Molecular Characterization of Natural Hybrids of *Phytophthora nicotianae* and *P. cactorum*

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ABSTRACT

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Hybrid isolates of *Phytophthora nicotianae* × *P. cactorum* from five different hosts (*Cyclamen*, *Lavandula*, *Lewisia*, *Primula*, and *Spathiphyllum* spp.) were identified by their atypical morphology and their well-defined heterozygous isozyme patterns. The hybrid nature of these isolates was tested by restriction fragment length polymorphism analysis of the internal transcribed spacer (ITS) region of rDNA, generating fragments typical for both *P. nicotianae* and *P. cactorum*. In hybrid isolates, polymerase chain reactions (PCR) with primers derived from unique parts of the ITS region (ITS-PCR) of both species yielded a combination of unique amplicons typical of both parental species. Eleven hybrid isolates, three isolates of each parental species and two atypical isolates from *Rhododendron* and *Idesia* spp. close to *P. cactorum*, were analyzed for amplified fragment length polymorphisms (AFLP). Consistent differences in AFLP patterns existed among the hybrid isolates, strongly indicating that these hybrids have arisen from independent hybridization events between *P. nicotianae* and *P. cactorum*. The two atypical isolates morphologically resembling *P. cactorum* were identical to the latter species in ITS-restriction fragment length polymorphism and response to the specific PCR primers but were intermediate between *P. nicotianae* × *P. cactorum* and *P. cactorum* in isozyme profiles and AFLP patterns. Since the introduction of hydroponic systems in greenhouses in the Netherlands, outbreaks of *Phytophthora* diseases are occurring in previously unaffected host species. This may be due to interspecific hybridization events resulting in novel pathogenic behavior.

The Oomycete genus *Phytophthora*, composed of some of the most destructive plant pathogens, causes considerable economic losses to food crops and ornamentals every year (13). More than 50 species have been described, and new species are still being discovered (16). Some species are highly specialized to a single host; *P. fragariae* var. *rubi*, for example, is only infectious to *Rubus idaeus*. Other species, such as *P. nicotianae*, *P. cactorum*, and *P. palmivora*, have wide host ranges. Several species have been placed on quarantine lists in and outside Europe.

Species identification is based primarily on the shape of the sporangia and the morphological features of the sexual structures. Other criteria widely used to distinguish species are cardinal growth temperature, growth rate, morphological (growth) characteristics in culture, and mating behavior. Due to intraspecific variation and overlapping characters, accurate identification of isolates is often difficult, even for specialists. Moreover, some species (e.g., *P. megasperma*, *P. cryptogea*) probably consist of species complexes rather than single taxa.

Interspecific hybridization in the genus *Phytophthora* has not been investigated intensively. Brasier (4) suggested the potential for zoospore fusion to produce hybrids. A few attempts to create hybrids in the laboratory were performed successfully (1,2,11,15). Proof of hybridization was also obtained by DNA analysis (11,12,15). Natural hybridization has never been proven but may have occurred with *P. meadii* (24). Due to their atypical morphology, natural hybrids of *Phytophthora* are difficult to detect by classical taxonomy and, hence, their existence has been hypothetical for a long time. Recently, however, molecular analysis of certain atypical isolates found in hydroponic systems in the Netherlands revealed that these isolates actually represent hybrids of *P. nicotianae* and *P. cactorum* (21). The hybrid nature of the isolates involved, isolated from *Spathiphyllum* and *Primula* spp., was confirmed using isozymes, random amplified polymorphic DNAs and Southern analyses. Brasier et al. (8) studied an unusual *Phytophthora* species from an *Alnus* sp. that also might be a hybrid, possibly involving *P. cambivora* as one parent. Based on chromosome karyotyping, internal transcribed spacer (ITS) sequence data, and amplified fragment length polymorphisms (AFLP) fingerprinting, Brasier et al. (5) concluded that these isolates represent a heterogeneous population of polyploid hybrids between a *P. cambivora*-like species and an unknown species related to *P. fragariae*.

In the present study a number of new atypical *Phytophthora* isolates from various host species (*Lavandula*, *Lewisia*, and *Cyclamen* spp.) was characterized culturally and morphologically, as well as by isozyme analysis, analysis of the ITS region of the ribosomal DNA gene repeat (rDNA), and AFLP polymorphisms (27) to confirm the hybrid status of the isolates. The hypothesis of whether different hybridization events took place or whether the hybrid isolates belong to a single clonal lineage was tested with AFLP analysis. Two atypical isolates from *Idesia* and *Rhododendron* spp. that morphologically resemble *P. cactorum* were included as well.

MATERIALS AND METHODS

Sources and cultivation of isolates. Fungal isolates used in this study and some of their characteristics are listed in Table 1. Isolates PD 93/1339 and PD 94/1166 were inoculated on a *Spatiphyllum* sp., and several reisolates from the plants were recovered. Isolates of all *Phytophthora* spp. were grown in 50 ml of V8 juice medium: 125 ml of V8, and 11.5 g of oatmeal were

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added to 500 ml of demineralized water, boiled for 5 min, and clarified by centrifugation (15 min at 1600 rpm). $CaCO_3$ (0.94 g) was added to solution and demineralized water was added to a final volume of 625 ml. The mixture was autoclaved for 30 min at 110°C. After incubation at 22°C for 15 to 30 days, mycelium was harvested by filtration, washed with demineralized water, freeze-dried, and stored at –20°C until DNA isolation.

Biochemical, morphological, and cultural characteristics. Biochemical (isozyme analysis [MDHP and MDH]), morphological (dimensions of oogonia, oospores, and sporangia), and cultural (maximum growing temperature) characteristics were determined as described before (21).

Molecular techniques. *DNA isolation.* Freeze-dried mycelium (10 to 50 mg) was ground in microcentrifuge tubes with sterile sand and a pestle. DNA was isolated with the Puregene kit (Gentra/Biozym, Landgraaf, the Netherlands) according to the manufacturer's instructions.

ITS-PCR. ITS-PCR was performed with primers I1 and I4 (18) as previously described (3). PCR product (10 µl) was digested for 3 h at 37°C with the restriction enzymes *Alu*I, *Taq*I, *Msp*I, and *Hae*III according to the manufacturer's instructions (Boerhringer GmbH, Mannheim, Germany). After digestion, products were separated on 2.5% agarose gels in 0.5× TBE buffer (10× TBE = 0.9 M Tris-HCl [pH 8.0], 0.9 M Boric acid, 10 mM EDTA). Bands were visualized by ethidium bromide staining and UV illumination. Sizes of fragments were calculated using a 100-bp ladder (Boehringer) as the molecular weight standard. ITS-PCR products were cloned into a pGEM-T vector (Promega, Leiden, the Netherlands). Transformation was performed in JM109 competent cells (Promega). Plasmid DNA was isolated with Quiagen columns (Quiagen, Breda, the Netherlands). Sequence analysis was performed on an ABI3700 automatic sequencer (Perkin-Elmer, Nieuwerkerk a/d IJssel, the Netherlands). Some additional sequences from *P. cactorum* and *P. nicotianae* were described by Cooke and Duncan (9) and Crawford et al. (10). Primers for *P. cactorum* and *P. nicotianae* were developed based on consensus sequences.

Specific PCR conditions were: buffer (10 mM Tris/HCl, pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 60 µM dNTP, 0.6 µM each primer, 1 unit of *Taq*-polymerase (Boehringer) and 10 ng of fungal DNA in a final reaction volume of 25 µl. Reactions were performed in a PTC200 thermocycler (MJ Research/Biozym, Landgraaf, the Netherlands). The PCR profile was 2 min at 94°C for 35 cycles (cycle = 30 s at 94°C, 30 s at 57°C, 60 s at 72°C), a final extension of 10 min at 72°C, and cooling to 4°C. PCR amplicons were separated on 1.0% agarose gels and visualized as described above.

Radioactive AFLP. Two-hundred and fifty nanograms DNA was digested with *Eco*RI (10 U) and *Mse*1 (10 U) for 4 h at 37°C in RL buffer (5 mM Tris/HCl [pH 7.5], 5 mM MgAc, 25 mM KAc, 2.5 mM DTT, BSA at 25 ng/µl). Digestion was confirmed on agarose gels. Adapters (Table 2) were ligated to restriction fragments. Final concentrations were 2.5 U of ligase (Amersham Pharmacia Biotech, Roosendaal, the Netherlands), 0.1 µM *Eco*RI adapter, 1.0 µM *Mse*I adapter and 0.2 µM ATP. Ligation was performed overnight at 10 to 12°C. Ligation products were diluted 10 times with ultrapure water. Nonselective amplification was performed with 0-primers (Table 2) to amplify all restriction fragments. PCR conditions were as follows: buffer (10 mM Tris/HCl, pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 60 µM dNTP, 5 ng/µl of each primer, 1 U of *Taq*-polymerase (Boehringer) and 5 µl of diluted ligation product in a final reaction volume of 25 µl. Reactions were performed in a PTC200 thermocycler (MJ Research/Biozym). The PCR profile was 2 min at 94°C for 35 cycles (cycle = 30 s at 94°C, 30 s at 56°C, 90 s at 72°C), a final extension of 10 min at 72°C, and cooling to 4°C. After nonselective amplification, products were separated on 1.0% agarose gels. Bands were visualized by ethidium bromide staining and UV illumination. Products were diluted 20 times in ultrapure water and stored at –20°C until selective amplification. Selective PCR conditions were as described above, except with 200 µM dNTP, 5 ng of radioactively labeled *Eco*RI primer, 30 ng of *Mse*1 primer, and 5 µl of diluted (1:20) nonselective amplification product in a final reaction volume of 20 µl. *Eco*RI primer was radioactively labeled with ^{33}P-ATP using kinase (Amersham). AFLP reactions were performed in a PE 9600 thermocycler (Perkin Elmer). The cycling profile was 2 min at 94°C for 13 cycles (cycle = 30 s at 94°C, 30 s at 65°C, 60 s at 72°C; annealing temperature was lowered 0.7°C each cycle), followed by 23 cycles (of 30 s at 94°C, 30 s at 56°C, 60 s at 72°C), a final extension of

TABLE 1. Collection data and summary information about the isolates of *Phytophthora* used in this study

Species Isolate number	Host plant	Location	Isozyme genotype MDHP	 MDH	Sexual type	Amphigynous antheridia %	Maximum growth temperature (°C)
Phytophthora cactorum							
P6183	*Rubus idaeus*	New York	92/92	93/93	Homothallic	0	30–32
P1943	*Panax quinquefolius*	Wisconsin	92/92	93/93	Homothallic	0	30–32
P6187	*Fragariae* × *ananassa*	New York	92/92	93/93	Homothallic	0	30–32
P. nicotianae							
P582	*Nicotiana tabacum*	Kentucky	100/100	100/100	Heterothallic	100	35–36.5
P1753	*Nicotiana tabacum*	Australia	100/100	100/100	Heterothallic	100	35–36.5
P1923	*Vanilla planifolia*	French Polynesia	100/100	100/100	Heterothallic	100	35–36.5
P. cf. *cactorum*							
PD 95/5111	*Idesia*	Noord-Brabant[a]	92/100	93/93	Homothallic	10–20	30–32
PD 92/229	*Rhododendron*	Gelderland[a]	92/100	93/93	Homothallic	0	30–32
P. nicotianae × *P. cactorum*							
PD 93/1339	*Spathiphyllum* sp.	Zuid-Holland[a]	92/100	93/100	Homothallic	80–90	35–36.5
PD 94/1166	*Spathiphyllum* sp.	Zuid-Holland	92/100	93/100	Homothallic	80–90	35–36.5
PD 98/8734	*Spathiphyllum* sp.	Groningen[a]	92/100	93/100	Homothallic	80–90	35–36.5
PD 94/988	*Primula*	Gelderland	92/100	93/100	Homothallic	80–90	35–36.5
PD 97/8771	*Lavandula*	Noord-Holland[a]	92/100	93/100	Homothallic	80–90	35–36.5
PD 97/9389	*Lavandula*	Noord-Brabant	92/100	93/100	Homothallic	80–90	35–36.5
PD 98/9165	*Lavandula*	Noord-Brabant	92/100	93/100	Homothallic	80–90	35–36.5
PD 98 / 9105	*Lewisia*	Noord-Holland	92/100	93/100	Homothallic	80–90	35–36.5
PD 97 / 10235	*Cyclamen*	Noord-Holland	92/100	93/100	Homothallic	80–90	35–36.5
PD 99 / 1760	*Cyclamen*	Noord-Brabant	92/100	93/100	Homothallic	80–90	35–36.5
PD AN 99 / 3	*Cyclamen*	Noord-Holland	92/100	93/100	Homothallic	80–90	35–36.5

[a] The Netherlands.

10 min at 72°C, and cooling to 4°C. Selective amplification products were diluted with an equal volume of formamide dye (98% formamide, 10 mM EDTA, 0.1% brome-phenolblue, and 0.1% xylenecyanol), heated for 5 min at 95°C to denature, and immediately put on ice. Between 3 and 5 µl of the diluted product was loaded on a 6% denaturing polyacrylamide gel (20:1 acrylamide/bis, 7.5 M urea, 0.5× TBE buffer) or Sequagel (Biozym). Pre-electrophoresis with constant power (±75 W) for 20 min until the temperature was 50°C and electrophoresis at constant power (constant temperature was 50°C) was performed on a sequencing system (Gibco BRL, Breda, the Netherlands) until the dye was approximately 12 cm above the bottom of the gel. Gels were dried on Whatman-3MM paper (Whatman Inc., Clifton, NJ) covered with plastic wrap in a vacuum geldryer (Biorad, Veenendaal, the Netherlands). Dried gels were exposed to x-ray films (X-OMAT, Kodak, Odijk, the Netherlands) for 1 to 3 days.

Hybridization. Specific AFLP bands for *P. cactorum* and *P. nicotianae* were excised by positioning the autoradiogram carefully above the dried gel. Radioactive ink was used for correct positioning. Products were eluted from Whatman paper (Whatman Inc.) pieces by boiling in 50 µl of ultrapure water in microcentrifuge tubes. The boiling solution was separated from Whatman paper (Whatman Inc.) by centrifugation and stored in clean tubes at –20°C. Specific bands were reamplified using the 0-primers as described above. Reamplified specific bands were radioactively labeled (^{32}P) by random-prime labeling (Boehringer) and hybridized with Southern blots of genomic DNA digested with *Eco*RI as described before (21).

Fluorescent AFLP. Protocols for fluorescent AFLP analysis were the same as for radioactive AFLP, except for the incorporation of Cy5-labeled fluorescent primers (Amersham) in the selective amplification reaction. Samples were analyzed on Sequagel (Biozym) or Readymix (Amersham) polyacrylamide gels and run on an ALFexpress automatic sequencer (Amersham). Conditions were 1500 V, 60 mA, 35 W, and 55°C. Running buffer was 0.6× TBE. A fluorescently labeled 50-bp ladder (Amersham) was used as a molecular weight standard.

Data analysis. AFLP patterns were analyzed with ImageMaster software (Amersham). Presence or absence of the reproducible bands was converted in binary data and incorporated in the analysis. A similarity matrix was constructed using the method of Nei and Li (22). Unweighted pair group method cluster analysis of binary data was performed with Treecon software (26) and a dendrogram was constructed with a distance scale.

TABLE 2. DNA sequences of internal transcribed spacer (ITS) primers, amplified fragment length polymorphism (AFLP) primers, and AFLP adapters used in this study

Primer/Adapter	Sequence
ITS	
I1	5′-TCCGTAGGTGAACCTGCGG
I4	5′-TCCTCCGCTTATTGATATGC
CACTF1	5′-GGTGAGCCCTATCATGGC
CACTR1	5′-AGTCGGTCCGAAAACCAGC
NICF1	5′-AGTGAGCCCTATCAAAAAAAAG
NICR2.1	5′-GCATACCGAAGTACACATTAAGTT
AFLP	
E00	5′-GACTGCGTACCAATTC
M00	5′-GATGAGTCCTGAGTAA
E20	5′-GACTGCGTACCAATTCGC
E21	5′-GACTGCGTACCAATTCGG
M16	5′-GATGAGTCCTGAGTAACC
AFLP adapters:	
*Eco*RI	5′-CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA-5′
*Mse*I	5′-GACGATGAGTCCTGAG TACTCAGGACTCAT-5′

RESULTS

Biochemical, morphological, and cultural characteristics. Putative hybrid isolates were identified by their heterodimeric isozyme patterns using MDHP (malic enzyme) and MDH (malate dehydrogenase) (Table 1). These isolates were homothallic, had 80 to 90% amphigynous antheridia, and a high cardinal growth temperature (≈ 36°C). Two isolates from *Rhododendron* and *Idesia* spp. (PD 92/229 and PD 95/5111, respectively) were atypical in their isozyme patterns, which resembled the hybrids for only one of the enzymes (Table 1). They had a three-banded, heterodimeric, pattern with MDHP, similar to that of the putative hybrids, but the typical *P. cactorum* band with MDH (Table 1). These isolates were also homothallic but had largely paragynous antheridia, a lower cardinal growth temperature (30.5°C), and are presently classified as *Phytophthora* cf. *cactorum* because of their morphological resemblance to this species.

ITS-RFLP. The PCR amplification products of the *P. cactorum* isolates with primers I1 and I4 (880 bp) were slightly smaller than those of the *P. nicotianae* isolates (900 bp) (data not shown). This agreed with the total sequence of the I1/I4 PCR fragment (882 and 892 bp for *P. cactorum* and *P. nicotianae*, respectively). After digestion of the I1/I4 PCR product with *Alu*I (Fig. 1) a clear difference emerged between *P. cactorum* (lanes 2 through 4) and *P. nicotianae* (lanes 12 through 14) isolates. For T*aq*I and *Hae*III, the differences were only due to small length differences (data not shown). For *Msp*I (data not shown) and *Alu*1 (Fig. 1), the differences were due to restriction site changes. All *P. nicotianae* × *P. cactorum* hybrid isolates contained restriction fragments from both parental *Phytophthora* species. However, differences between hybrid isolates were also observed (Fig. 1). Isolate PD 94/988 (lane 7) gave a weak *P. cactorum* pattern and a strong *P. nicotianae* pattern compared with the other hybrid isolates. The

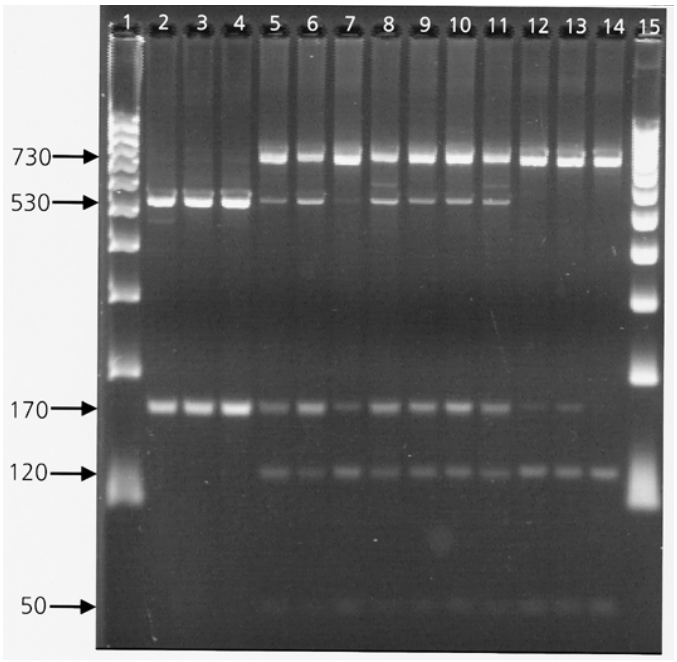

Fig. 1. Gel electrophoresis of polymerase chain reaction product with primers I1/I4 (Table 2) of DNA from several isolates of *Phytophthora* species digested with *Alu*I (lanes 2 through 14). Lanes 1 and 15: molecular weight markers (100-bp intervals); lanes 2 through 14: *P. cactorum* P6183, *P. cactorum* P1943, *P. cactorum* P6187, *P. nicotianae* × *P. cactorum* PD 93/1339, *P. nicotianae* × *P. cactorum* PD 93/1339 MZ, *P. nicotianae* × *P. cactorum* PD 94/988, *P. nicotianae* × *P. cactorum* PD 94/1166, *P. nicotianae* × *P. cactorum* PD 97/10235, *P. nicotianae* × *P. cactorum* PD 97/8771, *P. nicotianae* × *P. cactorum* PD 97/9389, *P. nicotianae* P582, *P. nicotianae* P1753, and *P. nicotianae* P1923, respectively. MZ: monozoospore culture.

ITS RFLP data for the *Phytophthora* cf. *cactorum* isolates were identical to *P. cactorum* (data not shown).

Specific PCR. Sequences of the I1/I4 PCR product of *several P. cactorum* and *P. nicotianae* isolates were determined or derived from databases; the consensus sequence was determined. Primers either specific for *P. cactorum* or for *P. nicotianae* were designed on the basis of sequence differences and are listed in Table 2. PCR reactions were performed with these primers using DNAs from several isolates. When using *P. cactorum* primers only, the *P. cactorum* isolates and the *P. nicotianae* × *P. cactorum* hybrid isolates showed a PCR product of the expected size (data not shown). The same was true when using the *P. nicotianae* primers. IN a multiplex PCR experiment (Fig. 2) using two *P. cactorum* and two *P. nicotianae* primers (Table 2), *P. cactorum* isolates (lanes 2 and 5) gave a small (370 bp) product (marked C*), P. nicotianae* isolates (lanes 3 and 6) gave a large (700 bp) product (marked N) and the *P. nicotianae* × *P. cactorum* hybrid isolates (lanes 4 and 7) amplified both products, proving that those isolates contained ITS sequences from both *Phytophthora* species. All *P. nicotianae* × *P. cactorum* hybrid isolates were screened with this multiplex PCR and all resulted in mixed patterns. The *Phytophthora* cf. *cactorum* isolates only showed the *P. cactorum* band (data not shown).

Radioactive AFLP. DNA from 14 isolates was subjected to radioactive AFLP analysis. The primer combination E21×M16 revealed bands specific for either *P. cactorum* or *P. nicotianae* (Fig. 3). These bands were also present in the putative hybrids (Fig. 3); the AFLP profiles of the *P. nicotianae* × *P. cactorum* hybrid isolates are the sum of the bands of *P. cactorum* and *P. nicotianae*. This was not due to mixed cultures: the AFLP pattern of isolate PD 93/1339 (lane 11) was identical to that of a monozoospore isolate of PD 93/1339 (lane 12). Some differences between the different hybrid isolates were observed. The patterns of isolates PD 93/1339 (lane 11) and PD 94/1166 (lane 14), both isolated from a *Spathiphyllum* sp., were almost identical, whereas the pattern of PD 94/988 (lane 13), isolated from a *Primula* sp., differed significantly. The AFLP patterns of *Phytophthora* cf. *cactorum* isolates (lanes 7 through 10) were comparable to *P.*

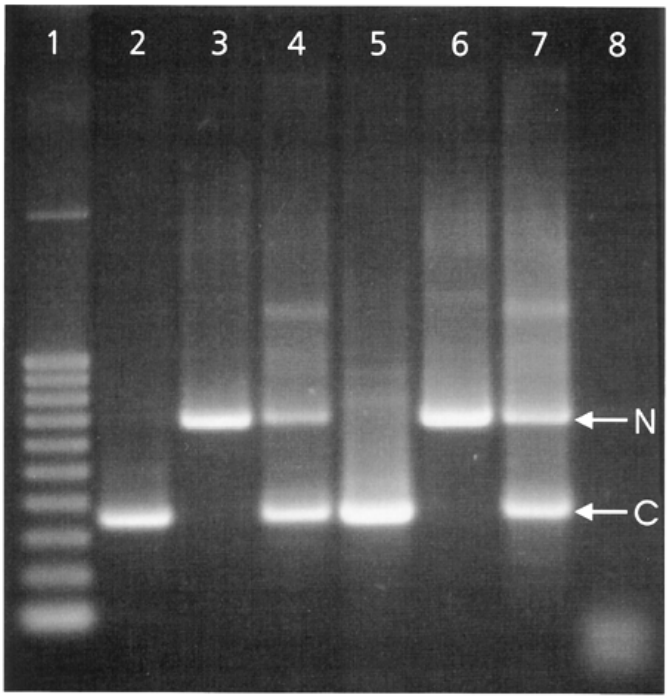

Fig. 2. Gel electrophoresis of multiplex polymerase chain reaction products with primers for *Phytophthora cactorum* (CACTF1 and CACTR1 [Table 2]) and for *P. nicotianae* (NICF1 and NICR2.1 [Table 2]) from the DNA of several *Phytophthora* isolates. Lane 1: molecular weight markers (100-bp intervals); lanes 2 through 7: *P. cactorum* P1943; *P. nicotianae* P582; *P. nicotianae* × *P. cactorum* PD 94/1166; *P. cactorum* P6183; *P. nicotianae* P1753; and *P. nicotianae* × *P. cactorum* PD 93/1339, respectively; lane 8: ultrapure water. PCR products specific for *P. nicotianae* and *P. cactorum* are designated with N and C respectively.

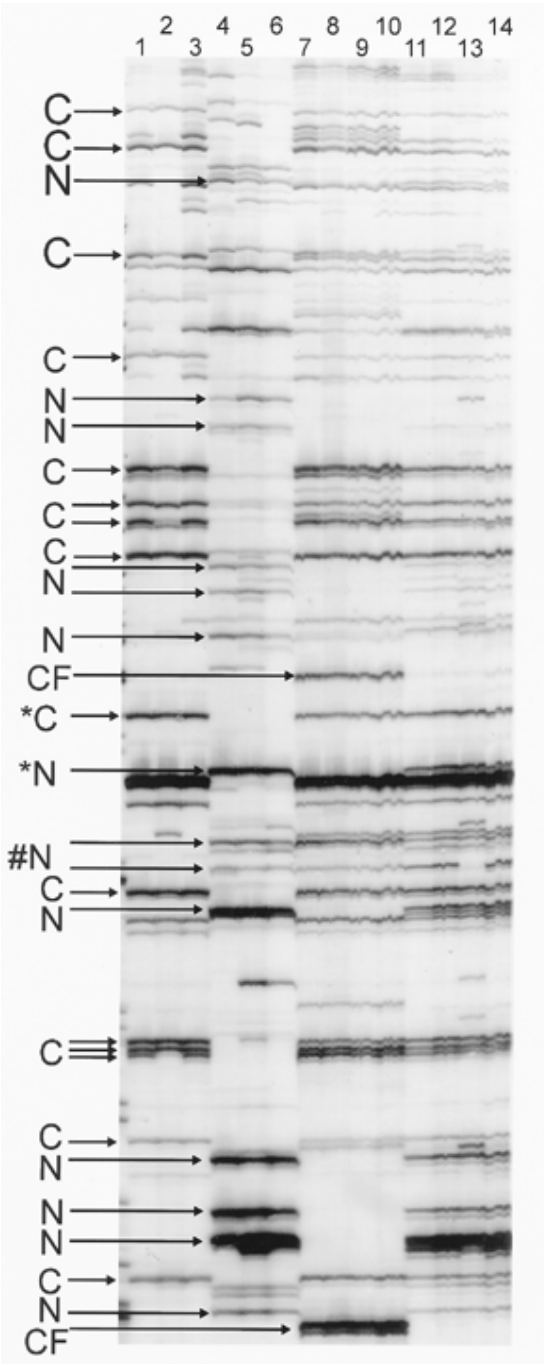

Fig. 3. Radioactive amplified fragment length polymorphic (AFLP) DNA fingerprint patterns of several *Phytophthora* isolates with primer combination E21×M16. Lanes 1 through 3: *P. cactorum* isolates P6183, P1943, and P6187, respectively; lanes 4 through 6: *P. nicotianae* isolates P582, P1753, and P1923, respectively; lanes 7 through 10: *Phytophthora* cf. *cactorum* isolates PD 95/511, PD 92/229 PC-vanc, PD 92/229 PC, and PD 92/229 MZ, respectively; lanes 11 through 14: *P. nicotianae* × *P. cactorum* isolates PD 93/1339 PC, PD 93/1339 MZ, PD 94/988, and PD 94/1166, respectively. PC-vanc: parent culture grown on vancomycine; PC: parent culture; MZ: monozoospore culture. Arrows indicate *P. cactorum* (C)-, *P. nicotianae* (N)- or *P.* cf. *cactorum* (CF)-specific AFLP fragments and *P. nicotianae* bands present in *P.* cf. *cactorum* isolates (#N). *C and *N indicate AFLP bands selected for Southern hybridization experiments.

cactorum, although they also contained a small number of bands comigrating with *P. nicotianae* bands (marked with #N) and some unique AFLP bands (marked CF).

Cluster analysis of 100 reproducible AFLP bands showed that isolates of *P. nicotianae* × *P. cactorum* were grouped between both parental species (Fig. 4). Isolates of *Phytophthora* cf. *cactorum* grouped between the hybrids and *P. cactorum*. The different clustering of isolate PD 94/988 is clearly shown. Other primer combinations showed the same results (data not shown).

Hybridization. A *P. cactorum*-specific AFLP fragment (Fig. 3, *C) was excised from the radioactive AFLP gel and used in Southern analysis (Fig. 5). Results showed several RFLP bands in the *P. cactorum* isolate (lane 1) that were also present in the *P. nicotianae* × *P. cactorum* hybrid isolate (lane 4). The same was true for the RFLP band present in the *P. nicotianae* isolate (lane 2). The *Phytophthora* cf. *cactorum* isolate (lane 3) only showed the *P. cactorum* RFLP bands. Hybridization with a *P. nicotianae*-specific AFLP fragment (Fig. 3, *N) showed a similar pattern (data not shown).

Fluorescent AFLP. Similar results were obtained using fluorescent AFLP analysis on 17 isolates (Fig. 6). Again, the *P. nicotianae* × *P. cactorum* isolates showed bands specific for *P. cactorum* and *P. nicotianae*. Eight new *Phytophthora* isolates (PD 97/10235, PD 97/8771, PD 97/9389, PD 98/8734, PD 98/9165, PD 98/9105, PD 99/1760, and PD AN 99/3) were discovered and were hybrid by nature (Fig. 6), with *P. nicotianae* and *P. cactorum* as parental species. They were isolated from new hosts (*Lavendula*, *Cyclamen*, and *Lewisia* spp.).

Cluster analysis of 350 reproducible AFLP bands from 2 primer combinations (E21×M16 and E20×M16) showed that the *P. nicotianae* × *P. cactorum* hybrid isolates again grouped between the two parental *Phytophthora* species (Fig. 7). Other primer combinations gave similar results (data not shown). There was some variation among the hybrid isolates. Isolates PD 98/9165 and PD 94/988 differed from the other hybrid isolates. Isolates PD 93/1339 and PD 98/8734, both from a *Spathiphyllum* sp., clustered together, although they were isolated from plants from different regions in the Netherlands. The same was true for the *Lavandula* sp. isolates PD 97/8771 and PD 97/9389, and for the *Cyclamen* sp. isolates PD 97/10235 and PD 99/1760. *Spathiphyllum* sp. isolate PD 94/1166 clustered between the other hybrid isolates, although it clustered together with the other *Spathiphyllum* sp. isolates in the radioactive analysis (Fig. 4). Reisolations made from inoculated *Spathiphyllum* sp. plants showed exactly the same AFLP pattern as the inoculated isolate (data not shown).

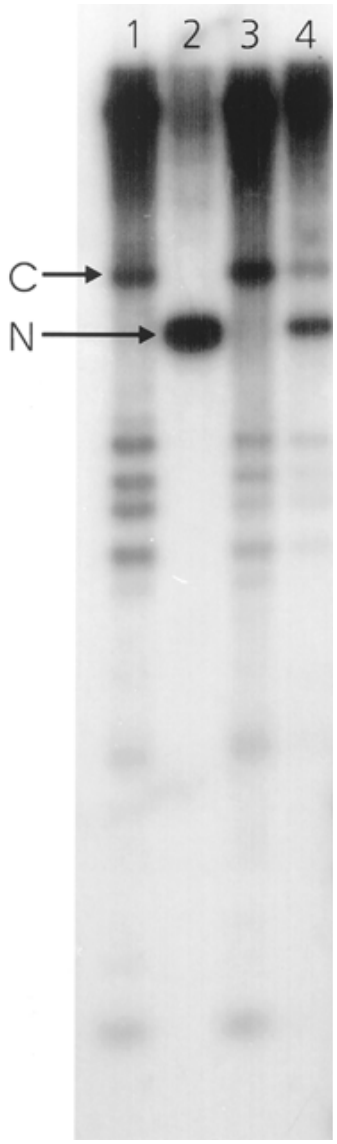

Fig. 5. Southern blot from *Phytophthora* DNA digested with *Eco*RI and hybridized with amplified fragment length polymorphism band *C (Fig. 3). Lane 1: *P. cactorum* P6183; lane 2: *P. nicotianae* P1753; lane 3: *P.* cf. *cactorum* PD 92/229; lane 4: *P. nicotianae* × *P. cactorum* PD 93/1339. C and N indicate *P. cactorum*- and *P. nicotianae*-specific restriction fragment length polymorphism bands, respectively.

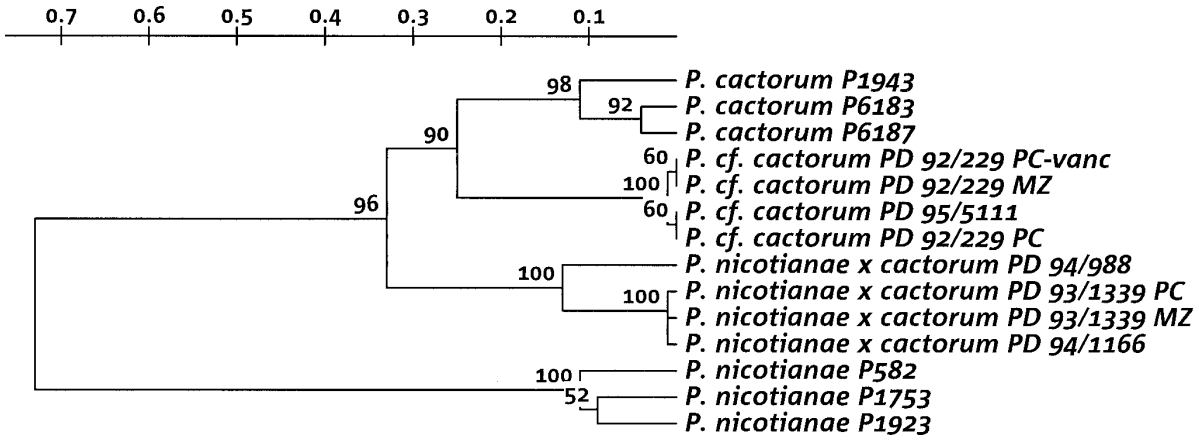

Fig. 4. Unweighted pair group method cluster analysis of binary data dendrogram constructed from 100 bands of amplified fragment length polymorphism fingerprint patterns of *Phytophthora* isolates with primer combination E21×M16, as shown in Figure 3. Distance scale (in fixed mutations per site) is drawn. Bootstrap values of 1,000 replications are shown in percentages above branches.

DISCUSSION

Little is known about interspecific hybridization in the genus *Phytophthora*. If exchange of genetic material occurs in the hybrid oospores, interspecific crosses may be an important source of variability. Demonstration of interspecific hybridization in the laboratory is difficult due to the very low percentage of germination of hybrid oospores (15).

The occurrence of natural *P. nicotianae* × *P. cactorum* hybrids was recently demonstrated by isozyme analysis, RAPD profiling, and hybridization of Southern blots with species-specific RAPD bands (21) on three isolates. The current molecular analysis (ITS-RFLP, ITS-PCR, and AFLP) provide additional evidence for the hybrid nature of these and more atypical *Phytophthora* isolates. In the course of several years, a total of 13 hybrid isolates have been detected associated with disease symptoms on 5 hosts (*Spathiphyllum*, *Primula*, *Lavandula*, *Cyclamen*, and *Lewisia* spp.). Apparently, hybrids are not extremely rare in greenhouses in the Netherlands, where hydroponic systems and diseases caused by *Phytophthora* are common in ornamental cultures.

The two atypical *Phytophthora* isolates (*Phytophthora* cf. *cactorum* PD 92/229 and PD 95/5111) need not be hybrids between *P.*

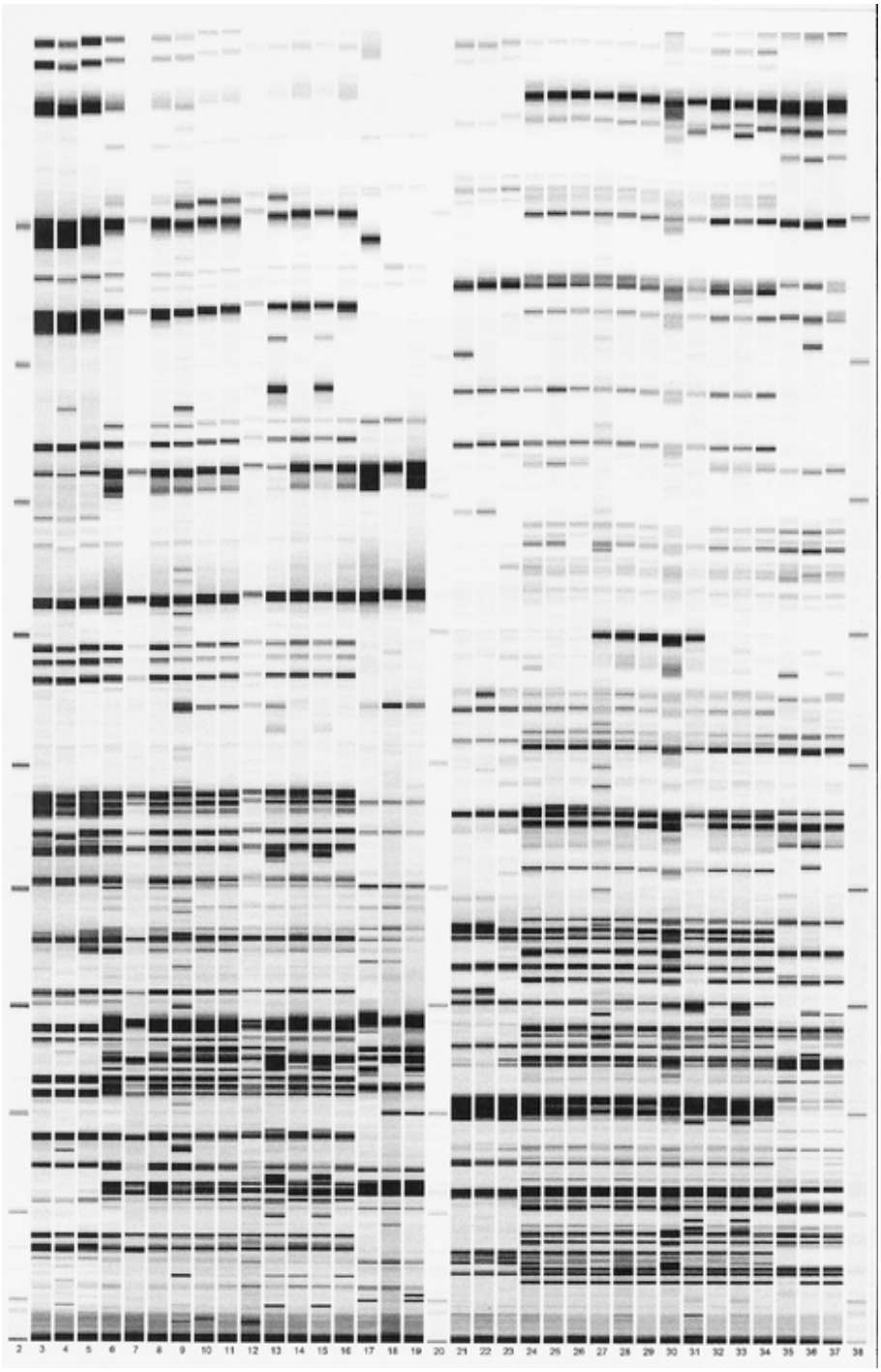

Fig. 6. Fluorescent amplified fragment length polymorphism DNA fingerprint patterns of several *Phytophthora* isolates with primer combinations E21xM16 (lanes 3 through 19) and E20×M16 (lanes 21 through 37). Lanes 2, 20, and 38: 50-bp marker; lanes 3 through 5 and 21 through 23: *P. cactorum* P6183, P1943, and P6187, respectively; lanes 6 through 16 and 24 through 34: *P. nicotianae* × *P. cactorum* PD 93/1339, PD 94/1166, PD 98/8734, PD 94/988, PD 97/8771, PD 97/9389, PD 98/9165, PD 98/9105, PD 97/10235, PD AN 99/3, and PD 98/1760, respectively; lanes 17 through 19 and 35 through 37: *P. nicotianae* P582, P1753, and P1923, respectively.

cactorum and *P. nicotianae*. Analyses of the mtDNA of these two isolates with two restriction enzymes (*Hind*II and *Scrf*I) generated a unique banding pattern, different from both *P. nicotianae* and *P. cactorum* and from the *P. nicotianae* × *P. cactorum* hybrid isolates (20,21). The possibility, however, that a third parental species, in addition to *P. nicotianae* and *P. cactorum*, was involved has not been ruled out. The deviating mtDNA haplotype may be explained in other ways. Several haplotypes of mtDNA may exist in *P. cactorum*, as in *P. nicotianae* (17), and it was reported previously that *P. cactorum* was not a homogeneous species (19,23). The two *Phytophthora* cf. *cactorum* isolates, thus, could represent an evolutionary lineage within *P. cactorum* with a different isozyme pattern for MDHP. At this moment, no final conclusion can be drawn with regard to the origin and taxonomic status of these two isolates.

In 1995, Brasier et al. (8) suggested that a new *Phytophthora* species isolated from an *Alnus* sp. might be a hybrid. Recently Brasier et al. (5) analyzed several *Phytophthora* isolates from an *Alnus* sp. Except for the Swedish variants, all isolates possessed ornamented oogonia, a feature typical for *P. cambivora*. However, the alder *Phytophthora* isolates differed from *P. cambivora* by their homothallism, colony morphology, lower cardinal temperatures for growth, and unusually high chromosome numbers and dimorphic ITS arrays, especially in the UK variants. It was concluded that the UK variants represented a heterogeneous group of interspecific hybrids between a *P. cambivora*-like species and an unknown species closely related to *P. fragariae*.

P. meadii also was suggested to be a hybrid (24). Possible parental isolates were hypothesized to be *P. palmivora* and *P. capsici* or the heterothallic *P. botryosa* and the homothallic *P. heveae*, both of which occur on rubber trees in Southeast Asia. Brasier also suggested that *P. citrophthora* originated as a hybrid of *P. nicotianae* on citrus and *P. capsici* on cocoa (7). In the laboratory, hybrids were created between *P. nicotianae* and *P. capsici* by zoospore fusion (11). Some of the resulting hybrid isolates had an extended host range. Hence, it was hypothesized that interspecific hybridization in nature may result in extended host ranges. However, a reduced host range was found by Goodwin and Fry (15) in interspecific hybrids between *P. infestans* and *P. mirabilis*. Both of these results may be possible depending on the type of host–pathogen interaction involved. Our *P. nicotianae* × *P. cactorum* hybrid isolates, from a *Cyclamen* sp., may be an example of extended host range, because neither of the parental species was reported on this host previously (13; Plant Protection Service, Wageningen, the Netherlands, *unpublished data*).

A detailed analysis of AFLP profiles revealed differences among hybrid isolates. Isolate PD 94/988 differed consistently from all other hybrid isolates, and this difference was also shown in the ITS-RFLP pattern. The ITS-RFLP bands of *P. cactorum* were weaker than the *P. nicotianae* bands, suggesting that isolate PD 94/988 had fewer copies of the ribosomal genes of *P. cactorum*. Isolates from the same host in most cases were more similar in AFLP profile to each other than to isolates from other hosts. This suggests that, dispersed as a clonal lineage, different hybrid isolates possibly specialized on different hosts. More data have to be collected to support this hypothesis. These differences indicate that the hybrid isolates may be not a single clonal lineage but, instead, may be the result of multiple hybridization events. Multiple hybridization events could be explained by the current sympatric occurence of *Phytophthora* isolates in Dutch horticulture. Phylogenetically closely related, but formerly allopatric, isolates of *P. cactorum* from Central Europe and isolates *P. nicotianae* from South America (9) can occur in such a system. More than one species of *Phytophthora* is often isolated from the same host or crop system (6), e.g., *P. palmivora* and *P. megakarya* on cocoa.

Phytophthora species are either homothallic or heterothallic, with two mating types, A1 and A2, in the latter case. Mating types are universal in that an A1 of one species is compatible with A2 of another species. Homothallic species contain at least one, but mostly both, mating hormones and their compatible receptors, and hence, outcrossing between homothallic species cannot be excluded theoretically. In fact, it has been proven that in homothallic *P. sojae* outcrossing actually does occur (25,28). Hybridization, therefore, is possible between the heterothallic *P. nicotianae* and the homothallic *P. cactorum*. All hybrid isolates described in this paper are homothallic and, hence, produce oospores autonomously. Although abortive oospores were observed in all isolates, apparently significant numbers seem to be well developed. The fertility of the hybrid oospores is currently under investigation. Possible exchange of genetic materials in hybrid oospores may be an important factor in the evolution of natural *Phytophthora* populations. Nuclei of different ploidy levels may occur in the same mycelium as was shown for *P. meadii* (24) and may lead to genetic instability of the fungus. Our hybrid isolates showed AFLP patterns with an increased number of fragments compared

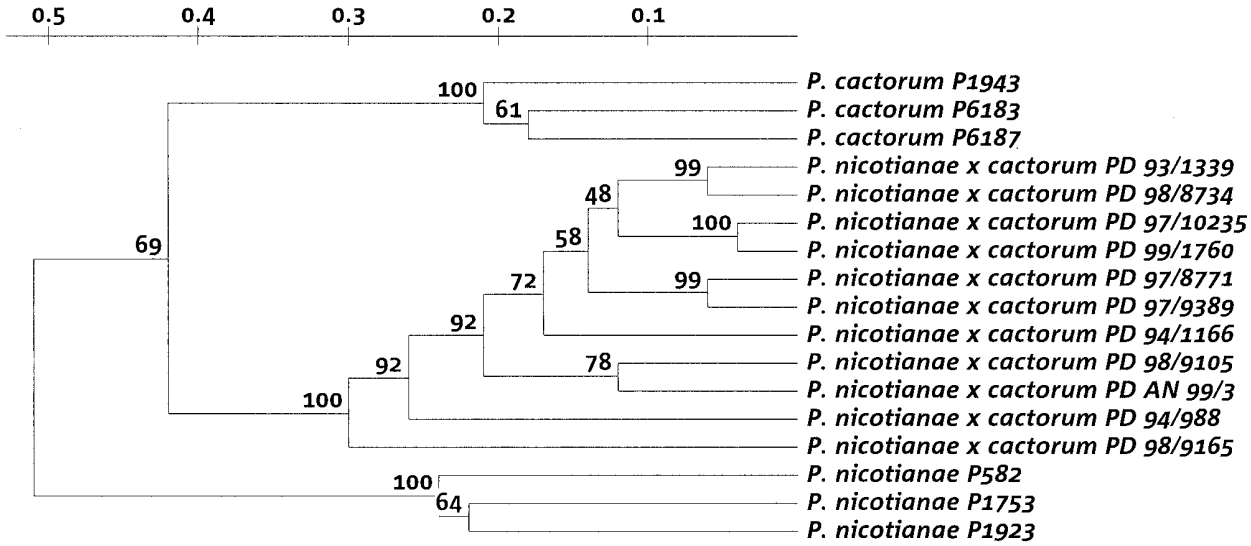

Fig. 7. Unweighted pair group method dendrogram constructed from 350 bands of amplified fragment length polymorphism fingerprint patterns of *Phytophthora* isolates with two primer combinations (E21×M16 and E20×M16) as shown in Figure 6. Distance scale (in fixed mutations per site) is drawn. Bootstrap values of 1,000 replications are shown in percentages above branches.

with the parental isolates, which suggests polyploidy. The *Alnus* sp. isolates of *Phytophthora* also contained a greater number of AFLP bands (5) compared with single-species isolates suggesting different levels of ploidy.

The present paper has shown that natural *P. nicotianae* × *P. cactorum* hybrid isolates do not belong to a single clonal lineage and may pose a serious threat to agriculture. Even a clonally spreading, single-hybrid isolate with a new host range may be fairly dangerous. For comparison, the migration of a single clone of the A1 mating type of *P. infestans* from the Americas to Europe in the 19th century caused tremendous damage all over the world (14). The danger would be even greater if the hybrid isolates were not of clonal origin. This is likely the case with the genetically diverse *P. cambivora*-like hybrid population from an *Alnus* discovered by Brasier et al. (5).

Also, it is risky to rely on one molecular technique to establish the identity of atypical *Phytophthora* spp. isolates. If, for example, one specific PCR primer set had been used, the hybrid isolates would have been misidentified. The existence of several *Phytophthora* species hybrids will undoubtedly complicate correct identification, particularly if new species hybrids are discovered in the future.

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