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Genetic characterization of *Phytophthora nicotianae* by the analysis of polymorphic regions of the mitochondrial DNA

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ABSTRACT

A new method based on the analysis of mitochondrial intergenic regions characterized by intraspecific variation in DNA sequences was developed and applied to the study of the plant pathogen *Phytophthora nicotianae*. Two regions flanked by genes *trnY* and *rns* and *trnW* and *cox2* were identified by comparing the whole mitochondrial genomes of *Phytophthora infestans*, *Phytophthora ramorum*, and *Phytophthora sojae* and amplified using primers designed from the flanking conserved genes. These regions were sequenced from 51 isolates of *P. nicotianae* of both A1 and A2 mating type recovered from different hosts and geographic regions. Amplicon length varied from 429 bp to 443 bp (*trnY/rns*) and 322 bp to 373 bp (*trnW/cox2*) with intraspecific variation due to single nucleotide polymorphisms and indels. Seventeen, seven and 20 different haplotypes were detected by individually analyzing regions *trnY-rns*, *trnW-cox2* and the combined data set of sequences from both regions, respectively. Phylogenetic analysis inferred with three different methods enabled the grouping of isolates in five clades, each containing different mitochondrial haplotypes and revealed diversity in the mitochondrial genome of *P. nicotianae*. The majority of isolates from citrus grouped in a single clade indicating either movement of isolates on planting stock or an association of particular isolates with this host. Phylogenetic groups were not correlated with the radial growth rate of the isolates or the rapidity of apple flesh colonization. The method developed in the present study represents an innovative molecular tool for the characterization of natural populations of *P. nicotianae* and should be easily expanded to other species of *Phytophthora* as well as other plant pathogens. It can be used to track specific haplotypes and, thanks to its high genetic resolution, it could be standardized and applied in a DNA barcoding like strategy for the precise identification of sub-specific taxa. Compared to alternative molecular methods, a major advantage is that results are unbiased (a list of nucleotides) and highly reproducible, thus enabling the comparison of data from different laboratories and time periods. Furthermore, the method could be further enhanced by the identification of additional variable mitochondrial and/or nuclear genomic regions.

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Introduction

Phytophthora nicotianae van Breda de Haan (=*Phytophthora parasitica* Dastur) (1896) stands out among plant pathogens since it is a threat to plant productivity on a global scale for a broad range of hosts (Erwin & Ribeiro 1996). Hickman (1958) reported the host range of *P. nicotianae* included 72 plant genera and 298 plants species, but in the subsequent 50 y since this report the number of hosts has increased to 255 plant genera in 90 families (Cline *et al.* 2008). This pathogen has been widely studied for its impact on tobacco (*Nicotiana tabacum* L.) since it is responsible for a disease commonly named black shank that can account for severe annual losses. Host resistance and crop rotations are the most economic measures to control black shank, but they are not always effective (Shew 1987; Johnson *et al.* 2002). Similarly, productivity in the major citrus growing regions across the world is heavily affected by citrus root rot and gummosis, whose main causal agents are *P. nicotianae* and *Phytophthora citrophthora* (Menge & Nemec 1997; Cacciola & Magnano di San Lio 2008). When severely affected, plants show a lower yield, fruit are smaller and trees progressively decline until death. Control strategies, which include the use of resistant rootstocks, chemical products, and/or fumigants, and proper management of the orchards, are not always sufficiently effective (Menge & Nemec 1997).

Apart from *N. tabacum* and *Citrus* species, *P. nicotianae* is responsible for heavy losses on a number of other economically important species including fruit trees and herbaceous hosts (Erwin & Ribeiro 1996). Recent surveys have revealed that this species is one of the most common pathogens on ornamental plants, the cultivation and sale of which has been recognized as a principal pathway for the introduction and spread of invasive plant pathogens (Cacciola *et al.* 1997, 2001; Reichard & White 2001; Pane *et al.* 2005; Moralejo *et al.* 2009). Commonly, ornamental nurseries are intensive farming systems in which many plant species are simultaneously and repeatedly cultivated with limited crop rotation. In such conditions multiple generations of the pathogen can occur and different genotypes, including opposite mating types, can come in contact thereby increasing the potential for sexual outcrossing and evolutionary divergence.

Despite the relevance of *P. nicotianae*, specific studies to evaluate intraspecific genetic variability and to establish the possible pathways by which the pathogen has been introduced and distributed to new areas are quite limited and mainly restricted to populations from tobacco. Random Amplified Polymorphic DNAs (RAPDs) were utilized to study the variability among seven populations of *P. nicotianae* from different tobacco fields (Zhang *et al.* 2003). Populations were genotypically and phenotypically variable, but no distinct genotypic differences were identified among populations from the seven locations. The same technique was applied to differentiate isolates causing black shank (Zhang *et al.* 2001) and to identify markers linked to the dominant black shank resistance gene (Johnson *et al.* 2002). Amplified Fragment Length Polymorphism (AFLP), utilized by Lamour *et al.* (2003) to study a population from different floricultural hosts and production sites, enabled the identification of six clonal lineages. Although RAPD-PCR and AFLP have proved valuable within a particular study, results obtained with such fingerprinting tools are not always easily reproducible in different laboratories (Cooke & Lees 2004).

Several alternative molecular approaches have been proposed to study intraspecific variability of *Phytophthora* species (Cooke *et al.* 2007). Microsatellites or simple sequence repeats (SSRs) have been recognized as one of the most powerful choices, but their main limitation is the need for knowledge of the DNA sequence of the SSR flanking regions in order to design specific primers. As a consequence, microsatellites have been widely utilized for those species whose genome has been partially or completely sequenced (Dobrowolski *et al.* 2003; Ivors *et al.* 2006; Prospero *et al.* 2007; Weng *et al.* 2007; Widmark *et al.* 2007), but their application to other *Phytophthora* species remains challenging despite recent attempts to create a comprehensive dataset of candidate SSRs for a range of species (Schena *et al.* 2008).

Accurate analysis of mitochondrial and nuclear DNA has elucidated the phylogenetic relationships within the genus *Phytophthora* with a grouping of ten genetically related clades (Cooke *et al.* 2000; Martin & Tooley 2003; Kroon *et al.* 2004; Blair *et al.* 2008). However, these studies were based on genes commonly conserved within a species and therefore unsuitable to characterize intraspecific variability. Recently, the analysis of different intergenic regions of the mitochondrial DNA (mt-IGS) from 31 *Phytophthora* species, representing the breadth of diversity in the genus, revealed the existence of regions too variable to be used for broad scale phylogenetic analyses. However, it was suggested these markers were suitable for the examination of intraspecific variation and the analysis of closely related species (Schena & Cooke 2006). Intraspecific polymorphisms in mitochondrial DNA have been useful for characterizing populations by mitochondrial haplotypes for *Phytophthora infestans* (Gómez-Alpizar *et al.* 2007) and the recent classification of mitochondrial haplotypes in *Phytophthora ramorum* should be useful for this species as well (Martin 2008).

In the present study, variable mitochondrial intergenic regions were identified by comparing the whole mitochondrial genomes of *P. infestans*, *P. ramorum*, and *Phytophthora sojae* in GenBank and primers were designed to amplify these regions to characterize a population of A1 and A2 mating types of *P. nicotianae* from different hosts and geographic origins. Isolates were also characterized by measuring the radial growth rate on agar medium and the ability to colonize the flesh of artificially inoculated apples.

Materials and methods

Phytophthora nicotianae *isolates*

Fifty-one isolates from different geographic regions and international culture collections were used in this study (Table 1). Isolates were stored on oatmeal agar at 15 °C and grown on potato dextrose agar (PDA) for routine stock cultures.

Identification of easily amplifiable and variable mtDNA intergenic regions

The complete mitochondrial genome from four different haplotypes of *Phytophthora infestans* (accession numbers NC_002387,

Table 1 – Isolates of *Phytophthora nicotianae* included in the study, their designation, origin, year of collection, and mating type. Amplicon size, accession numbers and haplotypes are reported for the two mitochondrial regions (*trnY-rns* and *trnW-cox2*) sequenced in the present study. Isolates were listed accordingly to the last two column reporting haplotypes (Fig 2) and phylogenetic groups (Fig 3) respectively.

Isolate code	Origin			Mating Type	*trnY-rns* region			*trnW-cox2* region			*trnY-rns* + *trnW-cox2*	
	Host	Region and Country	Year		Amplicon size (bp)	Accession number	Haplotype	Amplicon size (bp)	Accession number	Haplotype	Haplotype	Phylogenetic group
Albicocco9	*Prunus armeniaca*	Calabria (Southern Italy)	2005	A2	430	GU938492	1	373	GU938586	1	H1	N1
IMI 268688	*Citrus* sp.	Trinidad-Tobago	1982	A1	431	GU938493	16	373	GU938585	1	H2	N1
Dodonea Col1	*Dodonaea viscosa*	Sicily (Southern Italy)	2005	A2	429	GU938495	8	373	GU938591	1	H3	N1
Dodonea Rad1	*Dodonaea viscosa*	Sicily (Southern Italy)	2005	A2	429	GU938494	8	373	GU938592	1	H3	N1
Correa5	*Correa reflexa*	Sicily (Southern Italy)	2004	A1	431	GU938498	7	373	GU938590	1	H4	N1
Correa3	*Correa reflexa*	Sicily (Southern Italy)	2004	A1	432	GU938496	6	373	GU938588	1	H5	N1
Correa8	*Correa reflexa*	Sicily (Southern Italy)	2004	A1	431	GU938497	6	373	GU938589	1	H5	N1
Ciclamino1	*Cyclamen* sp.	Sicily (Southern Italy)	2004	A1	432	GU938499	5	373	GU938580	5	H6	N1
Ph168	*Citrus* sp. (root)	Tunisia	2003	A1	443	GU938542	4	373	GU938593	1	H7	N2
STA24	*Rhamnus alaternus*	Sicily (Southern Italy)	2000	A2	443	GU938540	4	373	GU938587	1	H7	N2
Ph440/00	*Cyclamen* sp.	Liguria (Northern Italy)	2004	A2	443	GU938539	4	373	GU938584	4	H8	N2
KVB	*Howea* sp.	Sicily (Southern Italy)	2000	A2	443	GU938541	4	373	GU938582	4	H8	N2
IRF26/2	*Impatiens wallerana*	Liguria (Northern Italy)	2007	A2	443	GU938512	4	373	GU938583	4	H8	N2
Ceanothus	*Ceanothus sp.*	Sicily (Southern Italy)	2002	A2	443	GU938538	4	373	GU938581	4	H8	N2
Ph3	*Citrus clementina* (fruit)	n.d.	2001	A1	431	GU938534	9	373	GU938551	6	H9	N3
Ph87	*Citrus aurantium* (root)	Apulia (Southern Italy)	2000	A1	431	GU938531	9	373	GU938550	6	H9	N3
Ph195	*Citrus* sp.	Tartaus (Syria)	2003	A1	431	GU938535	9	373	GU938543	6	H9	N3
Ferrara R11	*Citrus aurantium* (root)	Sicily (Southern Italy)	2004	A1	431	GU938529	9	373	GU938547	6	H9	N3
Ferrara R3	*Citrus aurantium* (root)	Sicily (Southern Italy)	2004	A1	431	GU938533	9	373	GU938549	6	H9	N3
Ferrara R8	*Citrus aurantium* (root)	Sicily (Southern Italy)	2004	A1	431	GU938536	9	373	GU938548	6	H9	N3
Serravalle 1	*Citrus aurantium* (root)	Sicily (Southern Italy)	2004	A1	431	GU938530	9	373	GU938544	6	H9	N3
Ph342/03	*Limonium sinensis*	Liguria (Northern Italy)	2004	A2	431	GU938532	9	373	GU938545	6	H9	N3
Ph9	*Citrus* sp. *(soil)*	Apulia (Southern Italy)	n.d.	A1	432	GU938528	10	373	GU938552	6	H10	N3
Ph142	*Poncirus trifoliata*	Valona (Albania)	2001	A1	432	GU938527	10	373	GU938553	6	H10	N3
Serravalle 3	*Citrus* sp. (root)	Sicily (Southern Italy)	2004	A1	430	GU938537	11	373	GU938546	6	H11	N3
SCRP462	*Fragaria x ananassa*	India	1998	A1	431	GU938522	15	366	GU938563	7	H12	N4
Ph653/03	*Choisya ternata*	Liguria (Northern Italy)	2004	A2	431	GU938518	15	373	GU938570	3	H13	N4
Ph647/b03	*Phormium tenax*	Liguria (Northern Italy)	2004	A2	431	GU938519	15	373	GU938568	3	H13	N4
Ph5	*Citrus* sp. (root)	Basilicata (Southern Italy)	2000	A1	431	GU938520	15	373	GU938572	3	H13	N4
Lavanda4	*Lavandula angustifolia*	Sicily (Southern Italy)	2002	A2	431	GU938516	15	373	GU938569	3	H13	N4
IMI 379626	*Lycopersicum esculentum*	Chile	n.d.	A1	431	GU938521	15	373	GU938571	3	H13	N4
Melanzana1	*Solanum melongena*	Sicily (Southern Italy)	1998	A2	431	GU938517	15	373	GU938564	3	H13	N4
Lavanda1	*Lavandula angustifolia*	Sicily (Southern Italy)	2002	A2	432	GU938514	14	373	GU938574	3	H14	N4
Mirtus3	*Myrtus communis*	Sicily (Southern Italy)	2002	A1	432	GU938515	14	373	GU938566	3	H14	N4
IRF5	*Polygala myrtifolia*	Liguria (Northern Italy)	2007	A2	433	GU938526	13	373	GU938573	3	H15	N4
TL8VP	*Lavandula angustifolia*	Piedimont (Northern Italy)	2000	A2	433	GU938524	3	373	GU938575	3	H16	N4
Nic8Vasi	n.d.	Sicily (Southern Italy)	2000	A2	433	GU938525	3	373	GU938576	3	H16	N4
C88	*Simmondsia chinensis*	Apulia (Southern Italy)	1984	A2	433	GU938523	3	373	GU938567	3	H16	N4
IMI 207770	*Durio zibethinus*	Malaysia	1976	A2	431	GU938508	12	373	GU938577	3	H17	N5

IRF3	*Polygala myrtifolia*	Liguria (Northern Italy)	2007	A2	431	GU938502	12	373	GU938565	3	H17	N5
C301	*Myrtus communis*	Sicily (Southern Italy)	1991	A2	430	GU938500	17	322	GU938557	2	H18	N5
IRF27	*Agapanthus* sp.	Liguria (Northern Italy)	2007	A2	431	GU938501	12	322	GU938561	2	H19	N5
IRF8	*Anemone americana*	Liguria (Northern Italy)	2007	A2	431	GU938503	12	322	GU938578	2	H19	N5
Peperone GJ	*Capsicum annuum*	Calabria (Southern Italy)	2000	A1	431	GU938506	12	322	GU938558	2	H19	N5
Peperone RC	*Capsicum annuum*	Calabria (Southern Italy)	2000	A2	431	GU938507	12	322	GU938560	2	H19	N5
Pomodoro	*Lycopersicum esculentum*	Sicily (Southern Italy)	2000	n.d.	431	GU938504	12	322	GU938555	2	H19	N5
Mirto p5	*Myrtus communis*	Sicily (Southern Italy)	2007	A2	431	GU938509	12	322	GU938579	2	H19	N5
Pittosporo	*Pittosporum* sp.	Sicily (Southern Italy)	1996	A1	431	GU938505	12	322	GU938559	2	H19	N5
Anthurium	*Anthurium* sp.	Sicily (Southern Italy)	2005	A1	432	GU938510	2	322	GU938554	2	H20	N5
Hybiscus B	*Hybiscus* sp.	Calabria (Southern Italy)	2004	A2	432	GU938511	2	322	GU938556	2	H20	N5
Pandorea2C	*Pandorea jasminoides*	Sicily (Southern Italy)	2005	A2	432	GU938513	2	322	GU938562	2	H20	N5

AY894835, AY898627, AY898628), *Phytophthora ramorum* (accession number DQ832718) and *Phytophthora sojae* (accession number DQ832717) were aligned using ClustalX (http://www.clustal.org/) and manually analyzed to select variable intergenic regions flanked by conserved sequences on both sides. The latter condition was necessary to design primers suitable to amplify selected intergenic regions from other *Phytophthora* species, including *Phytophthora nicotianae*. Two intergenic regions appeared to be of particular interest, one was flanked by genes *trnY* and *rns* (*trnY/rns*) and had been previously sequenced from a number of other *Phytophthora* species using primers Mt2F-Mt5R (Schena & Cooke 2006). A second region, flanked by genes *trnW* and *cox2* (*trnW/cox2*) was amplified with two primers (Mt17F AAATACTTTTTAACAAAAGGGAATTTA and Mt12R TGGAGTTGCTGGATCTTGAA) selected among six candidates during preliminary tests to identify the best primer combinations and amplification conditions. All primers were designed using the Primer3 Software (Rozen & Skaletsky 2000).

DNA amplification and sequencing

Total DNA was extracted from all isolates according to the procedure described by Ippolito *et al.* (2002). Amplification conditions consisted of one cycle of 94 °C for 3 min followed by 35 cycles of 94 °C for 45 s, 54 °C for 30 s, 72 °C for 45 s and by a final extension cycle of 72 °C for 10 min. Reactions were performed in a total volume of 30 μl containing 5 ng of genomic DNA, 1× PCR buffer, 0.1 mM dNTPs, 3 mM $MgCl_2$, 1 unit Taq polymerase (Invitrogen, CA, USA) and 10 μM for each primer. Amplicons were analyzed by electrophoresis in 1.5 % agarose gels containing SYBR Safe™ DNA gel stain (Invitrogen, CA, USA) in Tris-Borate-EDTA (TBE) buffer and visualized with UV light. PCR products were purified using Centri Spin™ Columns (Applied Biosystems, Foster City, USA) to remove excess primers and nucleotides and sequenced in both directions with the corresponding amplification primers using the BigDye sequencing kit (Applied Biosystems, Foster City, USA) on ABI 310 DNA Analyzer (Applied Biosystems, Foster City, USA).

Sequence analysis

The 'ChromasPro version1.5' software (http://www.technelysium.com.au/) was utilized to evaluate reliability of sequences and to create consensus sequences. Non-reliable sequences in which either forward or reverse sequences contained doubtful bases were sequenced again. Consensus sequences from both mitochondrial regions were aligned, analyzed, and edited manually for checking indels and single nucleotide polymorphisms (SNPs) using Bioedit 7.0 software (Hall 1999). Prior to analysis, sequences of primers were removed.

Haplotype analysis and networks

Haplotypes were identified by aligning sequences from all 51 isolates with Bioedit 7.0 (Hall 1999) and confirmed using the DnaSP ver. 5.10.01 software (Librado & Rozas 2009). To infer intraspecific evolution a network of haplotypes was constructed using a statistical parsimony algorithm implemented in TCS ver. 1.21 (Clement *et al.* 2000) individually for each of the mitochondrial regions and for the combined data set. This program

applies a statistical parsimony method to infer unrooted cladograms based on Templeton's 95 % parsimony connection limit (Templeton *et al.* 1992). Haplotypes were directly connected without dots when differing by a single change. Every additional putative change was indicated by adding a dot.

Phylogenetic analysis

The two regions were analyzed individually and combined in a single data set. To test the homogeneity of the combined data set for the phylogenetic analysis a partition homogeneity test (PHT) (Farris *et al.* 1995) was performed using heuristic search with 1000 number of replicates in PAUP v4.0b10 (D. Swofford, Sinauer Associates, Sunderland, MA). To generate compatible alignments for the phylogenetic analysis, indels were recoded manually to minimize errors due to the length of the gaps and ensure the treatment of all indels as a single event of mutation.

A maximum parsimony analysis was performed in PAUP v4.0b10 using a heuristic search algorithm with random stepwise addition of taxa (ten replicates), tree bisection reconnection (TBR) branch swapping and multiple trees option. The statistical support was determined by bootstrap values for 1000 replicates. Maximum likelihood analysis was inferred using the TrN (Tamura & Nei 1993) +I (proportion of infinite sites model) while Bayesian method was inferred using the HKY+I (Hasegawa *et al.* 1985). The substitution models that best fit our data were selected with Jmodeltest 0.1.1 (Posada 2008). Maximum likelihood analysis was conducted using PhyML ver 2.4.5 (Guindon & Gascuel 2003) implemented in TOPALi v2 (Milne *et al.* 2009) with 100 bootstrap replicates. Bayesian analysis was performed in TOPALi using MrBayes ver 3.1.1; four runs were conducted simultaneously for 1 000 000 generations with 10 % sampling frequency and burn in of 25 %.

Biological tests

All isolates were analyzed to assess their mating type by pairing each isolate with known A1 and A2 strains on V8 juice agar medium according to standard procedures (Erwin & Ribeiro 1996). Isolates that did not produce oospores were considered sterile. The growth rate of the isolates was evaluated by transferring PDA agar plugs (∅ 5 mm) containing actively growing mycelium into Petri dishes with PDA and incubating at 24 °C in the dark. Colony diameter was measured daily until the complete colonization of the dish. Three replicate dishes were used for each isolate.

Isolates were also compared by evaluating their rapidity in colonizing the flesh of 'Golden Delicious' apples. Uniform fruits for size and ripeness were surface sterilized by immersion for 1 min in a 2 % of sodium hypochlorite solution, washed with tap water, air dried, wounded in the equatorial zone with a nail (∅ 0.5 mm) and inoculated by placing on each wound a PDA agar plug (∅ 5 mm) containing actively growing mycelium. Inoculated apples (five per each isolate) were placed in plastic boxes to create high relative humidity and incubated at 20 °C. The extension of decaying tissues on the apple surface was measured daily for 7 d, after which the fruit was cut perpendicularly along the inoculation site in order to measure length and width of internal flesh rotted areas. Data were subjected to analysis of variance (ANOVA) and mean values were compared using Tukey test.

Results

Characterization of polymorphic mtDNA regions

Two different primer pairs were utilized to amplify mitochondrial regions *trnY/rns* and *trnW/cox2* from 51 isolates of *Phytophthora nicotianae* from different hosts and geographic origins (Table 1). Amplicons exhibited variable length ranging from 429 bp to 443 bp (*trnY/rns*) and 322 bp to 373 bp (*trnW/cox2*) (Table 1). Intraspecific variability was observed as SNPs, short indels (1–4 bp), long indels (a 19 bp indel and two 7 and 51 bp indels were revealed in the *trnY/rns* and *trnW/cox2* regions, respectively) and length variations in a homopolimeric T region in the *trnY-rns* spacer (Fig 1). For the *trnY-rns* region nine SNPs were identified; three were transversions and six were transitions with eight of these parsimony informative. When combined with data for indels 17 different haplotypes were observed. The *trnW-cox2* region was less polymorphic with four SNPs (three transversions and one transition) and a single parsimony informative site; when combined with data for indels seven haplotypes were present. Combining DNA sequences from the two intergenic regions identified 20 different haplotypes (Table 1).

The *P. nicotianae trnY/rns* region had a length comparable to that of the homologous region from *Phytophthora ramorum* (353 bp; DQ832718), but was significantly shorter compared to homologous regions in *Phytophthora sojae* (952 bp; DQ832717) and *Phytophthora infestans* haplotype IIa (2785 bp; AY898627),

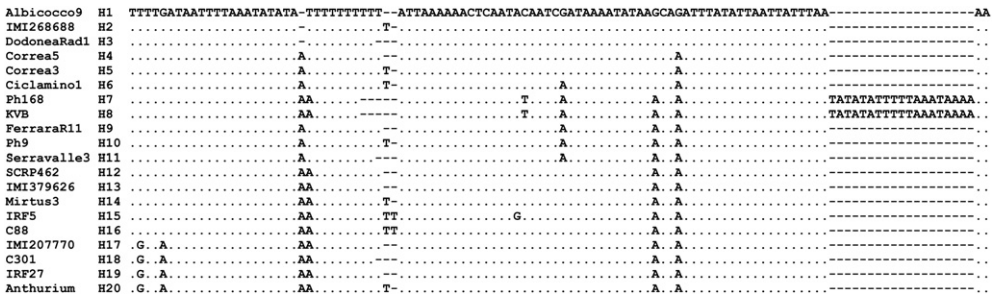

Fig 1 – Sequence alignment of portion of the *trnY-rns* region of 20 representative *Phytophthora nicotianae* haplotypes.

IIb (2729 bp; AY898628), Ia (904 bp; AY894835) and Ib (904 bp; NC_002387). Similarly, the *trnW/cox2* region in *P. nicotianae* was shorter compared to homologous sequences from *P. ramorum* (846 bp; DQ832718), *P. sojae* (725 bp; DQ832717) and *P. infestans* haplotype IIa (1007 bp; AY898627), IIb (849 bp; AY898628) and Ia (814 bp; AY894835) and Ib (850 bp; NC_002387).

Haplotype analysis and networks

Seventeen and seven haplotypes were identified by individually analyzing regions *trnY/rns* and *trnW/cox2*, respectively; the combined data set of sequences revealed 20 different haplotypes (Table 1). The haplotype network (Fig 2) of the combined data set confirmed the presence of five different genetic groups defined by the phylogenetic analyses (Fig 3). The N2 group was more distant compared to the other groups while the N3 group, which was almost exclusively represented by citrus isolates, presented the most frequent haplotype in the network. No patterns of geographic association were revealed among the most frequent haplotypes. The network has shown cases where identical haplotypes were present within isolates with opposite mating types. In particular, the haplotypes H14, H19, and H20 had opposite mating types in the same geographic location (Table 1). Four cases of homoplasy were observed in the combined data set network (Fig 2). This homoplasy was also confirmed in the analysis of the network for the two intergenic region considered individually (data not shown).

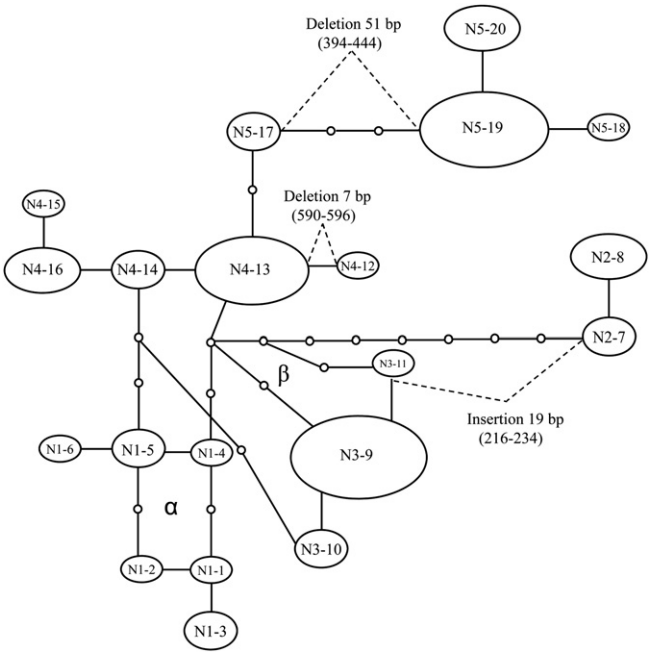

Fig 2 – Parsimony haplotype network of *Phytophthora nicotianae* isolates constructed using combined sequence data from *trnW-cox2* and *trnY-rns* regions. The letter codes identify the single or the groups of haplotypes with N referring to the phylogenetic clade in Fig 3 and the number after the '-' the final mitochondrial haplotype. The size of each oval represents the relative frequencies of haplotypes in the pool of isolates considered in this study. Greek letters (α and β) indicate possible event of homoplasy. Haplotypes were directly connected without dots when differing by a single change. Every additional putative change was indicated by adding a dot.

Phylogenetic analyses

In the PHT a significant congruence ($P = 0.74$) between the two mitochondrial regions was observed so the concatenated file of the two regions was used for analysis with the three different phylogenetic methods (maximum parsimony, maximum likelihood, and Bayesian analysis). A tree with the same five major clades was observed with each type of analysis (Fig 3), a grouping that was also observed when the two regions were analyzed separately (although a less accurate discrimination among isolates was possible, data not shown).

The first clade (N1) was comprised of seven isolates from Southern Italy (six from Sicily and one from Calabria) and an isolate (IMI 268688) obtained from citrus in Trinidad (Table 1; Fig 3). This clade contained six mitochondrial haplotypes distributed in three branches. Isolates of this clade shared differences that clearly distinguished them from the other clades such as a SNP in position 193 of the *trnY-rns* region. The isolate Ciclamino1 constituted an individual haplotype branch within this group. No mating type association within this clade was revealed. Six out of the eight isolates were recovered from host plants of the *Sapindales* order (*Correa reflexa*, *Dodonea viscosa*, and *Citrus* sp.).

Clade N2 was well differentiated from other clades with two distinct mitochondrial haplotypes clustered in this group (Fig 3). Except for the citrus isolate Ph168, all isolates were mating type A2 (Table 1). Isolates of this clade had a 19 bp insertion in the *trnY-rns* region that differentiated them from all the other isolates analyzed in this work.

Clade N3 was almost exclusively represented by isolates from citrus recovered from different regions of southern Italy, Syria (isolate Ph195), and Albania (isolate Ph142). This clade also contained an isolate from *Limonium sinensis* that, unlike all other isolates, was an A2 mating type (Table 1; Fig 3). Three different mitochondrial haplotypes clustered in this group that differed in base numbers in a homopolymeric thymine region in the *trnY-rns* intergenic region (Fig 1).

Five mitochondrial haplotypes clustered in clade N4 (Fig 3). This group of isolates was heterogeneous for geographic origin, host, and mating type. Only two SNPs (pos. 148 and 394) differentiate this group of isolates from the N3 clade of isolates from citrus. Except for the isolate IRF5 from *Polygala myrtifolia* that constituted an individual haplotype branch within this group (SNP in position 175 in *trnY-rns* region) and the isolate SCRP462 that had a 7 bp deletion in the *trnW-cox2* region, the other isolates within this group were distinguished by differences in length variation in the homopolymeric thymine region in the *trnY-rns* intergenic spacer.

Clade N5 was represented by 13 isolates divided in four mitochondrial haplotypes; no specific associations were observed with either geographic origin or host. All isolates of this clade shared a 51 bp indel in the *trnW-cox2* intergenic region.

Biological tests

The characterization of 51 isolates of *Phytophthora nicotianae* from different geographic regions and hosts showed that 23

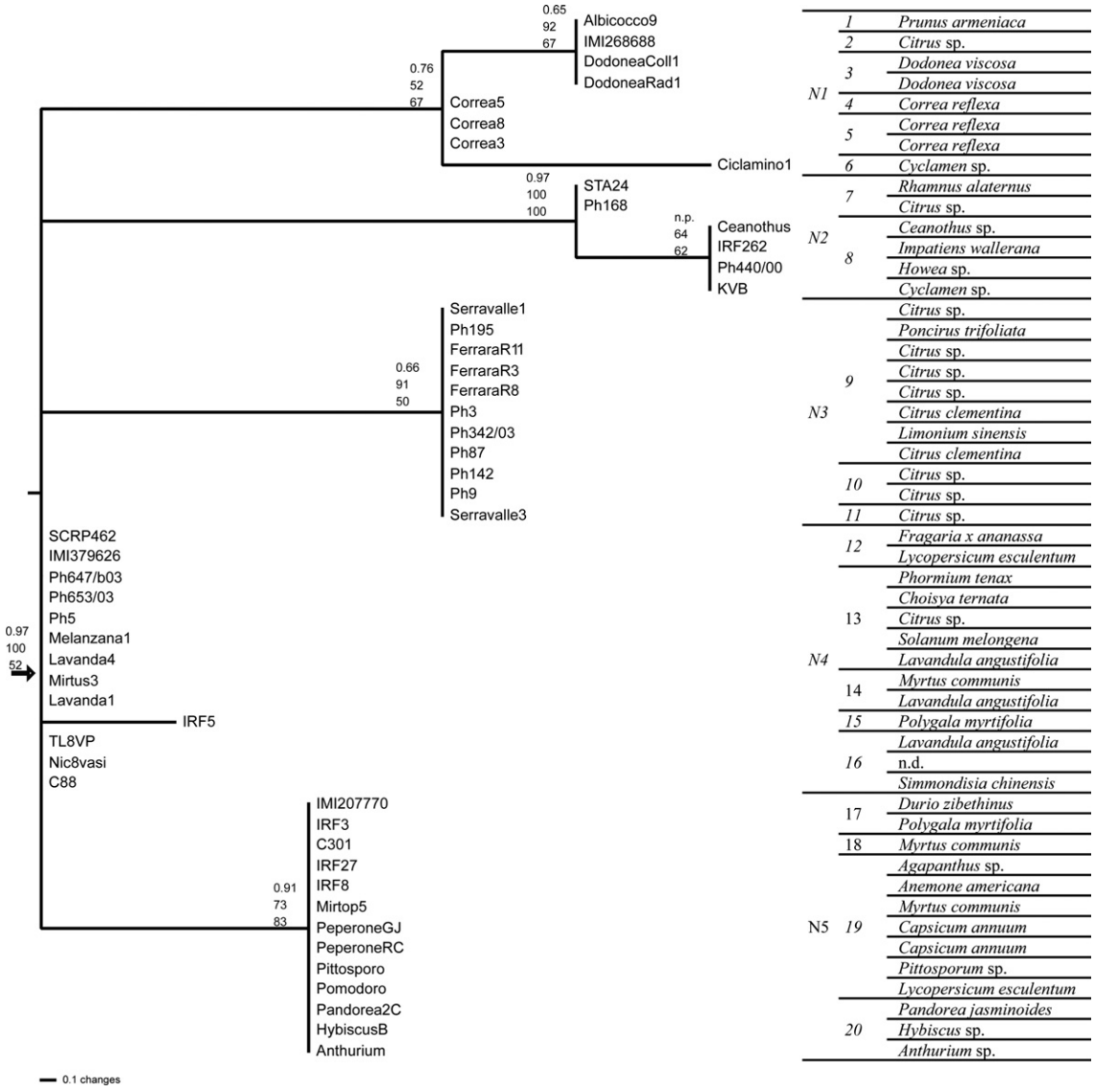

Fig 3 – Phylogenetic relationships between *Phytophthora nicotianae* isolates based on the combined data set of sequences from the two mitochondrial intergenic region *trnY-rns* and *trnW-cox2*. The tree was midpoint rooted. Numbers on nodes represent the statistical support for the Bayesian method (posterior probabilities, top number), maximum likelihood (100 replicates, middle number) and maximum parsimony (1000 bootstrap replicates, bottom number). Table on the right of the tree reports phylogenetic clades (first column; N followed by a number) distinctive haplotypes (second column; number only) and plant host from which isolates were obtained (third column).

and 27 isolates were A1 and A2 mating type, respectively (Table 1). Among these, all isolates from citrus were of mating type A1. One isolate from tomato did not form oospores when paired with the two reference mating types.

Growth rates of isolates on PDA ranged from 1.7 mm d^{-1} to 9.1 mm d^{-1} and differences among isolates were significant ($P \leq 0.05$; Fig 4). Similarly, significant differences were also observed in the colonization rates of apple tissues. After 5 d of incubation the extent of colonization of apple flesh by the pathogen ranged from 1.7 mm^2 to 33 mm^2 (Fig 4). The extension of decaying tissues on the apple surface reflected internal colonization and after 5 d of incubation ranged from 3.2 mm^2 to 44.5 mm^2 (data not shown). Although differences among isolates for both growth rates on PDA and apple flesh colonization were significant, no correlations were found between these two parameters or with host, geographic origin or molecular group based on mitochondrial haplotype analyses (Fig 4).

Discussion

Fifty-one isolates of *Phytophthora nicotianae* from different hosts and geographic origins were characterized using two variable intergenic regions of the mitochondrial DNA to evaluate mitochondrial haplotypes and their phylogenetic relationships. The analysis of haplotypes exhibited a different level of variation between the mitochondrial regions used. The *trnY-rns* intergenic region was more variable with nine

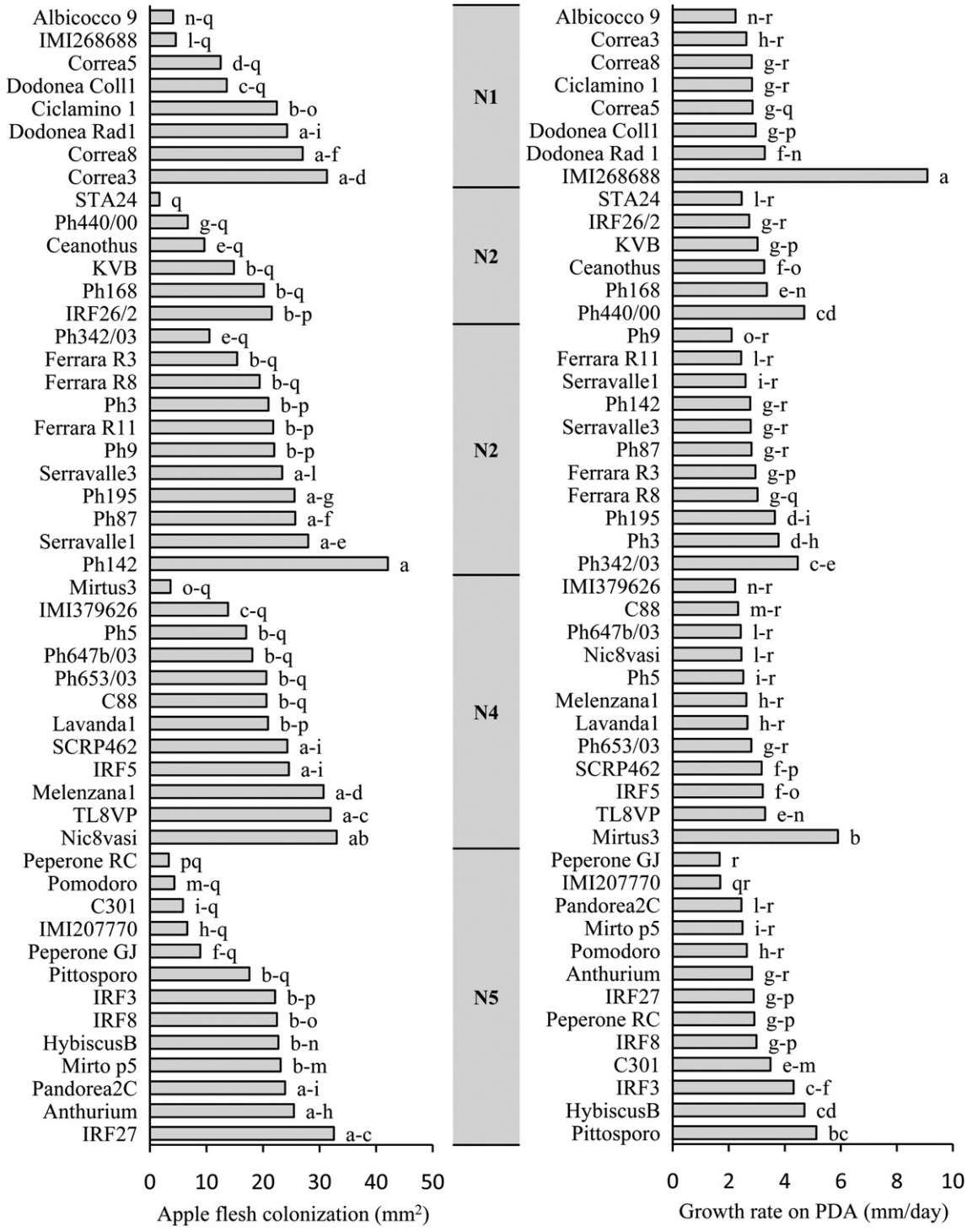

Fig 4 – Comparison between apple flesh colonization after 5 d of incubation (left) and growth rate on PDA (right) of *Phytophthora nicotianae* isolates grouped according to phylogenetic clades (centre). In both charts isolates were primarily listed accordingly to their phylogenetic groups (Fig 2) and secondly (in each group) according to a decreasing order. Letters on the right of columns report statistical analysis; values not sharing common letters are statistically different according to Tukey test ($P \leq 0.05$).

SNPs, three indels (29 bp) and length variations in a homopolymeric thymine region that differentiated 17 haplotypes. In contrast, for the *trnW-cox2* region four SNPs and two indels (58 bp) differentiated isolates into seven mitochondrial haplotypes. The combined data set for both regions revealed a total of 20 mitochondrial haplotypes.

No consistent association of haplotypes with the geographic location of isolation or host from which the isolates

were recovered was observed. The majority of isolates from citrus had similar haplotypes (H9, H10, and H11) and differed only in the number of bases in a homopolymeric thymine region (haplotypes with nine, ten or 11 thymine bases). Similar length variations in homopolymeric stretches of 'T' were also identified in other groupings of haplotypes (H13, H14, H16, and H18, H19, H20; Fig 2). It is likely these differences were generated by slippage events during DNA replication, which is known as source of length polymorphisms in sequence stretches in human populations (Schlötterer & Tautz 1992). The fact that isolates from citrus recovered from different geographic regions had the same mitochondrial haplotype (or very similar) could be due to the nursery trade distributing infected plant material, which could also explain the presence of identical haplotypes for isolates from ornamental and horticultural plants from different production areas (H8, H13, H17, and H19).

Another explanation for shared mitochondrial haplotype among citrus isolates is there may be a preferential association between these isolates and citrus hosts since subgroups of *P. nicotianae* showing some host specificity have been reported for some host species (Philips & Baker 1962; Bonnet *et al.* 1978; Erwin & Ribeiro 1996; Allagui & Lepoivre 2000). It was demonstrated that isolates from *Citrus* species were more virulent on roots of rough lemon than isolates from petunia, tomato, walnut, silk tree, jojoba, hibiscus, and peach. Also, tomato plants exhibited high susceptibility to many isolates including citrus isolates (Matheron & Matejka 1990). Furthermore, the analysis of both mitochondrial and nuclear DNA restriction fragments distinguished isolates causing black shank in tobacco from other *P. nicotianae* isolates (Colas *et al.* 1997). The observation that an isolate recovered from *Limonium sinensis* (Ph342/03) had the same mitochondrial haplotype as most citrus isolates could be due to its recovery from a nursery in which many ornamental species, including ornamental citrus, were also grown. Furthermore, while some *P. nicotianae* groups may have a preferential host, most studies have demonstrated that they can still infect other hosts (Erwin & Ribeiro 1996). Additional experimentation evaluating virulence of citrus isolates on *Citrus* and several other hosts is needed to confirm if there is a preferential host association.

The use of mitochondrial markers, which are maternally inherited, would be useful to study clonally reproducing populations of the pathogen. Clonal populations of *P. nicotianae* were identified as a component of field populations from the same tobacco field in four consecutive years (Sullivan *et al.* 2010) and for isolates from different ornamental plants and production sites (Lamour *et al.* 2003). It was suggested that isolates within the same ornamental nurseries spread by asexually generated propagules, such as hyphal fragments, sporangia, chlamydospores or zoospores (Lamour *et al.* 2003). Furthermore, it was concluded that, at least in citrus orchards in Italy, *P. nicotianae* reproduced primarily asexually since in the majority of citrus orchards examined only the A1 mating type was found (Cacciola & Magnano di San Lio 1998). However, the heterothallic mating behaviour of this pathogen can generate a sexual recombining population when both mating types are present and therefore nuclear markers should be included when analyzing field populations in much the same way as observed for *Phytophthora infestans* (Flier *et al.* 2007; Widmark *et al.* 2007). The observations that opposite mating types were found within the same mitochondrial haplotype (H7, H9, H13, H14, H19, and H20 with isolates from the last three haplotypes having opposite mating types in the same geographic region) suggest that sexual recombination has occurred in the past.

These two mitochondrial intergenic regions were used also in phylogenetic analyses and highlighted evolutionary divergences at mitochondrial genome level. The phylogenetic analysis of the combined sequences identified five phylogenetic clades, a result that was consistent with three different methods of analysis (maximum parsimony, maximum likelihood, and Bayesian analysis).

Different haplotypes were distributed in each of the five clades and did not exhibit a consistent clustering based on geographic origin, mating type or host. However, the majority of haplotypes representing citrus isolates (10/13) grouped together in clade N3, reinforcing the idea that these haplotypes came from the same ancestral mitochondrial genome and indicating that the differences found at level of homopolymeric T region (as for some isolates of N4 and N5 clades), were meaningless in a phylogenetic sense. A divergent evolutionary pattern was shown in particular for clade N2 as shown also in the parsimony haplotype network (Fig 2). Two haplotypes (H7, H8) were identified in the N2 clade by a different number of polymorphisms. In particular, three SNPs that were parsimony informative (SNPs specific of this clade), a 19 bp insertion and 4 bp deletions differentiated this clade from the others. In contrast, the lower amount of genetic variation between the N3 clade and the N4, clade N5 and N4, N1 and N4 suggested a more recent evolutionary divergence among these groups of haplotypes. In particular, only two SNPs that were parsimony informative differentiated the clade N5 (haplotype H17 of clade N5, Fig 2) from the clade N4 (haplotype H13 of clade N4); three SNPs that were parsimony informative discriminated clade N1 (haplotype H5, Fig 2) and the citrus clade N3 from clade N4 (from haplotypes H14 and H13 of clade N4). Furthermore, as shown in the haplotype network analysis, the homoplasy revealed between haplotypes could explain the reduction of evolutionary resolution, in particular, between the citrus clade N3 and clade N4. These results do not exclude the possibility of recombination between different mitochondrial haplotypes, although given the uniparental inheritance of mitochondrial genomes in sexual outcrossing this would be unlikely (Forester & Coffey 1990). Taking into consideration that the majority of these isolates come from nurseries, the combined effect of rare mutation in mitochondrial DNA and possible drift of haplotypes due to commerce could be involved in establishing new divergent haplotypes populations.

Whereas mitochondrial DNA was one of the markers more available in the studies of population evolution, we need to bear in mind that phylogenetic analysis based solely on mitochondrial markers could have some important limitations. Importantly, mitochondrial DNA represents the historical map of the maternal lineage, ignoring part of the genetic history present in the population (Zhang & Hewitt 2003). Therefore, a phylogenetic analysis based only on this cytoplasmatic marker may only partially help to answer questions on the evolution of populations and resulting evolutionary relationships may be biased. However, these two intergenic regions give an idea, even partly, of the evolutionary relationships

between the haplotypes identified in this study. A larger number of isolates representing the range of diversity of *P. nicotianae* is required to verify the usefulness of these mitochondrial intergenic regions to highlight different evolutionary pathways.

Mitochondrial haplotype and phylogenetic clades were not correlated with phenotypic traits, such as growth rate and ability to colonize apple tissues. This result was partially expected since, according to previous reports, biological and pathogenicity tests are key traits for studying and differentiating closely related strains of *P. nicotianae*, however they provided only limited information on the actual diversity and genetic potential of pathogen populations since they are likely influenced by a number of background factors, including *in vitro* culture duration, storage conditions and culturing media (Powers & Lucas 1952; Apple 1957). Many cases of mutation or adaptive changes leading to variability have been reported for *Phytophthora* species (Erwin and Ribeiro 1996) and specifically for isolates of *P. nicotianae* that lose virulence with continuous culturing (Apple 1957).

In conclusion, a new molecular approach to characterize intraspecific variability in *P. nicotianae* is proposed. This method may be further improved by the identification of new target regions and could be easily extended to other species of the genus *Phytophthora* as well as other plant pathogens. Compared to alternative molecular methods, such as RAPD-PCR and AFLP, a major advantage of this approach is that results are objective (a list of nucleotides) and can be highly reproducible because the results are not affected by a number of factors such as the purity of target DNA, amplification reagents, thermocyclers, etc. This aspect is particularly important since it would enable the comparison of data from different research groups or time periods and could be implemented as soon as a molecular database is available from submission of sequences to GenBank. Application of this method could also be used in standardized protocols to develop a DNA barcoding like strategy for the precise identification of sub-specific taxa in *P. nicotianae* as well as in other species of *Phytophthora*. In fact, although more variable than the loci commonly used for barcode analysis of *Phytophthora* species (ITS, *cox1*; C. A. Levesque, pers. comm.), the regions used in this experimentation possess many of the important characteristics of a desirable locus for DNA barcoding since they are present in most of the taxa of interest, can be amplified without species-specific PCR primers and are short enough to be easily sequenced with current technology (Kress & Erickson 2008). A limitation to the wide exploitation of the method proposed in the present study could be represented by the cost of sequencing multiple target genes from a large number of isolates. However, the increasing development of high throughput sequencing equipment and the consequent reduction of sequencing costs recorded in recent years are encouraging.

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