids (Man in 't Veld et al 1998, Brasier et al 2004) will remain valuable in the future.

TAXONOMY

Phytophthora rubi (Wilcox & Duncan) Man in 't Veld, comb. nov.

Basionym *Phytophthora fragariae* var. *rubi* Wilcox & Duncan, Mycological Research 97(7):830. 1993.

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