Natural Hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* Demonstrated by Isozyme Analysis and Random Amplified Polymorphic DNA

Willem A. Man in 't Veld, Wil J. Veenbaas-Rijks, Elena Ilieva,
Arthur W. A. M. de Cock, Peter J. M. Bonants, and Rob Pieters

First, second, third, and sixth authors: Plant Protection Service, Department of Mycology, P.O. Box 9102, NL 6700 HC Wageningen, the Netherlands; third author: Plant Protection Institute, Kostinbröd 2230, Sofia, Bulgaria; fourth author: Centraalbureau voor Schimmelcultures, Yeast Division, Julianalaan 67, NL 2628 BC Delft, the Netherlands; and fifth author: Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW Wageningen, the Netherlands.

Accepted for publication 4 June 1998.

ABSTRACT

Man in 't Veld, W. A., Veenbaas-Rijks, W. J., Ilieva, E., de Cock, A. W. A. M., Bonants, P. J. M., and Pieters, R. 1998. Natural hybrids of *Phytophthora nicotianae* and *P. cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. *Phytopathology* 88:922-929.

Three similar isolates of *Phytophthora* (*Phytophthora* sp-h) were obtained from diseased *Spathiphyllum* and *Primula* plants. Cultural characteristics did not fit any known description of *Phytophthora* species. The *Phytophthora* sp-h isolates are papillate, are homothallic, possess 80 to 86% amphigynous antheridia, and have a maximum temperature for growth of 36.5°C. Isozyme analysis of the *Phytophthora* sp-h isolates revealed a three-banded pattern with malic enzyme and a three-banded pattern with malate dehydrogenase on the second putative locus. The fastest band at both enzyme loci comigrated with the single *P. nicotianae* band, the slowest band comigrated with the single *P. cactorum* (and also *P. pseudotsugae*)

band, and one band in between was concluded to represent the heterodimeric isozyme. The random amplified polymorphic DNA patterns of the *Phytophthora* sp-h isolates almost exclusively consisted of bands that were also present in either *P. nicotianae* or *P. cactorum*. Southern hybridization showed that bands specific for *P. nicotianae* were present as comigrating bands in the *Phytophthora* sp-h isolates. The same was found for species-specific bands of *P. cactorum*. It is concluded that the three *Phytophthora* sp-h isolates represent interspecific hybrids, *P. nicotianae* being the one parent and *P. cactorum* the other. Analysis of mitochondrial DNA with restriction enzymes revealed banding patterns in all the *Phytophthora* sp-h isolates identical with those of *P. nicotianae*, confirming that indeed *P. nicotianae* was one of the parents.

Additional keywords: morphology.

The genus *Phytophthora* currently consists of more than 50 recognized species (25). These species can be divided into six main groups based on the morphology of sporangia and the attachment of the antheridium (25,28). Groups I and II are composed of the species with papillate sporangia, and groups III and IV are composed of the semipapillate species; whereas species belonging to groups V and VI have no papilla. The attachment of the antheridia can be paragynous (groups I, III, and V) or amphigynous (groups II, IV, and VI).

One of the activities of the Dutch Plant Protection Service is the monitoring of plant-pathogenic fungi like *Phytophthora* species. The identification of *Phytophthora* species is notoriously difficult, due to the paucity of suitable morphological characters. A number of biochemical techniques are available to help in the identification process (e.g., random amplified polymorphic DNA [RAPD], ribosomal DNA internal transcribed spacer region [ITS], and mitochondrial [mt] DNA). We used isozyme analysis to confirm the outcome of microscopical examination of the morphological structures. Many alleles of several isozyme loci in the genus *Phytophthora* are known from the literature (19,20). Moreover, the advantage of isozyme analysis over other techniques is that, in employing dimeric enzymes, it is possible to distinguish physical mixtures from crossings; only in the latter case, heterodimeric bands are present (10).

The question of naturally occurring, interspecific hybrids of *Phytophthora* has been scarcely addressed in the literature (2,3). Probably because of their presumed atypical appearance, they are hard to detect and, moreover, they may be rare (2). Nevertheless, the question of hybridization in *Phytophthora* is important, since hybridization may be responsible for the introduction in nature of new variants in pathogen populations.

We have obtained three similar *Phytophthora* isolates (*Phytophthora* sp-h) from diseased *Spathiphyllum* and *Primula* plants grown in hydroponic systems. These systems facilitate physical encounters between different *Phytophthora* species, since in many greenhouses different crops are serviced by the same hydroponic source.

All three isolates have papillate sporangia, are homothallic, possess mainly amphigynous antheridia, and have a maximum growing temperature of 36.5°C. Using the tabular key of Stamps et al. (25), they could not be assigned to a known *Phytophthora* species unambiguously.

Diseases caused by *Phytophthora* species are common in ornamentals grown in greenhouses in the Netherlands. In the last decades, 21 different *Phytophthora* species have been recorded altogether; two species were observed on *Spathiphyllum* (*P. drechsleri* and *P. nicotianae*) and three on *Primula* (*P. nicotianae*, *P. palmivora*, and *P. primulae*). Generally, these *Phytophthora* species cause severe root rot and blackening of stems of the host. The three homothallic *Phytophthora* sp-h isolates were obtained from diseased *Spathiphyllum* and *Primula* plants grown in hydroponic systems.

Symptoms caused by these isolates were identical to those caused by the *Phytophthora* species previously mentioned. In all three cases of *Phytophthora* sp-h, oospores were observed in the stems

Corresponding author: W. A. Man in 't Veld
E-mail address: w.a.man.in.'t.veld@pd.agro.nl

of all diseased plants. So far, observations from growers gave no indication that these isolates showed increased aggressiveness towards their respective hosts.

Preliminary experiments using isozyme analysis indicated that the three isolates of *Phytophthora* sp-h might possibly represent hybrids of *P. nicotianae* van Breda de Haan and *P. cactorum* (Lebert & Cohn) J. Schröter.

The purpose of this paper was to test the hypothesis that the three *Phytophthora* sp-h isolates concerned were hybrids, with *P. nicotianae* and *P. cactorum* as presumed parental species, by performing a comparative study of morphology, isozyme patterns, and Southern analysis of RAPD of most species of groups I and II.

MATERIALS AND METHODS

Fungal isolates and study of morphology. Fungal isolates and their sources are listed in Table 1. Cultures were maintained on cornmeal-agar slants. Isolates were cultivated on V8 agar at 20°C in the dark to study the morphological features (8). The formation of sporangia was stimulated by growing the mycelium, taken from the margin of V8 cultures, in the vicinity of pepper seeds in pond water. The dimensions of oogonia, oospores, and sporangia were measured. For all characteristics studied, at least 25 measurements were made for each isolate, and the average value was calculated. The attachment of the antheridia was only scored in clear situations.

The maximum growing temperature was determined by incubating the colonies at different temperatures (31 to 37°C) with intervals of 0.5°C. The highest temperature at which growth occurred was considered to be the maximum growing temperature.

To be certain that the *Phytophthora* sp-h isolates do not represent a physical mixture of species, we established five strains by isolating zoospores from isolate PD 93/1339. Reisolates from artificially infected *Spathiphyllum* plants using PD 93/1339 and PD 94/1166 were labeled PD 93/1339-R' and PD 94/1166-R', respectively.

Isozyme analysis. *Cultivation of mycelium.* Isolates of all *Phytophthora* spp. were grown in 50 ml of tryptone soy broth medium in 250-ml Erlenmeyer flasks on a rotary shaker at 40 rpm in the dark. Each flask had been inoculated with three V8-agar discs (diameter 5 mm) with mycelium taken from actively growing colony margins of cultures (3 days old). Cultures were incubated at 23°C. After 7 days, 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 -20°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 EDTA, 10% polyvinylpyrrolidone (wt/vol), 50 µg of soybean trypsin inhibitor per ml, 0.1 mM phenylmethylsulfonyl fluoride, and 5% glycerol (vol/vol). All mycelia were ground for 3 min. The homogenate was transferred to Eppendorf tubes and centrifuged for 10 min at 16,000 × g (4°C). The supernatant (40 to 80 µl) was collected and stored at -70°C before use.

Electrophoresis and enzyme staining. Electrophoresis was carried out at 4°C using the automated PhastSystem of Pharmacia (Roosendaal, the Netherlands). Crude extracts, obtained as described before, were routinely analyzed for malic enzyme (MDHP; EC 1.1.1.40)

TABLE 1. Isolates of *Phytophthora* used in this study, their origins (host plant and location), reference numbers, and biochemical methods of analysis

<i>Phytophthora</i> species	Group	Host plant	Location	Isolate number ^a	Methods of analysis ^b		
					Isozymes	RAPD ^c	mtDNA ^d
<i>P. cactorum</i>	I	Cactus	Unknown	CBS 108.09	—	+	+
<i>P. cactorum</i>	I	<i>Prunus armeniaca</i>	India	CBS 279.37	—	—	+
<i>P. cactorum</i>	I	<i>Rubus idaeus</i>	New York (USA)	P6183	+	—	+
<i>P. cactorum</i>	I	<i>Panax quinquefolius</i>	Wisconsin (USA)	P1943	+	—	—
<i>P. cactorum</i>	I	<i>Fragaria × ananassa</i>	New York (USA)	P6187	+	+	+
<i>P. clandestina</i>	I	<i>Trifolium subterraneum</i>	Victoria (Australia)	CBS 347.86	—	+	—
<i>P. idaei</i>	I	<i>Rubus idaeus</i>	Great Britain	CBS 971.95*	+	+	—
<i>P. iranica</i>	I	<i>Solanum melongena</i>	Iran	CBS 374.72*	+	+	—
<i>P. pseudotsugae</i>	I	<i>Pseudotsuga menziesii</i>	Oregon (USA)	CBS 444.84*	+	+	—
<i>P. tentaculata</i>	I	<i>Chrysanthemum frutescens</i>	Germany	CBS 412.96	—	+	—
<i>P. boehmeriae</i>	II	<i>Boehmeria nivea</i>	Japan	CBS 291.29	—	+	—
<i>P. botryosa</i>	II	<i>Hevea brasiliensis</i>	Malaysia	CBS 581.69	—	+	—
<i>P. capsici</i>	II	<i>Capsicum annuum</i>	New Mexico (USA)	CBS 128.23*	—	+	—
<i>P. citrophthora</i>	II	<i>Citrus</i> sp.	California (USA)	CBS 950.87	—	+	—
<i>P. heveae</i>	II	<i>Hevea brasiliensis</i>	Malaysia	CBS 296.29	—	+	—
<i>P. infestans</i>	II	<i>Solanum tuberosum</i>	The Netherlands	CBS 366.51	—	+	—
<i>P. katsurae</i>	II	Soil	Taiwan	CBS 587.85	—	+	—
<i>P. meadii</i>	II	<i>Hevea brasiliensis</i>	India	CBS 219.88	—	+	—
<i>P. megakarya</i>	II	<i>Theobroma cacao</i>	Cameroon	CBS 238.83	—	+	—
<i>P. nicotianae</i>	II	<i>Nicotiana tabacum</i>	Puerto Rico	CBS 303.29	—	—	+
<i>P. nicotianae</i>	II	<i>Nicotiana tabacum</i>	Taiwan	CBS 305.29	—	+	+
<i>P. nicotianae</i>	II	<i>Nicotiana tabacum</i>	North Carolina (USA)	CBS 310.62	+	—	—
<i>P. nicotianae</i>	II	<i>Nicotiana tabacum</i>	Kentucky (USA)	P582	+	+	+
<i>P. nicotianae</i>	II	<i>Nicotiana tabacum</i>	Australia	P1753	+	+	+
<i>P. nicotianae</i>	II	<i>Nicotiana tabacum</i>	North Carolina (USA)	P1349	+	—	—
<i>P. nicotianae</i>	II	<i>Vanilla planifolia</i>	French Polynesia	P1923	+	—	—
<i>P. nicotianae</i>	II	<i>Spathiphyllum</i> sp.	The Netherlands	PD 96/4943	—	+	—
<i>P. palmivora</i>	II	<i>Theobroma cacao</i>	Trinidad	CBS 298.29	—	+	—
<i>Phytophthora</i> sp-h		<i>Spathiphyllum</i> sp.	The Netherlands	PD 93/1339	+	+	+
<i>Phytophthora</i> sp-h		<i>Spathiphyllum</i> sp.	The Netherlands	PD 93/1339-MZ	+	+	—
<i>Phytophthora</i> sp-h		<i>Spathiphyllum</i> sp.	The Netherlands	PD 93/1339-R'	+	—	—
<i>Phytophthora</i> sp-h		<i>Primula</i> sp.	The Netherlands	PD 94/988	+	+	+
<i>Phytophthora</i> sp-h		<i>Spathiphyllum</i> sp.	The Netherlands	PD 94/1166	+	+	+
<i>Phytophthora</i> sp-h		<i>Spathiphyllum</i> sp.	The Netherlands	PD 94/1166-R'	+	—	—

^a * = Type strains. Letters in isolate numbers refer to the collections of CBS = Centraalbureau voor Schimmelcultures, Baarn, the Netherlands; PD = Plant Protection Service, Wageningen, the Netherlands; and P = University of California, Riverside, CA.

^b + = analyzed, and — = not analyzed.

^c Random amplified polymorphic DNA.

^d Mitochondrial DNA.

on native, 12.5% homogeneous polyacrylamide gels and for malate dehydrogenase (MDH; EC 1.1.1.37) on native, 8 to 25% gradient polyacrylamide gels. 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). Glycine was added to the samples to a final concentration of 0.22 M. Extracts assayed for MDH activity had to be diluted 5 to 10 times to avoid smearing.

At the completion of electrophoresis, gels were immersed in freshly prepared staining solutions in the dark at 37°C for 5 and 20 min for MDH and MDHP, respectively.

Two enzymatic stains tested, notably those for MDH and MDHP, gave clearly interpretable bands. The reaction ingredients for each enzyme were as follows. MDH contained 25 ml of 0.2 M Tris-HCl, pH 8.0; 440 mg of L-malic acid (disodium salt); 12.5 mg of NAD; 7.5 mg of nitroblue tetrazolium (NBT) (N 6876; Sigma Chemical Co., Zwijndrecht, the Netherlands); and 1 mg of phenazine methyl sulfate (PMS) (P9625; Sigma Chemical Co.). MDHP contained 25 ml of 0.2 M Tris-HCl, pH 8.0; 440 mg of L-malic acid (disodium salt); 12.5 mg of NADP; 7.5 mg of NBT; and 1 mg of PMS.

Alleles were scored according to the method of Spielman et al. (24). Arbitrarily, 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 (21). Both MDH (15) and MDHP (18) are known to be dimeric enzymes in *Phytophthora* spp. 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.

Analysis of RAPD. *Cultivation of mycelium for DNA isolation.* Fungal isolates were grown in pea broth medium (filtered decoction of 200 g of frozen peas boiled in 1 liter of distilled water for 1 h, to which 5 g of glucose was added) in petri dishes for 5 to 10 days at 25°C in the dark. Mycelia were harvested by vacuum-filtration, washed twice with distilled water, frozen at -20°C, and freeze-dried overnight. Dry mycelia were stored at -20°C when not used immediately.

Isolation of DNA. DNA was extracted after the method of Möller et al. (16) with slight modifications (13). Freeze-dried mycelium (30 mg) was extracted in 1 ml of TES buffer (100 mM Tris-HCl, 25 mM EDTA, and 2% sodium dodecyl sulfate [SDS], pH 8.0) with 100 to 200 µg of proteinase K at 55°C for 30 min with mixing at 5-min intervals. After adding NaCl (280 µl, 5 M) and 138 µl of cetyltrimethylammonium bromide/NaCl (10%/0.7 M), the mixture was incubated for 10 min at 65°C. The suspension was centrifuged (16,000 × g) for 10 min at 4°C to remove cell debris. One milliliter of supernatant was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) for 30 min on ice, centrifuged (16,000 × g) for 10 min at 4°C, and the supernatant was extracted again by adding 450 µl of 5 M ammonium acetate and incubating for 30 min on ice. The mixture was centrifuged at 16,000 × g for 10 min at 4°C. DNA was precipitated from 1,300 µl of the supernatant by adding 715 µl of isopropanol. The solution was centrifuged at 16,000 × g for 15 min, and the DNA pellet was washed for 15 min with 800 µl of 70% ethanol at ambient temperature. The pellet was dried and dissolved in 100 µl of TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0).

Polymerase chain reaction (PCR). PCR was performed in 50-µl volumes containing 10 mM Tris-HCl (pH 9.5), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 200 µM of each deoxy-nucleotide triphosphate, 10 pmol of a single primer, and 0.5 U of *Thermus thermophilus* DNA polymerase (Super Taq; Sphaero Q, Leiden, the Netherlands). Primers tested for RAPD were (i) random 10-mer primers (each of the 20 random primers of kit A of Operon Technologies, Inc., Alameda, CA) and (ii) 15-mer primer ((ATG)₅ and the core sequence of M13 [GAGGGTGGCGGTTCT]) (26). Template DNA consisted of 50 ng of a crude nucleic acid extract pre-

pared as described above. In the case of 10-mer primers, 40 amplification cycles of 1 min at 94°C, 1 min at 34°C, and 2 min at 72°C were performed in a Biomed thermocycler (type 60; Biomed, Theres, Germany). For 15-mer primers, conditions were the same, except that denaturing was 20 s at 94°C and annealing was 1 min at 48°C.

Southern blot/probe/hybridization. *Southern blot.* For Southern blot analysis (23), 1 µl of each RAPD reaction mixture was run on 1.7% agarose gel in TAE buffer (40 mM Tris-HCl and 2 mM EDTA, pH 8.0) at 4 V/cm for 3 h. Bands were visualized with ethidium bromide (0.5 µg/ml) and photographed under 302-nm light. After electrophoresis, gels were rinsed for 10 min with 0.25 N HCl, two times for 15 min with denaturation buffer (0.5 N NaOH and 1.5 M NaCl), and finally two times for 15 min with neutralization buffer (0.5 M Tris-HCl, pH 7.5, and 1.5 M NaCl). Fragments were blotted onto Hybond N (Amersham, 's-Hertogenbosch, the Netherlands) overnight with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). After blotting, blots were rinsed with 2× SSC and dried. Finally, the blots were heated for 2 h at 80°C.

Probe. To prove that RAPD bands present in *Phytophthora* sp-h that comigrate with bands specific for *P. nicotianae* and *P. cactorum* contain identical sequences, fragments were excised from RAPD gels and incubated overnight with 50 µl of TE buffer, pH 8.0. One microliter of the supernatant was reamplified with RAPD primer (described above).

The reamplification mixture (2 to 3 µl) was random prime-labeled with ³²P-dATP (random prime labeling kit of Boehringer GmbH, Mannheim, Germany). Labeled probe was separated from free ³²P-dATP using Sephadex G-50 column chromatography.

Hybridization. Blots were hybridized with labeled probe overnight at 65°C in hybridization buffer (5× SSPE [1× SSPE is 0.18 M NaCl, 10 mM NaPO₄, and 1 mM EDTA, pH 7.7], 5× Denhardt's solution, 0.1% SDS, and 20 µg of salmon sperm DNA per ml).

After hybridization, blots were washed twice with 2× SSPE and 0.1% SDS at room temperature for 10 min and with 1× SSPE and 0.1% SDS at 65°C for 15 min. Finally, blots were exposed to KODAK X-OMAT AR films (Kodak, Driebergen, the Netherlands) at -70°C. Blots were stripped with 0.2 N NaOH and 0.1% SDS at 37°C for probing again.

Analysis of mtDNA. *Isolation of mtDNA.* For DNA isolation, isolates were grown and harvested as described above for RAPD analysis. The mycelium was freeze-dried, and 5 g of fresh weight was finely ground with sand in a mortar with a pestle for 1 h and then incubated in 25 ml of TES buffer containing 0.5 mg of proteinase K at 55°C. The suspension was then extracted with 15 ml of chloroform/isoamyl alcohol (24:1, vol/vol), cooled on ice for at least 15 min, and centrifuged at 4°C at 100,000 × g for 45 min. The supernatant was then centrifuged at 4°C at 100,000 × g for 45 min to remove polysaccharides. DNA in the supernatant was precipitated with 1/50 volume of 5 M NaCl and one volume of isopropanol for 1 h at room temperature, followed by centrifugation (3,200 × g) for 15 min at 20°C. Pellets were washed with 10 ml of 70% ethanol, dried, and dissolved in 8 ml of TE buffer. Finally, 8.8 g of CsCl and 60 µl of bisbenzimidazole (Hoechst 33258; Hoechst, Amsterdam; stock solution 1 mg/ml) were added to the DNA-containing solution that was then centrifuged at 135,000 × g at 20°C for 40 h. Of the resulting two fluorescent bands, the upper one, containing the mtDNA, was collected, and bisbenzimidazole was removed by extraction with isopropanol saturated with CsCl/TE buffer. After dialysis against 2 liters of TE buffer overnight at room temperature, the DNA was precipitated with NaCl/isopropanol, washed with 70% ethanol, dried, and dissolved in TE buffer (17).

Restriction enzyme analysis. To see which mtDNA was inherited in the *Phytophthora* sp-h isolates, preparations of mtDNA were digested with *Hae*III, *Sac*II, and *Hind*II according to the instructions of the supplier (Boehringer GmbH). Digested mtDNAs were analyzed by electrophoresis in 1% agarose gels prepared in TAE buffer at 2 V/cm for 15 h, stained with ethidium bromide (0.5 µg/ml), and photographed in 302-nm light.

Fig. 1. Random amplified polymorphic DNA banding patterns of *Phytophthora* isolates belonging to group I and group II with **A**, primer M13 and **B**, OPA 13 (reference isolate numbers shown in Table 1).

RESULTS

Characterization of single-zoospore progeny. We established five strains by isolating single zoospores of PD 93/1339. The RAPD patterns of PD 93/1339-MZ were identical to those of isolate PD 93/1339, from which the zoospores originated (Fig. 1A and B). All five monozoosporic isolates of PD 93/1339 generated isozyme patterns that were identical to those of the isolate PD 93/1339, from which the zoospores originated (Fig. 2A and B, lanes 8 and 9). This confirmed that isolate PD 93/1339 was not a physical mixture.

Fig. 2. A, Malic enzyme (MDHP) patterns and B, malate dehydrogenase (MDH) patterns generated in several *Phytophthora* strains (assigned genotypes shown in Table 2).

Isozyme analysis. Isozyme analysis of MDHP showed a single band in the isolates of *P. cactorum*, *P. idaei*, *P. iranica*, *P. pseudotsugae* (all group I species), and *P. nicotianae*. The single *P. pseudotsugae* band comigrated with that of *P. cactorum* (Fig. 2A, Table 2). All three isolates of *Phytophthora* sp-h showed three MDHP bands, the upper one comigrating with *P. cactorum* and *P. pseudotsugae*, and the lower one comigrating with *P. nicotianae* and one intermediate band. The single *P. nicotianae* band was assigned *Mdh*¹⁰⁰, and all other bands were calculated relative to this band.

Oudemans and Coffey (20) assigned the MDH enzyme bands they found in *P. nicotianae* on starch gels to two different putative loci. The first locus (*Mdh-1*) was of heterogeneous composition, whereas *Mdh-2* consisted of one band in *P. nicotianae*. We found that, on native polyacrylamide gels, only the second locus could be resolved; the bands belonging to the first locus formed a long smear (Fig. 2B). In *P. cactorum*, the first putative locus also could not be analyzed in our system, due to smearing. Therefore, only the second MDH locus was analyzed.

Isozyme analysis of *Mdh-2* showed a single band in the isolates of *P. idaei*, *P. nicotianae*, *P. cactorum*, and *P. pseudotsugae* with unique migration distance, except for *P. pseudotsugae*, which comigrated with *P. cactorum*. *P. iranica* generated a three-banded pattern with unique migration distances, the upper band being very faint indeed (Fig. 2B). In *Phytophthora* sp-h isolates, three bands were found also at *Mdh-2*, the upper one comigrating with *P. cactorum* and *P. pseudotsugae*, and the lower one comigrating with *P. nicotianae* and one band in between (Fig. 2B). The single *P. nicotianae* band was assigned *Mdh-2*¹⁰⁰, and all other bands were calculated relative to this band.

RAPD. The RAPD patterns of isolates of *Phytophthora* sp-h and their putative parents (*P. cactorum* and *P. nicotianae*) as well as most other papillate species generated with primers M13 core-sequence and OPA 13 are shown in Figure 1A and B, respectively. All isolates of *P. nicotianae* showed almost identical banding patterns of PCR products with the two primers used (PD 96/4943 reacted poorly with primer M13); the same was found for all strains of *P. cactorum*. Patterns of different species, however, differed considerably from each other, showing only few, if any, common bands, except for some species in group I. *P. pseudotsugae* and, to a lesser degree, *P. idaei* shared some bands with *P. cactorum*. The three *Phytophthora* sp-h strains showed almost identical patterns. Bands in these patterns comigrated with species-specific bands of *P. nicotianae* (N₁ to N₈) on the one hand and species-specific bands of *P. cactorum* (C₁ to C₁₃) on the other (Fig. 1A and B). Actually, patterns of *Phytophthora* sp-h almost exclusively consisted of bands also present in either *P. cactorum* or *P. nicotianae*. This was confirmed by the results of most of the other primers used (some primers did not perform well).

TABLE 2. Features of *Phytophthora* sp-h, *P. cactorum*, *P. idaei*, *P. iranica*, *P. nicotianae*, and *P. pseudotsugae* isolates

Species, isolate	Oogonia dimensions (µm)		Oospore dimensions (µm)		Sporangia length/breadth (µm)		Length/breadth ratio	Amphigynous antheridia	Sexual type	Maximum growing temp. (°C)	Genotype	
	Range	Average	Range	Average	Range	Average					<i>Mdh</i> ^p	<i>Mdh-2</i>
<i>P. idaei</i> , CBS 971.95 ^a	27–38	32.1	23–31	27.0	41–55 × 30–44	48.9 × 34.4	1.35:1	~0%	Homothallic	27.0	87/87	99/99
<i>P. iranica</i> , CBS 374.72 ^b	21–45	34.0	15–37	29.3	30–72 × 22–51	47.9 × 36.8	1.30:1	~0%	Homothallic	35.0	98/98	87/97
<i>P. pseudotsugae</i> , CBS 444.84 ^c	24–45	35.0	27–33	24.0	34–45 × 28–35	39.0 × 32.0	1.23:1	~0%	Homothallic	30.0	92/92	93/93
<i>P. cactorum</i> ^d	23–35	27.4	18–27	24.0	24–55 × 19–40	35.7 × 26.8	1.33:1	0%	Homothallic	32.0		
<i>P. cactorum</i> , P6183	25–30	27.8	22–25	23.6	35–40 × 26–30	36.6 × 28.0	1.31:1	0%	Homothallic	32.0	92/92	93/93
<i>Phytophthora</i> sp-h, PD 93/1339	34–38	35.8	29–32	31.0	30–52 × 23–40	42.7 × 33.1	1.29:1	84%	Homothallic	36.5	92/100	93/100
<i>Phytophthora</i> sp-h, PD 94/988	34–40	36.1	28–34	30.7	36–48 × 29–38	41.3 × 32.8	1.26:1	86%	Homothallic	36.5	92/100	93/100
<i>Phytophthora</i> sp-h, PD 94/1166	34–38	35.7	30–34	31.8	34–46 × 30–36	41.0 × 32.9	1.25:1	80%	Homothallic	36.5	92/100	93/100
<i>P. nicotianae</i> , P582	24–30	26.7	22–26	23.4	29–54 × 25–39	43.1 × 31.4	1.37:1	100%	Heterothallic	36.5	100/100	100/100
<i>P. nicotianae</i> ^d	25–41	31.8	20–31	26.7	23–79 × 16–54	43.9 × 33.0	1.33:1	100%	Heterothallic	36.0		

^a General features according to the description of Kennedy and Duncan (12) (except isozyme genotypes).

^b General features according to the description of Ershad (7), except isozyme genotypes.

^c General features according to the description of Hamm and Hansen (11), except isozyme genotypes.

^d General features according to the description of Kröber (14).

Southern blot/hybridization. To prove unambiguously the homologous nature of comigrating bands, bands were excised from RAPD gels and used as probes on a Southern blot from these RAPD gels. Figure 3 shows the hybridization patterns of the Southern blot of the RAPD gel (primer M13) hybridized with band C₇ (Fig. 3A) and N₂ (Fig. 3B) and the hybridization patterns of the Southern blot of the RAPD gel (primer OPA 13) hybridized with band C₁₃ (Fig. 3C) and N₄ (Fig. 3D). All four hybridization patterns show the presence of both unique *P. cactorum* or *P. nicotianae* sequences in the *Phytophthora* sp-h strains. All other species did not hybridize with any of the four probes used, except *P. idaei*, which hybridized weakly with C₇ at a different position on the gel, and *P. pseudotsugae*, which hybridized weakly with C₇ and C₁₃ at different positions on the gel.

Characterization of mtDNA. In Figure 4, restriction enzyme patterns are shown generated in mtDNA from *P. nicotianae*, *P. cactorum*, and *Phytophthora* sp-h. With three restriction enzymes, *Hae*III (data not shown), *Hind*II (Fig. 4A), and *Scr*FI (Fig. 4B), the patterns in all three *Phytophthora* sp-h isolates were identical to those of *P. nicotianae*.

Fig. 3. Hybridization patterns of blot RAPD/M13 probed with **A**, C₇ and **B**, N₂ and blot RAPD/OPA 13 probed with **C**, C₁₃ and **D**, N₄.

Morphology. The cultural characters of the three *Phytophthora* sp-h strains, some group I species, and *P. nicotianae* are displayed in Table 2. The three *Phytophthora* sp-h strains are papillate, are homothallic, have predominantly amphigynous antheridia, and have a maximum temperature for growth of 36.5°C.

Dimensions of oogonia, oospores, and sporangia of *P. nicotianae* (P582) and *P. cactorum* (P6183) were comparable to those determined by Kröber (14), although dimensions from his isolates were somewhat larger.

DISCUSSION

The RAPD patterns of the three *Phytophthora* sp-h strains seemed almost exclusively to represent the sum of the patterns of *P. nicotianae* and *P. cactorum*, although *P. idaei* and *P. pseudotsugae* showed some comigrating bands with both *P. cactorum* and the *Phytophthora* sp-h strains as well (Fig. 1A and B). However, Southern analysis showed that the three strains of *Phytophthora* sp-h indeed had a hybrid origin, with *P. nicotianae* the one parent and *P. cactorum* the other.

Hybrids of *P. nicotianae* and *P. cactorum* should have the *Mdh*¹⁰⁰ and *Mdh*-2¹⁰⁰ alleles of *P. nicotianae* and the *Mdh*⁹² and

Fig. 4. Banding patterns of mitochondrial DNA digested with **A**, *Hind*II and **B**, *Scr*FI.

*Mdh-2*²³ alleles of *P. cactorum*. Both enzymes are known to be dimeric (15,18); consequently, two homodimeric bands and one intermediate heterodimeric band at both enzyme loci should be present in *Phytophthora* sp-h strains (10). Oudemans and Coffey (19,20) found the MDHP and MDH enzyme patterns to be consistent within 47 *P. cactorum* isolates and within 60 *P. nicotianae* isolates. They found two *Mdh* alleles in *P. nicotianae*, whose gene products, however, comigrate in our gel system (data not shown).

The three-banded pattern at *Mdh-2* of *P. iranica* was interpreted as heterozygous; the faint upper band was hypothesized to be caused by so-called null allelism. Null alleles produce faint, or not visible, homodimeric bands, but strong heterodimeric bands (21). If this interpretation is correct (fixed heterozygosity through tandem gene duplication might be an alternative explanation), this is peculiar, considering the homothallic nature of *P. iranica*. Homothallic species should be homozygous after constant selfing.

Since hybrids are difficult to detect, it is not clear whether interspecific hybridization in *Phytophthora* is a common event in nature. As noted before by Brasier (2), it is not very likely that hybrids will be detected by orthodox taxonomy, since, because of their appearance, they will be labeled as atypical representatives of a species or as unidentified. Indeed, based on cultural characters, it is not possible to detect the hybrid nature of the three *Phytophthora* sp-h strains nor to assign their putative parents unambiguously.

Only mtDNA of *P. nicotianae* was found in the hybrids described here (Fig. 4A and B), confirming the parenthood of *P. nicotianae*. Apparently, *P. nicotianae* has acted as the oogonial strain in all three hybrid strains.

Interspecific hybridization has been reported for a few other species of *Phytophthora* only. Boccas (1) attempted to cross different *Phytophthora* species, and out of 220 progeny, he regarded one as an interspecific hybrid between *P. capsici* and *P. palmivora*, as deduced from general protein patterns; in all other cases, oospores, presumed to be the result of interspecific hybridization, were concluded to be the result of selfing.

Two genetic forms (polyploid and diploid) of *P. meadii* are known to exist. Based upon the observation that the diploid form of *P. meadii* produced small oogonia, which degenerated during meiosis resulting in many empty oogonia, Sansome et al. (22) concluded that the diploid form of *P. meadii* might represent a species hybrid.

Brasier et al. (3) speculated upon a hybrid nature of isolates belonging to group O (unknown taxa), which have similarities to *P. gonapodyides* and *P. cryptogea* with respect to morphology and general protein patterns.

Goodwin and Fry (9) crossed the closely related *P. infestans* and *P. mirabilis*, both having a limited host range. Isozyme analysis proved that 79 out of 86 progeny were interspecific hybrids. DNA fingerprint analysis confirmed the hybrid nature of the strains created. Most of the hybrids had lost the ability to infect the hosts that were infected by the parents.

Érsek et al. (6) created interspecific hybrids between *P. capsici* and *P. nicotianae* by zoospore fusion. This was confirmed by the occurrence of DNA sequences unique to both *P. capsici* and *P. nicotianae* in the putative hybrids. In contrast to the results of Goodwin and Fry (9), some hybrids created by Érsek et al. (6) had expanded host ranges.

Except for the putative hybrid nature of the diploid form of *P. meadii* and isolates belonging to group O (22), all hybrids reported in literature are laboratory made. The isolates described in the current paper seem to be the first reported hybrids of natural origin.

Since the introduction of hydroponic systems in greenhouses in the Netherlands, an increase of *Phytophthora* outbreaks has been recorded. In many greenhouses, different crops are serviced by the same hydroponic system and, hence, different *Phytophthora* species, colonizing different hosts, can be present simultaneously in the hydroponic system. Hybrids could have been formed by anastomosis or through interspecific mating. The latter requires that the homothallic *P. cactorum* has served as the opposite mating

type, since *P. nicotianae* is heterothallic. Stephenson et al. (27) showed that hyphal anastomosis occurs in *P. capsici*. Outcrossing has not been demonstrated before for *P. cactorum*. However, it has been shown by Whisson et al. (29) that, in homothallic isolates of *P. sojae*, outcrossing can occur.

Artificial inoculation of *Spathiphyllum* plants with PD 93/1339 and PD 94/1166 in hydroponic systems caused the same symptoms (blackening of stems) with which the hybrids originally were associated (A. Kerssies and A. Numansen, *personal communication*). After reisolation (six reisolations), the fungus showed the same isozyme patterns (Fig. 2A and B, lanes 4 to 7) and morphology as the isolates that were used for the infection, thus confirming the postulates of Koch.

Oospores were observed in the stems of diseased *Spathiphyllum* plants. At this moment, it is not clear whether they are fertile.

The hybrids described in this paper probably survived vegetatively after formation, through chlamydospores and as mycelium in infected plant tissue.

The intraspecific consistency of both *P. nicotianae* and *P. cactorum* indicates that they are well defined and distinct species (19, 20). The existence of hybrids between the two species suggests that they recently derived from a common ancestor. Based on ITS sequences, Cooke and colleagues (4,5) concluded that *P. nicotianae* is closely related to *P. cactorum*. In the course of evolution, the two species diverged, but their divergence apparently has not yet reached the phase at which the two genomes are no longer compatible.

The phytopathological and taxonomical consequences of interspecific hybridization in *Phytophthora* are not yet fully understood. When using molecular markers for identifying isolates of *Phytophthora* spp. (e.g., restriction enzyme analysis of mtDNA), one should be aware of the possible occurrence of hybrid forms.

In view of the change in host specificity of *Phytophthora* hybrids compared with their parental strains (6,9), interspecific hybridization might be an important means of extension of host ranges or exploration of new hosts in *Phytophthora* and, thus, in the rapid evolution of new species.

ACKNOWLEDGMENTS

We thank M. Coffey for his generous gift of some *Phytophthora nicotianae* and *P. cactorum* isolates, and H. de Gruyter and G. Karssen for critically reading the manuscript. We thank J. de Cock, T. de Haas, and T. van den Bosch for skillful technical assistance. We also thank P. Sassen and R. Wolbert for their stimulating discussions.

LITERATURE CITED

- Boccas, B. R. 1981. Interspecific crosses between closely related heterothallic *Phytophthora* species. *Phytopathology* 71:60-65.
- Brasier, C. M. 1991. Current questions in *Phytophthora* systematics. Pages 104-128 in: *Phytophthora*. Cambridge University Press, Cambridge.
- Brasier, C. M., Hamm, P. B., and Hansen, E. M. 1993. Cultural characters, protein patterns and unusual mating behaviour of *Phytophthora gonapodyides* isolates from Britain and North America. *Mycol. Res.* 97: 1287-1298.
- Cooke, D. E. L., and Duncan, J. M. 1997. Phylogenetic analysis of *Phytophthora* species based on ITS1 and ITS2 sequences of the ribosomal RNA gene repeat. *Mycol. Res.* 101(6):667-677.
- Cooke, D. E. L., Kennedy, D. M., Guy, D. C., Russell, J., Unkles, S. E., and Duncan, J. M. 1996. Relatedness of group I species of *Phytophthora* as assessed by random amplified DNA (RAPDs) and sequences of ribosomal DNA. *Mycol. Res.* 100(3):297-303.
- Érsek, T., English, J. T., and Schoelz, J. E. 1995. Creation of species hybrids of *Phytophthora* with modified host ranges by zoospore fusion. *Phytopathology* 85:1343-1347.
- Ershad, D. 1971. Beitrag zur Kenntnis der *Phytophthora* Arten in Iran und ihrer Phytopathologischen Bedeutung (Contribution to the knowledge of *Phytophthora* species in Iran and their phytopathogenic importance). *Mitt. Biol. Bundesanst. Land Forstwirtschaft. Berl. Dahlem* 140.
- Gams, W., van der Aa, H. A., van der Plaats-Niterink, A. J., Samson, R. A., and Stalpers, J. A. 1987. CBA Course of Mycology. CBS (Centraal-bureau voor Schimmelcultures) Institute, Baarn, the Netherlands.

9. Goodwin, S. B., and Fry, W. E. 1994. Genetic analysis of interspecific hybrids between *Phytophthora infestans* and *Phytophthora mirabilis*. Exp. Mycol. 18:20-32.
10. Goodwin, S. B., Webster, R. K., and Allard, R. W. 1994. Evidence for mutation and migration as sources of genetic variation in populations of *Rhynchosporium secalis*. Phytopathology 84:1047-1053.
11. Hamm, P. B., and Hansen, E. M. 1983. *Phytophthora pseudotsugae* a new species causing root rot of Douglas-fir. Can. J. Bot. 61:2626-2631.
12. Kennedy, D. M., and Duncan, J. M. 1995. A papillate *Phytophthora* species with specificity to *Rubus*. Mycol. Res. 99:57-68.
13. Klassen, G. R., Balcerzak, M., and de Cock, A. W. A. M. 1996. 5S ribosomal RNA gene spacers as species-specific probes for eight species of *Pythium*. Phytopathology 86:581-587.
14. Kröber, H. 1985. Erfahrungen mit *Phytophthora* de Bary und *Pythium* Pringsheim. Mitt. Biol. Bundesanst. Land Forstwirtschaft. Berl. Dahlem 225.
15. Manchenko, G. P. 1988. Subunit structure of enzymes: Allozymic data. Isozyme Bull. 12:144-158.
16. Möller, E. M., Bahnweg, G., Sandermann, H., and Geiger, H. H. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 20:6115-6116.
17. Möller, E. M., de Cock, A. W. A. M., and Prell, H. H. 1993. Mitochondrial and nuclear DNA restriction enzyme analysis of the closely related *Phytophthora* species *P. infestans*, *P. mirabilis*, and *P. phaseoli*. J. Phytopathol. 139:309-321.
18. Mosa, A. A., Kobayashi, K., Ogoshi, A., Kato, M., and Sato, N. 1993. Isoenzyme polymorphism and segregation in isolates of *Phytophthora infestans* from Japan. Plant Pathol. 42:26-34.
19. Oudemans, P., and Coffey, M. D. 1991. Isozyme comparison within and among worldwide sources of three morphologically distinct species of *Phytophthora*. Mycol. Res. 95:19-30.
20. Oudemans, P., and Coffey, M. D. 1991. A revised systematics of twelve papillate *Phytophthora* species based on isozyme analysis. Mycol. Res. 95:1025-1046.
21. Richardson, B. J., Baverstock, P. R., and Adams, M. 1986. Electrophoresis. Page 23 in: Allozyme Electrophoresis. Academic Press, New York.
22. Sansome, E., Brasier, C. M., and Hamm, P. B. 1991. *Phytophthora meadii* may be a species hybrid. Mycol. Res. 95:273-277.
23. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503-517.
24. Spielman, L. J., Sweigard, J. A., Shattock, R. C., and Fry, W. E. 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. Exp. Mycol. 14:57-69.
25. Stamps, D. J., Waterhouse, G. M., Newhook, F. J., and Hall, G. S. 1990. Revised tabular key to the species of *Phytophthora*. Mycol. Pap. No. 162. International Mycological Institute, Kew, United Kingdom.
26. Stenlid, J., Karlsson, J. O., and Högborg, N. 1994. Intraspecific genetic variation in *Heterobasidion annosum* revealed by amplification of mini-satellite DNA. Mycol. Res. 98:57-63.
27. Stephenson, L. W., Erwin, D. C., and Leary, J. V. 1974. Hyphal anastomosis in *Phytophthora capsici*. Phytopathology 64:149-150.
28. Waterhouse, G. M. 1963. Key to the species of *Phytophthora* de Bary. Mycol. Pap. No. 92. International Mycological Institute, Kew, United Kingdom.
29. Whisson, S. C., Drenth, A., Maclean, D. J., and Irwin, J. A. G. 1994. Evidence for outcrossing in *Phytophthora sojae* and linkage of a DNA marker to two avirulence genes. Curr. Genet. 27:77-82.