Genetic Analysis of *Phytophthora nicotianae* Populations from Different Hosts Using Microsatellite Markers

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ABSTRACT

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In all, 231 isolates of *Phytophthora nicotianae* representing 14 populations from different host genera, including agricultural crops (*Citrus*, *Nicotiana*, and *Lycopersicon*), potted ornamental species in nurseries (*Lavandula*, *Convolvulus*, *Myrtus*, *Correa*, and *Ruta*), and other plant genera were characterized using simple-sequence repeat markers. In total, 99 multilocus genotypes (MLG) were identified, revealing a strong association between genetic grouping and host of recovery, with most MLG being associated with a single host genus. Significant differences in the structure of populations were revealed but clonality prevailed in all populations. Isolates from *Citrus* were found to be genetically related regardless of

their geographic origin and were characterized by high genetic uniformity and high inbreeding coefficients. Higher variability was observed for other populations and a significant geographical structuring was determined for isolates from *Nicotiana*. Detected differences were related to the propagation and cultivation systems of different crops. Isolates obtained from *Citrus* spp. are more likely to be distributed worldwide with infected plant material whereas *Nicotiana* and *Lycopersicon* spp. are propagated by seed, which would not contribute to the spread of the pathogen and result in a greater chance for geographic isolation of lineages. With regard to ornamental species in nurseries, the high genetic variation is likely the result of the admixture of diverse pathogen genotypes through the trade of infected plant material from various geographic origins, the presence of several hosts in the same nursery, and genetic recombination through sexual reproduction of this heterothallic species.

In a recent survey to query the scientific community for their ranking of plant-pathogenic oomycete species based on both scientific and economic importance, *Phytophthora nicotianae* Breda de Haan (*P. parasitica* Dastur) was listed eighth, and it is likely to gain importance in the foreseeable future (Kamoun et al. 2015). This oomycete is a soilborne plant pathogen with a wide host range comprising more than 250 plant genera (Cline et al. 2008; Panabières et al. 2016). It is particularly known for its damage on the genera *Nicotiana* and *Citrus*, as the causal agent of black shank and citrus root rot and gummosis, respectively, but it is responsible for heavy losses on many other economically important crops such as fruit trees and vegetable and ornamental crops (Erwin and Ribeiro 1996; Ippolito et al. 2004; Moralejo et al. 2009). A number of surveys revealed that *P. nicotianae* is one of the most common pathogens in nurseries, especially on ornamental plants, where it commonly completes several disease cycles per year due to the repeated culture of different host species (Cacciola et al. 1997, 2001; Moralejo et al. 2009; Pane et al. 2005; Prigigallo et al. 2015, 2016; Reichard and White 2001). This pathogen produces various types of propagules, including sporangia, chlamydospores, and oospores, which can be formed when the mycelium of sexually

compatible mating types (A1 and A2) comes into contact. Sporangia can germinate directly as mycelium or differentiate into zoospores that are motile. Oospores constitute a potential source of genetic variation and, with resting chlamydospores, contribute to survival under unfavorable conditions in soil or within infected plant tissues.

P. nicotianae might be considered a candidate species for studying migration pathways of *Phytophthora* spp. and other soilborne pathogens on a global scale. Its polyphagia and prominence in nurseries of potted ornamentals and fruit tree species are important factors that favor a rapid distribution on a global scale. Indeed, the global nursery trade represents one of the most efficient diffusion pathways for soilborne pathogens (Abad et al. 2014; Jung et al. 2016; Moralejo et al. 2009; Olson and Benson 2011; Parke and Grünwald 2012). Furthermore, the ornamental industry extensively uses antioomycete chemicals that can hide the presence of the pathogen and increase the risk of rapid spread of resistant strains to new areas (Brasier 2008; Olson et al. 2013). Moreover, *P. nicotianae* will likely benefit from the warming climate because its host range is generally similar to other species of prime economic importance such as *P. infestans*, *P. capsici*, and *P. citrophthora* but generally requires warmer conditions than these other species (Erwin and Ribeiro 1996; Kamoun et al. 2015).

Despite the great economic relevance of *P. nicotianae*, significant information on the biology and ecology of this pathogen is still lacking. Few studies are currently available on the genetic structure of *P. nicotianae* populations and most of them were focused on either specific hosts (mainly tobacco) or geographic areas. The characterization of seven different populations from tobacco using a random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) approach revealed a high level of variability within a

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population but no distinct genotypes were associated with specific populations (Zhang et al. 2003). The same technique was used to differentiate isolates causing black shank from isolates that did not cause this disease (Zhang et al. 2001) and to identify markers linked to the dominant black shank resistance gene (Johnson et al. 2002a). Amplified fragment length polymorphism analysis enabled the identification of six clonal lineages in a population from different floricultural hosts and production sites (Lamour et al. 2003). Sullivan and coworkers (2010) found that tobacco populations were able to evolve and adapt to the host genotypes deployed in the field.

Recently, polymorphic mitochondrial and nuclear regions were utilized to study genetic diversity in a worldwide collection of *P. nicotianae* isolates (Mammella et al. 2011, 2013). Single-nucleotide polymorphisms (SNP) detected in both mitochondrial and nuclear markers revealed a high level of dispersal of isolates and a low geographic clustering of populations. Nonetheless, a specific association was observed between the host of origin and genetic cluster for some hosts and, thus, it was suggested that nursery populations played an important role not only for increasing genetic recombination within the species but also in terms of providing a means of dispersal of genotypes worldwide via movement of infected plant material. However, mitochondrial analyses conducted with an outcrossing species such as *P. nicotianae* may produce incomplete results, and the three nuclear markers investigated were not variable enough to provide discrimination among closely related isolates (Mammella et al. 2013).

Microsatellites, or simple-sequence repeats (SSR), are largely accepted as a powerful molecular tool for investigating intraspecific variability in a large number of eukaryotic species (Oliveira et al. 2006; Gonthier et al. 2015). In oomycetes, SSR have been used in a number of important applications, including diagnosis and determination of mating type, genetic structure and disease dynamics, and population genetics (Garnica et al. 2006; Schena et al. 2008). Relevant studies have been conducted on several *Phytophthora* spp., including *P. infestans* (Cooke and Lees 2004; Goss et al. 2014; Li et al. 2013), *P. alni* (Ioos et al. 2007), *P. ramorum* (Goss et al. 2009; Prospero et al. 2007), *P. sojae* (Stewart et al. 2014), and *P. capsici* (Hu et al. 2013), as well as *P. plurivora*, *P. multivora*, and *P. pini* (Schoebel et al. 2013, 2014). Furthermore, a set of nine specific SSR markers for *P. nicotianae* has been recently developed (Biasi et al. 2015).

The objective of the present study was to genotype isolates of *P. nicotianae* obtained from different hosts and geographic localities using recently validated SSR markers (Biasi et al. 2015) to determine whether there were associations of particular genotypes with host of recovery or location of collection.

MATERIALS AND METHODS

Isolate sampling, identification, and mating type determination.

In total, 231 isolates of *P. nicotianae* were obtained from soil, roots, and basal stem samples from different hosts and geographic regions (Supplementary Table S1). Isolates were sampled from six continents, although most of them were sourced from Italy ($n = 163$), Vietnam ($n = 36$), the United States ($n = 16$), and Australia ($n = 6$). Isolates were mainly obtained from *Citrus* spp. ($n = 90$), several horticultural and ornamental crops ($n = 121$), and *Nicotiana* spp. ($n = 20$). All isolates from ornamental cultures were sampled from potted plants in nurseries.

Isolates were identified by means of morphological criteria and sequencing of the internal transcribed spacer (ITS) region of the ribosomal DNA. For ITS sequencing, total DNA was extracted as described by Ippolito et al. (2002), amplified with the universal primers ITS6 and ITS4, and sequenced with the same primers using an external service (Macrogen). Isolates were determined to be *P. nicotianae* based on their phylogenetic clustering with reference isolates of the species (Robideau et al. 2011). Mating type was determined by pairing each isolate with known A1 and A2 strains on V8 juice agar medium, as described by Erwin and Ribeiro (1996).

SSR genotyping. Nine validated polymorphic SSR markers were utilized to genotype isolates (Biasi et al. 2015). Forward primers were labeled with the fluorescent dyes 6-FAM or HEX, while all reverse primers were modified with a 5' PIG tail "GTTT" to reduce the phenomenon of stutter peaks (Brownstein et al. 1996). Eight of the nine forward primers were labeled with two different fluorophores (HEX and 6-FAM) and assembled into four multiplex PCR sets for simultaneous amplicon sizing (Table 1). Reaction mixtures and amplification conditions were as described by Biasi et al. (2015). The data were collected using the software Data Collection (v.2.0; Applied Biosystems) and analyzed by Gene Mapper (v. 4.1; Applied Biosystems) to derive the size of the labeled DNA fragments using the GeneScan 500 LIZ Size Standard (Life Technologies).

Data analysis. Analysis focused on multilocus genotypes (MLG) to infer genotypic diversity in populations. To perform analyses, isolates were grouped according to the genus of the host from which they were obtained. The R package *poppr* (Kamvar et al. 2014) was used to calculate basic population statistics, including (i) Stoddart and Taylor's index, $G = 1/\sum_i p_i^2$, where p_i is the observed frequency of i th genotype (Stoddart and Taylor 1988) and the normalized G' index which removes bias due to the sample size (Chen et al. 1994), and it is particularly appropriate when comparing populations showing high diversity; (ii) evenness, $E_5 = (1/\lambda) - 1/e^{H'} - 1$, where λ is Simpson's index and H' is Shannon-Wiener's index; and (iii) Bruvo's distance (Bruvo et al. 2004). E_5 is a preferred index of evenness because it is less dependent on the number of genotypes in a sample (Grünwald et al. 2003). Bruvo's distance enabled the measurement of the genetic distances among isolates by calculating the minimum distance across all combinations of possible pairs of alleles at a single locus and then averaging that distance across all loci. Bruvo's distance was utilized to construct minimum spanning networks (MSN) to graphically show the possible evolutionary relationships among MLG. MSN contain a set of pairwise distances that describe the degree of dissimilarity among individuals and allow inference of population structure analogous to a phylogenetic analysis (Ronquist and Huelsenbeck 2003; Salipante and Hall 2011). Lynch's distances (Lynch 1990) was used to perform a principal coordinate analysis (PCA) using the R package POLYSAT (Clark and Jasieniuk 2011). Lynch's distance is a simple measure of similarity and corresponds to two times the number of common alleles divided by the total number of alleles of the two genotypes considered.

Furthermore, F statistics (F_{IS} and F_{ST}) were calculated using the software GENODIVE (Meirmans and van Tienderen 2004), in order to quantify the level of divergence between observed and expected heterozygosity. More specifically, the inbreeding coefficient F_{IS} was used to scale the deviation of genotypic frequencies from expected panmictic frequencies in terms of heterozygous deficiency or excess. The fixation index F_{ST} was used to examine the overall genetic differentiation among populations.

TABLE 1. Number of alleles, range of amplicon size, and number of repeats detected in the complete panel of *Phytophthora nicotianae* isolates analyzed in the present study using a validated set of nine simple-sequence repeats^a

Locus ^b	Number of alleles	Dye	Product size (bp)	Number of repeats
P5 ₍₁₎	13	HEX	186–266	4–24
P15 ₍₂₎	14	FAM	66–123	8–27
P17 ₍₁₎	14	FAM	102–165	10–31
P643 ₍₃₎	17	FAM	148–202	9–36
P788 ₍₃₎	7	HEX	127–143	9–17
P1129 ₍₄₎	7	HEX	133–163	7–17
P1509 ₍₅₎	23	FAM	116–176	12–42
P2039 ₍₄₎	4	FAM	99–120	5–12
P2040 ₍₂₎	7	HEX	149–167	8–14

^a Biasi et al. (2015).

^b Each number in parentheses indicates the markers paired for multiplex polymerase chain reaction.

RESULTS

SSR genotyping. Considering all possible combinations among investigated isolates and the nine SSR loci, only 2 of 2,079 amplicons (locus P1509 for isolates P1495 and M1f1h) could not be sized because no amplification occurred (Supplementary Tables S1 and S2). In total, 99 MLG were detected among the 231 isolates and most genotypes were observed only once or at low frequency. The number of alleles at each locus varied from a minimum of 4 (locus 2039) to a maximum of 23 (locus P1509) while the number of repeats ranged from 4 (locus P5) to 42 (locus P1509) (Table 1).

TABLE 2. List of multilocus genotypes (MLG) characterized in the present study along with host and geographic origin of isolates^a

MLG	N ^b	Host	Geographic origin
1	1	<i>Nicotiana tabacum</i>	Australia
2	1	<i>Myrtus communis</i>	Sardinia
3	4	<i>Lavandula stoechas</i>	Sicily
4	1	<i>L. stoechas</i>	Sicily
5	1	<i>Chamaleucium uncinatum</i>	Apulia
6	3	<i>Convolvulus mauritanicus</i>	Apulia
7	1	<i>C. mauritanicus</i>	Apulia
8	1	<i>C. mauritanicus</i>	Apulia
9	1	<i>Hebe</i> × <i>Veronica myrtifolia</i>	Sicily
10	1	<i>L. officinalis</i>	Apulia
11	1	<i>L. officinalis</i>	Apulia
12	2	<i>M. communis</i>	Sardinia
13	1	<i>M. communis</i>	Sardinia
14	1	<i>Chamaleucium uncinatum</i>	Sicily
15	8	<i>Ruta graveolens</i>	Sicily
16	1	<i>R. graveolens</i>	Sicily
17	3	<i>Lycopersicon esculentum</i> (2); <i>R. graveolens</i> (1)	Lazio (2); Sicily (1)
18	1	<i>L. esculentum</i>	Lazio
19	1	<i>L. esculentum</i>	Lazio
20	2	<i>Lavandula officinalis</i>	Sicily (1); Liguria (1)
21	1	<i>L. officinalis</i>	Liguria
22	1	<i>Capsicum annum</i>	Spain
23	2	<i>Citrus maxima</i>	Mo Cay
24	3	<i>C. aurantium</i>	Sicily
25	1	<i>C. aurantium</i>	Sicily
26	1	<i>C. aurantium</i>	Sicily
27	1	<i>C. aurantium</i>	Sicily
28	1	<i>C. aurantium</i>	Sicily
29	1	<i>C. aurantium</i>	Tunisia
30	1	<i>C. aurantium</i>	Sicily
31	1	<i>C. aurantium</i>	Sicily
32	1	<i>C. aurantium</i>	Sicily
33	1	<i>Polygala myrtifolia</i>	Liguria
34	1	<i>Lavandula</i> sp.	Apulia
35	1	<i>M. communis</i>	Liguria
36	1	<i>Convolvulus mauritanicus</i>	Apulia
37	3	<i>C. mauritanicus</i>	Apulia
38	1	<i>Lycopersicon esculentum</i>	Spain
39	1	<i>L. esculentum</i>	Lazio
40	1	<i>L. esculentum</i>	Lazio
41	1	<i>L. esculentum</i>	Sicily
42	1	<i>L. esculentum</i>	Lazio
43	1	<i>C. sinensis</i> × <i>P. trifoliata</i>	California
44	2	<i>Lavandula angustifolia</i>	Piedmont
45	2	<i>Capsicum annum</i> (1); <i>M. communis</i> (1)	Calabria (1); Sicily (1)
46	1	<i>Lycopersicon esculentum</i>	Chile
47	1	<i>C. annum</i>	Calabria
48	19	<i>Citrus maxima</i> (18); <i>C. reticulata</i> (1)	Mo Cay (10); Thot Not (7); Dong Nai (2)
49	1	<i>C. sinensis</i> × <i>P. trifoliata</i>	California
50	1	<i>C. maxima</i>	Mo Cay

(continued in next column)

^a Numbers within parentheses indicate isolates sharing the same MLG from each host and geographic locality. Sicily, Apulia, Sardinia, Lazio, Liguria, Piedmont, Calabria, and Sardinia are Italian regions and Mo Cay, Dong Nai, Thot Not, Binh Duong, and Cao Phong are Vietnamese provinces.

^b Number of isolates.

MLG52 was observed most frequently, because it was shared in 38 isolates, which were all recovered from *Citrus* spp. (Table 2). In all, 6 of these isolates were collected from three different districts in Vietnam, 1 from the Philippines, and 31 from three Italian regions (27 in Sicily, 3 in Calabria, and 1 in Apulia). Interestingly, the second most numerous MLG (MLG48) was also shared among *Citrus* strains ($n = 19$), although most of them were obtained from a single host species (*Citrus maxima*), with a single isolate sampled from *C. reticulata*. All isolates with this MLG were recovered from southern Vietnam. MLG71 and MLG66 were represented by 12 and 11 isolates, respectively, and all isolates of these genotypes were recovered from the same geographic region (Sardinia, Italy) and host

TABLE 2. (continued from preceding column)

MLG	N ^b	Host	Geographic origin
51	1	<i>C. maxima</i>	Mo Cay
52	38	<i>C. sinensis</i> × <i>P. trifoliata</i> (1); <i>C. aurantifolia</i> (1); <i>C. aurantium</i> (23); <i>C. jambhiri</i> (1); <i>C. maxima</i> (4); <i>C. medica</i> (8)	Sicily (27); Calabria (3); Binh Duong (2); Cao Phong (2); Dong Nai (2); Apulia (1); Philippines (1)
53	1	<i>C. aurantium</i>	Sicily
54	1	<i>C. maxima</i>	Dong Nai
55	2	<i>C. maxima</i>	Dong Nai
56	1	<i>C. sinensis</i> × <i>P. trifoliata</i>	California
57	1	<i>M. communis</i>	Sardinia
58	1	<i>C. aurantium</i>	Syria
59	1	<i>C. aurantium</i>	Apulia
60	1	<i>C. aurantium</i>	Apulia
61	1	<i>C. aurantium</i>	Sicily
62	6	<i>Lavandula officinalis</i> (4); <i>P. myrtifolia</i> (2)	Sicily (4); Liguria (2)
63	1	<i>L. officinalis</i>	Sicily
64	2	<i>C. aurantium</i>	Sicily
65	6	<i>Hebe</i> × <i>Veronica</i> <i>buxifolia</i> (2); <i>L. officinalis</i> (4)	Sicily (6)
66	11	<i>M. communis</i>	Sardinia
67	1	<i>M. communis</i>	Sardinia
68	1	<i>M. communis</i>	Sardinia
69	3	<i>M. communis</i>	Sardinia
70	1	<i>M. communis</i>	Sardinia
71	12	<i>M. communis</i>	Sardinia
72	1	<i>M. communis</i>	Sardinia
73	1	<i>Rosmarinus officinalis</i>	Sicily
74	2	<i>Chamaleucium</i> <i>uncinatum</i> (1); <i>L. officinalis</i> (1)	Sicily
75	5	<i>Dodonaea purpurea</i>	Sicily
76	1	<i>N. tabacum</i>	Kentucky
77	3	<i>N. tabacum</i>	Virginia
78	1	<i>N. tabacum</i>	Australia
79	1	<i>N. tabacum</i>	Australia
80	1	<i>N. tabacum</i>	Australia
81	1	<i>N. tabacum</i>	Australia
82	1	<i>N. tabacum</i>	Australia
83	1	<i>N. tabacum</i>	Virginia
84	1	<i>N. tabacum</i>	Virginia
85	2	<i>N. tabacum</i>	Virginia
86	1	<i>Rosmarinus</i> sp.	Sicily
87	1	<i>N. tabacum</i>	Kentucky
88	1	<i>N. tabacum</i>	Georgia
89	1	<i>Citrus</i> sp.	Trinidad and Tobago
90	1	<i>N. tabacum</i>	North Carolina
91	1	<i>N. tabacum</i>	Georgia
92	1	<i>N. tabacum</i>	Greece
93	1	<i>N. tabacum</i>	Virginia
94	10	<i>Correa reflexa</i>	Sicily
95	1	<i>C. reflexa</i>	Sicily
96	2	<i>Rosmarinus</i> sp.	Sicily
97	1	<i>Citrus aurantium</i>	Ham Yen
98	1	<i>C. aurantium</i>	Ham Yen
99	2	<i>C. aurantium</i> (1); <i>C. reticulata</i> (1)	Ham Yen (1); Cao Phong (1)

(*Myrtus* spp.). Similarly, MLG94 was identified in 10 isolates from the same region (Sicily, southern Italy) and host (*Correa reflexa*).

The great majority of MLG were associated with isolates from a single host genus. Exceptions were observed in MLG17 (*Ruta* and *Lycopersicon*), MLG45 (*Myrtus* and *Capsicum*), MLG62 (*Polygala* and *Lavandula*), MLG65 (*Hebe* and *Lavandula*), and ML74 (*Chamaeleucium* and *Lavandula*).

Fifteen isolates showed unexpected ploidy; that is, more than two alleles were detected in one or more SSR loci. For 14 of the isolates, triploidy was identified in one of five loci (P15, P788, P1129, P1509, and P2039), while tetraploidy was observed for locus P17 in isolate Ph168; this isolate also was triploid for loci P15 and P1129. All isolates were the only representatives in their MLG (MLG 1, 8, 9, 26, 29, 31, 32, 33, 40, 51, 53, 70, 88, and 92) with the exception of MGL44, which had two isolates collected from the same host and location.

Population genetic analysis. The 231 isolates sampled globally were grouped into 14 populations according to the host genus sampled or into 15 populations by geographic origin to determine whether populations were differentiated by host or geographic origin (Table 3). Analyses revealed a prevalence of clonal reproduction in *P. nicotianae*, with positive F_{IS} values ranging from 0.260 to 1 (Table 3). This was particularly evident for isolates collected from *Citrus* spp. ($F_{IS} = 0.721$), which represented the most abundant group. MSN constructed using Bruvo's distance (Bruvo et al. 2004) revealed that isolates from *Citrus* constituted a distinct group regardless of their geographic origin (Figs. 1 and 2). Two subgroups, mainly represented by MLG48 (19 isolates) and MLG52 (38 isolates), were identified within the *Citrus* population. The first subgroup contained exclusively isolates from Vietnam, while the latter subgroup included isolates from three different continents (Europe, North America, and Asia) (Figs. 1 and 2). The only *Citrus* isolate outside of these groups was IMI268688 (MLG 89), which grouped close to the tobacco isolates from Kentucky and Virginia (MLG 76, 77, 83, 84, and 85). The A1 mating type occurred at high frequency (82 of 90) in *Citrus* spp. and the A2 isolates were mainly found in Vietnam (6 of 8). The PCA, based on Lynch's distance, further confirmed that isolates from *Citrus* spp. constituted a separate cluster, independent of geographic provenance (Fig. 3). In agreement between MSN and PCA, statistical analyses revealed a higher level of uniformity ($G' = 0.049$, $E_5 = 0.4$, $F_{IS} = 0.72$) in the population from *Citrus* spp. (Table 3). Furthermore, F_{ST} values showed a relevant genetic divergence among *Citrus* isolates and other populations (Table 4).

A different population structure was observed for isolates from *Nicotiana* spp. These isolates clustered into three different groups (Fig. 1). Two of these groups were clearly associated with their geographic origin because they contained only isolates from Virginia and Kentucky (MLG 76, 77, 83, 84, and 85, all of which are A2 mating type) or Australia (MLG 78, 79, 80, 81, and 82; all except MLG82 were A1 mating type), respectively (Fig. 2). On the other hand, a third group (MLG 87, 88, 90, 91, 92, and 93; both A1 and A2 mating types) included isolates from Georgia, North Carolina, Kentucky, Virginia, and Greece (Fig. 2). According to the G' values observed, a significant lower genetic uniformity was determined in the *Nicotiana* population (0.714) as compared with the *Citrus* population. Furthermore, a higher level of variability was found based on E_5 (0.896) and F_{IS} (0.26) coefficients (Table 3).

Isolates from *Lycopersicon* spp. were predominantly A1 and were found to be more distantly related, with all but one (MLG 19) distributed in the central part of the network connected by thin gray lines, indicating a high genetic distance among isolates (Fig. 1). This is reflected by this population having the highest values of G' and E_5 (0.833 and 0.952, respectively) among those analyzed (Table 3). Furthermore, it had a significantly lower F_{IS} (0.511) as compared with the *Citrus* population, indicating a lower level of inbreeding.

A high genetic diversity was revealed for isolates from some of the ornamental species. For example, the *Lavandula* population

(8 A1 and 14 A2 isolates) was separated into several genetically distant groups, despite the fact that all isolates were recovered from Italy (Figs. 1 and 2; Table 1). Similarly, isolates from genera *Convolvulus* and *Myrtus* (both with all A2 isolates) clustered into three and two genetically distant groups, respectively, although they were recovered from single nurseries located in Apulia and Sardinia, respectively. Statistical parameters confirmed a high level of genetic variation (Table 3). Genetically uniform populations were associated with isolates recovered from the ornamental species of *Correa* and *Ruta* (Fig. 1; Tables 3 and 4). However, this result was likely to be affected by the limited number of isolates examined (11 and 10, respectively) and the fact that all isolates were recovered from the same nursery. Isolates from other plant genera were represented by a limited number of samples, preventing an accurate statistical analyses (Table 1). These isolates mainly scattered in the central part of the MSN (Fig. 1).

DISCUSSION

In the present study, a set of nine polymorphic microsatellite markers (Biasi et al. 2015) was used to analyze the genetic diversity within 231 isolates of *P. nicotianae* grouped into distinct populations representing a wide host range and several geographic locations. The selected markers proved to be very consistent, with a percentage of amplification close to 100%; only 2 of 2,079 isolate-marker combinations did not produce an amplicon. In contrast, considerably higher levels of failures in sizing amplicons at SSR loci have been reported for other *Phytophthora* spp. For example, two of the nine loci selected for the assessment of the variability in *P. infestans* failed in producing positive amplification with 30% of the isolates (Brurberg et al. 2011). Our analyses further revealed a high level of polymorphism, with a total number of 4 (locus P2039) and 23 (locus P1509) alleles detected per locus. Unexpectedly, the number of bases in the repeat unit was not inversely correlated with the number of genotypes observed (Shinde et al. 2003). For example, the 4-bp motif analyzed in the present study (locus P5) yielded 16 different alleles and was more polymorphic than four other loci with a 3-bp motif. Dinucleotides were the markers with the highest number of alleles. On the whole, our data confirm that the selected markers were appropriate for the accurate characterization of populations of *P. nicotianae*. Furthermore, labeling primers for each locus with different fluorophores (HEX and FAM) significantly increased the throughput of the method and enabled fast, accurate, and cost-effective genotyping (Biasi et al. 2015).

The genotyping of 231 isolates allowed identification of 99 MLG, with a significant number of isolates exhibiting a unique genetic fingerprint. Interestingly, an unexpected genetic framework for a diploid organism like *P. nicotianae* was revealed by the detection of 16 examples of triploidy and 1 of tetraploidy. The presence of polyploidy in *P. nicotianae* was previously observed by SNP analyses

TABLE 3. Origin, diversity statistics, and mating type frequency in *Phytophthora nicotianae* populations investigated in the present study^a

Population	N	MLG	G'	E_5	F_{IS}	Mating type		
						A1	A2	ND
<i>Convolvulus</i>	9	5	0.429	0.859	0.464	0	9	0
<i>Lycopersicon</i>	10	9	0.833	0.952	0.511	1	7	2
<i>Ruta</i>	10	3	0.152	0.576	0.858	10	0	0
<i>Correa</i>	11	2	0.109	0.556	1	8	2	1
<i>Nicotiana</i>	20	17	0.714	0.896	0.260	7	12	1
<i>Lavandula</i>	23	12	0.365	0.832	0.369	8	14	1
<i>Myrtus</i>	37	13	0.129	0.614	0.624	0	37	0
<i>Citrus</i>	90	29	0.049	0.400	0.721	82	8	0

^a N = number of isolates, MLG = number of multilocus genotypes identified in each population, G' = normalized Stoddart and Taylor's index, E_5 = evenness, F_{IS} = inbreeding coefficient, and ND = not determined.

(Mammella et al. 2013). It is possible that this variation in ploidy was generated through sexual recombination because non-Mendelian recombination patterns in progeny have been reported (Förster and Coffey 1990). The characterization of *P. cinnamomi* using micro-satellite markers also revealed a large proportion of non-Mendelian inheritance (Dobrowolski et al. 2002). This aberrant nuclear genetic condition was best explained by nondisjunction at meiosis in the trisomic parents, because aneuploid progeny were also identified. Ivors et al. (2006) observed conditions of trisomy for some isolates of *P. ramorum*. Initially, they hypothesized that multiple alleles resulted from horizontal gene transfer occurring after introgression of genes from other *Phytophthora* spp.; however, the high homology of flanking regions suggested that trisomy was due to gene duplication. Examples of different levels of ploidy for SSR regions were also documented for *P. infestans* (Brurberg et al. 2011; Goss et al. 2014; Lees et al. 2006; van der Lee et al. 2001). In the present study, Bruvo's distances were utilized to construct MSN and evaluated for 14 or 15 populations identified according to the genera of the plant hosts or

geographic origin. The use of this function was important because it enabled calculation of genetic distances without considering ploidy of a locus and, thus, permitted interpretation of heterogeneous data (Cooke et al. 2012).

Analyses revealed significant differences in the structure of populations associated with different host species, although a prevalence of clonality was observed for all populations. Furthermore, the most abundant MLG detected were associated with a single host genus, suggesting a strong association among *P. nicotianae* genotypes and select plant hosts. According to both MSN and PCA analyses, *Citrus* isolates clustered together regardless of their geographic origin. In agreement with this clustering, all indexes used for statistical analyses confirmed a higher genetic uniformity in this group. This *Citrus* population was characterized by a low evenness (E_s) value and by a heterozygote deficiency, especially when compared with other populations. Furthermore, the index of association (F_{ST}) confirmed a clear separation of the *Citrus* population that maintained its own genotypic uniqueness. These results are

Fig. 1. Minimum spanning network of *Phytophthora nicotianae* isolates describing the relationship among multilocus genotypes (MLG) detected based on their host genus. Numbers along nodes and genus of origin correspond to MLG. Nodes represent unique MLG and are scaled proportionally to the number of individuals sharing the same MLG. Thickness and black intensity of lines connecting nodes are inversely proportional to Bruvo's distance (the larger the distance between two nodes, the thinner and less black intense is the line that connects them).

particularly relevant considering that *Citrus* isolates were recovered from several different countries and represented the most abundant population analyzed in the present study. Our results are in agreement with previous reports based on the analysis of mitochondrial and nuclear SNP (Mammella et al. 2011, 2013). In this study, 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. The existence of a marked preference among certain *P. nicotianae* genotypes for *Citrus* spp. is in agreement with the great degree of virulence specialization seen in *P. nicotianae* to a given host (Erwin and Ribeiro 1996). For instance, an isolate from okra was not pathogenic to *Citrus* spp. and vice versa (Erwin 1964). 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 (Bonnet et al. 1978; Matheron and Matejka 1990). Furthermore, a mating type (A1) was largely prevalent on *Citrus*, although

six and two isolates with A2 mating type were recovered in Vietnam and Italy, respectively. The absence of a geographic clustering and the concurrent existence of a significant correlation with the host could be indicative of extensive migration of the isolates via plant material or host adaptation. It can be speculated 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 seems to be played by the globalization of the nursery trade, with particular emphasis on container-grown and bare root plants.

The identification of two subgroups within the *Citrus* population may also be related to the considerations discussed above. Indeed, one of the subgroups was represented by isolates exclusively recovered from Vietnam, mainly from *Citrus maxima*, while the other subgroup comprised isolates from several different countries and *Citrus* spp. Considering that *C. maxima* (commonly named “pommelo”) is a native species of Southeast Asia and that the introduction of plant material of this species into Vietnam from other countries is very limited, a specific coevolution of *P. nicotianae* with *Citrus* in Vietnam

Fig. 2. Minimum spanning network of *Phytophthora nicotianae* isolates describing the relationship among multilocus genotypes (MLG) detected based on their country of origin. Numbers along nodes and country of origin correspond to MLG. Nodes represent unique MLG and are scaled proportionally to the number of individuals sharing the same MLG. Thickness and black intensity of lines connecting nodes are inversely proportional to Bruvo's distance (the larger the distance between two nodes, the thinner and less black intense is the line that connects them).

can be hypothesized. An understanding of this phenomenon could allow unraveling of the molecular basis of interactions between host and *P. nicotianae*, developing a picture of how life history traits of both host and pathogen interaction shape evolutionary trajectories (Burdon and Thrall 2009).

A marked association among molecular groups and host of recovery was also revealed for isolates from *Nicotiana*, although a different population structure was observed. Indeed, isolates clustered into three genetically distinct groups, generally corresponding to different geographic areas and, in agreement with the presence of both mating types (7 A1 and 12 A2 isolates), a significantly lower genetic uniformity was revealed. Although a preferential virulence association between *Nicotiana* spp. and *P. nicotianae* has been reported (Colas et al. 1998), the commercial production system utilized for this crop is likely to have played a role in determining its geographical structuring. In fact, tobacco is propagated by seed, which does not contribute to the spread of the pathogen, and plantlets are very rarely transplanted into areas different from those in which they have been produced. In this situation, different pathogen lineages can evolve into geographically separated areas on their own host because migration does not play a factor. Furthermore, the tobacco population may have been shaped by cultivar resistance and other control means. According to Sullivan and coworkers (2005), the continuous planting of varieties with *Php* and *Phl* genes caused a shift in pathogen population from race 0 to race 1. On the other hand, prolonged cultivation of varieties with partial resistance to all races may cause an increased level of pathogen aggressiveness (Sullivan et al. 2005). Similarly, cultural practices, crop rotation, and chemicals commonly used in integrated control approaches have an impact on pathogen fitness and play a role in modeling populations (Bittner and Mila 2016).

A similar situation may occur for isolates from *Lycopersicon*. Indeed, isolates characterized in the present study were found to be

genetically related, although high genetic variation was revealed within this population. Because tomato is propagated via seed and transplants produced in nurseries are generally utilized in the same area of production, the geographic isolation of lineages and their separate coevolution with the host can be hypothesized.

High levels of genetic variation and low inbreeding coefficients were observed for three of the analyzed populations from potted ornamental species in nurseries (*Lavandula*, *Convolvulus*, and *Myrtus* spp.). Although statistical analyses did not support a higher variability when compared with *Nicotiana* and *Lycopersicon* populations, it must be highlighted that isolates from these ornamental species were recovered from limited areas represented by few nurseries in Italy (*Lavandula*) or even single nurseries (*Convolvulus* and *Myrtus*). The prevalence or the exclusive presence of the A2 mating type in these populations deserves further investigation. Furthermore, the high genetic uniformity revealed in two other ornamental genera (*Correa* and *Ruta*) does not seem remarkable because of the limited number of isolates, all recovered from the same nurseries. The production system of nursery ornamentals is different from that of *Citrus* plants. Usually, *Citrus* plants produced in nurseries are planted in commercial orchards. In contrast, potted ornamental plants are produced in large production nurseries and then distributed to smaller ones which, in turn, sell them to retail garden centers or shops. As a consequence, new *Phytophthora* genotypes are rapidly spread on a large scale. Several components may determine the genetic variability of a pathogen population, including the genetic variability of the original population or the introduction of a few new genotypes from other populations (founder effect), mutations, selection pressure by the host, and sexual recombination. In ornamental nurseries, the cultivation of many plant species and varieties from different geographic areas may favor the introduction of exotic new *Phytophthora* spp., as demonstrated for *P. ramorum* (Goss et al. 2009; Ivors et al. 2006; Vercauteren et al. 2011), and the intermixing of complementary mating types of heterothallic species, thus increasing the

Fig. 3. Principal coordinate analysis based on Lynch distances of *Phytophthora nicotianae* isolates analyzed in the present study according to the host genus of origin.

TABLE 4. Genetic differentiation (pairwise F_{ST}) estimates of simple-sequence repeats for *Phytophthora nicotianae* isolates grouped by host genus

Host	<i>Citrus</i>	<i>Convolvulus</i>	<i>Correa</i>	<i>Lavandula</i>	<i>Lycopersicon</i>	<i>Myrtus</i>	<i>Nicotiana</i>	<i>Ruta</i>
<i>Citrus</i>	...	...	...	...	...	...	...	...
<i>Convolvulus</i>	0.138	...	...	...	...	...	...	...
<i>Correa</i>	0.073	0.25	...	...	...	...	...	...
<i>Lavandula</i>	0.090	0.023	0.059	...	...	...	...	...
<i>Lycopersicon</i>	0.116	0.037	0.058	0.037	...	...	...	...
<i>Myrtus</i>	0.085	0.053	0.084	0.041	0.067	...	...	...
<i>Nicotiana</i>	0.094	0.051	0.066	0.042	0.051	0.059	...	...
<i>Ruta</i>	0.125	0.060	0.087	0.048	0.043	0.087	0.073	...

opportunity of sexual recombination. Furthermore, interspecific hybrids of *P. nicotianae* are well documented (Faedda et al. 2013). Natural hybrids of *P. nicotianae* and *P. cactorum* were first reported in The Netherlands and they are now considered well established on several hosts all over the world (Faedda et al. 2013; Man in't Veld et al. 1998; Szigethy et al. 2013).

In agreement with the above considerations, the few MLG shared by isolates from different plant hosts were detected in isolates from the ornamental species (*Polygala* and *Lavandula*, *Hebe* and *Lavandula*, and *Chamaeleucium* and *Lavandula*) or in isolates from ornamental and horticultural species (*Ruta* and *Lycopersicon* or *Myrtus* and *Capsicum*). Furthermore, considering the total number of analyzed isolates, a conspicuously higher number of MLG was found in nurseries as compared with *Citrus* and tobacco.

In conclusion, the present study provides an important advancement in our understanding of the population structure and evolutionary history of *P. nicotianae*. Although new analyses focusing on specific hosts and on a larger number of isolates collected with a hierarchical approach are necessary to accurately define *P. nicotianae* genetic groups, a strong association among pathogen genotypes and host species is clearly supported, suggesting that the speciation process of this pathogen may be driven by host selection pressure and type of propagation. In general, it seems that host specialization of a *P. nicotianae* population occurs more frequently in intensive cropping systems. This finding, in agreement with the data available on the host-specific virulence of *P. nicotianae* isolates (Erwin and Ribeiro 1996; Taylor et al. 2012) and with previous molecular data (Mammella et al. 2011, 2013), may suggest the need for reconsideration of the concept of species and the consequent identification of specific pathotypes or subspecies within *P. nicotianae* sensu lato. Genome-wide analyses currently in progress on several *P. nicotianae* strains of diverse origins may greatly contribute to enabling a comparative analysis of genes that determine host range (Kamoun et al. 2015). An analysis of the corresponding pathogenicity and virulence gene diversity may provide additional information necessary to draw firmer conclusions about factors involved in host specialization.

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