Analyses of the Population Structure in a Global Collection of *Phytophthora nicotianae* Isolates Inferred from Mitochondrial and Nuclear DNA Sequences

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ABSTRACT

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Genetic variation within the heterothallic cosmopolitan plant pathogen *Phytophthora nicotianae* was determined in 96 isolates from a wide range of hosts and geographic locations by characterizing four mitochondrial (10% of the genome) and three nuclear loci. In all, 52 single-nucleotide polymorphisms (SNPs) (an average of 1 every 58 bp) and 313 sites with gaps representing 5,450 bases enabled the identification of 50 different multilocus mitochondrial haplotypes. Similarly, 24 SNPs (an average of 1 every 69 bp), with heterozygosity observed at each locus, were observed in three nuclear regions (*hyp*, *scp*, and *β-tub*) differentiating 40 multilocus nuclear genotypes. Both mitochondrial and nuclear markers revealed a high level of dispersal of isolates and an inconsistent geographic structuring of populations. However, a specific association was observed for host of origin and genetic grouping with both nuclear and mitochondrial sequences. In particular, the majority of citrus isolates from Italy, California, Florida, Syria, Albania, and the Philippines clustered in the same mitochondrial group and shared at least one nuclear allele. A similar association was also observed for isolates recovered from *Nicotiana* and *Solanum* spp. The present study suggests an important role of nursery populations in increasing genetic recombination within the species and the existence of extensive phenomena of migration of isolates that have been likely spread worldwide with infected plant material.

Additional keywords: intraspecific variability.

The oomycete *Phytophthora nicotianae* Breda de Haan (*P. parasitica* Dastur) is distributed worldwide and has a broad host range infecting herbaceous and perennial host plants, causing foliar and fruit diseases as well as root and crown rots (15). The pathogen is particularly known as the causal agent of tobacco black shank and citrus root rot and gummosis but it can cause severe damage on many other plants, including ornamental, horticultural and fruit tree species (15). In particular, *P. nicotianae* is a major causal agent of root rot in nurseries of ornamental plants in Mediterranean regions (2,7,47,49).

Despite the economic importance of *P. nicotianae*, little is known about the structure of its population. The current state of knowledge is mainly restricted to populations from tobacco and are the results of studies of physiological aspects, such as resistance to fungicides (mainly the phenylamides mefenoxam and metalaxyl) and differential pathogenicity on tobacco cultivars (29,50). In the last 10 years, several molecular investigations have been conducted to analyze intraspecific variability within *P. nicotianae* populations; however, most of them focused on isolates from a single host (tobacco) and were designed to study isolates within specific fields or from the same geographic region (34,59, 65). Furthermore, the majority of these studies have been conducted using the random amplified polymorphic DNA (RAPD) technique, which does not readily enable the comparison of data from different laboratories.

Other electrophoretic markers such as restriction fragment length polymorphisms, amplified fragment length polymorphisms (AFLPs), and simple-sequence repeats (SSRs) have been utilized to study the population biology of *Phytophthora* spp. (12,42). In particular, AFLP was utilized to characterize a large number of *P. nicotianae* isolates from ornamental plants in different nurseries in Tennessee and identified six clonal lineages isolated from the same facility (36). However, such electrophoretic markers are not optimal for phylogenetic and genealogical reconstruction because comigrating bands shared by two individuals do not necessarily reflect descent from a common ancestor (27). In contrast, nucleotide sequence data offer the possibility of reconstructing patterns of descent among genotypes within a species or among populations of one or more species (24). In recent years, such approaches have been effectively utilized to study population structure, evolution, center of origin, and patterns of migration in *P. infestans* (8,22,26), *P. ramorum* (24,25), and *P. capsici* (20, 31,32).

Single-nucleotide polymorphisms (SNPs) have lower mutation rates (10^{-8} to 10^{-9}) compared with SSRs (10^{-2} to 10^{-6}) and, because multiple mutation events are improbable on the same site, most SNPs are bi-allelic and, therefore, appropriate for high-throughput genotyping of a diploid organism like *P. nicotianae* (5,55). SNPs are ideal for evolutionary studies because they are widespread in the genome and several unlinked nuclear loci are required to estimate population genetic parameters with statistical

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confidence (1). Furthermore, SNPs are easier to screen compared with SSRs. Even when, as in the case of *P. nicotianae*, genomic data are not available, SNPs can be identified by comparative genomic approaches designing primers to conserved flanking regions of related species. Using this strategy, a panel of mitochondrial and nuclear SNPs were discovered for a number of *Phytophthora* spp. representative of the genus (53,54).

In a recent study, 20 different mitochondrial haplotypes of *P. nicotianae* grouping in five clades were identified from 51 isolates mainly recovered in Italy (39) by analyzing two polymorphic intergenic regions flanked by genes *trn*Y and *rns* and by gene *trn*W and *cox2*. However, the different modes of inheritance and model of evolution in mitochondrial compared with nuclear DNA can evaluate different genetic components of variability. Some evolutionary processes can be explained only through the analyses of nuclear markers because mitochondrial DNA is uniparentally inherited and is much smaller compared with nuclear DNA (21). In particular, mitochondrial analyses conducted alone with an outcrossing species like *P. nicotianae* may produce incomplete results (64). The combined analysis of nuclear and mitochondrial genomes provides a more comprehensive insight into the evolutionary forces acting on natural populations.

In the present study, four mitochondrial and three nuclear loci containing SNPs were analyzed to investigate the structure of *P. nicotianae* populations in relation to geographic origin and hosts and to acquire information about prevalent reproduction systems and mechanisms that have facilitated its global distribution.

MATERIALS AND METHODS

Isolates of *P. nicotianae* and DNA extraction. In total, 96 isolates of *P. nicotianae* from six continents and various hosts (mainly citrus, tobacco, and ornamental species in nurseries) were evaluated (Table 1). Of these, 51 isolates were also analyzed by Mammella et al. (39), with an additional 45 new isolates from the World *Phytophthora* Genetic Resources Collection at the University of California, Riverside (http://phytophthora.ucr.edu) added to the analysis. DNA from these was extracted as described by Blair et al. (3).

Selection of mitochondrial and nuclear markers. Polymorphic mitochondrial DNA regions potentially containing SNPs were identified by aligning and comparing the complete mitochondrial genome of *P. nicotianae* (F. Martin, *unpublished data*) with the mitochondrial genomes of *P. infestans* (NC_002387, AY894835, AY898627, and AY898628), *P. ramorum* (DQ832718), and *P. sojae* (DQ832717). Sequences were aligned using Clustal W implemented in the software Accelrys Gene (ver. 2.5; Accelrys Inc., San Diego, CA). Several potentially useful regions were manually identified and screened (amplified and sequenced) for the presence of SNPs using 15 isolates representative of five different clades identified within *P. nicotianae* in a previous study (39). Mitochondrial regions selected for in-depth analysis (*trnG-rns*, *rns-cox2*, *cox2*+spacer, and *atp1-nad5*) represented 10% of the mitochondrial genome of *P. nicotianae*, had a length appropriate for polymerase chain reaction (PCR) amplification and sequencing, and were characterized by conserved flanking regions from which primers were designed (Fig. 1; Table 2). Additional internal primers were used for the sequencing of *cox2*+spacer and *atp1-nad5* regions from some isolates that did not produce clean reads with primers used for the amplification (Table 2).

Several reported nuclear genes were evaluated for the presence of SNPs using a restricted number of representative isolates of *P. nicotianae.* Selected regions represented a hypothetical conserved protein (*hyp*) amplified with primers I11F-I12R (53), the Scp-like protein (*scp*) amplified with primers NscpF-NscpR (35), and the β-tubulin (*β-tub*) gene (3). Additional primers to amplify the *β-tub* gene (NtubF1-NtubR1) were designed because those reported by Blair et al. (3) did not perfectly match the sequence of the gene of *P. nicotianae* (EU080504). Furthermore, nested sequencing primers (NtubF2-NtubR2) were utilized with a limited number isolates to improve the quality of sequences (Table 2).

Amplification and sequencing of target genes. Amplifications were run in a total volume of 25 µl containing 1 µl of genomic DNA (approximately 10 ng), 1× PCR buffer, 0.1 mM dNTPs, 1 unit of Taq polymerase (Applied Biosystems, Carlsbad, CA), and 0.5 µM each primer. The concentration of $MgCl_2$ and the annealing temperature were optimized for each primer pair (Table 2). Amplification conditions were 95°C for 3 min followed by 40 cycles of 1 min at 95°C, 1 min at the annealing temperature (Table 2), and 1 min at 72°C. All reactions ended with a final extension of 5 min at 72°C. Amplicons were analyzed by electrophoresis in 1.5% agarose gels containing SYBR Safe DNA gel stain (Invitrogen, Carlsbad, CA) in 1× Tris-borate-EDTA buffer and visualized with UV light. PCR products were purified using ExoSap (USB, Cleveland) in accordance with manufacturer's instructions and sent to the Nucleic Acid Sequencing Facility at Penn State University (University Park, PA) for sequencing. For each nuclear region where heterozygous bases were identified, amplicons from three different isolates were selected and cloned using the TOPO easy vector kit according to manufacturer's protocol (Invitrogen). For each isolate, 10 different clones were sequenced as described above but using standard T7 and T3 primers in order to confirm the presence of heterozygosity and determine haplotypes.

Analysis of sequences. Sequence chromatograms were evaluated for reliability and utilized to generate a consensus sequence using Sequencher 4.7 (Gene Codes, Ann Arbor, MI). Unreliable sequences were reamplified and sequenced using nested primers. Consensus sequences were aligned and manually analyzed for the presence of SNPs and indels in MacClade (ver. 4.02; Sinauer Associates, Sunderland, MA). All polymorphic sites observed in the alignment were confirmed in the chromatograms. Heterozygous SNPs were identified in the nuclear coding regions by the presence of double peaks in both forward and reverse sequences and were marked with standard degeneracy codes.

Genetic diversity. Mitochondrial and nuclear genetic diversity was evaluated for each individual region and for the combined data set of sequences (Table 3). The number of polymorphic sites, haplotype or allele reconstruction, and nucleotide diversity (π), were analyzed using DnaSP ver. 5.10.01 (38) with gaps included in the analysis. The number of multilocus haplotypes and genotypes was determined manually and using an SNP allele and position-calling program (MJBv1.2Gba) edited in python (N. Feau and G. Bilodeau, *unpublished data*).

Nucleotide and allele diversity were determined for the nuclear regions *hyp* and *β-tub* (Table 3) but not for the *scp* gene, because the sequencing of cloned amplicons from this latter gene for some isolates revealed more than two different alleles. For the *hyp* region, alleles were determined through a simple clone subtraction approach using sequences of cloned isolates (P1452, IRF5, and P7330) as known alleles. To determine alleles of the *β-tub* region, the software PHASE v2.1.1 with input file automatically formatted in SeqPHASE (18) was used, because this gene contained a higher number of heterozygous SNPs. The model for nonintragenic recombination was applied for the analysis. Standard search settings were 100 number of iterations, 1 thinning interval, and 100 as burn-in. Cloned and sequenced alleles from isolates P6915, P6113, and P7346 were treated as known alleles. The analysis was run five times for the same data set using a different starting seed.

The positions of synonymous and nonsynonymous substitution were inferred using a specific GenBank *P. nicotianae* sequence (EU080504) for the *β-tub* gene and the orthologous sequences retrieved from the *P. infestans* genome database (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans) for *hyp* (PITG_18320.2) and *scp* (PITG_10036.1) genes.

Phylogenetic analysis and population structure. Phylogenetic analyses were conducted for the combined data set of mitochondrial sequences using maximum parsimony, maximum likelihood, and Bayesian methods (Fig. 2). A partition homogeneity test (17) was performed on the concatenated data set with PAUP v4.0b10 (Sinaur Associates) to test the congruence of data using a heuristic search with 1,000 replicates. The maximum parsimony analysis was performed in PAUP using a heuristic search algorithm with random stepwise addition of taxa (10 replicates), tree bisection reconnection branch swapping, and multiple trees option. The statistical support was determined by bootstrap values for 1,000 replicates. TOPALi v2 (46) was used to determine the substitution model that best fit the data. The model HKY+I+G was selected for the Bayesian and maximum likelihood phylogenetic analysis using MrBayes version 3.1.1 (52) and PhyML version 2.4.5 (28), respectively, implemented in TOPALi. Bayesian analysis was performed with four runs conducted simultaneously for 500,000 generations, with 10% sampling frequency and burn-in of 30%. Maximum likelihood was performed with 100 bootstrap replicates.

Phylogenetic analysis was not possible for the nuclear data due to the high level of heterozygosity. However, the concatenated data set of nuclear sequences was utilized to construct a neighbor-joining tree and evaluate relationships among different multilocus genotypes. The tree was constructed using absolute measure of distances and a random input order of sequences in PAUP v4.0b10.

The population structure of *P. nicotianae* was analyzed by generating networks of isolates with the statistical parsimony algorithm implemented in TCS ver. 1.21 (10). Networks were

TABLE 1. Isolates of *Phytophthora nicotianae* included in the study, their host of recovery, geographic origin, mating type, and summary of the results obtained from the analysis of mitochondrial and nuclear DNA

				Mitochondrial DNA[c]									Nuclear DNA[e]			
				trnG-rns		*rns-cox2*		*cox2*+spacer		*atp1-nad5*						
Isolates[a]	Host	Origin	MT[b]	MH	Size	MH	Size	MH	Size	MH	Size	MMH[d]	*Hyp*	*Scp*	*β-tub*	MNG[f]
Albicocco9*	*Prunus armeniaca*	Southern Italy	A2	h1	606	h1	577	h1	910	h1	876	H1	g1 (a1)	g1	g1 (a1/a2)	G1
P7449	*Chrysanthemum* sp.	India	A1	h2	608	h2	577	h2	910	h2	788	H2	g2 (a1/a3)	g2	g2 (a1)	G2
P1495	*Nicotiana tabacum*	Australia	A2	h3	607	h2	577	h2	910	h3	788	H3	g1 (a1)	g3	g3 (a4)	G3
P1955	*N. tabacum*	South Africa	A1	h2	608	h2	577	h2	910	h3	788	H4	g1 (a1)	g2	g4 (a4/a5)	G4
P6115	*Gypsophila* sp.	Japan	A2	h2	608	h2	577	h2	910	h3	788	H4	g1 (a1)	g3	g5 (a2/a4)	G5
Ciclamino1*	*Cyclamen* sp.	Southern Italy	A1	h4	608	h2	577	h2	910	h2	788	H5	g1 (a1)	g4	g6 (a2/a3)	G6
Correa5*	*Correa reflexa*	Southern Italy	A1	h5	607	h1	577	h2	910	h4	785	H6	g1 (a1)	g1	g7 (a1/a3)	G7
Correa8*	*C. reflexa*	Southern Italy	A1	h6	608	h1	577	h2	910	h5	785	H7	g1 (a1)	g1	g7 (a1/a3)	G7
Correa3*	*C. reflexa*	Southern Italy	A1	h6	608	h1	577	h2	910	h5	785	H7	g1 (a1)	g1	g7 (a1/a3)	G7
Dodonea Rad1*	*Dodonaea viscosa*	Southern Italy	A2	h7	605	h1	577	h2	910	h1	876	H8	g1 (a1)	g1	g1 (a1/a2)	G1
Dodonea Col1*	*D. viscosa*	Southern Italy	A2	h7	605	h1	577	h2	910	h1	876	H8	g1 (a1)	g1	g1 (a1/a2)	G1
IMI 268688	*Citrus* sp.	Trinidad	A1	h8	607	h1	577	h3	910	h6	876	H9	g1 (a1)	g5	g8 (a4/a6)	G8
P6915	*Dieffenbachia maculata*	Germany	A2	h9	607	h3	464	h4	910	h7	882	H10	g1 (a1)	g1	g9 (a4/a7)	G9
P10297	*D. maculata*	Florida	A2	h9	607	h3	464	h4	910	h7	882	H10	g1 (a1)	g1	g9 (a4/a7)	G9
P1492	*N. tabacum*	Australia	A2	h10	619	h1	577	h5	910	h8	890	H11	g3 (a1/a2)	g1	g7 (a1/a3)	G10
P1751	*N. tabacum*	Australia	A1	h10	619	h1	577	h5	910	h8	890	H11	g3 (a1/a2)	g1	g10 (a3)	G11
P1752	*N. tabacum*	Australia	A1	h10	619	h1	577	h5	910	h8	890	H11	g3 (a1/a2)	g1	g7 (a1/a3)	G10
P1753	*N. tabacum*	Australia	A1	h10	619	h1	577	h5	910	h8	890	H11	g3 (a1/a2)	g1	g7 (a1/a3)	G10
P7387	*Hippeastrum* sp.	Netherlands	A2	h10	619	h1	577	h5	910	h8	890	H11	g2 (a1/a3)	g1	g11 (a1/a5)	G12
P7330	*Lavandula* sp.	Australia	A2	h10	619	h1	577	h5	910	h8	890	H11	g3 (a1/a2)	g1	g7 (a1/a3)	G10
P7665	*Leucodendron* sp.	Australia	A1	h10	619	h1	577	h5	910	h8	890	H11	g3 (a1/a2)	g1	g7 (a1/a3)	G10
STA24*	*Rhamnus alaternus*	Southern Italy	A2	h10	619	h1	577	h5	910	h8	890	H11	g2 (a1/a3)	g1	g11 (a1/a5)	G12
P6113	*Lilium* sp.	Japan	A2	h11	619	h4	577	h5	910	h9	898	H12	g1 (a1)	g4	g5 (a2/a4)	G13
P3813	*Catharanthus* sp.	California	A1	h10	619	h1	577	h5	910	h10	890	H13	g3 (a1/a2)	g1	g2 (a1)	G14
P16824	*Catharanthus roseus*	Japan	A1	h10	619	h1	577	h5	910	h10	890	H13	g3 (a1/a2)	g1	g10 (a3)	G11
P3458	*Carthamus tinctorius*	Venezuela	A1	h12	620	h1	577	h6	910	h11	890	H14	g4 (a2)	g6	g2 (a1)	G15
Ceanothus*	*Ceanothus* sp.	Southern Italy	A2	h13	619	h5	577	h7	910	h12	891	H15	g1 (a1)	g7	g7 (a1/a3)	G16
KVB*	*Howea* sp.	Southern Italy	A2	h13	619	h5	577	h7	910	h12	891	H15	g1 (a1)	g7	g7 (a1/a3)	G16
IRF26/2*	*Impatiens* sp.	Northern Italy	A2	h13	619	h5	577	h7	910	h12	891	H15	g1 (a1)	g7	g7 (a1/a3)	G16
Ph440/00*	*Cyclamen* sp.	Northern Italy	A2	h13	619	h5	577	h7	910	h12	891	H15	g1 (a1)	g7	g7 (a1/a3)	G16
Ph168	*Citrus* sp.	Tunisia	A1	h13	619	h1	577	h8	910	h13	894	H16	g1 (a1)	g4	g3 (a4)	G17
P0582	*N. tabacum*	Kentucky	A2	h14	617	h1	577	h8	910	h14	894	H17	g5 (a3)	g1	g3 (a4)	G18
P1335	*N. tabacum*	Virginia	A2	h14	617	h1	577	h8	910	h14	894	H17	g5 (a3)	g1	g3 (a4)	G18
P1334	*N. tabacum*	Virginia	A2	h14	617	h1	577	h8	910	h14	894	H17	g5 (a3)	g1	g3 (a4)	G18
P1333	*N. tabacum*	Virginia	A2	h14	617	h1	577	h8	910	h14	894	H17	g5 (a3)	g1	g3 (a4)	G18
P7522	*Catharanthus roseus*	California	A2	h15	607	h6	577	h9	910	h15	893	H18	g3 (a1/a2)	g4	g7 (a1/a3)	G19
P1452	*Citrus* sp.	California	A1	h16	607	h6	577	h9	910	h16	892	H19	g2 (a1/a3)	g4	g7 (a1/a3)	G20
P7561	*Citrus jambiri*	Philippines	A1	h16	608	h6	577	h9	910	h17	891	H20	g5 (a3)	g4	g7 (a1/a3)	G21
P1569	*Citrus* sp.	California	A1	h16	607	h6	577	h9	910	h18	893	H21	g2 (a1/a3)	g4	g7 (a1/a3)	G20
Ph195	*Citrus* sp.	Syria	A1	h16	607	h6	577	h9	910	h18	893	H21	g5 (a3)	g4	g7 (a1/a3)	G21
Ph3	*C. clementina*	Italy	A1	h16	607	h6	577	h9	910	h18	893	H21	g5 (a3)	g4	g7 (a1/a3)	G21
P1325	*Citrus* sp.	California	A2	h17	608	h6	577	h9	910	h16	892	H22	g2 (a1/a3)	g4	g7 (a1/a3)	G20
Ferrara R11	*C. aurantium*	Southern Italy	A1	h16	607	h6	577	h9	910	h19	893	H23	g5 (a3)	g4	g7 (a1/a3)	G21

(*continued on next page*)

[a] Isolates were primarily listed accordingly to their multilocus mitochondrial haplotypes (MMHs) and secondly accordingly to multilocus nuclear genotypes (MNGs). Isolates labeled with an asterisk (*) were sourced from potted ornamental species in nursery.

[b] MT = mating type.

[c] MH = mitochondrial haplotype, and Size = length of the amplified fragments in base pairs.

[d] MMHs determined for the combined mitochondrial data set of sequences (*trnG-rns, rns-cox2, cox2*+spacer, and *atp1-nad5*).

[e] Nuclear genotypes. Alleles (a) were determined for *hyp* and *β-tub* genes but not for the *scp* gene, since more than two different alleles were identified in some strains. In homozygote strains a single allele is indicated;

[f] MNGs for the combined data set of sequences for the three nuclear regions (*hyp*, *scp*, and *β-tub*). n.d. = not determined

generated for individual mitochondrial (*trnG-rns, rns-cox2, cox2*+spacer, and *atp1-nad5*) and nuclear (*hyp* and *β-tub*) regions as well as for the combined data set of mitochondrial sequences.

To determine the presence of significant genetic structuring among geographic origin (Africa, America, Asia, Australia, and Europe) and among originating host genus, combined mitochondrial haplotypes and nuclear alleles from *β-tub* and *hyp* genes where analyzed by analysis of molecular variance (AMOVA) using Arlequin 3.1 software (16). Furthermore, the Wright's inbreeding coefficients (F_{IS}) (62) were calculated using nuclear alleles to analyze the mating system within populations from three field crops (*Citrus*, *Nicotiana*, and *Solanum* spp.) and from potted ornamental species in nursery (Table 1). Isolates from *Solanum* spp. were not included in the analysis with the *hyp* gene because all alleles where identical.

RESULTS

Amplification and sequencing. Four mitochondrial (t*rnG-rns, rns-cox2, cox2*+spacer, and *atp1-nad5*) and three nuclear (*hyp, scp*, and *β-tub*) regions were selected because they contained intraspecific variation and their amplification and sequencing was possible from the complete panel of 96 isolates (Table 1). A single exception was represented by the *β-tub* region of isolate P7622 because it was not possible to obtain a high-quality sequence. DNA sequences were deposited in GenBank with the following accession numbers: JF706726-JF706821 (*atp1-nad5*), JF706822-JF706917 (*trnG-rns*), JF706918-JF707013 (*rns-cox2*), JF707014-JF707109 (*cox2*+spacer), JF707110-JF707204 (*β-tub*), JF707205-JF707300 (*scp*), and JF707301-JF707396 (*hyp*). Sequence alignments from both combined mitochondrial and

TABLE 1. (*continued from preceding page*)

				Mitochondrial DNA[c]												
				trnG-rns		*rns-cox2*		*cox2*+spacer		*atp1-nad5*			Nuclear DNA[e]			
Isolates[a]	Host	Origin	MT[b]	MH	Size	MH	Size	MH	Size	MH	Size	MMH[d]	*Hyp*	*Scp*	*β-tub*	MNG[f]
Ferrara R3	*C. aurantium*	Southern Italy	A1	h16	607	h6	577	h9	910	h19	893	H23	g5 (a3)	g4	g7 (a1/a3)	G21
Ferrara R8	*C. aurantium*	Southern Italy	A1	h16	607	h6	577	h9	910	h19	893	H23	g5 (a3)	g4	g7 (a1/a3)	G21
Serravalle1	*C. aurantium*	Southern Italy	A1	h16	607	h6	577	h9	910	h19	893	H23	g5 (a3)	g4	g7 (a1/a3)	G21
Ph87	*C. aurantium*	Southern Italy	A1	h16	607	h6	577	h9	910	h19	893	H23	g5 (a3)	g4	g7 (a1/a3)	G21
Ph342/03*	*Limonium sinensis*	Northern Italy	A2	h16	607	h6	577	h9	910	h19	893	H23	g5 (a3)	g2	g7 (a1/a3)	G22
Ph142	*Poncirus trifoliata*	Albania	A1	h17	608	h6	577	h9	910	h19	893	H24	g5 (a3)	g4	g7 (a1/a3)	G21
Ph9	*Citrus* sp.	Southern Italy	A1	h17	608	h6	577	h9	910	h19	893	H24	g5 (a3)	g4	g7 (a1/a3)	G21
Pn17	*Citrus* sp.	Florida	n.d.	h18	609	h6	577	h9	910	h19	893	H25	g5 (a3)	g1	g7 (a1/a3)	G23
Serravalle3	*Citrus* sp.	Southern Italy	A1	h19	606	h6	577	h9	910	h19	893	H26	g5 (a3)	g4	g7 (a1/a3)	G21
C88	*Simmondsia chinensis*	Southern Italy	n.d.	h20	609	h7	577	h9	910	h20	881	H27	g1 (a1)	g2	g12 (a1/a4)	G24
Nic8Vasi*	*Lavandula angustifolia*	Southern Italy	A2	h20	609	h7	577	h9	910	h20	881	H27	g1 (a1)	g2	g12 (a1/a4)	G24
TL8VP*	*L. angustifolia*	Southern Italy	A2	h20	609	h7	577	h9	910	h20	881	H27	g1 (a1)	g2	g12 (a1/a4)	G24
P1350	*N. tabacum*	North Carolina	A1	h20	609	h7	577	h9	910	h21	880	H28	g5 (a3)	g1	g3 (a4)	G18
P0583	*N. tabacum*	Kentucky	A2	h21	608	h7	577	h10	876	h21	880	H29	g6 (a2/a3)	g1	g12 (a1/a4)	G25
P6832	*Cyclamen* sp.	Greece	A2	h20	609	h7	577	h9	910	h22	881	H30	g1 (a1)	g4	g3 (a4)	G17
P3461	*Solanum lycopersicum*	UK	A2	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g2	g3 (a4)	G26
P3118	*S. lycopersicum*	Australia	A2	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g2	g3 (a4)	G26
Ph5	*Citrus* sp.	Southern Italy	A1	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g8	g10 (a3)	G27
Ph653/03*	*Choisya ternata*	Northern Italy	A2	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g2	g3 (a4)	G26
Ph647b/03*	*Phormium tenax*	Northern Italy	A2	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g4	g12 (a1/a4)	G28
IMI 379626	*S. lycopersicum*	Chile	A1	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g2	g3 (a4)	G26
Melanzana1	*S. melongena*	Southern Italy	A2	h9	607	h7	577	h9	910	h20	881	H31	g1 (a1)	g2	g3 (a4)	G26
IRF5*	*Polygala myrtifolia*	Northern Italy	A2	h22	609	h7	577	h9	910	h22	881	H32	g7 (a1/a4)	g9	g12 (a1/a4)	G29
Lavanda1*	*L. angustifolia*	Southern Italy	A2	h21	608	h7	577	h9	910	h20	881	H33	g1 (a1)	g4	g12 (a1/a4)	G28
Mirtus3*	*Myrtus communis*	Southern Italy	A1	h21	609	h7	577	h9	910	h20	881	H33	g1 (a1)	g4	g7 (a1/a3)	G30
Lavanda4*	*L. angustifolia*	Southern Italy	A2	h23	606	h7	577	h9	910	h20	881	H34	g1 (a1)	g4	g12 (a1/a4)	G28
SCRP462	*Fragaria × ananassa*	India	A1	h9	607	h8	570	h9	910	h23	895	H35	g1 (a1)	g2	g3 (a4)	G26
Anthurium*	*Anthurium* sp.	Southern Italy	A1	h24	608	h9	526	h9	910	h24	893	H36	g1 (a1)	g3	g12 (a1/a4)	G31
Pandorea2C*	*Pandorea jasminoides*	Southern Italy	A2	h24	608	h9	526	h9	910	h24	893	H36	g1 (a1)	g3	g12 (a1/a4)	G31
C301*	*Myrtus communis*	Southern Italy	A2	h25	606	h9	526	h9	910	h24	893	H37	g1 (a1)	g2	g3 (a4)	G26
P3815	*Rosa hybrida*	United States	A2	h25	606	h9	526	h9	910	h24	893	H37	g2 (a1/a3)	g10	g7 (a1/a3)	G32
Peperone GJ	*Capsicum annum*	Southern Italy	A1	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g6	g4 (a4/a5)	G33
Peperone RC	*C. annum*	Southern Italy	A2	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g4	g4 (a4/a5)	G34
Pomodoro	*S. lycopersicum*	Southern Italy	n.d.	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g2	g11 (a1/a5)	G35
P7622	*Gypsophila* sp.	South Africa	A1	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g2	n.d.	n.d.
IRF27*	*Agapanthus* sp.	Northern Italy	A2	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g2	g11 (a1/a5)	G35
IRF8*	*Anemone americana*	Northern Italy	A2	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g2	g11 (a1/a5)	G35
P16870	*S. lycopersicum*	Spain	A2	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g6	g4 (a4/a5)	G32
P16883	*S. lycopersicum*	Spain	A2	h26	607	h9	526	h9	910	h24	893	H38	g1 (a1)	g4	g13 (a3/a5)	G36
P1083	*Gypsophila* sp.	California	A1	h26	607	h9	526	h9	910	h25	893	H39	g1 (a1)	g8	g2 (a1)	G37
P16823	*Karankoe* sp.	Japan	A2	h27	609	h9	526	h9	910	h24	893	H40	g1 (a1)	g4	g14 (a3/a4)	G38
Hybiscus B*	*Hibiscus* sp.	Southern Italy	A2	h24	608	h9	526	h11	910	h26	892	H41	g1 (a1)	g7	g7 (a1/a3)	G16
MirtoP5*	*Myrtus communis*	Southern Italy	A2	h26	607	h9	526	h9	910	h27	893	H42	g1 (a1)	g4	g12 (a1/a4)	G28
Pittosporo*	*Pittosporum* sp.	Southern Italy	A1	h26	607	h9	526	h9	910	h27	893	H42	g1 (a1)	g4	g2 (a1)	G39
IRF3*	*Polygala myrtifolia*	Northern Italy	A2	h26	607	h7	577	h12	910	h28	893	H43	g1 (a1)	g4	g5 (a2/a4)	G13
P3456	*Hibiscus* sp.	Pakistan	A2	h28	607	h7	577	h9	910	h28	893	H44	g1 (a1)	g1	g10 (a3)	G40
P3549	*Aphelandra* sp.	Florida	A2	h25	606	h7	577	h9	910	h29	893	H45	g1 (a1)	g1	g7 (a1/a3)	G7
P3234	*Hibiscus* sp.	China	A2	h26	607	h7	577	h9	910	h28	893	H46	g1 (a1)	g1	g10 (a3)	G40
P1577	*Citrus* sp.	California	A1	h24	608	h6	577	h13	912	h30	894	H47	g1 (a1)	g4	g3 (a4)	G17
P0700	*S. lycopersicum*	Ponape	A2	h24	607	h7	577	h9	910	h31	893	H48	g1 (a1)	g1	g10 (a3)	G40
P7346	*Choisya ternata*	United Kingdom	A1	h26	607	h9	526	h9	910	h28	893	H49	g1 (a1)	g2	g11 (a1/a5)	G35
IMI 207770	*Durio zibethinus*	Malaysia	A1	h26	607	h7	577	h9	910	h31	893	H50	g1 (a1)	g1	g10 (a3)	G40
P10802	*Dianthus caryophyllus*	Japan	A2	h26	607	h7	577	h9	910	h31	893	H50	g1 (a1)	g1	g10 (a3)	G40

nuclear sequences have been deposited in TreeBASE (submission ID 12393).

Three other mitochondrial regions (*secY*, *rps10*, and *rpl5*) (43) (F. Martin, *unpublished*) and two nuclear genes (elicitin [33] and ras-related protein *Ypt1*) [9]) were investigated and discarded during preliminary screenings because they did not exhibit enough variation within a representative panel of 15 isolates. Furthermore, another potentially interesting target, the necrosis-inducing protein *Pp1* gene (51), was discarded because it did not yield reliable sequences from all isolates.

Analysis of genetic diversity. *Mitochondrial DNA*. The analyses of mitochondrial regions revealed the presence of intraspecific polymorphisms in the four loci sequenced (Tables 1 and 3). The *rns-cox2* region (length variable from 464 to 577 bp) was the least variable region, with π of 0.0018 (Table 3). The analyzed region consisted of the entire intergenic region and portions of the flanking coding regions of the *rns* and *cox2* genes (39 and 58 bp, respectively) (Fig. 1). No variation was observed in these coding regions and in the *trnW* (cca) tRNA that is within the intergenic region.

The *cox2*+spacer region had a length between 876 and 912 bp (Table 1) and π of 0.0022. This region consisted of almost the entire reading frame for the *cox2* gene (92 bases at the 5′ end were not included), two intergenic spacers (197 bp) flanking the putative *orf32*, and a small portion (31 bp) of the *cox1* gene (Fig. 1). In the coding region of the *cox2* gene, eight SNPs were identified (position 64, 114, 133, 206, 226, 547, 634, and 680) and six of them led to a nonsynonymous change in the protein sequence (Supplemental Table 1). In particular, two nonsynonymous mutations in nucleotide position 64 (Val to Leu) and 547 (Glu to Lys) differentiated two isolates (P10297 and P6915) isolated from *Dieffenbachia maculata* in Florida and Germany from all other isolates (Table 1).

The *trnG-rns* intergenic region (length of 605 to 620 bp) contained 12 SNPs and 33 sites with gaps giving π of 0.0051 (Table 3); no variation was present in the *trnG* and *trnY* genes flanking the intergenic region. The *atp1-nad5* intergenic region (length of 785–895 bp) was the most variable region examined, with π of 0.0077 as a result of 23 SNPs and 124 sites with gaps (Table 3). No variation was present in coding portions of this gene (Fig. 1). On the whole, 52 SNPs (average of 1 SNP every 59 bp) and 313 sites with gaps representing 5,450 bases were observed in the combined dataset for the four mitochondrial regions (Table 3).

Nuclear DNA. Intraspecific variability was detected in the three nuclear regions analyzed (Tables 1 and 3). In all, 3, 10, and 11 SNPs differentiated 7, 10, and 14 genotypes in the *hyp, scp*, and *β-tub* nuclear loci, respectively. In the combined data set of nuclear sequences (1,654 bp), 24 SNPs were identified (average of 1 SNP every 69 bp) and heterozygosity was observed at each SNP locus (Table 4). In total, 40 genotypes were observed for the combined nuclear data set and 21 of them were unique (Table 4).

A high level of nucleotide and allele diversity was determined for the *hyp* and *β-tub* genes (Table 3). The *scp* region was excluded from this analysis because more than two alleles were observed in two of the representative isolates by cloning and sequencing of the respective amplicons. In particular, five and four alleles were revealed for isolates Anthurium and IMI268688, respectively, revealing an unexpected genetic framework for a diploid organism like *P. nicotianae*.

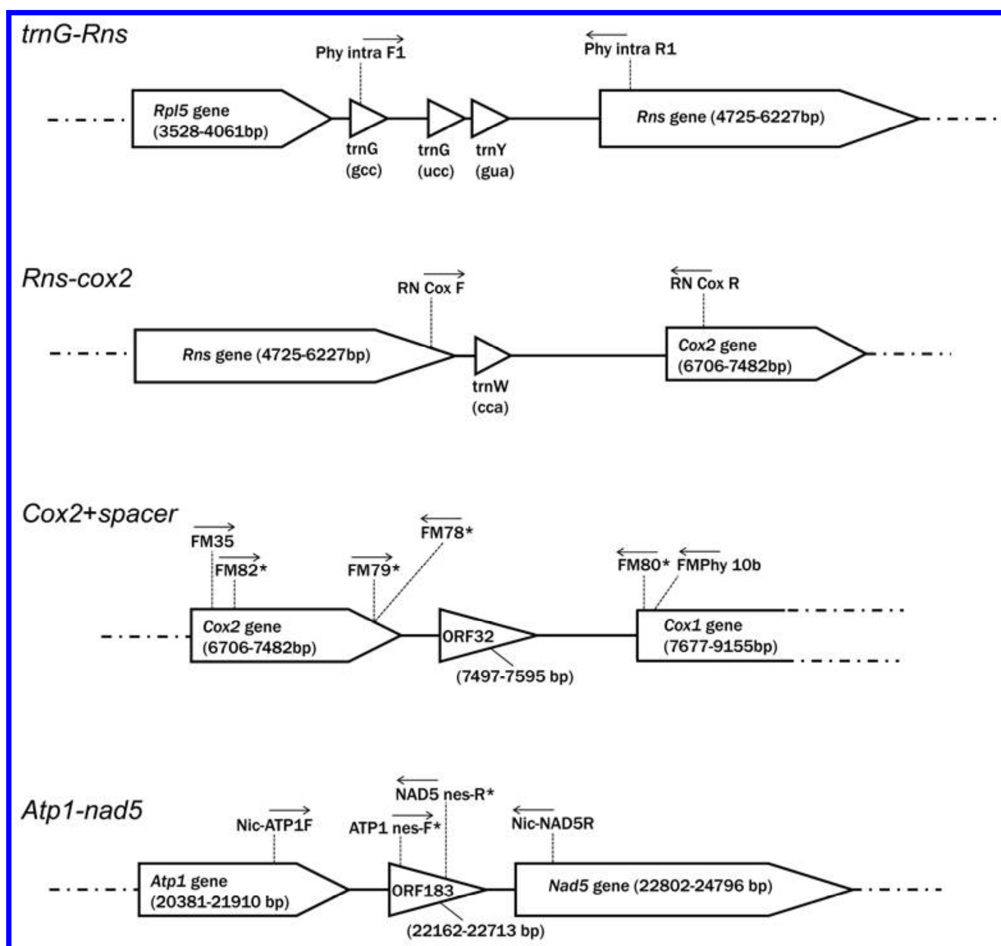

Fig. 1. Schematic representation of the mitochondrial regions examined in this study with location of selected primers. Arrows on primers indicate their orientation. Primers labeled with the symbol (*) were used for the sequencing of few isolates that did not produce reliable sequences with primers used for the amplification.

Nonsynonymous substitutions were identified in the *hyp* locus, with 27 isolates having an SNP in position 101, leading to a change from Cys to Ser. Similarly, 41 isolates had a nonsynonymous change in position 238 of the *scp* locus leading to a change from Ser to Gly (Supplemental Table 2). Furthermore, a single SNP in position 397 led to a change from Ala to Thr in isolate P3815. Nonsynonymous substitutions were not observed in the *β-tub* gene.

Haplotype analyses. *Mitochondrial DNA*. Polymorphisms in the four mitochondrial regions partitioned the 96 isolates of the panel into 28, 9, 13, and 31 haplotypes for regions *trnG-rns*, *rns-cox2*, *cox2*+spacer, and *atp1-nad5,* respectively (Tables 1 and 3). Haplotype diversity was 0.6243–0.9414 (Table 3) and eight haplotypes were also identified in the coding region of *cox2* gene. The combined data set of the four mitochondrial regions enabled the identification of 50 haplotypes with a high haplotype diversity (0.9735), mainly due to the large number of unique multilocus haplotypes (31). Haplotypes H11 and H38 were the most frequently encountered (Table 1). Based on haplotypes and π, the intergenic spacers *trnG-rns* and *atp1-nad5* were the most variable regions. In all, 47 of 50 haplotypes were identified analyzing only these two regions.

Nuclear DNA. Four alleles were identified for the *hyp* region, with a haplotype diversity of 0.4515 (Tables 1 and 3). A higher number of alleles (n = 7) were identified for the *β-tub* region (Tables 1 and 3). The higher haplotype diversity in the *β-tub* gene was mainly determined by heterozygous individuals showing low-frequency alleles (Table 1). The confidence probability score for each SNP position in the haplotypes determined using PHASE was 1 or 0.97 (in a single case) for the five different runs, indicating a high accuracy of the test.

Phylogenetic analyses. *Mitochondrial DNA*. Phylogenetic analyses were conducted using the concatenated data set of the four mitochondrial regions because the partition homogeneity test was not significant (P = 0.06). The three methods of analysis (maximum parsimony, maximum likelihood, and Bayesian) generated trees with a similar topology (Fig. 2). The 96 isolates (50 haplotypes) were distributed in six main clades largely

TABLE 2. Primers and polymerase chain reaction amplification conditions used in the present study to amplify and sequence mitochondrial and genomic regions of *Phytophthora nicotianae*

Target DNA	Primers[a]	Sequence (5′–3′)[b]	Amplification conditions
Mitochondrial DNA			
TrnG-rns	Phy intra-F1[c]	GGTAGAGTATAACCTTGC	…
	Phy intra-R1[c]	ATAGCATTTATTCTGAGCCA	3 mM Mg, 57°C
Rns-cox2	RN-CoxF[d]	GATGAAGTCGTAACAAGGTA	…
	RN-CoxR[d]	AAACCTAATTGCCAAGGTTC	3 mM Mg, 64°C
Cox2+spacer	FM35[e]	CAGAACCTTGGCAATTAGG	…
	FMphy-10b[e]	GCAAAAGCACTAAAAATTAAATATAA	…
	FM78[f]	(ACAAATTTCACTACATTGTCC)	…
	FM79[f]	(GGACAATGTAGTGAAATTTGT)	…
	FM 82[f]	(TTGGCAATTAGGTTTTCAAGATCC)	…
	FM 80[f]	(AATATCTTTATGATTTGTTGAAA)	3 mM Mg, 54°C
Atp1-nad5	Nic-ATP1F	AACTAATCATTCTAATATTTTAGAA	…
	Nic-NAD5R	CAGAACCTTTACGTCCAATAT	…
	ATP1nes-F	(AAACAATTTAGATTACGTGGA)	…
	NAD5 nes-R	(CAAATATCTATTGGTGTTAACAT)	3 mM Mg, 54°C
Nuclear DNA			
hyp	I11F[g]	TCGTCBGTGTCCTCBACGTC	…
	I12R[g]	ACCAGCATCTTRTTCTGRGCAG	1 mM Mg, 55°C
scp	NscpF	TGTGCGGTGATGTCTGTGC	…
	NscpR	TCACCACCTTTGCGAARCC	1 mM Mg, 60°C
β-tub	BtubF1[h]	GCCAAGTTCTGGGAGGTCATC	…
	BtubR1[h]	CCTGGTACTGCTGGTACTCAG	…
	NtubF1	(ACGCTTCTTATCTCGAAGATT)	…
	NtubR1	(CTTACGCAGGTCCGAGTTC)	…
	NtubF2	(CTCGGACCTGCAGCTGGA)	…
	NtubR2	(CGTAAACTGTTCGGACACAC)	2.5 mM Mg, 60°C

[a] Primers not designed in the present study were from other sources, as indicated below.
[b] Primers reported in parenthesis were only used for sequencing.
[c] Martin and Coffey (43).
[d] F. N. Martin, *unpublished*.
[e] Martin (41).
[f] Martin and Tooley (44).
[g] Schena et al. (53).
[h] Blair et al. (3).

TABLE 3. Summary table showing results from the analyses of mitochondrial and nuclear sequences of *Phytophthora nicotianae*[a]

	Mitochondrial DNA					Nuclear DNA			
Parameters	*trnG-rns*	*rns-cox2*	*cox2*-spacer	*atp1-nad5*	CMS	*hyp*	*scp*	*β-tub*	CNS
Isolates	96	96	96	96	96	96	96	95	95
Alignment length (bp)	629	577	912	905	3,023	241	544	869	1,654
Haplotype/allele number	28	9	13	31	50	4	n.d.	7	n.d.
Haplotype/allele diversity	0.9325	0.8132	0.6243	0.9414	0.9735	0.4515	n.d.	0.7334	n.d.
Polymorphic sites[b]	12	4	13	23	52	3	10	11	24
Site with gaps	33	120	36	124	313	0	0	0	0
Parsimony informative sites	9	3	9	18	39	2	2	11	23
Nucleotide diversity (π)	0.0051	0.0018	0.0022	0.0077	0.0043	0.0020	n.d.	0.0020	n.d.

[a] CMS = combined mitochondrial sequences, CNS = combined nuclear sequences, and n.d. = not determined.
[b] Gaps were excluded from the analysis of polymorphic sites.

corresponding to groups observed in the mitochondrial network (Fig. 3).

Clade N1 consisted of nine haplotypes representing 12 isolates, mainly from ornamental species, that could be separated into two subclades, 1a and 1b. Among isolates of this group there were 15 SNPs (12 parsimony informative) and 149 sites with gaps. Haplotype diversity within clade N1 was 0.9545. Two different haplotypes for tobacco isolates from Australia (P1495) and South Africa (P1955) clustered in this group.

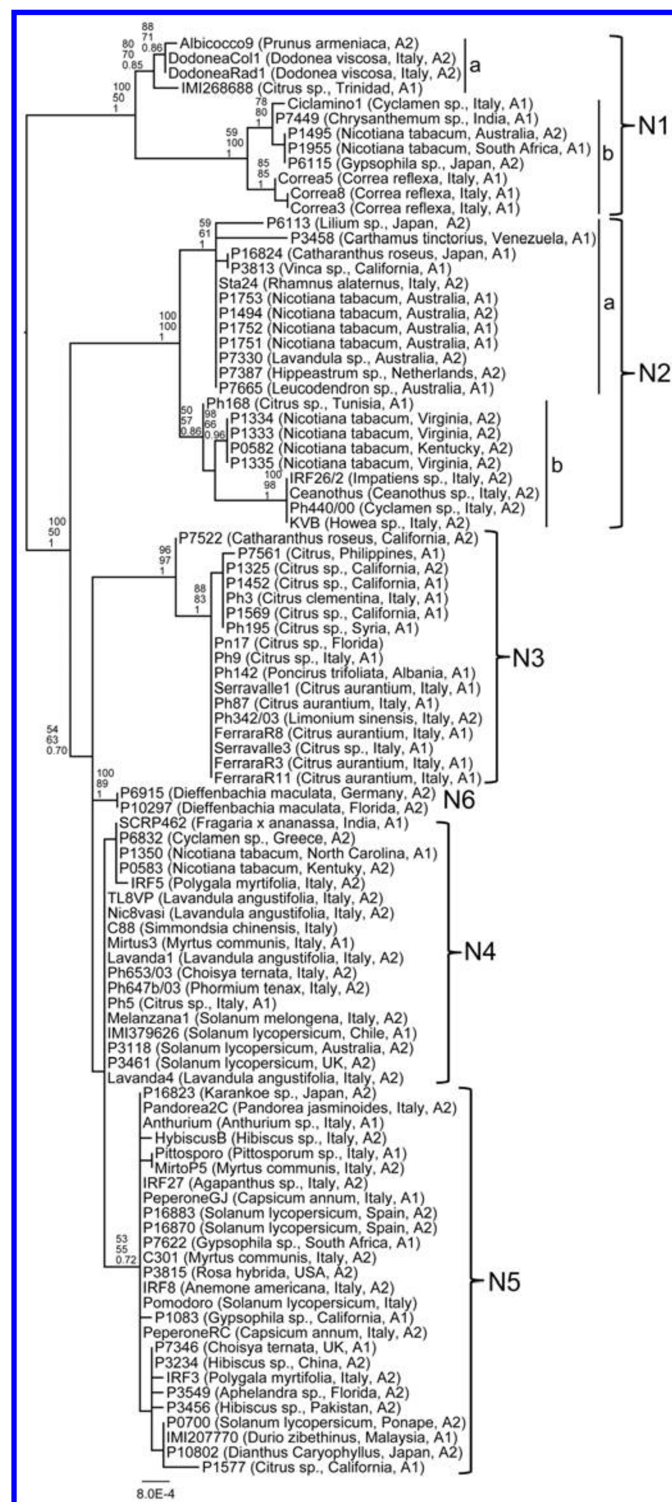

Fig. 2. Phylogenetic relationships among isolates of *Phytophthora nicotianae* based on the combined data set of sequences of the four mitochondrial regions (*trnG-rns, rns-cox2, cox2*+spacer, and *atp1-nad5*). Numbers on nodes represent the statistical support for maximum likelihood (100 replicates, top number), maximum parsimony (1,000 bootstrap replicates, middle number), and Bayesian method (posterior probabilities, bottom number). Per each isolate host, country of origin and mating type are indicated. Numbers preceded by "N" on the right of the tree indicate phylogenetic clades as identified in this study (and correlating as much as possible with clade numbering by Mammella et al. [39]).

Clade N2 consisted of seven haplotypes representing 21 isolates. In all, 18 SNPs (10 parsimony informative) and 30 sites with gaps were observed in this clade, with a haplotype diversity of 0.805. Isolates from various geographic locations and hosts clustered in this clade, including the majority of isolates from tobacco (8 of 12 isolates) from Australia and the United States (Kentucky and Virginia). Seven SNPs differentiated Australian tobacco isolates (subclade 2a) from those recovered from the United States (subclade 2b) (Fig. 2).

In clade N3, there were nine haplotypes (17 isolates), mainly recovered from citrus (15 isolates) from different geographic locations; two other isolates were from *Limonium sinensis* (Ph342/03) and *Catharanthus roseus* (P7522). Isolate Ph342/03 had the same multilocus mitochondrial haplotype (H23) as five isolates from citrus but was the only isolate of this haplotype showing an A2 mating type (Fig. 2). Isolate P7522 represented a unique haplotype and clustered in a single branch basal to the rest of the clade. In total, five SNPs (one parsimony informative) differentiated isolates of this clade, with a haplotype diversity of 0.8603. However, only two SNPs were observed (one parsimony informative) in the *atp1-nad5* region and haplotype diversity dropped to 0.8417 by excluding isolate P7522 from the analysis.

Clades N4 and N5 were very heterogeneous for geographic origin, host, and mating type (Fig. 2). The first clade was represented by 18 isolates (9 haplotypes) differentiated by two SNPs and was characterized by a haplotype diversity of 0.8366. Clade N5 consisted of 15 haplotypes representing 26 isolates and had a haplotype diversity of 0.9015. Haplotypes in this clade were differentiated by 10 SNPs (2 SNPs parsimony informative) and 89 sites with gaps. Most isolates in clades 4 and 5 were from ornamental and horticultural crops. In particular, all isolates from *Solanum* spp. clustered within these groups. Clade N6 corresponded to a single haplotype branch for two isolates from *D. maculata* recovered from Florida and Germany.

Nuclear DNA. The concatenated data set of nuclear sequences utilized to construct a neighbor-joining tree did not reveal any significant clustering with regards to geographic origin of isolates (data not shown). Analysis of distance focused on the host confirmed mitochondrial results, with the clustering of the majority of citrus isolates in a common group. In particular, 12 of 15 citrus isolates grouped in the mitochondrial clade N3 (Fig. 2) also constituted a very uniform group in the nuclear analysis (data not shown). The other three isolates (P1325, P1452, and P1569) of clade N3 shared a common root with the other citrus isolates.

Network analysis. *Mitochondrial DNA*. The network analysis of haplotypes using the combined data set of mitochondrial sequences showed an inconsistent correlation among haplotype groups and geographic origin of isolates (Table 1; Fig. 3). Interestingly, some haplogroups (H4, H11, H23, H31, H33, H36, H38, and H50) contained isolates of both mating types from different geographic locations (Table 1). Similarly, four isolates from southern Italy (PeperoneGJ and PeperoneRC from Calabria and Lavanda1 and Mirtus3 from Sicily) had the same mitochondrial haplotype but opposite mating types.

A relevant association was observed among hosts of recovery and haplogroups. For example, the majority of isolates from citrus species grouped together, even if recovered from different geographic regions (Italy, California, Florida, Philippines, Syria, and Albania) (Table 1; Fig. 3). Haplotype diversity within all isolates from citrus was 0.9181 (π of 0.00219) and dropped to 0.8667 (π of 0.00022) when examining only isolates that clustered together. The majority of tobacco isolates grouped in two similar haplotypes (H11 and H17). Other examples of association among host

of recovery and haplogroups was observed with isolates P10297 and P6915 from *D. maculata* from Florida and Germany (haplotype H10), isolates P3813 and P16824 recovered from *Catharanthus* spp. from Japan and California (haplotype H13), and isolates P3461 and P3118 recovered from *Solanum lycopersicum* from the United Kingdom and Australia (haplotype H31) (Fig. 3).

Nuclear DNA. Analysis of the network generated using data from the *hyp* nuclear region revealed the presence of four alleles differentiated by single steps (Fig. 4A). The dominant allele (a1) was identified in 76 isolates of *P. nicotianae* from different geographic origins and hosts (Table 1; Fig. 4A). Of these isolates, 59 were homozygous and 17 were heterozygous (Table 1). The majority of citrus isolates had the same allele, a3. Three of the California isolates (P1325, P1452, and P1569) were heterozygous (a1/a3) and shared allele a1 with the other four citrus isolates (Ph168, Ph5, P1577, and IMI268688) that did not group together in the mitochondrial network (Table 1; Fig. 4A). Tobacco isolates from the United States all had the same *hyp* allele (a3). One of these isolates from Kentucky (P0583) was heterozygous (a2/a3) and shared allele a2 with tobacco isolates from Australia that were all heterozygous (a1/a2). These latter isolates shared allele a1 with the two tobacco isolates (P1495 and P1955) from Australia and South Africa, respectively, that grouped separately based on mitochondrial analysis. A unique allele (a4) was found for a heterozygous (a1/a4) isolate collected in Italy from *Polygala myrtifolia* (Table 1; Fig. 4A).

Seven alleles, separated by one or two steps (in most of the cases) or five steps (a6 and a7), were identified with the *β-tub* gene (Fig. 4B). The distribution of alleles was not related to the geographic origin of isolates. The most frequent allele (a1) was shared by 57 isolates, and only 5 of these were homozygous (Table 1). All citrus isolates grouping together in the mitochondrial network were heterozygous and shared the same alleles (a1/a3). Allele a3 joined this citrus group as a single homozygous citrus isolate (Ph5) that grouped separately based on mitochondrial analysis. Two other citrus isolates (P1577 and Ph168) were homozygous (a4) and shared this allele with the heterozygous isolate IMI268688 (a4/a6) that contained the unique allele a6 (Table 1; Fig. 4B). The tobacco isolates from Australia were almost all heterozygous (a1/a3), except for P1751 (a3) and P1495 (a4). This latter allele joined homozygous tobacco isolates from the United States (βtubH4) and a single heterozygous (βtubH1/βtubH4) isolate (P0583) from Kentucky. The two isolates from *D. maculata* recovered from Florida and Germany were heterozygous (a4/a7) and had the unique allele a7 (Table 1).

Population structure and inbreeding analysis. A significant genetic structure was determined with AMOVA among host of recovery with both combined mitochondrial haplotypes and *β-tub*

TABLE 4. List of multilocus nuclear genotypes differentiated by 24 single-nucleotide polymorphisms (SNPs) identified in the pool of 96 isolates of *Phytophthora nicotianae* by the analysis of hypothetical protein (*hyp*), SCP-like extra cellular protein (*scp*), and β-tubulin (*β-tub*) genes[a]

		SNP sites																							
		hyp			*scp*										*β-tub*										
Gen	Fr	85	101	181	331	337	451	479	496	502	574	638	691	727	814	862	886	1147	1228	1252	1375	1420	1558	1567	1588
G1	3	G	T	C	C	A	G	A	C	G	G	G	G	G	C	C	C	G	G	C	C	R	G	T	G
G2	1	.	W	.	.	G	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.
G3	1	.	.	.	Y	R	.	R	Y	R	R	.	R	K	T	.	.	.	.	.	T	G	.	.	A
G4	1	.	.	.	.	G	.	G	.	.	.	.	A	.	Y	Y	Y	.	.	.	Y	G	.	.	R
G5	1	.	.	.	Y	R	.	R	Y	R	R	.	R	K	Y	.	.	.	.	.	Y	.	.	.	R
G6	1	.	.	.	.	R	.	R	.	.	.	.	R	.	.	.	.	.	.	.	Y	.	.	.	.
G7	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	G	.	.	.
G8	1	.	.	.	.	R	R	R	.	.	.	.	R	.	T	.	.	.	.	.	Y	G	.	.	R
G9	2	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	R	R	Y	Y	G	R	Y	R
G10	5	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	G	.	.	.
G11	2	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.
G12	2	.	W	.	.	.	.	.	.	.	.	.	.	.	.	Y	Y	.	.	.	.	G	.	.	.
G13	2	.	.	.	.	R	.	R	.	.	.	.	R	.	Y	.	.	.	.	.	Y	.	.	.	R
G14	1	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.
G15	1	A	.	.	.	R	.	R	.	.	.	.	A	.	.	.	.	.	.	.	.	G	.	.	.
G16	5	.	.	.	.	R	.	R	.	.	.	.	.	.	.	.	.	.	.	.	Y	G	.	.	.
G17	3	.	.	.	.	R	.	R	.	.	.	.	R	.	T	.	.	.	.	.	T	G	.	.	A
G18	5	.	A	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	G	.	.	A
G19	1	R	.	.	.	R	.	R	.	.	.	.	R	.	.	.	.	.	.	.	Y	G	.	.	.
G20	3	.	W	.	.	R	.	R	.	.	.	.	R	.	.	.	.	.	.	.	Y	G	.	.	.
G22	1	.	A	.	.	G	.	G	.	.	.	.	A	.	.	.	.	.	.	.	Y	G	.	.	.
G23	1	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	Y	G	.	.	.
G24	3	.	.	.	.	G	.	G	.	.	.	.	A	.	Y	.	.	.	.	.	Y	G	.	.	R
G25	1	R	W	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	Y	G	.	.	R
G26	7	.	.	.	.	G	.	G	.	.	.	.	A	.	T	.	.	.	.	.	T	G	.	.	A
G27	1	.	.	.	.	G	.	G	.	.	.	.	R	.	.	.	.	.	.	.	T	G	.	.	.
G28	4	.	.	.	.	R	.	R	.	.	.	.	R	.	.	.	.	.	.	.	Y	G	.	.	R
G29	1	.	W	Y	.	R	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	Y	G	.	.	R
G30	1	.	.	.	.	R	.	R	.	.	.	.	R	.	.	.	.	.	.	.	Y	G	.	.	.
G31	2	.	.	.	Y	R	.	R	Y	R	R	.	R	K	Y	.	.	.	.	.	Y	G	.	.	R
G32	1	.	W	.	.	G	.	R	.	.	.	R	R	.	.	.	.	.	.	.	Y	G	.	.	.
G33	2	.	.	.	.	R	.	R	.	.	.	.	A	.	Y	Y	Y	.	.	.	Y	G	.	.	R
G34	1	.	.	.	.	R	.	R	.	.	.	.	R	.	Y	Y	Y	.	.	.	Y	G	.	.	R
G35	4	.	.	.	.	G	.	G	.	.	.	.	A	.	.	Y	Y	.	.	.	.	G	.	.	.
G36	1	.	.	.	.	R	.	R	.	.	.	.	R	.	.	Y	Y	.	.	.	Y	G	.	.	.
G37	1	.	.	.	.	G	.	G	.	.	.	.	R	.	.	.	.	.	.	.	.	G	.	.	.
G38	1	.	.	.	.	R	.	R	.	.	.	.	R	.	Y	.	.	.	.	.	T	G	.	.	R
G39	1	.	.	.	.	.	.	R	.	.	.	.	R	.	T	.	.	.	.	.	T	G	.	.	A
G40	5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	G	.	.	.
Obs	…	0.10	0.09	0.01	0.04	0.45	0.01	0.46	0.04	0.04	0.04	0.01	0.40	0.04	0.20	0.11	0.11	0.02	0.02	0.02	0.58	0.07	0.02	0.02	0.23

[a] Order and nucleotide position of the loci refer to the combined nuclear sequences. Gen = genotype, Fr = frequency, and Obs = observed heterozygosity.

and *hyp* nuclear alleles which explained 44.82, 12.07, and 29.6% of the total variability, respectively (Tables 5 and 6). By contrast, only 6.52% (mitochondrial haplotypes), 4.65% (*β-tub*), and 11.19% (*hyp*) of the variability was explained by the geographic origin of the isolates (Tables 5 and 6).

Inbreeding coefficients (F_{IS}) were slightly positive, with both gene alleles for populations from specialized field crops (*Citrus*, *Nicotiana*, and *Solanum* spp.) (Table 7). Significantly lower F_{IS} values were observed for potted ornamental species in the nursery, suggesting a higher level of outbreeding within this population (Table 7).

DISCUSSION

Four mitochondrial markers and three nuclear coding regions were used in this research to study 96 isolates of *Phytophthora nicotianae* representing a wide range of hosts and geographic locations. Among mitochondrial regions, the *rns-cox2* and *trnG-rns* markers spanned regions (*trnW-cox2* and *trnY-rns*) analyzed in our previous work (39) but included an additional 262 and 275 bp of data, respectively. For the *rns-cox2* locus, the additional sequence data did not improve haplotype identification, whereas the additional sequence data from the *trnG-rns* region improved resolution of haplotypes. The *cox2*+spacer region identified 13 different haplotypes, with 8 of them separated by eight SNPs in the coding region. Intraspecific variability within this gene has been identified for *Phytophthora* spp., including two isolates of *P. nicotianae* (44). In all, 9 of 14 species with multiple isolates showed genetic variability at an intraspecific level for the *cox2* gene (44). The *atp1-nad5* region was the most variable among the four mitochondrial regions analyzed in the present work because it allowed the identification of 31 haplotypes differentiated by 23 SNPs as well as 124 sites with gaps and length variations in homopolymeric regions.

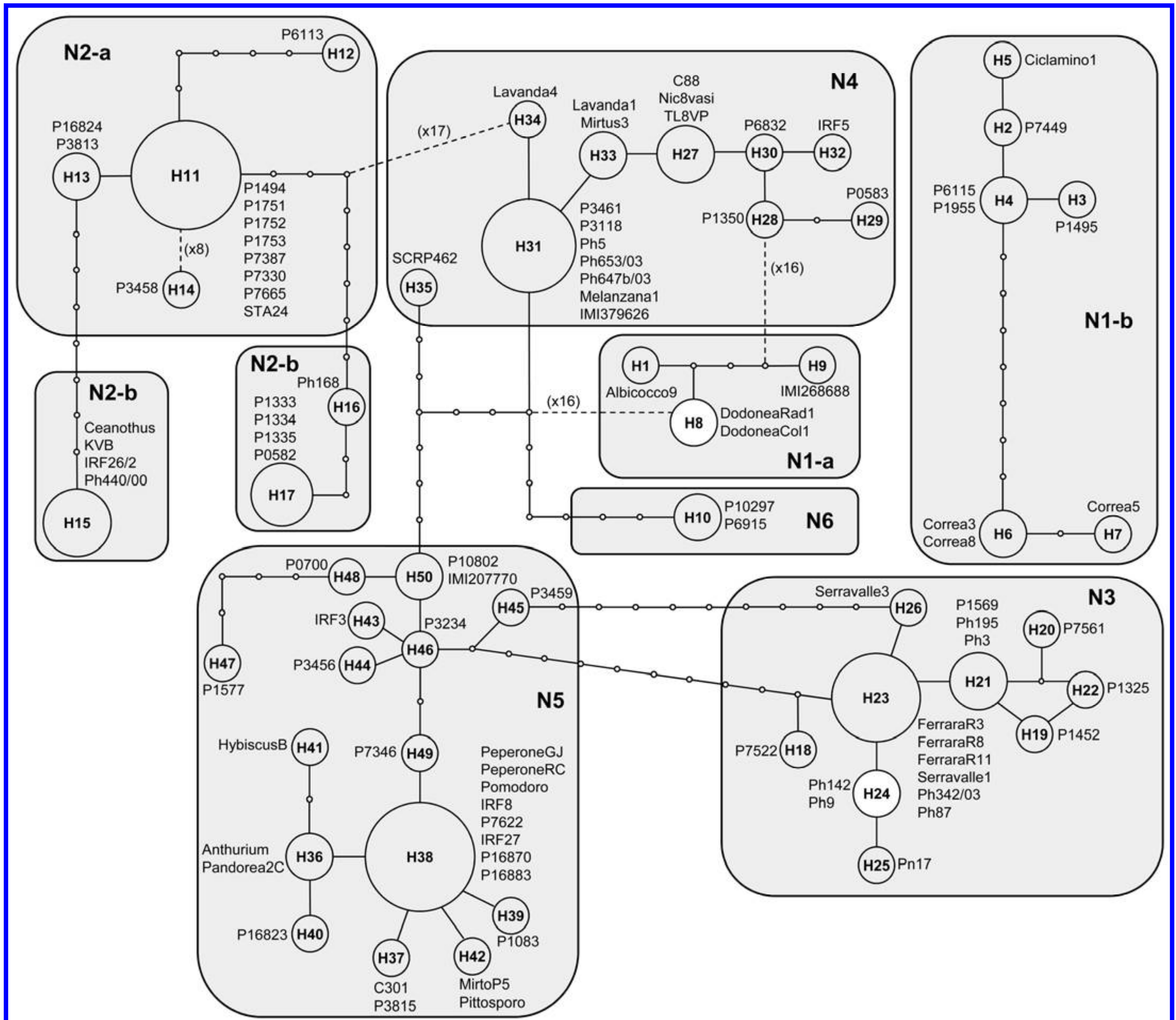

Fig. 3. Haplotype network based on the combined sequences of the mitochondrial data (*trnG-rns, rns-cox2, cox2*+spacer, and *atp1-nad5*) of the 96 *Phytophthora nicotianae* isolates analyzed in the present study. The network was constructed using a statistical parsimony algorithm implemented in TCS 1.21 (10). Per each haplotype (H), the size of each circle represents its relative frequency and the corresponding isolates are indicated. Delimitated groups of haplotypes marked with the letter code "N" represents genetic groups according to the phylogenetic analysis (Fig. 2). The haplotypes were directly connected without dots when differing by a single change. Every additional putative change was indicated by adding a dot or by indicating the number of steps on dotted lines. Haplotypes grouping in the subclade N1-b (Fig. 2) were not linked to any other group of the network, considering a connection limit of 95%.

The phylogenetic analysis of the combined mitochondrial data revealed the existence of separate phylogenetic groups that were consistent with three different phylogenetic methods and produced clades largely confirming those reported by Mammella et al. (39). However, the analysis of a larger portion of the genome (10% of the total) and the increased number of isolates enabled the identification of a higher number of mitochondrial haplotypes (50 haplotypes) and a more accurate delineation of genetic groups with the identification of a new clade (N6) and some subclades. It is worth noting that almost all the observed haplotypes (47 haplotypes) were identified by analyzing two mitochondrial regions (*trnG-rns* and *atp1-nad5*). These two regions could represent a valuable tool for the analysis of the worldwide distribution of *P. nicotianae* haplotypes without the need for extensive sequencing of additional markers.

The analysis of both mitochondrial and nuclear markers revealed the absence of a relevant geographic structure, with *P. nicotianae* haplotypes or alleles shared among different locations as in a typical panmictic distribution resulting from recurring events of migration. In particular, AMOVA analysis of mitochondrial haplotypes showed a very limited contribution of the geographic origin of isolate to the observed variability, with a fixation index (F_{ST}) of 0.0652. In agreement with AMOVA, all identified mitochondrial phylogenetic and network groups contained isolates from different countries and the most frequent haplotypes (H11 and H38) were shared among isolates from different geographic origins. Mitochondrial results were largely confirmed by the analysis of three nuclear loci, although these markers were less effective in differentiating closely related isolates. In particular, AMOVA applied to nuclear alleles confirmed a negligible role of geographic origin in determining total variability even if a quite high F_{ST} value was obtained with the *hyp* gene. However, *hyp* was the least variable gene among those analyzed in the present study and its F_{ST} value was indirectly influenced by the host, considering that the Australian population had a major role in determining the final value and was largely sourced from a single host (tobacco). In agreement with AMOVA analyses the nuclear gene networks exhibited alleles widely distributed among isolates from different geographic origins, even if they were differentiated by few steps. For instance, the most frequent alleles (*hyp* a1 and *β-tub* a1) were represented by isolates from different regions. Furthermore, although an accurate phylogenetic analysis was not possible for the concatenated data set of nuclear sequences due to their high level of heterozygosity, the constructed neighbor-joining tree confirmed the absence of significant clustering as far as the geographic origin of isolates was concerned.

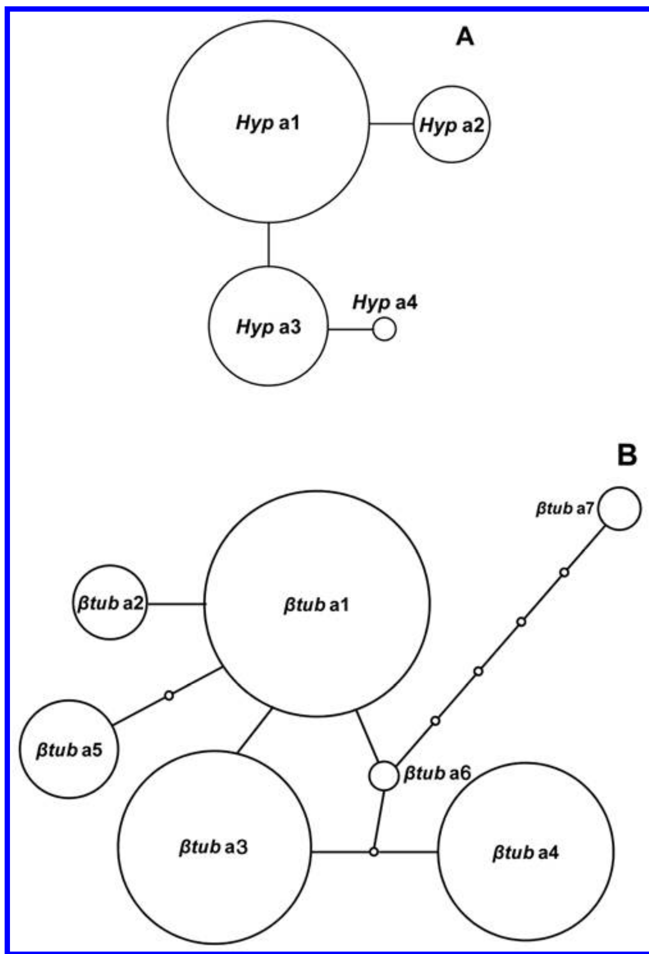

Fig. 4. Network of alleles generated for the **A,** nuclear hypothetical conserved protein (*hyp*) and **B,** β-tubulin (*β-tub*) genes of *Phytophthora nicotianae*, using the statistical parsimony algorithm implemented in TCS 1.21 (10). Isolate codes belonging to each group are listed in Table 1.

A significant structuring of populations was revealed with mitochondrial and nuclear markers in relation to the host. A high F_{ST} value (0.4482) was obtained from the AMOVA analyses of mitochondrial haplotypes but significant values were also obtained with *β-tub* and *hyp* alleles. Furthermore, the mitochondrial phylogenetic tree and network analysis showed a relevant grouping of isolates from the same host species. The majority of citrus isolates (15 of 19) clustered together, regardless of their geographic origin (Philippines, Syria, Albania, California, Florida, and Italy). These isolates were characterized by a low level of π that was limited to two SNPs in the *atp1-nad5* region and length variation in some homopolymeric T regions. A similar clustering of isolates recovered from the same host was observed for eight isolates from tobacco originating from Australia and United States (Kentucky and Virginia), although a higher genetic variation (seven SNPs) differentiated these isolates. Concordant results were also obtained for isolate from *Solanum* spp. that clustered in the two related clades 4 and 5 and for different ornamental species, despite the limited number of isolates from each host that prevented accurate evaluations. In agreement with mitochondrial data, the *hyp* region revealed that the majority of citrus isolates clustering in the N3 mitochondrial group also shared the same *hyp* a3 allele. Only three isolates from California were heterozygous (a1/a3) and shared the additional allele *hyp* a1 with other four citrus isolates that did not group together in the mitochondrial DNA analysis. These data reinforced the association among genetic grouping and host of origin, because the few citrus isolates that appeared genetically distant on the basis of the mitochondrial DNA were actually linked at the *hyp* nuclear level.

TABLE 5. Analysis of molecular variance (AMOVA) of *Phytophthora nicotianae* populations based on multilocus mitochondrial genotypes

Source of variation[a]	df	Sum of squares	Variance components	Variation (%)	Fixation index (F_{ST})	*P* value
Host genus						
Among all populations	37	2,032.943	15.25412	44.82	0.4482	0.0000
Within populations	57	1,070.299	18.7771	55.18	…	…
Geographic origin						
Among all populations	4	259.508	2.19087	6.52	0.0652	0.00489
Within populations	91	2,856.169	31.38647	93.48	…	…

[a] AMOVA considering originating host genus (Table 1) or geographic origin of isolates (Africa, America, Asia, and Europe).

Similar results were also obtained with the *β-tub* nuclear region; all citrus isolates that grouped together in the mitochondrial network were heterozygous (a1/a3) and shared the allele *β-tub* a3 with the homozygous citrus isolate Ph5 that grouped separately in the mitochondrial analysis. In accordance with previous results, the neighbor-joining tree constructed for the concatenated nuclear data set confirmed the clustering of the majority of citrus isolates in a common group. Tobacco isolates were also genetically similar because they shared at least one allele of the *hyp* gene. With the *β-tub* gene, a single tobacco isolate from Australia did not share any allele with other isolates.

The existence of a significant structuring based on the host of recovery could appear in contrast with the abovementioned inconsistent geographic structuring and polyphagy of *P. nicotianae*. However, a specific association among molecular groups and host of recovery has been reported for isolates causing black shank in tobacco that were differentiated from other *P. nicotianae* isolates (11). Furthermore, considerable evidence supports host preference by some isolates of *P. nicotianae* (15). For instance, an isolate from okra was not pathogenic to *Citrus* spp. and vice versa (14). Similarly, isolates from *Citrus* spp. were more virulent on roots of rough lemon than isolates from petunia, tomato, walnut, silk tree, jojoba, hibiscus, and peach although, in another study, tomato plants exhibited high susceptibility to many isolates, including *Citrus* isolates (4,45). These authors suggested that the degree of susceptibility among hosts is not clear but the greatest degree of virulence is commonly shown by isolates on their own host.

The absence of a geographic structuring and the concurrent existence of a significant structuring in relation to the hosts or origin could be indicative of extensive phenomena of migration of the isolates via plant material or host adaptation. Although specific pathogenicity tests are needed to evaluate the virulence of groups identified in the present study on different hosts and determine whether molecular groupings can be taken as evidence of physiological races or pathotypes, it can be hypothesized that *P. nicotianae* isolates have been spread worldwide with infected plant material and, afterward, lineages may have progressively diverged. In this context, a major role could have been played by the globalization of the nursery trade, with particular emphasis to the sector of potted ornaments. For example, isolates from *D. maculata* from Germany and Florida shared the same mitochondrial haplotype and nuclear multilocus genotype. The role of the nursery trade in the spreading of *Phytophthora* inoculum has been investigated for *P. ramorum*, the causative agent of sudden oak death (25,58), and similar routes are likely for other *Phytophthora* spp. In particular, *P. nicotianae* has been widely recovered in nurseries during recent surveys and its long-distance movement can likely be facilitated by its broad host range, currently with more than 250 plant genera in 90 families, including a large number of ornamentals (7,30,47,49,56,63).

The high number of nuclear genotypes identified ($n = 40$), with more than half of them ($n = 21$) unique, and the heterozygosity observed in all loci analyzed seem to indicate a quite high frequency of genetic exchange in *P. nicotianae*, with at least some of the isolates examined in this study originating from a sexually reproducing population. The worldwide diffusion of this species and its broad range of host surely play an important role in favoring this high level of recombination. Indeed, lower levels of diversity were reported for other heterothallic species of *Phytophthora*, including *P. infestans* (1) and *P. capsici* (19,20). In this study, isolates from field crops (*Citrus, Nicotiana*, and *Solanum* spp.) were characterized by slightly positive inbreeding coefficients (F_{IS}) with both *β-tub* and *hyp* markers. This result is in line with expectations for a heterothallic species such as *P. nicotianae* (23) but seems to be in contrast with previous reports, indicating that sexual recombination is unlikely to serve as a major mechanism enhancing the genetic diversity of the pathogen in tobacco fields (36,50). Similar data are available for populations of *P. nicotianae* in citrus groves, where A1 is the prevalent and often the sole mating type (6). However, it can be hypothesized that isolates from specialized cultivation are the result of asexually propagated heterozygous clones, adapted to a specific host. For example, most citrus isolates from southern Italy had the same multilocus genotype (homozygous and heterozygous for *hyp* and *β-tub* gene, respectively), were all mating type A1, and all had very closely related mitochondrial haplotypes, suggesting that homozygous and heterozygous loci were fixed in a clonal population. Although larger numbers of isolates collected from each specific host species would be necessary to confirm these speculations, fixed heterozygosity in clonal populations has been already identified in *Phytophthora* spp. The analyses of six polymorphic SNP loci in *P. capsici* isolates from costal Peru revealed the same heterozygous genome (PcPE-1) among the population, suggesting the widespread diffusion of a single clonal lineage (32) that was also predominant in the Amazonian high jungle of Peru (31). Similarly, the analysis of a *P. capsici* population in Argentina indicated that 87% of the isolates had the same multilocus genotype, which was fixed for heterozygosity at seven of the eight SNP sites (20). Completely different populations were found in United States and South Africa with many unique genotypes, suggesting a primary role of sexual recombination (19,37).

A different behavior was revealed for *P. nicotianae* isolates collected from potted ornamentals in nursery production, where many different plant species are grown together, favoring the meeting of different genetically distant isolates of *P. nicotianae*.

TABLE 7. Inbreeding coefficients (F_{IS}) calculated for three different *Phytophthora nicotianae* populations sourced from open field cultures (*Citrus*, *Nicotiana*, and *Solanum* spp.) and for isolates from potted ornamental species in the nursery (Ornamentals)

	F_{IS}	
Population	*β-tub* gene	*hyp* genes
Citrus spp.	0.11561	0.63265
Nicotianae spp.	0.40976	0.43103
Solanum spp.	0.42149	n.d.[a]
Ornamentals	–0.10557	0.37857

[a] n.d. = not determined.

TABLE 6. Analysis of molecular variance (AMOVA) of *Phytophthora nicotianae* populations based on the β-tubulin and hypothetical protein (in parenthesis values) genotypes

Source of variation[a]	df	Sum of squares	Variance components	Variation (%)	Fixation index (F_{ST})	*P* value
Host genus						
Among all populations	38 (39)	49.279 (22.565)	0.10485 (0.07401)	12.07 (29.60)	0.12066 (0.29598)	0.54936 (0.03030)
Among individuals within populations	56 (57)	45.500 (14.132)	0.04835 (0.07189)	5.56 (28.75)	…	…
Within individuals	95 (96)	68.000 (10.000)	0.71579 (0.10417)	82.37 (41.66)	…	…
Geographic origin						
Among all populations	4 (4)	8.703 (4.894)	0.04076 (0.02859)	4.65 (11.19)	0.04648 (0.11193)	0.09482 (0.01271)
Among individuals within populations	90 (91)	86.076 (31.804)	0.12031 (0.12266)	13.72 (48.02)	…	…
Within individuals	95 (96)	68.000 (10.000)	0.71579 (0.10417)	81.63 (40.78)	…	…

[a] AMOVA considering originating host genus (Table 1) or geographic origin of isolates (Africa, America, Asia, and Europe).

These isolates were characterized by a lower inbreeding coefficient (higher level of heterozygosis) compared with isolate groups from *Citrus, Nicotiana*, and *Solanum* spp. In particular, the inbreeding coefficient determined with the *β-tub* gene was negative, indicating heterozygote excess as compared with the Hardy-Weinberg expectations. In agreement with the considerations above, some isolates recovered from ornamentals in Sicily (southern Italy) were characterized by opposite mating type, the same mitochondrial haplotype, and different multilocus genotypes. These results suggest that nursery populations significantly increase genetic recombination within *P. nicotianae* and play an important role in the evolution of the species.

In the present study, PCR products cloned and sequenced for the *scp* region showed an unexpected genetic framework for a diploid organism such as *P. nicotianae* because more than two alleles were identified for isolates recovered from Anthurium (five alleles observed) and IMI268688 (four alleles). A possible explanation could be that *scp* belongs to a gene family, as already reported for other *P. nicotianae* genes (57). However, a single locus (PPTG_11765.1) with a high homology (99% identity) was retrieved by blasting *scp* sequences obtained in the present study against the complete set of *P. nicotianae* transcripts from the genome project (http://www.broadinstitute.org/annotation/genome/Phytophthora_parasitica). Outcrossing could also have played a role in determining the unexpected framework. Recently, the genetic characterization of *P. ramorum* single-oospore progeny of crosses between a European A1 isolate and North American or European A2 isolates revealed a considerable number of non-Mendelian inheritance events (61). This included inheritance of more than two alleles at a locus and noninheritance of alleles from one parent at another locus. Single-oospore progenies displayed aberrant genomic and phenotypic variation due to meiotic irregularities but also differences in DNA content of single-oospore progeny and variation as a result of post-meiotic genomic rearrangements. Similarly, trisomy and multiple alleles for coding region were observed in *P. infestans* (48,60) and *P. cinnamomi* (13). The possibility of different meiotic rearrangements has been also demonstrated in the related *Pythium sylvaticum* (40).

In conclusion, a combined analysis of mitochondrial and nuclear markers has been applied in the present study to characterize *Phytophthora nicotianae*. Using these approaches, it has been possible to generate an overview about its global population structure in relation to geographic origin and hosts, leading to the formulation of hypotheses about preferential reproduction systems and the role of nurseries and trading of propagation material in the evolution and distribution of the species. Considering the level of variation identified in the approximately 1,600 bp screened in the present study, the recent release of the whole genome of *P. nicotianae* (http://www.broadinstitute.org/annotation/genome/Phytophthora_parasitica) would provide an inexhaustible resource for marker development. The future development of new markers in association with the analysis of a large number of isolates sampled according to specific schemes will enable the characterization of the genetic structure of individual populations of *P. nicotianae* and accurately determine migration pathways and the center of origin of this cosmopolitan plant pathogen. Furthermore, repetitive sampling of the same population over longer time frames could be useful to determine the incidence of sexual and clonal reproduction within specific sites, as well as to track introductions of new genotypes and their effect on the established population.

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