Phenotypic and molecular characterization of species hybrids derived from induced fusion of zoospores of *Phytophthora capsici* and *Phytophthora nicotianae*

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Phenotypes of species hybrids created from *in vitro* fusion of zoospores from *Phytophthora nicotianae* and *P. capsici* were characterized and compared. The species hybrids were created as part of a study of sources of genetic variation in populations of the parent species that are pathogenic over a similar range of plants. Four isolates of species hybrids proved to be similar to both *P. capsici* and *P. nicotianae* in relation to vegetative and reproductive morphologies. As in a previous study, DNA of *P. capsici* was detected more readily than that of *P. nicotianae* in all hybrid isolates. In the present study, DNA of *P. nicotianae* was detected in three of four hybrids by hybridization of RAPD-PCR products with species-specific DNA from *P. nicotianae*. By thermal denaturation analyses, DNA melting temperatures and GC contents of parent species and species hybrids were similar. The mean GC content of 47·2% was similar to GC contents reported for other *Phytophthora* spp. Additionally, the distributions of GC-rich regions of hybrids were more similar to the distribution in *P. capsici* than in *P. nicotianae*. By these molecular analyses, the hybrids were shown to be more similar to *P. capsici* than to *P. nicotianae*. Even though interspecific somatic fusion is likely to occur rarely under natural conditions, it could contribute to the genetic diversity of heterothallic species of *Phytophthora*.

Despite best management efforts, many soilborne, plant pathogenic fungi cannot be eradicated from soil. Often, the most effective methods of disease control, such as plant resistance and fungicide application, break down after repeated use. Ultimately, sustainable control of pathogenic fungi depends on a better understanding of temporal and spatial aspects of the genetic variability in fungal populations. This is true for *Phytophthora*, an important genus of soilborne pathogens that affects hundreds of plant species world-wide. Goodwin, Sujkowski & Fry (1995), for example, described the importance of characterizing race variability of *P. infestans* to understand the geographic occurrence of potato late blight. These authors emphasized, in particular, the possibility of several intraspecific genetic mechanisms by which pathogenicity might evolve within clonal lineages of this pathogenic species. These types of insights combined with information on pathogen migration have provided an important framework for developing disease management strategies.

In addition to intraspecific mechanisms, interspecific hybridization has been suggested by several authors as a means by which genetic variability can be generated in populations of *Phytophthora* spp. (Vorob'eva & Gridnev, 1984; Brasier, 1992). For instance, in laboratory studies, Goodwin & Fry (1994) were able to induce sexual crosses of *P. infestans* and *P. mirabilis* to create viable species hybrids. Also in laboratory studies, Boccas (1981) detected one possible species hybrid from a number of induced sexual crosses among heterothallic *Phytophthora* species.

Phytophthora capsici Leonian and *P. nicotianae* Breda de Haan (syn. *P. parasitica*) are heterothallic pathogens that inhabit a wide range of soil habitats. In many agroecosystems, however, it is rare that both compatibility types needed for sexual reproduction are present, and consequently, genetic variability generated via sexual processes is rare. More commonly, in either species, reproduction occurs asexually via production of zoospores. *Phytophthora capsici* and *P. nicotianae* can occur in the same geographical region and in the same field (Satour & Butler, 1967). On rare occasions, the two organisms might co-infect a single plant and come into contact with each other. If somatic fusion occurs between the two pathogens, genetic exchange might follow. It is difficult to detect the occurrence of these or other rare events that generate new pathogen genotypes. Consequently, it has been difficult to study population-level processes that influence the success or failure of new and initially rare genotypes to compete and become established among existing populations of these species.

To overcome the obstacle of rare events, we established methods by which genetic variants can be created in the form of species hybrids of *P. capsici* and *P. nicotianae* (Érsek, English & Schoelz, 1995). The four hybrids, created by fusion of zoospores, were confirmed to be interspecific by the detection of DNA for each parent species in fusion isolates. In particular, DNA of *P. capsici* was detected readily in the hybrid isolates; whereas, DNA of *P. nicotianae* was more difficult to detect. The hybrids exhibited modified host ranges compared to the parent isolates. For example, two hybrid isolates exhibited an

expanded host range that included radish and lemon, hosts that are susceptible to either *P. capsici* or *P. nicotianae*, respectively. In contrast, a third isolate was not able to infect either of these hosts but still could infect tomato, a plant susceptible to both parent species. All of these hybrids have remained stable in modified pathogenicity for more than two years.

This report extends the phenotypic characterizations of these four hybrids and confirms the morphological similarities to parent species. We also firmly establish the occurrence of DNA of *P. nicotianae* in the hybrid isolates by use of RAPD-PCR.

MATERIALS AND METHODS

Fungal isolates

Isolates of *P. capsici* Mex^{r}5 (compatibility type A2) and *P. nicotianae* Fpa^{r}10 (compatibility type A2) were derived from wild-type isolates as described previously (Érsek *et al*., 1995). During two years of periodic transfer on media without selective drugs, *P. capsici* Mex^{r}5 remained resistant to 60 μg ml^{-1} metalaxyl (Novartis, Greensboro, NC) and *P. nicotianae* Fpa^{r}10 remained resistant to 300 μg ml^{-1} p-fluorophenylalanine (Sigma, St Louis, MO). The creation of the four species hybrids, H0, H1, H3 and H4, by zoospore fusion of isolates Mex^{r}5 and Fpa^{r}10 of *P. capsici* and *P. nicotianae*, respectively, was described previously by Érsek *et al*. (1995).

Growth characterization and oospore formation

Cultures of parental species and hybrids were grown initially on 20% clarified V8-juice (V8C) agar (Tuite, 1969) in the dark. After four days, a plug of mycelium was removed from the margin of a colony with a no. 3 cork borer and transferred to the centre of a 5 cm Petri dish containing V8C agar amended with selected combinations of metalaxyl and fluorophenylalanine at concentrations of 0–60 and 0–300 μg ml^{-1}, respectively.

Inoculated cultures were incubated in the dark at 25 °C for 4 d. At that time, colony growth was determined as the average colony radius measured at two positions perpendicular to each other. Growth measurements were made for three replicate colonies per isolate. Differences in growth among isolates were evaluated by analysis of variance and Duncan's multiple range test. The growth experiment was repeated once.

Hybrid isolates were evaluated for their abilities to form oospores in crosses with representative isolates of the parent species. Each hybrid was paired with *P. capsici* 1794 (A1 mating type), *P. nicotianae* 34-3-9 (A1 mating type), *P. capsici* Mex^{r}5 (A2 mating type) and *P. nicotianae* Fpa^{r}10 (A2 mating type). In each pairing, an agar block of a hybrid isolate and an isolate of a parental species were placed at a distance of 2–2·5 cm from each other on V8C agar in a small Petri plate. Two replicate, inoculated plates for each isolate combination were incubated in the dark at 24° for 21 d. Oospores were counted at six random locations where the two colonies merged.

Molecular characterization of hybrids

Variability in genetic structure of parental and hybrid isolates was evaluated by RAPD-PCR (Williams *et al*., 1990). DNA of each isolate was extracted and purified as described previously (Érsek, Schoelz & English, 1994). Twenty-two, 10-base oligonucleotide primers (Operon Technologies, Inc., Alameda, CA) were selected randomly for these isolate comparisons. Each primer (20 pmoles) was mixed with reaction buffer, $MgCl_2$ (2 mM), dNTPs (200 mM each), *Taq* DNA polymerase (2·5 U), fungal DNA (100 ng), and sterile, glass-distilled water in a total volume of 50 μl. Reactions were cycled with an automated thermal cycler (Hybaid, model HB-TR1). The thermal cycler was programmed for 44 cycles of 94° for 1 min, 36° for 1 min and 72° for 2 min, preceded by one cycle with an extended 5-min denaturation at 94°. The amplification products were resolved by electrophoresis in 1·2% agarose gels and stained with ethidium bromide.

In a previous study, it was readily shown that DNA of *P. capsici* occurred in all species hybrids (Érsek *et al*., 1995). In contrast, DNA of *P. nicotianae* was detectable to only limited extents in these isolates. To further confirm the occurrence of DNA from *P. nicotianae* in species hybrids, selected amplification products of hybrids were probed for their uniqueness to that parent species. Amplification products of *P. nicotianae* Fpa^{r}10 that were equivalent in size to those of one or more hybrids were used as probes. From each PCR reaction based on primers OPG-02 and OPG-18, a putatively homologous band was cut from 0·8% low-melt agarose gel and was purified before being radiolabelled with (^{32}P) dCTP. Alternatively, hybrid amplification products of primer OPG-01 were probed with pPP33A, a plasmid that contained a 1000 bp repetitive DNA sequence shown previously to be specific to *P. nicotianae* (Érsek *et al*., 1994).

Conditions for hybridization of RAPD-PCR products and probes were as described by Sambrook, Fritsch & Maniatis (1989). All RAPD-PCR and hybridization experiments were performed at least twice.

Thermal denaturation of fungal DNA

The thermal denaturation patterns of DNA were evaluated to compare the base compositions of parent species and hybrids. DNA was extracted from a culture of each isolate grown in a 250 ml flask containing 50 ml of 20% V8 broth amended with either 10 μg ml^{-1} metalaxyl or 50 μg ml^{-1} *p*-fluorophenylalanine, or both. Five agar plugs of 5 mm diam. were used to initiate each culture which was then incubated at 25° in the dark for 8 d. The agar plugs used to initiate cultures were removed, and the mycelial mats were rinsed thoroughly with sterile, double-distilled water and lyophilized. Dried mycelia were pulverised by mortar and pestle and used for DNA extraction.

DNA was isolated from mycelia using a modification of the methods of Pitcher, Saunders & Owen (1989). One-hundred mg of powdered mycelium were suspended in 120 μl TE buffer (pH 8). To this was added 600 μl of a lysis solution of 5 M guanidine thiocyanate in 0·1 M EDTA (pH 8·0), containing 0·5% sodium dodecyl sulphate, and the mixture was shaken gently for 30 min. After lysis, 300 μl of 7·5 M ammonium

acetate were added, and the mixture was extracted 3–4 times against 600 µl of phenol-chloroform-isoamyl alcohol (25:24:1) and once against chloroform-isoamyl alcohol (24:1). After a final centrifugation at 10000 ***g*** for 4 min, DNA was precipitated with one-half volume of isopropyl alcohol. DNA was precipitated by centrifugation at 10000 ***g*** for 1 min, washed three times with 70% ethanol, and dried under vacuum. The pellet was resuspended in 100 µl TE buffer (pH 8) and treated with 50 µl RNAse A at 37° for 2 h. After a final treatment for 15 min with 400 µl lysis solution, 300 µl of 7·5 M ammonium acetate were added before extracting DNA once against 600 µl phenol-chloroform-isoamyl alcohol and twice against the same volume of chloroform-isoamyl. Purified DNA was precipitated and washed as above and resuspended in 0·1 × standard saline citrate (SSC).

In preparation for thermal denaturation, samples were adjusted to 30–40 µg DNA ml^{-1} SSC and inserted into a heating block of a Perkin-Elmer Lambda-2 UV/VIS spectrophotometer. Absorbance of DNA was recorded at 260 nm as the temperature was raised from 50 to 85° at a rate of 0·5° min^{-1}. Temperature change during measurement of optical density was controlled by the PTP-6 Peltier Temperature Programmer under the direction of Petemp-1 software (Perkin-Elmer, Norwalk, CT). Absorbance signals were logged at 0·1° intervals. All samples were analysed three times.

The melting temperature of DNA, T_m, was calculated from the point of zero slope of second derivatives of the absorbance (H) relative to temperature (T). The second derivatives were calculated with a temperature interval of 4·8° (Sly *et al.*, 1986). Based on the value of T_m, the percentage GC content was determined as:

$$\%\,\text{GC content} = 2{\cdot}08(T_m) - 106{\cdot}4 \text{ (Marmur \& Doty, 1962).}$$

DNA of *Escherichia coli* HB101 was used as the reference sample to verify precision of temperature measurement (Marmur & Doty, 1962).

Evaluations of DNA heterogeneity were based on ΔT, the denaturation temperature range over which 17–83% of hyperchromicity occurred, and by 2σ, the range of temperatures at one-half of the maximum value of the first-derivative curve. The first derivatives were calculated with a temperature interval of 3·6°. Increasing values of each of these variables represents a broader distribution of sequences with variable GC content (Blake & Lefoley, 1978).

All fine structure calculations were carried out using the PECSS computer software (Perkin-Elmer). Differences in values of DNA structural parameters among isolates were evaluated by analysis of variance. Significant differences among isolates were determined by the *T* method of Tukey (Sokal & Rohlf, 1981). Significance was assumed at the 99% confidence level.

RESULTS AND DISCUSSION

Growth characterization and oospore formation

There were obvious differences between the colony morphologies of *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10 when grown on both non-amended (control) or drug-amended V8C agar. On non-amended medium and media supplemented with metalaxyl, roseate colonies of *P. capsici* Mex^{r}5 typically produced submerged or appressed mycelium (Fig. 1). In contrast, dense, non-roseate colonies of *P. nicotianae* Fpa^{r}10 produced aerial mycelium. Both colony morphologies were typical of growth patterns of wild-type isolates of the respective species (Erwin & Ribeiro, 1996). Colony morphologies of the species hybrids were consistent over most drug concentrations tested and were intermediate to those of the parent organisms. Hybrid colonies were predominantly roseate, but they all produced some aerial mycelium.

After 4 d incubation, the average radius of colonies of *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10 on non-amended V8C agar was 23·3 and 22·6 mm, respectively (Table 1). Growth of species hybrids on non-amended medium was similar; however, only the growth of isolate H3 was significantly less than the growth of the parent species. The average colony radius of H3 was 18 mm, which was equivalent to 77% of the growth of *P. capsici* Mex^{r}5. In contrast, the average colony radius of hybrid H4 was 22 mm, or 94% of this parental isolate.

As the concentrations of the selective drugs were increased in the medium, the growth of *P. capsici* Mex^{r}5 and *P. nicotianae* Fpa^{r}10 decreased until no growth of either species occurred in the presence of 300 and 60 µg ml^{-1} of fluorophenylalanine and metalaxyl, respectively (Table 1). In contrast, all species hybrids grew well at the highest drug concentrations tested. At maximum concentrations, growth of species hybrids was 69–97% of their respective growth on non-amended medium. Additionally, on amended media, growth of hybrid isolates H1 and H3 was significantly less than the growth of hybrid isolates H0 and H4 (Table 1).

Oospore production among hybrid isolates varied with the paired parental species and mating type. For example, all hybrids formed abundant oospores when paired with the A1 mating type isolate *P. capcisi* 1794. In contrast, oospores formed rarely when any hybrid was paired with the A1 mating type isolate *P. nicotianae* 34-3-9. No oospores formed at all when hybrids were paired with A2 mating type isolates, *P. capsici* Mex^{r}5 or *P. nicotianae* Fpa^{r}10. Germination frequencies of oospores produced in pairings were not assessed.

Molecular characterization

Polymorphisms in amplification products were observed among hybrids for various primers. For example, the bands amplified by primer OPG-02 in isolate H1 differed slightly from those of isolates H0, H3, and H4, which appeared to be identical (Fig. 2). In the case of primer OPG-18, the amplification pattern of each hybrid isolate was unique (Fig. 4). As a final example, the bands amplified by primer OPG-01 were identical in species hybrids H3 and H4, but amplified bands in hybrids H0 and H1 were each unique (Fig. 6).

Amplification patterns of hybrids resembled those of *P. capsici* Mex^{r}5 more closely than those of *P. nicotianae* Fpa^{r}10. Typically, there were several amplification products of equivalent size for species hybrids and *P. capsici* Mex^{r}5 for each of the 22 random-base primers examined. Less often, amplification products that were unique to *P. nicotianae* Fpa^{r}10

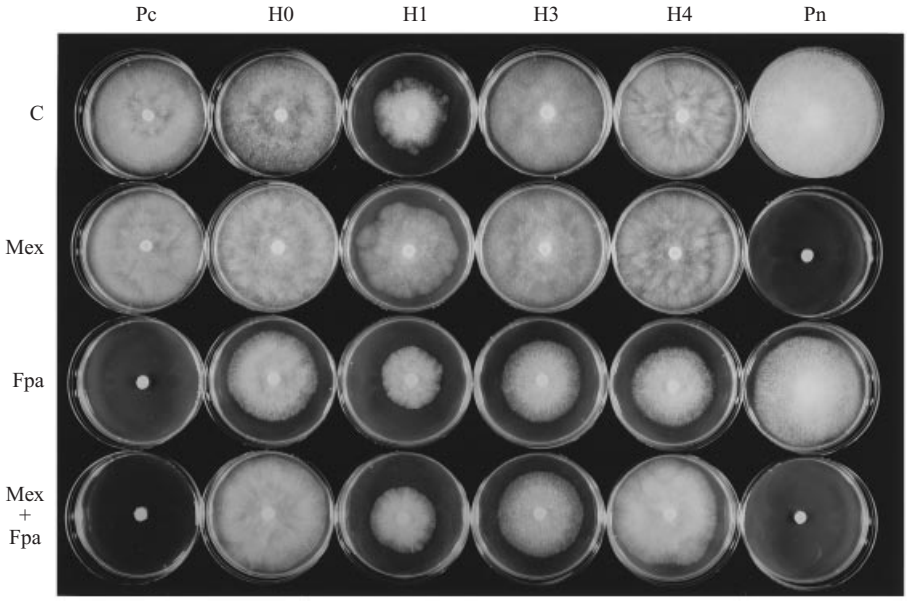


Fig. 1. Four-day-old colonies of interspecific hybrids, H0, H1, H3 and H4, and parental mutants, *P. capsici* Mex^{r}5 (Pc) and *P. nicotianae* Fpar^{r}10 (Pn), grown on V8C medium containing either no drugs (C) or supplemented with either 50 μg ml^{-1} metalaxyl (Mex), 200 μg ml^{-1} p-fluorophenylalanine (Fpa), or 30 μg ml^{-1} Mex plus 150 μg ml^{-1} Fpa (Mex + Fpa).

Table 1. Influence of selective drugs on growth of *P. capsici* Mex^{r}5, *P. nicotianae* Fpar^{r}10, and interspecific hybrids[a]

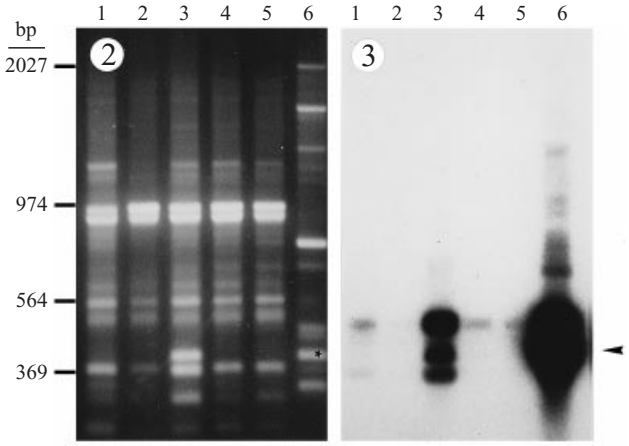
	Colony radius (mm) in presence Fpa/Mex[b] (μg ml^{-1})		
	0/0	150/30	300/60
P. capsici Mex^{r}5	23·3a[c]	0·3a	0[d]
P. nicotianae Fpar^{r}10	22·6a	0·3a	0
H0	19·3ab	18·7b	18·7a
H1	19·3ab	13·3c	13·3b
H3	18·0b	14·7c	13·7b
H4	22·0ab	18·0b	17·3a

[a] Measurements after 4 d incubation at 25° on V8C agar. The isolate of *P. capsici* was resistant to 60 μg ml^{-1} metalaxyl and the isolate of *P. nicotianae* was resistant to 300 μg ml^{-1} fluorophenylalanine.

[b] p-fluorophenylalanine (Fpa) or metalaxyl (Mex).

[c] Mean radial growth of three replicate colonies. Values followed by the same letter do not differ significantly as determined by Duncan's multiple range test ($P = 0·05$).

[d] No replicate colonies of *P. capsici* or *P. nicotianae* grew at these drug concentrations, and isolates were excluded from data analysis.



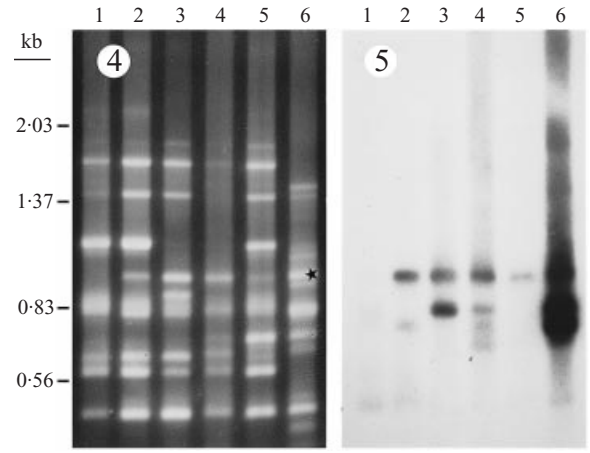
Figs 2, 3. PCR amplification products from *Phytophthora* spp. and hybrids using primer OPG-02. **Fig. 2.** Polymorphic amplification products of *P. capsici* Mex^{r}5 (lane 1), *P. nicotianae* Fpa^{r}10 (lane 6) and hybrids H0, H1, H3 and H4 (lanes 2, 3, 4 and 5, respectively) derived from RAPD-PCR with primer OPG-02. The *ca* 400-bp product that is characteristic of *P. nicotianae* Fpa^{r}10 and is present in H1 (lane 3) is indicated by the star. **Fig. 3.** Corresponding Southern-blot hybridized with the radiolabelled 400-bp product derived from *P. nicotianae* Fpa^{r}10. Arrowhead indicates homologous hybridization in *P. nicotianae* (lane 6) and in H1 (lane 3).

appeared in at least one hybrid isolate in the cases of primers OPG-01, OPG-02, OPG-06, OPG-18, OPK-07 and OPK-09.

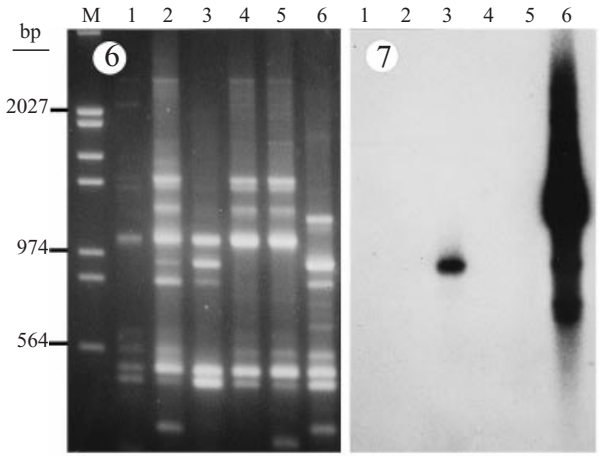
In a previous study, it was difficult to detect species-specific DNA of *P. nicotianae* in species hybrids (Érsek *et al.*, 1995). In the present study, however, DNA of *P. nicotianae* could be detected readily in three of four hybrids (H0, H1 and H3) by use of RAPD-PCR. For instance, after amplification using primer OPG-02, a *ca* 400-bp product that was diagnostic for *P. nicotianae* Fpa^{r}10 was detected in hybrid isolate H1 (Fig. 2, lane 3). To confirm the homology of these RAPD products from the two organisms, the 400-bp product from *P. nicotianae* Fpa^{r}10 was amplified and used as a probe against the RAPD products of the hybrids. Hybridization was observed with the 400-bp product in the parent species and hybrid (Fig. 3, lanes 3 and 6).

Similarly, in the case of OPG-18, a 1000-bp amplification product was observed in *P. nicotianae* Fpar10 that also was observed in three of four of the hybrid isolates, but not in *P. capsici* Mex^{r}5 (Fig. 4, lanes 2–4, 6). When the 1000-bp product of *P. nicotianae* Fpa^{r}10 was amplified and used as a probe, it hybridized strongly with three bands of equivalent size in *P. nicotianae* Fpa^{r}10 and hybrids H0, H1 and H3 (Fig. 5, lanes 2–4, 6).

Using primer OPG-01, only species hybrids H0 and H1 exhibited major amplification products that corresponded in size to products in *P. nicotianae* Fpa^{r}10 and not in *P. capsici*



Figs 4, 5. PCR amplification products from *Phytophthora* spp. and hybrids using primer OPG-18. **Fig. 4.** Polymorphic amplification products of *P. capsici* Mex^{r}5 (lane 1), *P. nicotianae* Fpa^{r}10 (lane 6) and hybrids H0, H1, H3 and H4 (lanes 2, 3, 4 and 5, respectively) derived from RAPD-PCR with primer OPG-18. The *ca* 1000-bp product that is characteristic of *P. nicotianae* Fpa^{r}10 and is present in hybrids is indicated by the star (lane 3). **Fig. 5.** Corresponding Southern-blot hybridized with the radiolabelled 1000-bp product derived from *P. nicotianae* Fpa^{r}10.

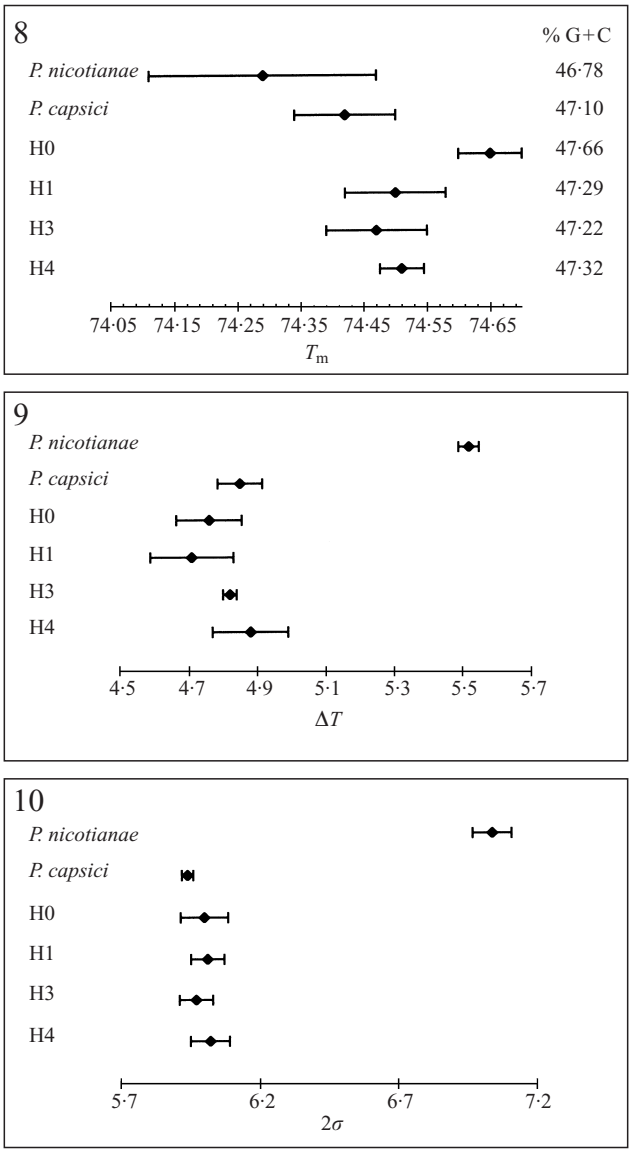


Figs 6, 7. PCR amplification products from *Phytophthora* spp. and hybrids using primer OPG-01. **Fig. 6.** Polymorphic amplification products of *P. capsici* Mex^{r}5 (lane 1), *P. nicotianae* Fpa^{r}10 (lane 6) and hybrids H0, H1, H3 and H4 (lanes 2, 3, 4 and 5, respectively) derived from RAPD-PCR with primer OPG-01. **Fig. 7.** Corresponding Southern-blot, probed with the radiolabelled *P. nicotianae*-specific repetitive sequence, P1000 that was amplified from pPP33A.

Mex^{r}5 (Fig. 6, lanes 2, 3 and 6). In this case, the hybrid bands were not hybridized with the corresponding homologous products of *P. nicotianae* Fpa^{r}10. Rather, a 1000-bp repetitive, species-specific sequence of DNA (plasmid pPP33A) from *P. nicotianae* Fpa^{r}10 (Érsek *et al.*, 1994) was used to probe the hybrid amplification products. This probe hybridized with a 910-bp product of species hybrid H1 only (Fig. 7, lane 3).

Thermal denaturation of DNA

DNA melting temperatures of *P. nicotianae* Fpa^{r}10 and *P. capsici* Mex^{r}5 did not differ significantly. Melting temperatures over all isolates varied from 74·3° for *P. nicotianae* Fpa^{r}10 to 74·6° for hybrid H0, respectively. The mean T_m for all isolates of species and hybrids was 74·5°. Isolate H0 was the only species hybrid that exhibited a melting temperature significantly higher than for either parent species (Fig. 8).



Figs 8–10. Thermal denaturation characteristics of DNA from *Phytophthora* spp. and hybrids. **Fig. 8.** Melting points (T_m) of DNAs and estimated percentage GC base compositions for *Phytophthora* spp. and hybrids. **Fig. 9.** Corresponding ΔT values of DNAs. **Fig. 10.** Corresponding values of 2σ. Bars = S.D.

The GC content of isolates varied between 46·8% and 47·7% for *P. nicotianae* Fpa^{r}10 and H0, respectively (Fig. 8). The mean GC content for all isolates was 47·2%. The GC contents of parent species and hybrids were similar to previous estimates for other *Phytophthora* spp. For example, Storck & Alexopoulos (1970) reported a GC content of 49% for *P. parasitica* (= *P. nicotianae*), and GC contents of 47·5 and 48% were reported for *P. infestans* (Clark *et al.*, 1968) and *P. megasperma* (Mao & Tyler, 1991), respectively. Storck & Alexopoulos (1970) also reported GC contents for 10 other species of *Phytophthora* that varied between 49 and 58%.

The fine structures of DNAs from hybrids were more similar to the DNA structure of *P. capsici* Mex^{r}5 than to *P.*

nicotianae Fpa^{r}10. For example, ΔT was significantly greater for *P. nicotianae* Fpa^{r}10 than for either *P. capsici* Mex^{r}5 or hybrids (Fig. 9), but it did not differ significantly among the hybrids and *P. capsici* Mex^{r}5. Similarly, the value of 2σ for *P. nicotianae* Fpa^{r}10 was significantly greater than values for all other isolates (Fig. 10). Again 2σ did not differ significantly among hybrid isolates or *P. capsici* Mex^{r}5.

These morphological, kinetic and molecular data suggest greater similarity of species hybrids to *P. capsici* than to *P. nicotianae*. Given the similarity of the hybrids to *P. capsici*, it was surprising the degree to which the hybrids varied in their pathogenicity and virulence traits (Érsek *et al.*, 1995). Even though interspecific somatic fusion is likely to occur rarely under natural conditions, it could contribute to the genetic diversity of heterothallic species of *Phytophthora*. The level of homogeneity in genotypes of these species under natural conditions bears further examination.

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