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Microsatellite analysis of the population structure in *Phytophthora nicotianae*

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Contents

Chapter 1

General Introduction

1.1 Introduction to Oomycetes and Phytophthora

The Oomycetes are fungus-like heterotrophs that are saprophytes or parasites of diverse hosts in marine, freshwater and terrestrial environments, although they are best known as devastating pathogens of plants (Johnson et al.,2002). They fall within the kingdom Stramenopila (= Chromista), which also includes golden brown algae, diatoms, and brown algae such as kelp (Baldauf et al., 2000). Oomycetes produce complex branching, tip-growing, hyphal systems and have modes of nutrition and ecological roles similar to the true fungi (Richards et al.,2006).

Phylogenetics has clearly demonstrated that Oomycetes are not Fungi but instead they are close relatives of heterokont algae; they have lost their plastids and have adopted a fungal-like lifestyle, especially for absorbing nutrients and the invasion of another organism for feeding (Lamour and Kamoun, 2008 libro). Some of the biochemical and cytological characteristics that distinguish oomycetes from fungi include having 1) cellulose as the principle component of the hyphal walls; 2) diploid thalli with meiosis preceding gamete formation, 3) vacuoles with phosphorylated β-(1,3) –mycolaminarin glucans and 4) mitochondria with tubular cristae (Beakes, 1989).

Despite their large diversity, more than 60% of Oomycetes are plant pathogens (Thines and Kamoun, 2010). However, the best-studied oomycetes are those of the genus *Phytophthora* (Kamoun, 2006). *Phytophthora* literally means "plant destroyer", a name coined in the 19th century by Anton de Bary when he investigated the causal agent of potato late blight, *Phytophthora infestans*, during the Great Irish Famine.

The genus *Phytophthora* contains a large diversity of devastating plant pathogens which occur in both natural and agricultural settings (Judelson and Blanco, 2005) and many species are able to infect a broad range of hosts. (Blair et al., 2008). In the last decade, the

number of validly described *Phytophthora* species reached about 116 species, 15 of which await valid publication, although for sure, this is an underrepresentation of the numer of species existing in nature (Kroon et al., 2012). This number of species is increasing yearly due to the availability of more sophisticated molecular tools for species delimitation and for the discovery of novel *Phytophthora* species in natural and agricultural settings.

The most important criteria used to distinguish species in the pre-molecular era included host range, sporangium morphology, presence or absence of chlamydospores, isozymes and hyphal swellings, optimal growth temperature, colony morphology, and oogonium and antheridium morphology (Stamps et al., 1990; Waterhouse, 1963; Oudemans and Coffey, 1991). More lately several DNA-based methods has been explored for *Phytophthora*, especially for clarifying the relationship among the species: ITS region (Cooke et al., 2000; Forster et al., 2000), cytochrome oxidase I and II of the mitochondrion (Martin and Tooley, 2003), or by using multiple loci from both the nuclear and mitochondrial genomes (Ivors et al., 2004; Donahoo et al., 2006). Using genomic sequence data from *P. ramorum* and *P. sojae*, and a large numbers of expressed sequence tags (EST) from *P. infestans*, *P. nicotianae* and others, Blair et al.(2008) was able to identify seven different molecular markers that can be phylogenetically informative when examining 234 isolates of 82 different species of *Phytophthora*. The analysis of the seven loci supported the division of the genus into 10 well-supported clades separated by short internal branches. A further analysis will complement the analysis effected by Blair et al (2008) with new 4 mitochondrial loci, in order to better examine 117 taxonomic entities of *Phytophthora* (Martin unpublished).

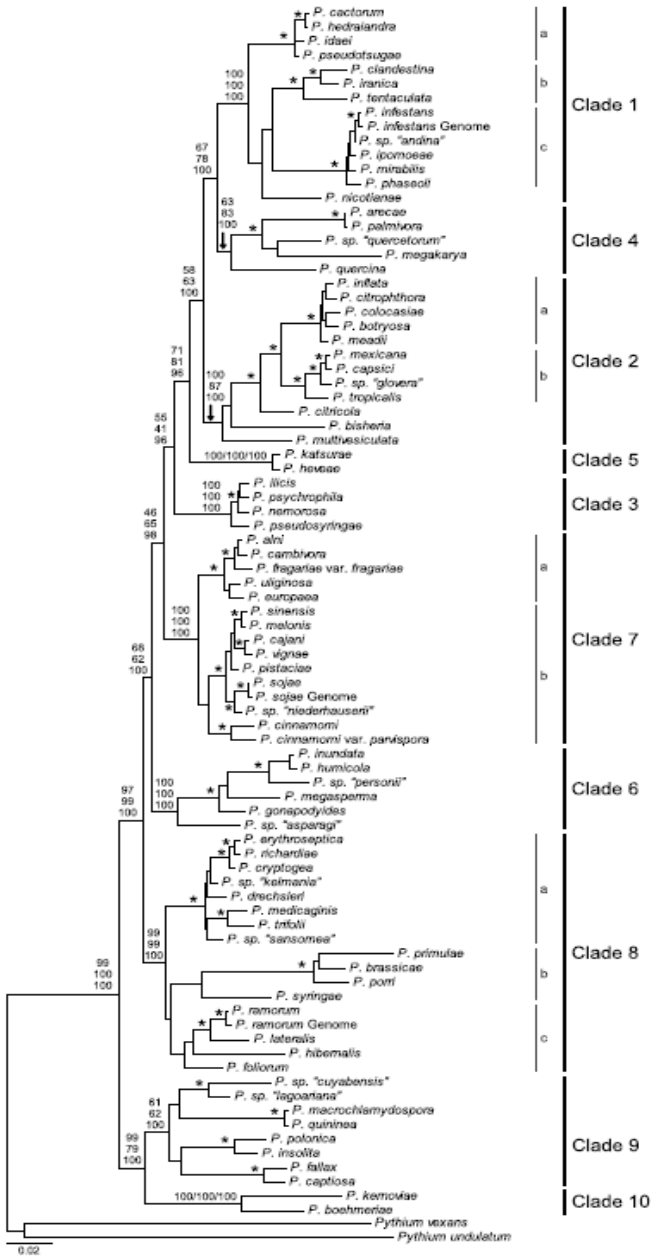

Figure 1: Phylogeny of the genus *Phytophthora* using seven different nuclear loci (Blair et al., 2008). Maximum likelihood branch lengths as shown. Number on nodes represent bootstrap support values for maximum likelihood (top) and maximum parsimony (middle), and Bayesian posterior probabilities presented as percentages (bottom), while nodes marked with an asterisk have been supported in all three analyses. Scale bar indicates the number of substitutions per site.

1.2 Biology and genetics of Phytophthora nicotianae

Phytophthora nicotianae van Breda de Haan (=*Phytophthora parasitica* Dastur) (1896) produces the typical structures characteristic of the genus. The pathogen is a heterothallic species and forms aplerotic oospores from amphigynous antheridia and septate oogonia. The mycelium is composed of long, branching, filamentous hyphae that are mostly coenocytic except in the old cultures, where septa can sometimes be seen. Asexual reproduction is characterized of the formation of ovoid, pear-shaped sporangia that produce kidney-shaped and biflagellate zoospores that are able to move in water for short distances. The pathogen can also produce chlamydospores, which are asexual and thick-walled structured that enable survival,in the soil for months to years. They are mostly dark and can be formed intercalary or terminal at the tip of the hyphae. These structures are able to germinate and infect plants when environmental conditions are favorable (Erwin and Ribeiro, 1996). *Phytophthora nicotianae* requires two mating types (A1 and A2) to produce an oospore. It is bisexual and self-incompatible (Erwin and Ribeiro, 1996), that is, the pathogen can be either the maternal or paternal parent during sexual crossing. The oospores are spherical and thick-walled (Ø 20 μm) and originate from the union of the two gametangia, an amphigynous antheridia (paternal) and a spherical oogonia (maternal). The haploid nuclei of the antheridia passes into the maternal gametangia and fuses with the haploid nuclei of oogonia to generate the diploid oospore.

1.3 Phytophthora nicotianae **diseases**

Phytophthora nicotianae 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). Cline et al. (2008) reported that the host range of *P. nicotianae* included 255 plant genera in 90 families.

Among the several plant species this pathogen can infect it is worth mentioning *Nicotianae* and *Citrus*spp., since *P. nicotianae* is causal agent of the Black Shank and the citrus root rot and gummosis, respectively (the latter is also caused by *P. citrophthora*) (Cacciola and Magnano di San Lio, 2008). Apart from *N. tabacum* and *Citrus* species, *P. nicotianae* is responsible for heavy losses on a number of other economically important species, such as fruit trees and herbaceous hosts (Erwin and Ribeiro, 1996). Recent surveys have revealed that this species is one of the most common pathogen 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 and White, 2001; Pane et al., 2005; Moralejo et al., 2009).

1.3.1 Black shank of tobacco

Black shank is among the most destructive and widespread of all tobacco diseases all over the world. It was described for the first time in Indonesia in 1896, and reported in North Carolina in Forsyth County by Tucker in 1931. It is a warm-weather disease, favored by temperatures ranging from 29 to 32 °C (Erwin and Ribeiro, 1996). This disease is characterized by a rapid yellowing and wilting followed by rapid death of the entire plant, with possible presence of lesions that can extend up the stalk or shank of the plant causing it to turn black. By splitting the stalk, it's usually revealed the blackened pith separated into discrete disks, although this feature could be associated to other factors. Roots and crowns are usually decayed.

Black shank affects tobacco plants at all growth stages. Disease begins on young seedlings or transplants once soil temperatures rise above 20 °C and often diseased plants are associated with wet soil, and losses may reach 100% in susceptible cultivars in years favorable for disease development (Shew and Lucas, 1991). In young and succulent seedlings,

disease develops very quickly and symptoms begin as wilting and yellowing of the leaves and development of stem lesions. Root tips and wounds represent the primary sites of infection by the pathogen: infected roots appear water-soaked, then rapidly become necrotic and the lesion expansion progresses rapidly into larger roots until all the root system is destroyed.

An effective disease management program for black shank requires an integration of cultural and chemical practices and planting resistant cultivars. Cultural practices include planting on raised beds in order to reduce the availability of free water so that the zoospore movement could be decreased, crop rotation for reducing the possibility of inoculum production on susceptible hosts of the pathogen, and stalk and root destruction after harvest to suppress inoculum buildup (Sullivan et al., 2005).

The most widely used method of control is planting of resistant varieties,, although continuous use of a resistance gene may cause the selection of more aggressive isolates of the pathogen. Four physiological races of *P. nicotianae* (0, 1, 2 and 3) have been identified, but race 0 is predominant. Race 0 (not pathogenic to *Nicotianae plumbaginifolia*) and race 1 (pathogenic to *N. plumbaginifolia*) occur in most tobacco growing areas of the world while race 2 is present in South Africa and race 3 occurs in Connecticut (Erwin and Ribeiro, 1996). Several varieties are available with different levels of resistance to black shank. Two single-gene sources of resistance have been incorporated into tobacco cultivars and both provide complete resistance to race 0 but no resistance to race of 1 of the pathogen. Nevertheless, this massive deployment of this resistance gene led to an increase in the prevalence of race 1, which is the dominant race of *P. nicotianae* in several areas. Because of this problem, integrated pest management approaches involving the use of metalaxyl or mefenoxam and resistant crop rotation scheme are suggested to reduce the development of the disease by the different races of black shank.. The use of soil fumigants such as chloropicrin may also reduce

pathogen population but due to cost may not be suitable for all cropping systems (Lucas, 1975).

1.3.2 Root rot of citrus

The most serious fungal diseases caused by *Phytophthora* spp. in citrus are root rot and gummosis (Graham and Timmer, 2000). In nurseries, gummosis can lead to the rapid death of young citrus trees, whereas on adult trees the disease can cause a chronic infection. In a mature tree, symptoms show as leaf chlorosis, philloptosis, dieback of twigs, small and poor colored fruit, offspring fruit production, twig dieback and withering of leaves during periods of drought if the infection affects more than 50% of the circumference of the trunk (Magnano and Cacciola, 2008). Root rot can be especially severe on susceptible rootstocks in infested nursery soil. The pathogen infects the root cortex, which turns soft and separates from the stele. *Phytophthora nicotianae* and *P. citrophthora* are the most common species causing disease in citrus production areas worldwide. *Phytophthora nicotianae* grows at higher temperatures (28-30°C with a maximum of 35-38°C) than *P. citrophthora* (25°C with a maximum of 30°C) and attacks mainly the rootlets, while *P. citrophothora* is the main causal agent of gummosis and brown rot of fruits (Cacciola and Magnano, 2008). The management of disease has mainly relied on the deployment of an integrated approach, including the selection and use of resistant rootstocks, grafting, and other practices useful for keeping the planting stock clean such as soil preparation, fertilizing and soil management and chemical control by fungicides (Cacciola and Magnano, 2008). Several biocontrol agents also have been tested for the management of this disease , in particular the ability of introduced antagonists to estabilish and colonize the rhizosphere soil can represent an important factor for the biological control of the disease (Graham, 2004) and can be coupled with the use of rootstocks resistant to *Phytophthora*, although the latter could besusceptible to other diseases or are horticulturally unsatisfactory (Ferguson et al., 1990) and therefore may have limited

utility for the growers. Sour orange is very resistant to *Phytophthora*, while other moderately resistant rootstocks are Cleopatra mandarin, Troyer citrange and Volkameriana.

Control by chemicals is attained with effective systemic fungicides like metalaxyl and Al-ethyl-phosphyte (or fosetyl-Al) (Timmer and Castle, 1985), although nowadays mefenoxam is more used instead of metalaxyl because it is effective at a lower dosage. For a preventive approach, the derivates of phosphorous acid are recommended since they are translocated up and down in the plant, while mefenoxam only moves upwards and must applied to the ground or to the bark to be effective. Both groups of fungicides will remain active in the plant tissue for 3-4 months (Matheron and Matejka, 1988).

1.4 Molecular markers for population studies

In the last two decades, several key advances in molecular genetics have greatly increased the impact of population genetics such as 1) the development of polymerase chain reaction (PCR); 2) the application of widely conserved PCR primers; and 3) the advent of routine DNA sequencing in biology laboratories (Sunnucks, 2000). Molecular marker technologies have been revolutionized the plant pathogen genomic analysis and have been extensively employed in many fields of molecular plant pathology. They offer the possibility of fast, accurate identification and early detection of plant pathogen (Bridge et al., 2003) and can answer many complex questions concerning molecular biology, such as the sources of inoculum and the changes in their population structure and the population dynamics of the disease they cause (Hernandez-Delgado, 2009). Diversity among organisms is a result of mutations resulting from substitution of single nucleotides, insertion or deletion of DNA fragments, duplication or inversion of DNA fragments and can be neutral or functional (Marsjan and Oldenbruek, 2007). It is important to mention that phylogenetic analysis at the

intraspecific level can be influenced by several phenomena such as recombination, parallel mutation and recurrent mutation that do not follow the typical phylogenetic bifurcating evolution as for the majority of interspecific analysis. As a consequence they require a multifurcate network to explain relationships among all the individuals (Bandelt et al., 1999).

1.4.1 RFLP

Restriction fragment length polymorphisms (RFLPs) is a technique wherein genomic DNA is treated with one or more restriction enzymes that cut the DNA whenever specific sequences of bases occurs, thus generating a number of fragments of the DNA of varying lengths (Panneerchelvam and Norazmi, 2003). Random changes in DNA cause one or more sites to be lost or gained, causing variation between individuals in the length of fragment that can be visualized by gel electrophoresis. RFLPs are characterized by a high heritability (Lowe et al., 2004). They are polymorphic and codominantly inherited, although they require relatively large amounts of un-degraded DNA; however, the process can be laborious and time-consuming.

1.4.2 RAPD

The standard Random Amplified Polymorphic DNA(RAPD) technology was introduced by Williams et al. (1990) and is based on arbitrarily amplifying DNA sequences during PCR without prior knowledge of the organism sequence by using a single 10 nucleotide primer and low annealing temperatures. PCR amplification with primers shorter than 10 nucleotides (DNA amplification fingerprinting (DAF)) has also been used producing more complex DNA fingerprinting profiles (Caetano-Annoles et al., 1991). At an appropriate

annealing temperature during the thermal cycle, if an oligoprimer of a random sequence binds to complementary sequences in the template genomic DNA that are close enough a discrete DNA band is produced. RAPD analysis can be used for a wide range of applications like genetic mapping, developing genetic markers linked to a trait in question, population and evolutionary genetics and plant and animal breeding. Although the method is fast and easy, the issue of reproducibility still remains unsolved since the RAPD reaction is sensitive to DNA concentration and reaction conditions (Bardakci, 2001; Welsh and McClelland, 1994). A major drawback of RAPD markers is that they are dominant, and this is an important issue for population genetic studies as this does not allow for the differentiation of a homozygote from heterozygote genotype in diploid organisms (McDonald et al., 1997).

1.4.3 AFLP

Amplified fragment length polymorphisms (AFLP) are PCR-based markers for the rapid screening of genetic diversity. This method is able to generate hundreds of highly replicable markers from the DNA of any organism, thereby allowing high-resolution genotyping. The AFLP procedure consists of two amplification steps on a subset of restriction enzyme-digested DNA fragments after ligation with specific adapters to the cohesive ends produced by the restriction enzyme (Vos et al., 1995). AFLP markers offer the ability to study the genotype of the organisms without the knowledge of the whole genome and they require minimal amounts of DNA. Furthermore, AFLP amplifications are performed under conditions of high stringency thus eliminating the problem of artifacts that is seen routinely in RAPD-PCR. The method is not time and money consuming and AFLP markers segregate in a Mendelian fashion, however these markers suffer from their general dominant nature because of the difficulty in identifying homologous alleles, rendering this method less useful for

studies that require precise assignment of allelic states, such an heterozygosity analyses (Muller and Wolfenbarger, 1999).

1.4.4 SNP

Single nucleotide polymorphisms are single base pair positions in genomic DNA at which different bases (alleles) exist in normal individuals in some population, wherein the least frequent allele has an abundance of at least 1% or greater (Jehan and Lakhanpaul, 2006). They represent the most abundant class of markers in the genome and due to their abundance in genome, they are extremely useful for creating high-density genetic map. Even though it is theoretically possible to have four nucleotides at a particular site, in reality only two of these four possibilities have been observed at the specific sites in a population, thus SNPs are largely biallelic in nature. This feature makes them less informative per locus examined than multiallelic markers such as RFLPs and microsatellites (Xing and Jin, 1999), nevertheless SNPs are very abundant and highly scattered all over the genome, thus it is possible the use of a higher number of loci (Kruglyak, 1997). SNPs are less mutable as compared to microsatellites, but they are excellent markers for studying complex genetic traits and for understanding the genomic evolution and suitable to follow in population studies.

1.4.5 Microsatellites

SSR (simple sequence repeats; also referred to as microsatellites) are genomic regions composed of tandem repeats usually ranging from 1 to 6 nucleotides found at high frequency in the nuclear genomes. A microsatellite locus typically varies in length between 5 and 40 repeats. Di-, tri- and tetranucleotide repeats are the most common choices for molecular

genetic studies, while mononucleotide repeats are less reliable because of problems with amplification (Li et al., 2002). Microsatellite repeat sequences have high-mutation rate (10^{-2}-10^{-6} mutations per locus per generation), that varies according to the length and to the nature of the motif itself. Microsatellite repeat sequences mutate frequently by slippage and proofreading errors during DNA replication that primarily change the number of repeats and thus, the length of the repeat string (Eisen, 1999). Microsatellites have become so popular because they are single locus, co-dominant markers for which many loci can be efficiently combined in the genotyping process to provide fast and inexpensive replicated sampling of the genome (Selkoe and Tonnen, 2006). Microsatellite polymorphisms derive mainly from variability in length rather than in the primary sequence and the genetic variation at many SSRs loci is characterized by high heterozygosity and the presence of multiple alleles, which in sharp contrast to unique DNA (Ellegren, 2004). Questions of paternity or clonal structure are well addressed using microsatellites with highest allelic diversity, which can provide every individual with a unique genotype "identification tag" using only a few loci (Queller et al., 1993). Similarly, the numerous alleles help to better study population structure and migration. Despite many advantages, these markers also have several challenges connected to the unclear mutation mechanism, like homoplasy. This phenomenon dampens the allelic diversity of populations: undetectable homoplasy occurs when two alleles are identical in sequence but not identical by descents, e.g. when there is a "back-mutation" to a previously existing size or when two unrelated alleles converge in sequence by changing repeat number in two different places in the sequence (Selkoe and Tonnen, 2006). Other problems are correlated to possible mutations that can occur in the primer region, thus in some individuals those alleles will fail to amplify (Paetkau and Strobeck, 1995).

1.4.6 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is one of the most popular population genetic markers and they are the most widely used in animals (Avise et al., 1987). The use of mtDNA is justify by the fact that it has a high level of variability, its clonal (maternal) inheritance, that is the whole genome behaves as a single, non recombining locus, and it's supposed nearly neutral mode of evolution. Other reasons for the adoption of mtDNA as marker of choice are correlated to the sensitivity of being amplify because it appears in multiple copies in the cell. The evolutionary rate of mtDNA has been frequently assumed to be clock-like, where in the absence of any mutations spreading through positive selection, only neutral mutations accumulate in time, so that mtDNA divergence levels should roughly reflect divergence times (Galtier et al., 2009). Other advantages of using the mtDNA markers concern the absence of interference from recombination events (Zhang and Hewitt, 2003), although they are rarely documented in fungi (Anderson et al., 2001). Mitochondrial markers were applied to assess intraspecificic variability in several *Phytophthora* species (Griffith and Shaw,1998; Martin, 2008), in *Verticillium dahliae* (Martin, 2010), and in *Ceratocystis fagacearum* (Kurdila et al., 1995) as well as in phylogeographic studies to estimate the genetic structure of populations of *Phaeosphaeria nodorum* (Sommerhalder et al., 2007) and *Phytophthora cinnamomi* (Martin and Coffey, 2012). Furthermore, intraspecific mtDNA variation in fungi has been useful for testing hypothesis on the evolutionary origins of *P. infestans* (Ristaino et al., 2001; Gomez-Alpizar et al., 2007) and for providing evidence of recombination in mitochondrial genome in fungi (Anderson et al., 2001; Saville et al., 1998).In spite of the robustness of these markers , it is recommended the use of mitochondrial markers complemented with nuclear markers, since only a small part of the evolutionary history of a species is revealed with the mtDNA (Martin et al., 2012).

1.5 Molecular techniques for population analysis applied for Phytophthora.

The advent of phylogenetic analysis has allowed for enhanced knowledge of evolutionary relationships in the genus *Phytophthora*. In fact, analysis with the ribosomal subunit (rDNA) sequences and cox2 gene elucidated the close affiliation of downy mildews and white rusts in the Peronosporales (Beaks and Sekimoto, 2009), although additional multigene analysis with other *Phytophthora* species would be required to better characterize this relationship. A comprehensive phylogenetic analysis with 7 different nuclear loci, allowed to Blair et al. (2008) to observe 10 different clades by using 82 species of *Phytophthora*, 2 more clades compared with analysis based on the internal transcribed spaces (ITS) done by Cooke et al. (2000)The same isolates were also used in a current mitochondrial multilocus analysis (Martin unpublished), and with a few exceptions, results were similar with the analysis of Blair et al. (2008).

The development of molecular resources, the presence of credible sequence databases available on the internet, the sequencing of nuclear genomes for several species and the use of molecular markers have provided a solid framework for studying the population biology and diversity of *Phytophtora* species (Martin et al., 2012).

RFLP analysis of digested genomic DNA has been useful for identification of subpopulations of several species. Förster and Coffey (1990) used these genetic markers to investigate sexual recombination in *Phytophthora nicotianae* during oospore formation; it was found that the majority of the oospore progeny from the crosses carried both of the parental markers. Mitochondrial RFLPs were also used to investigate the intraspecific variability of 87 *Phytophthora nicotianae* isolates. In addition, Lacourt et al. (1994) found eight mtDNA haplotypes, where one of them predominated and had the broadest geographical distribution, suggesting that the population derives mainly from the diffusion of one mtDNA lineage.

RAPDs were widely used for several species of *Phytophthora*, like *P. nicotianae* (Zhang et al., 2001), *P. infestans* (Abu-El Samen et al., 2003) and *P. cinnamomi* (Lindle et al., 1999), but also for investigating the genotypic diversity of several species in some geographical areas. The main limitation of these markers is that they are dominant markers, thus they do not allow the evaluation of the heterozygosity in diploid organisms like Oomycetes. Besides, reproducibility may also be a strong limitation since the results could vary between different labs and even between different thermalcyclers (Bardakci, 2001).

AFLP is another technique that generates dominant markers for population analysis and is more transferable among labs than RAPDs. This analysis allows to generate a large number of markers and has been used for comparison of isolates of the most important economic*Phytophthora* species. It is worth mentioning what Lamour et al. (2003) have done by analyzing isolates of *P. drechsleri* and *P. nicotianae*. They observed six and two clonal lineages, respectively, of the two species from eight floricultural hosts at 11 different production sites. Thus an important role played from asexual reproduction in epidemics and spread may occur between distant facilities via transplanting.

Mitochondrial RFLPs have been useful for population studies of *P.infestans* and differentiating haplotypes for several *Phytophthora* spp. (Gavino and Fry, 2002). By analyzing 31 different *Phytophthora* species, it has been possible to find out several intergenic regions extremely variable and suitable for the examination of intraspecific variation and the analysis of closely related species (Schena and Cooke, 2006). Comparing the mitochondrial genome of an isolate of *Phytophthora ramorum* from Europe to an isolate from California, Martin (2008) discovered 13 mitochondrial SNPs and a length difference of 180 bp due to an increase in the size of the spacer region between the *nad5* and *nad6* genes caused by a chimeric region containing duplication of the spacer sequence. By amplifying the regions where the SNPs were located 4 mitochondrial haplotypes were observed out of 40 isolates

collected all over the world. The alignment of the complete mitochondrial genome of four different haplotypes of *P.infestans*, *P.ramorum* and *P.sojae* allowed Mammella et al. (2011) to find out two variable intergenic regions useful to characterize a population of 51 isolates of *P.nicotianae* from different hosts and geographic origins. The combined data set for both regions revealed a total of 20 mitochondrial haplotypes grouped into 5 different clades. However, it is important to keep in mind that mitochondrial SNPs would not reflect nuclear genotypic differences in a sexually outcrossing population, thus nuclear markers should be implemented in the analysis with mitochondrial markers (Martin et al., 2012).

Single nucleotide polymorphisms (SNPs) can also be used to study pathogen subpopulations as well as for development of diagnostic molecular markers for their detection (Martin et al.., 2012). By amplifying and sequencing DNA in a worldwide panel of strains of *P. ramorum,* Bilodeau et al.(2010) discovered SNPs in two genes, β-tubulin and cellulose binding elicitor lectin (*CBEL*), that differentiated the North American from the European populations. A single nucleotide polymorphism (SNP)-based molecular marker system from *P. infestans* was developed by Abbott et al. (2010) by identifying polymorphisms in microsatellite flanking regions. Nucleotide diversity averaged 1 SNP per 426 bp and a number of homozygous loci were identified that could be used for genotyping isolates. SNP analysis has also been useful in population studies with *P. capsici* (Hulvey et al., 2010), where melt curve analysis instead of DNA sequencing was used to identify the SNPs.

Simple sequence repeats (SSRs) are also commonly used in population analysis and assessment of intraspecific variation. The advantage of this technique is that is possible to analyze codominant alleles, thus they are definitely more useful than RAPDs or AFLPs especially for diploid organisms, such as Oomycetes. The availability of sequenced genomes of several species has allowing the use of these markers, since the major drawback of microsatellites is the need of genomic DNA sequence data to identify potentially informative

SSR loci and design primers for their amplification (Martin et al., 2012). In addition, the knowledge of the genome is important for checking if the size differences of SSR loci are due to varying numbers of the repeats and not from length mutations in flanking regions. To overcome this issue, it is advisable to clone and sequence representative amplicons. However, possible multiple bands can be due to the slippage of the DNA polymerase. In fact, these extra bands can sometimes correspond to PCR products that were shorter by one or more repeat units than the main amplification product (Murray et al., 1993). This mechanism is explained with the slippage of the template and/or primer strands by one repeat that generates a single or more repeats deletion when the bulged-out repeat is in the template or primer strand, respectively (Olejniczak, 2006). These shadow bands can appear as "stutter peaks" when the capillary electrophoresis is used for classifying SSR bands. To optimize scoring of the length polymorphism, the 5' PIG tail "GTTT" is usually added to the reverse primer sequences (Brownstein et al., 1996). Another issue concerning *Phytophthora* spp. is the occurrence of more than two alleles, although Oomycetes are diploid (Brurberg et al., 2011), especially for when it involves data analysis since most of methods used are designed for examining diploid or haploid populations. Recently, new softwares are capable of appropriately interpreting SSR data in populations of mixed ploidy (Cooke et al., 2011). Despite their drawbacks, microsatellites are the markers the most used in the last years to investigate the geographic distribution and population structure of *Phytophthora*. In fact, they have been widely utilized for *Phytophthora* species whose genome has been partially or completely sequenced including P. *capsici, P. sojae, P. infestans and P. ramorum* (Ivors et al., 2006, Prospero et al., 2007, Wang et al., 2009, Gobena et al, 2012, Li et al., 2013).

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Chapter 2

Identification and validation of polymorphic microsatellite loci for the analysis of **Phytophthora nicotianae** *populations*

Abstract

A large number of SSRs were screened taking advantage of the recently sequenced genomes of *Phytophthora nicotianae* from 6 different isolates of this important plant pathogen. A panel of 9 different SSRs was selected and accurately evaluated by means of *in silico* and experimental lab approaches. Selected markers represent a new valuable tool for the intraspecific characterization of *P. nicotianae* since *they:* i) were found in the genome of all sequenced *P. nicotianae* isolates; ii) were highly polymorphic among different isolates and the observed variability was the result of a different number of SSRs rather than deletion/insertion events in flanking regions; iii) were easily amplified and sequenced from 5 representative tester isolates of *P. nicotianae*; and iv) primer annealing sites did not contain indels or point mutations that would hamper their amplification from a wide range of isolates. Particular attention was paid to the reduction of stutter peaks, which can greatly complicate genotyping and lead to ambiguous results, through an accurate optimization of amplification conditions and primer design. Markers were also optimized to be utilized in a multiplex approach by the use of primers labeled with different fluorescent dyes in order to reduce time and costs of SSRs analyses.

2.1 Introduction

Simple sequence repeats (SSRs) or microsatellites are very useful markers for the detection of the genetic diversity within specific species and a powerful tool in the study of *Phytophthora* population biology, epidemiology, ecology, genetics and evolution (Schena et al., 2008). They are tandemly repeated motifs of one to six bases which are scattered throughout the nuclear genome in all eukaryotics with a variable frequency among different organisms (Selkoe and Tonnen, 2006). SSRs exhibit a high degree of length polymorphism among related organisms due to stepwise mutations affecting the number of repeat units

(Mascheretti et al., 2008). Among the several advantages correlated to SSRs, it is worth mentioning: i) the possibility of detecting multiple SSR alleles at a single locus using a simple PCR-based screen, ii) SSRs are codominant, thus it is possible to discriminate heterozygote genotypes, iii) very small quantities of DNA are required for screening, and iv) results are objective and reproducible in different labs (Chistiakov et al., 2006).

Nowadays, microsatellites are largely accepted as the most powerful tool for investigating the genetic structure and the reproductive biology of *Phytophthora* species, although a major limitation to their wider exploitation is the need for knowledge of the DNA sequence of the SSR flanking regions to which specific primers have to be designed (Schena et al., 2008). Methods for the discovery of SSR loci have been based on constructing genomic DNA libraries enriched for SSR sequences. These methods were utilized for *P. cinnamomi* and *P. ramorum*, however they are time-consuming, and the specific sequencing of DNA libraries required is expensive (Dobrowolski et al., 2002; Prospero et al., 2004). However, in recent years, the use of SSR markers has sharply developed by successive technical advances, in particular by the advent of next-generation sequencing (NGS) technologies that are enabling the sequencing of an increasing number of genomes facilitating the identification of a large numbers of SSR loci at a reduced cost. The availability of entire genomes has allowed the use of effective SSR markers for the study of several *Phytophthora* species including *P. cinnamoni* (Dobrowolski et al., 2003), *P. infestans* (Li et al., 2013), *P. alni* (Ioos et al, 2007), *P. ramorum* (Prospero et al., 2007), and *P. plurivora* (Schoebel et al., in press). Thus far development of microsatellite markers remained challenging for *P. nicotianae* because the genome of this species had not been sequenced. However, in November of 2011 a 71Mb draft assembly of the entire genome achieved from data generated using the Illumina NGS technology (http://www.illumina.com/) was made available to the scientific community ("*Phytophthora parasitica* INRA-310 Sequencing Project, Broad Institute of Harvard and

MIT (http://www.broadinstitute.org/). This first genome of an isolate from Australia (INRA-310) has been then followed by other 5 genomes representative of the genetic diversity within the species. Even when genomic data are available to design primers for SSRs analyses, these markers need to be accurately selected and optimized to avoid a number of challenges that could limit their utility and/or confound results of the analyses. An important issue is the presence of homoplasy, that is a phenomenon that dampens the visible allelic diversity of populations and may inflate estimates of gene flow when mutation rate is high (Blankenship et al., 2002). In particular, it is impossible to detect two alleles that are identical in sequence but not identical by descent. Such non-identity occurs when there is a back-mutation to a previously existing size or when two unrelated alleles converge in sequence by changing repeat number in two different places in the sequence (Selkoe and Tonnen, 2006). Furthermore, the presence of mutations in the primer region and the quality of DNA may strongly decrease or lead to a failed amplification (Paetkau and Strobeck, 1995).

A possible issue one can encounter when working with SSRs markers concerns generating clean data with which to work with. In particular, stutter peaks, can represent a serious problem. They are minor peaks generated through strand slippage during amplification of the DNA by polymerase which generate incorrect amplicons, typically one base or one repeat unit length shorter or longer than the real allele fragment (Walsh et al., 1996). Stutter peaks can interfere with the data interpretation, for example by preventing the detection of an allele if it co-localizes in the same position on a chromatogram. Besides, some alleles can be underestimated because of an imbalance of the allele peak height or peak area ratios at heterozygous loci (Leclair et al, 2004).

The aim of this study was to analyze SSR loci within the *P. nicotianae* genome in order to identify and validate a number of effective SSRs markers for analysis of population structure. Primers designed to amplify SSRs in the genome of isolate INRA-310 were

manually analyzed within the 5 other *P. nicotianae* isolates by using Genious 5.5.9 (www.genious.com/download. If primer annealing sites were present then intervening sequences were examined to confirm if nonSSR insertion/deletions had occurred that would have an impact on the length of the amplified product. Selected primers were validated initially by cloning and sequencing target regions from 5 isolates representative of the genetic diversity within the species (Mammella et al., 2011; Mammella et al., 2013). Finally, the best selected primers were used to genotype 5 selected isolates using labeled primers in a multiplex approach. Multiplex PCR is an efficient approach in which several SSR loci can amplified in a single PCR reaction in order to reduce genotyping costs an increases its throughput (Hayden et al., 2008; Li et al., 2013).

2.2 *Materials and methods*

2.2.1 Identification of amplifiable SSR regions

All contigs (708) of the nuclear genome of the isolate INRA_310 of *P. nicotianae* were analyzed to screen for SSR loci and to design primers for their amplification using the Batch Primer3 software (You et al., 2009). Contigs were scanned for the presence of microsatellites defined as short tandem repeat motifs (SSRs) of 2-6 bp. SSRs were selected with a minimal acceptable length of 12 bp, for di-, tri- and tetranucleotide motifs, 15 bp for pentanucleotide motifs and 18 bp for esanucleotide motifs. Primers flanking all identified loci were designed using the same program with the following criteria: T_M of 50-60°C (optimum at 55°C), product size of 100-450 bp (optimum at 200 pb), GC content of 30-60% (optimum at 50%), and primer size of 18-21 bp (optimum at 20 bp).

Identified primers were aligned with the other available genomes of *P. nicotianae* (https://olive.broadinstitute.org/projects/phytophthora_parasitica) (Table 1) using the software Geneious 5.5.9 (www.genious.com/download) in order to evaluate the consistence of the

target regions in all available genomes. Primers with indels or point mutations in the target region in at least one of the tested genomes were discarded. Besides, the uniqueness of the primer annealing sites sequence in the genomes was checked using Bioedit in order to avoid mispriming and nonspecific amplifications (http://www.mbio.ncsu.edu/bioedit/bioedit.html).

2.2.2 DNA amplification and sequencing

Five isolates representative of different mitochondrial clades of *P. nicotianae* (Mammella et al., 2011; 2013) (Table 2) were utilized to experimentally evaluate selected SSR primers. Total DNA was extracted from *P. nicotianae* isolates as described by Ippolito et al. (2002) and subsequently quantified using a spectrophotometer (ND-2000, NanoDrop Technologies, Wilmington, DE) and adjusted to a final concentration of genomic DNA at 15 ng/µl. DNA was amplified with all selected primers using different $MgCl_2$ concentrations and annealing temperatures in order to optimize amplification conditions.

Table 1: Isolates included in the genome project of *Phytophthora nicotianae* (https://olive.broadinstitute.org/projects/phytophthora_parasitica) and utilized to identify SSRs and design primer

Sequenced isolates	***Host***	***Mating Type***	***Region and Country***
INRA-310	*Nicotiana tabacum*	A1	Australia
INRA-149	*Lycopersicon esculentum*	A2	Spain
INRA-PN475	*Capsicum annuum*	n.d.	Spain
INRA-364	*Theobroma cacao*	A2	Cuba
INRA-H02	*Vanilla spp*	n.d.	French Polynesia
INRA-329	*Nicotiana tabacum* Burley	A2	Greece

n.d.= not determined

Selected amplification conditions consisted of 1 cycle of 94°C for 3 min followed by 35 cycles of 94°C for 30s, 59°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 25 µl containing 15 ng of DNA, 1X PCR buffer, 0.2 mM dNTPs, 1 unit *Taq* polymerase (Invitrogen, CA, USA) and 0,1 µM for each primer. PCR products were separated on 3% agarose gels in 1× Tris-borate-EDTA

buffer and visualized with UV light after staining in ethidium bromide. The intensity of fluorescence of amplicons were visually inspected to identify primers generating stronger amplifications of PCR fragments of the expected size and to have preliminary information on the level of intraspecific polymorphism. Selected PCR products were cloned with the TOPO® TA CloningTM kit (Invitrogen) and utilized to transform One Shot® TOP10 *Escherichia coli* cells (Invitrogen), following the manufacturer's instruction. At least 10 *E. coli* recombinant colonies per isolate were randomly selected for colony PCR in order to screen the presence of the insert into the plasmid. Each colony was amplified combining a plasmid's and an amplicon specific primer using the same conditions as described above. Amplified products were separated and visualized by electrophoresis and sequenced in both directions using the primers T7 and T3. In some circumstances new primers were designed to amplify specific SSR's according to the alignment of the detected sequences.

Table 2: Isolates of *Phytophthora nicotianae* representative of the genetic diversity within the species (Mammella et al., 2011; 2013) utilized to validate designed SSRs primers

"Tester" isolates	***Host***	***Mating Type***	***Region and Country***
Albicocco 9	*Prunus armeniaca*	A2	Sicily (Italy)
Ferrara r3	*Citrus aurantium*	A1	Sicily (Italy)
Ceanothus	*Ceanothus spp*	A2	Sicily (Italy)
Hibiscus B	*Hibiscus rosa-chinensis*	A2	Calabria (Italy)
TL8VP	*Lavandula spp*	A2	Piedmont (Italy)

2.2.3 Evaluation of selected SSRs primers using a Genetic Analyzer

Selected primers were labeled and further assessed in automated genotype assays with five isolates representing the genetic diversity within the species (Table 2). Forward primers for each SSR locus were marked with different fluorescent dyes (6-FAM or HEX) to be used in multiplex analyses (Table 11). Labeled primers were purchased from IDT (Integrated DNA Technologies). The assessment consisted in the evaluation of the SSRs size through the analysis of the fluorescent signal and in determining fluorescence intensity and presence of

stutter peaks in the chromatogram. For reducing the phenomenon of stutter peaks, reverse primers contained a 5' PIG tail "GTTT" according to Brownstein et al. (1996). These modified primers were assessed in comparison with primers without any tail. Amplifications were performed in a total volume of 25 µl containing 15 ng of DNA, 1X PCR buffer, 0.2 mM dNTPs, 1 unit *Taq* polymerase (Invitrogen) and 0,1 µM for each primer, with the following amplification profile: 94°C for 3 min followed by 30 cycles of 94°C for 30s, 59°C for 30 s, 72°C for 45 s and a final extension cycle of 72°C for 10 min. The optimal dilution of the amplification for each primer pairs was determined empirically. Initially, the PCR products were diluted 50, 100, 250 and 500 times and run through the genetic analyzer; subsequently, the best dilution was chosen by visual inspection according to the signal achieved. Trials were conducted with the same "testers" isolates utilized in cloning and sequencing trials (Table 2).

Fluorescent PCR products were genotyped using an ABI3500 DNA Analyzer (Applied Biosystems), which is an automated 8-capillary instrument designed for a wide range of sequencing and fragment analysis applications. Two µl of diluted PCR product were mixed with 8.5 µl of Hi-Di formamide (used as injection solvent and facilitate the denaturation of the sample) and 0.5 µl of Gene Scan 600 LIZ, which is a five dye-labeled high density solution designed for sizing DNA fragments in the 20-600 nucleotides range. Analyses were carried out with the instrument supporting software according to the manufacturer instructions.

2.3 Results

2.3.1 Identification of SSRs within the genome isolate INRA-310 of P. nicotianae

The bioinformatics tool Batch Primer 3 (You et al., 2009) was used to identify tandem repeats in the draft genome sequence of isolate INRA-310 of *P. nicotianae*. Using the above mentioned parameters, a total of 5118 tandem repeats were identified from 708 scaffolds of the INRA-310 genome and primers were designed in their flanking regions. Surprisingly, tri-nucleotides represented the most abundant microsatellites (51,6%), followed by tetra-

(25,6%), di-(14,05%), esa-(4,6%) and pentanucleotides (4,2%) (Table 3; Table 4). The most widespread motif in the genome was $(AAG)_n$, (observed 138 times), followed by $(TTC)_n$ (observed126 times), $(GAA)_n$ (observed 120 times, and $(CAG)_n$ and $(GCT)_n$, observed 99 times (Table 5).

Table 3: SSR loci identified within the genome of *Phytophthora nicotianae*, isolate INRA-310

Motif lenght	***Count***	***Percentage***	***Rel.abundance****	***Rel.density*****
di	720	14,05	10,02	211,88
tri	2641	51,60	36,82	483,69
tetra	1308	25,56	18,24	240,79
penta	215	4,20	3,00	46,21
esa	235	4,59	3,28	62,82

* Rel. abundance = total number of SSRs per Mb of sequence analyzed

** Rel. density = total sequence length (bp) contributed by each SSR per Mb of total analyzed DNA sequences

Table 4: Longest SSR motifs identified within the genome of isolate INRA-310 of *Phytophthora nicotianae*

Di	**tri**	**tetra**	**penta**	**Esa**
(CA)45	(TAC) 27	(ATAG)24	(ATTTT)5	(GCTGTT)10
(GA)42	(AAC)23	(TGTA)24	(TAGGC)5	(CAAGCT)8
(GT)41	(TAG)21	(TCTG)22	(TTATT)5	(TGTTGC)8
(TC)41	(CTA)20	(TTAG)22	(ACAAG)4	(CAAGCT)5

Table 5: Most common repeat motifs identified within the genome of isolate INRA-310 of *Phytophthora nicotianae*

Motif	**Number of SSR**	**Percentage**
AAG	138	2,70
TTC	126	2,46
GAA	120	2,34
CAG	99	1,93
GCT	99	1,93
AT	92	1,80
AG	91	1,78
CTT	91	1,78
CTG	88	1,72
TCT	87	1,70
TGC	86	1,68
TG	84	1,64

2.3.2 In silico evaluation of SSRs by comparison of available genomes

Among the complete panel of SSR loci within the genome of isolate INRA-310 identified using Batch Primer 3 software, a limited number of SSR loci were selected based on the number of repeats. Selected SSRs (approximately 200) had a minimum of 5, 7, 8 or 16 repeats for pentanucleotides, tetranucleotides, trinucleotides, and dinucleotides, respectively. These SSRs were aligned with homologous sequences within the genomes of the other 5 isolates of *P. nicotianae* using the software Geneious (version 5.5.9). The analysis enabled the evaluation of the level of polymorphism within the target region of primer annealing sites to identify indels that would hamper the use of a loci for genotyping large populations of isolates. This *in silico* analysis enabled the selection of 18 putative SSR loci which included dinucleotides (11), trinucleotides (5) and tetranucleotides (2) (Table 6).

Table 6: List of selected SSRs and primers experimentally assessed along with optimized $MgCl_2$ concentration for PCR amplifications.

Locus	SSR motif	Product size range (bp)	Primer sequence	$MgCl^2$ (mM)
P5	TGTC	188-224	F-CAAGCCCGCTGAGGTTGAA	2
			R-CTCCGAGGTCCAAATGTGAT	
P15	TAC	66-114	F-AGCTTCTGCAGTAACGGTAA	2
			R-CGATCAAAGATTACTGCAACT	
P16	CT	101-133	F-GTCATACTCCACCTTCCACCA	2
			R-GCTAAGCCTGAAGCACAGAGC	
P17	AAC	126-147	F-GTCCTCAGGGATCAGCACAT	2
			R-TGGATATCGTTCCCGTTGTT	
P44	TC	173-209	F-TTCCTCCTGACCAGACGAGT	3
			R-TTCCGCTGCCAAAGAAGCWCG	
P334	CA	129-159	F-TCCGCAGTCTTCAYGAGTAA	2
			R-TCACCGCAAGAATCGAGTCAT	
P493	CT	125-173	F-CCGATTGAGGCCATGTGAAA	2
			R-AAGAGTATGTTGGTGAACAC	
P643	GT	162-172	F-TTTCAATCGTTTGACCATGC	2
			R-CAAGTCCAAACCGTCCTGTC	
P788	GA	123-135	F-GATGGCAAACCGCCCGACTT	3
			R-CGAGAAGCAGCAGAAGAAGC	
P853	TCTG	138-186	F- TTGAAGCTAGGGCCATTATCA	2
			R-CCAATCAACAGTCCGGAAAT	
P1129	GTA	138-168	F-TTCGTTTATGACAGCCTCCA	3
			R-TGTTAGGGGTCTCCAACTGC	
P1509	GT	118-128	F-CTAAGCCTAGCCAATCCAAAC	2
			R-CCAGCTTGACGCCGGGATTA	
P1511	TG	140-170	F-CAACAACGTGTGTCTGGTACG	3
			R-CTAGGACGTGCTCGGAAATC	
P1512	CT	144-178	F-GTCACCGGCATTGCTAAACT	2
			R-CAAACGGGAGTTTCGTTATCA	
P2039	CGA	96-120	F-GCAGTCGGTTGGATTGATCA	2
			R- TGAACCTTGTCCAGATTATTG	
P2040	AGT	153-162	F-ACGAGTTTGGGCATCGTTTA	3
			R-ATTTTCGCACGGAGGAGAT	
P2459	CT	137-151	F-GCTGGTCGACCTAACGTCTC	3
			R-CATCGTCCCGGTAAACAAAG	
P4560	TC	98-136	F-AGAAGACGCTGCGTGAATTT	3
			R-CACCTACAGCAGACGAGCTG	

The annealing sites for amplification primers did not contain polymorphisms that would prevent amplification or indels that would generate length differences not associated

with differences in the number of repeats in the SSR loci. All loci exhibited polymorphisms among the 6 analyzed genomes (at least three different alleles were identified) (Table 7, 8).

Table 7: Number of repeats determined for 18 selected SSR loci within the genome of six different isolates of *Phytophthora nicotianae*

Locus	Motif	Number of repeats					
		INRA-310	INRA-149	INRA-329	INRA-364	INRA-PN47	INRA-H02
P5	TGTC	24	6	5	5	8	7
P15	TAC	26	11	14	18	nf*	12
P16	CT	37	27	16	28	Inc**	20
P17	AAC	27	14	19	19	14	14
P44	TC	32	11	21	11	10	10
P334	CA	23	12	8	11	nf*	11
P493	CT	18	28	21	18	inc**	24
P643	GT	18	18	20	15	16	11
P788	GT	16	12	10	11	nf*	13
P853	TCTG	11	7	12	6	6	12
P1129	GTA	12	5	9	10	nf*	5
P1509	GT	23	12	20	16	nf*	13
P1511	TG	20	23	11	20	16	12
P1512	CT	18	15	21	18	nf*	19
P2039	CGA	11	4	8	4	4	4
P2040	AGT	10	inc**	8	6	8	11
P2459	CT	23	15	23	10	10	15
P4560	TC	30	21	19	14	nf*	21

*nf = locus not found in the genome
**inc = locus has been found but divided in two different contigs

The different loci showed different levels of polymorphism, although the evaluation was not always complete since some of the selected SSR loci were not identified in the complete panel of sequenced genomes (Table 7; 8). In particular, isolate PN47 did not contain some of the markers due to its limited sequence coverage. Furthermore, for a limited number of loci the analysis of the SSRs was not accurate because the analyzed regions were split into two different contigs/nodes or the SSR region was redundant within two fragments (Table 6). On the whole, the number of alleles detected within the six analyzed genomes ranged between

three (loci P17, P2039 and P 2059) and five (loci P5, P15, P16, P643, P788, P1129, P1509, P1511) (Table 7, 8, 9)

Table 8: Length of SSRs determined for 18 selected SSR loci within the genome of six different genome-sequenced isolates of *P. nicotianae*.

Locus	Motif	SSR length (in bp)					
		INRA-310	INRA-149	INRA-329	INRA-364	INRA-PN47	INRA-H02
P5	TGTC	264	192	188	188	inc**	196
P15	TAC	120	75	63	96	nf*	78
P16	CT	151	131	inc**	133	nf*	inc**
P17	AAC	147	108	123	123	108	108
P44	TC	inc**	177	197	177	nf*	175
P334	CA	155	133	125	131	nf*	131
P493	CT	141	161	147	141	nf*	153
P643	GT	166	166	170	160	nf*	inc**
P788	GT	141	133	129	131	inc**	135
P853	TCTG	146	162	166	142	nf*	166
P1129	GTA	159	138	150	153	nf*	138
P1509	GT	138	116	132	118	nf*	118
P1511	TG	156	162	160	156	148	140
P1512	CT	156	150	inc**	inc**	inc**	158
P2039	CGA	120	99	111	99	99	99
P2040	AGT	153	inc**	147	141	156	147
P2459	CT	151	135	151	125	inc**	inc**
P4560	TC	140	122	118	108	inc**	122

*nf = locus not found in the genome
**inc = locus has been found but split into two different contigs

Table 9: Example of results obtained by the comparison of the sequenced genomes for 6 isolates of *Phytophthora nicotianae* for a specific locus (P1511). Identical analyses were performed for all selected loci.

Isolate	Prod size	Motif	SSR length	Number of Repeat	node /contig
310	156	TG	40	20	1.7
149	162	TG	46	23	30611
329	138	TG	22	11	69182/22624
364	156	TG	40	20	2693
H02	140	TG	24	12	11664/5852
PN47	148	TG	32	16	26903/21000

2.3.3 DNA amplification and sequencing of selected SSR loci

Primers pairs designed with BatchPrimer3 to amplify the 18 *in silico* selected SSRs were experimentally evaluated using 5 isolates of *P. nicotianae* representative of different clades according to Mammella et al. (2011; 2013) (Table 2). All selected primers pairs produced a positive amplifications with PCR fragments of the expected size from the panel of the 5 isolates analyzed. Figures 1 and 2 represents analyses conducted with one of the selected loci (locus P1511), but almost identical analyses were conducted for all loci. After PCR amplification between 2 and 5 isolates were chosen for cloning and sequencing. Fragments for cloning were selected giving more emphasis to longer amplicons to confirm that the length was due to a higher number of repeats. Interestingly, several isolates were found to be heterozygote with a number of alleles ranging from 1 to 4 per each locus/isolate (Table 11). As a consequence many isolates showed a number of alleles higher than the expected number (2) for a diploid organism like *P. nicotianae*. For example, 4, 2 and 4 different alleles were identified for locus P1511 within isolate Ceanothus, TL8V, and Hibiscus, respectively (Figure 2). Nonetheless, these additional alleles could be due to the presence of stutter peaks produced from the slippage of the DNA polymerase, although cases of mixed ploidy (loci with more than two alleles) have been already contemplated for SSR analysis on *Phytophthora spp*. (Cooke et al., 2011). On the whole, the size of tested markers ranged from 66 bp (locus P15) and 224 pb (locus P5), whereas the number of alleles identified in the five tested isolates varied between 5 (loci P1129, P1512, P2039, P2040) and 11 alleles (locus P1511).

Cloning and sequencing also revealed amplification of non-specific fragments for 4 different loci (P12, P493, P1041 and P1509; data not shown). For these SSR loci new primers with enhanced specificity were designed and validated. Similarly, new primers were designed for the locus P5 and P15 since insertions of 5 and 18 bp, respectively, were detected for at least one the sequenced clones. Finally, a high percentage of single nucleotide polymorphisms

(SNPs) were identified between the target region of primers and SSRs. However these point mutations were not considered negatively since they were not localized in the primer annealing sites

Figure 1: Electrophoretic gel showing the results of the amplification of the 5 tester isolates of *Phytophthora nicotianae* (Albicocco9, Ceanothus, Ferrara r3, HibiscusB, and TL8V) with specific primers for the locus P1511. Note as 4 out of 5 isolates are characterized by two polymorphic PCR fragments.

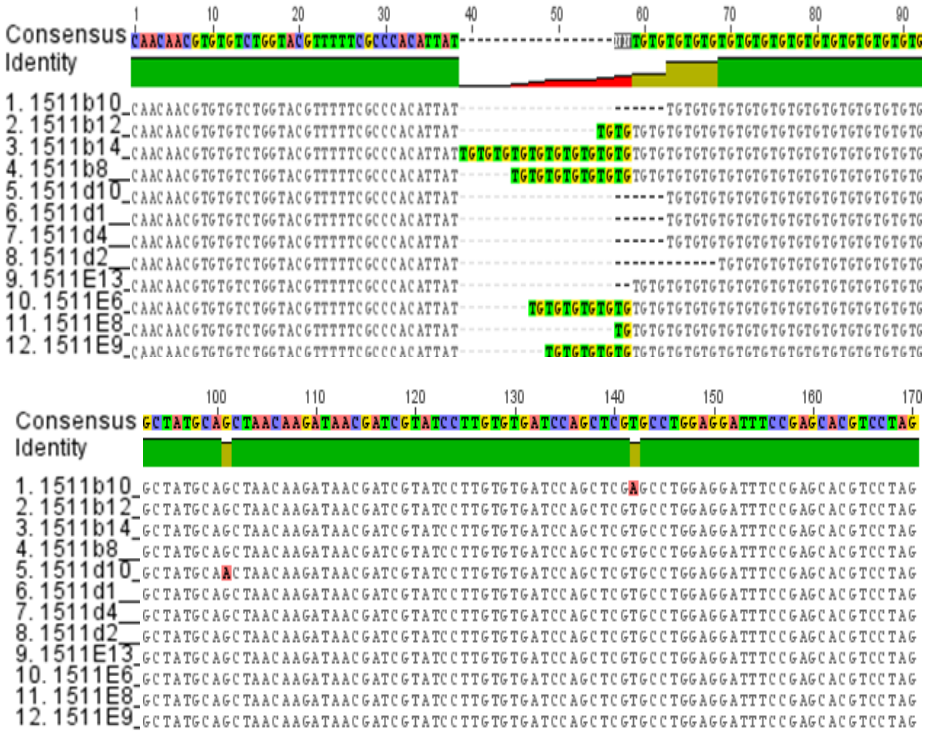

Figure 2. Alignment containing sequences from four clones of the tester isolates Ceanothus (B) , Hibiscus B (D) and TL8V (E) amplified with primers for the marker P1511.

2.3.4 Assessment of the SSRs primers by Genetic Analyzer

The reliability of the 18 selected primer pairs was evaluated in practical conditions to genotype 5 *P. nicotianae* isolates representative of the genetic diversity within the species (Table 2) using an ABI Genetic Analyzer 3500 (Applied Biosystems) and fluorescent labeled primer (Table 10). In preliminary analyses most of the primer pairs proved to be suitable for genotyping although significant differences in fluorescence intensity were revealed. A single primer pair (P2459) did not produce any amplification for the tester isolate TL8V at all the assessed dilutions and another locus (P853) did not produce fluorescent signals, probably because of the denaturation of the fluorophore utilized to label primers (data not shown). Based on this preliminary analysis a further selection of primers was done and 9 of them were utilized to genotype tester isolates (Table 10).

Table 10. SSRs labeled primers utilized to genotype tester isolates of *Phytophthora nicotianae* with an ABI Genetic Analyzer 3500

Locus	Dye	SSR motif	Product size (bp)*
P5	HEX	TGTC	188-264
P15	FAM	TAC	63-120
P17	FAM	AAC	108-147
P643	HEX	GT	160-190
P788	FAM	GA	127-141
P1129	HEX	GTA	138-159
P1509	FAM	GT	116-170
P2039	FAM	CGA	99-120
P2040	HEX	AGT	141-162

*The range of these loci does not consider the addiction of bases probably caused by PIG tail

Specific tests were also conducted to evaluate the presence of stutter peaks. In particular, reverse primers with and without 5'PIG tails "GTTT" (Brownstein et al., 1997) were compared, to evaluate if the presence of the tail was functional to reduce the phenomenon. In general the PIG tail dramatically increases the quality of signals and amplifications although for some primer pairs the presence of stutter peaks was not completely avoided (Figure 3). As an example, in the case of locus P1509, stutter products for

an allele with “n” dinucleotide repeats were observed at n-2, n-4 and n+2 positions, and this phenomenon was more evident for the lowest dilution (50 times). In general, stutter bands were more evident for SSR with a 2-bp motif, less evident for SSRs with a 3-bp motif and completely absent in the unique SSR with a 4-bp motif. The comparison between alleles revealed by cloning and sequencing with those detected with the Genetic Analyzer revealed important differences, although sequencing was not performed for all isolate/locus combinations (Table 11). In particular, data indicate that several alleles detected by sequencing were actually the result of the slippage of the DNA polymerase since they were not detected with the genetic analyzer using primers with the PIG tail. For example the triploidy detected by sequencing of locus 15 in isolates Albicocco 9 and Ceanothus was not confirmed with the fragment analysis (Table 11); smaller amplicons detected by sequencing appeared to be stutter bands of the real SSR region.

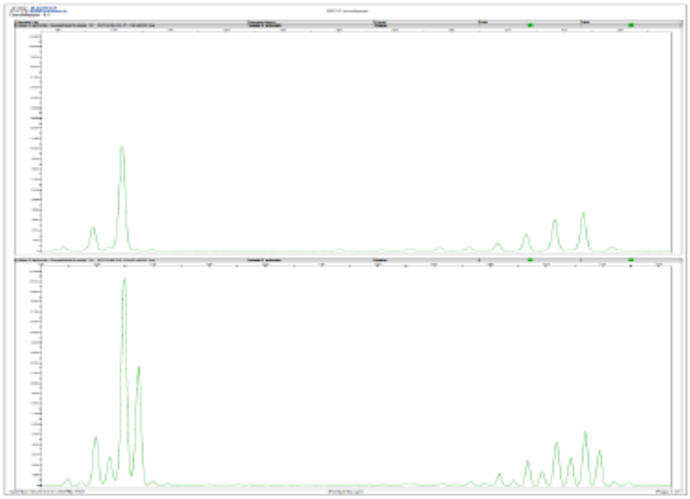

Figure 3: Example of differences in fluorescence signals obtained for the locus P1509 due to the presence of stutter peaks using a reverse primer with 5’PIG tail (top) as compared with the same primer without tail (bottom).

A similar finding was observed with locus 643 (for isolate Albicocco 9) locus P17 (isolate Ferrara r3), locus P788 (isolate Hibiscus B) and locus P2039 (isolate Ferrara r3). The presence of a relevant number of cases in which the slippage of the polymerase occurred was

also confirmed by the presence of differences in the length of alleles for several loci determined with the two analyses (Table 12). Most of these differences were in agreement with the SSR motif since they were multiple or sub-multiple of the motif length itself. Few cases were also found in which the Genetic Analyzer revealed alleles 1 bp longer as compared with the sequencing. For this phenomenon, the adding of a base pair carried by the PIG tail could be speculated due to the complementarity of the bases closely flanking the SSR region. Finally, an additional allele was detected with the Genetic Analyzer as compared to the sequencing results for locus 1129/albicocco9 and for locus 2039/TL8V, confirming a higher accuracy of the fragment analysis compared with the traditional sequencing

Table 11. Results of the SSRs analysis of 5 representative isolates of *Phytophthora. nicotianae* (Albicocco 9, Ceanothus, Ferrara r3, Hibiscus B, and TL8V) using a cloning/sequencing approach and a Genetic Analyzer with fluorescent labeled and 5'PIG tailed primers.

Locus	Motif	Albicocco 9		Ceanothus		Ferrara r3	
		Sequencing	Gen.Analyzer	sequencing	Gen.Analyzer	sequencing	Gen.Analyzer
P5	TGTC	nd	190/234	188/212/224	190/226	188	190
P15	TAC	90/93/114	93/111	84/87/96	93/96	66/75	66/75
P17	AAC	nd	126	nd	129/144	126/132/144	129/144
P643	GT	164/170/172	166/174	nd	160/168	nd	148/162
P788	GA	125/135	127/137	nd	129	nd	127/129
P1129	GTA	153	151/154	nd	151/154	138/168	139
P1509	GT	126/128	132	nd	116	118	124
P2039	CGA	nd	111	99/120	99/120	96/99/114	99/114
P2040	AGT	nd	158	153/162	155/164	156/162	158/164

Locus	Motif	Hibiscus B		TL8V	
		sequencing	Gen.Analyzer	sequencing	Gen.Analyzer
P5	TGTC	188/200	190/202	192/208	194/210
P15	TAC	78/90	78/90	78/96	78/96
P17	AAC	nd	126/144	126/147	105/129
P643	GT	nd	166/190	162/164	164/166
P788	GA	123/127/133	129/137	nd	135/139
P1129	GTA	nd	154	150	151
P1509	GT	nd	116	nd	118/168/170
P2039	CGA	nd	99	111	111/120
P2040	AGT	nd	155/158	nd	164

2.4 Discussion

The aim of the present study was the development of a new molecular approach to characterize intraspecific variability in *P. nicotianae* by using microsatellites. To this aim a large number of SSRs were screened taking advantage of the recently sequenced genomes of *P. nicotianae* from 6 different isolates of this important plant pathogen (http://www.broadinstitute.org). Selected SSRs and respective flanking primers were accurately evaluated using *in silico* and experimental approaches in order to identify 18 valuable markers. These markers showed to be all polymorphic although with a different level of variation within analyzed isolates. The locus 1511 was the most variable (11 alleles) followed by loci 15, 4560, and 493 (10 alleles), loci 5, 44, and 788 (8 alleles), locus 16 (7 alleles), loci 17, 334, 643, 853, 1509 and 2459 (6 alleles) and loci 1129, 1512, 2039 and 2040 (5 alleles). For some instances, the level of variability within selected loci was underestimated because SNPs in the flanking regions (excluding the target region of primers) was not taken into consideration. This type of variation was not considered when looking at population analysis using SSRs since it is a different type of mutation. The abundant presence of SNPs in the flanking regions of SSRs has been reported and was utilized to develop a SNP-based approach to characterize *Phytophthora infestans* (Abbott et al., 2010).

Although all 18 selected markers proved to be suitable for genotyping *P. nicotianae* isolates a further screening was performed to select 9 markers based on the results of analyses conducted using a Genetic Analyzer and labeled primer. This further selection of markers was primarily performed to select a panel of primers that could be used for massive automated SSRs analyses and was possible because, according to recent reports (Brurberg et al., 2011; Schoebel et al., in press), nine different markers seem to be enough to accurately characterize wide populations of *Phytophthora* species. However, the number of the markers was not previously planned but it is the result of the assessment of the amplifications in reference

isolates, the level of polymorphisms observed and the probable presence of indels in the flanking regions. These top selected markers behaved well in regards to fluorescence intensity and the limited presence of stutter peaks and were appropriate to be used in a multiplex approach which is important to reduce time and costs of SSRs analyses (Li et al., 2010; 2013) .The discovery of nine SSRs markers scattered over the genome can ensure good coverage of the entire genome of the pathogen, thus deepen some features concerning the population structure for a broad analysis that could include reproduction system, geographic origin, host and type of cultivation (open field or nursery), etc. Indeed, tester isolates utilized in the present study were representative of different clades identified within a population of 96 isolates from 5 continents and had been characterized using a single nucleotide polymorphism (SNP) approach (Mammella et al., 2013).

A relevant aspect of the present study is the accurate selection and optimization of SSRs in order to avoid major drawbacks related to the use of these molecular markers. In particular, the presence of stutter peaks due to DNA polymerase slippage was significantly reduced by selecting the best performing SSRs, by optimizing amplification conditions (annealing temperature and $MgCl_2$ concentration), by identifying the most appropriate dilution of amplicons to detect fluorescence and by adding an oligo-tail to the 5' end of the reverse primer. Stutter peaks can greatly complicate genotyping, and in extreme cases can lead to ambiguous results (Leclair et al., 2004). In the present study the existence of a high incidence of incorrect alleles was also deduced by comparing alleles detected using traditional cloning and the automated analysis of SSRs using labeled primers and a Genetic Analyzer. Indeed several alleles reveled by sequencing were found to be the results of errors during PCR amplification with minor products differing in size from the main product by multiples or sub-multiple of the repeat unit length. Accurate quantitative experiments have shown that the *Taq* polymerase slippage rate increases with the number of repeat units and is inversely correlated

with repeat unit length (Shinde et al., 2003). This feature was confirmed by our results since stutter peaks were prevalent within amplicons containing a 2-bp SSR motif, while no background signals were detected in the electropherogram for the tetranucleotide marker. It has been reported that the phenomenon of *Taq* polymerase slippage can be reduced by decreasing the denaturation temperature close to 85°C (Olejniczak, 2008), however a more reliable approach seem to be the addition of an oligo-tail to the 5' end of the reverse primer (Brownstein et al., 1996). This small tail can significantly decrease the formation of misalignments due to an incorrect position of the nascent strand with the template, which could generate secondary structures, such as loop, and contribute to the slippage of the DNA polymerase either in the active site of the enzyme or before the substrate binds to the enzyme (Kunkel and Bebenek, 2000). In agreement with previous reports the addition of a PIG tail dramatically increases the quality of signals and reduced stutter peaks increasing reliability of the analyses.

In conclusion, results of the present study indicate that selected markers are appropriate for the characterization of broad populations of *P. nicotianae* since they: i) were found in the genome of all *P. nicotianae*-sequenced isolates; ii) were highly polymorphic among different isolates and observed variability was the results of a different number of SSRs as loci containing deletion/insertions were preliminarily excluded; iii) were easily amplified and sequenced from 5 representative tester isolates of *P. nicotianae*; and iv) flanking regions in which specific primers were designed did not contain indels or point mutations that could hamper their amplification from genetically different isolates. Considering the great potential of SSRs in genotyping plant pathogen populations (Dobrowolski et al., 2003; Li et al., 2013; Ioos et al, 2007; Prospero et al., 2007), markers selected and optimized in the present study should represent a powerful tool for future investigations related to the study of *P. nicotianae* in order to improve our current knowledge

about ecology and epidemiology of this species that has been primarily based on less powerful approaches including RAPD PCR, AFLP and SNPs analyses (Zhang et al. 2001; 2003 Lamour et al. 2003; Mammella et al., 2011; 2013

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Chapter 3

Analysis of a wide population of **Phytophthora nicotianae** *using microsatellite markers*

Abstract

Nine validated SSRs (Cfr. chapter II) were used to characterize 268 isolates from a broad range of hosts and geographic localities. A total of 129 multilocus genotypes (MLG) were identified with markers showing polymorphisms ranging from 4 (locus P2039) to 24 alleles (locus 1509). Analyses revealed a preferential clonal reproduction in field orchards while sexual reproduction seemed to be more diffused in nurseries. A strong association between genetic groups and host of recovery was revealed for most isolates although this association was less evident for isolates from nurseries. In contrast, a significant geographical structuring was recovered only for isolates from tobacco (sourced in Australia and United States) and from *Citrus maxima* (sourced in Vietnam), while a typical panmictic distribution characterized the majority of isolates including those from other *Citrus* species. These difference were ascribed to the different propagation and cultivation systems. Isolates obtained from potted ornamental and citrus (excepted pommelo) were likely to be diffused worldwide with infected plant material (mainly potted plants). Conversely, tobacco is propagated by seeds which do not contribute to the spread of the pathogen and plantlets are very rarely transplanted in areas different from those in which have been produced. As regards to *C. maxima*, this species is a native plant of Vietnam and plant material was not introduced from other countries suggesting a specific co-evolution of *P. nicotianae* and *C. maxima*.

3.1 Introduction

Phytophthora nicotianae is a soil-borne, hemibiotrophic plant pathogen with a wide host range comprising more than 250 plant genera (Cline et al., 2008). This pathogen is particularly known for its damage on the genera *Nicotianae* and *Citrus*, since it is the causal agent of Black Shank and *Citrus* root rot and gummosis, respectively (Cacciola and Magnano

di San Lio, 2008). However, *P. nicotianae* is responsible for heavy losses on many other economically important species such as fruit trees, herbaceous hosts and ornamental plants (Erwin and Ribeiro, 1996, Moralejo et al., 2009). In particular, a number of surveys have revealed that this specie is one of the most common pathogen on ornamental plants (Cacciola et al., 1997, 2001; Reichard and White, 2001; Pane et al., 2005; Moralejo et al., 2009). In nurseries, the pathogen commonly completes several disease cycles per year due to the repeated growing of different host species. In contrast, a single cycle commonly occur on perennial plants on which root and crown rots are the most common symptoms (Hu et al., 2008).

Phytophthora nicotianae produce various types of propagules: i) sporangia which can germinate directly through a germ tube or indirectly releasing, motile biflagellate zoospores which lose the flagellum and encyst on contact with the surface of the host and germinate; ii) chlamydospores, resistant structures which allow the pathogen to survive in unfavourable conditions; iii) gametangia, called respectively antheridium (male gametangium) and oogonium (female gametangium); and iv) oospores, which are formed after sexual reproduction and act also as organs of preservation. *Phytophthora nicotianae* is heterothallic, producing oospores only if the mycelium of the two different sexually compatible mating types (A1 and A2) come into contact.

Although recently it has been suggested that the reproduction behavior of *P. nicotianae* can be significantly influenced by environmental conditions and hosts (Mammella et al., 2013) it can be generally stated that natural infections are most frequently caused by zoospores and occasionally by direct germination of sporangia (Klotz & De Wolfe, 1960). The primary source of inoculum is the rhizosphere soil, where the pathogen survives in the roots in the form of mycelium, chlamydospores and oospores. Sporangia can form in water saturated conditions and are transported on new plants by rain, irrigation water and wind. In

some specific environments such as nurseries, the dissemination of the pathogen to neighboring plants can be greatly increased by the recycled irrigation water (Benson and von Broembsen, 2001) making the disease management very difficult once water, plants and/or growing media are contaminated. As a consequence the rapid and accurate detection of the pathogen in the irrigation water is very important to develop effective control strategies (Kong et al., 2003).

The importance of *P. nicotianae* as an important pathogen has been highlighted by its recent inclusion among the "Top 10" oomycetes plant pathogens by the journal "Molecular Plant Pathology" (L. Schena, personal communication). However, complete information on the biology and ecology of this important pathogen is still lacking. Indeed studies are greatly complicated by the global diffusion of the pathogen and its wide range of hosts, As a consequence few studies are currently available on the genetic structure of *P. nicotianae* populations and most of them were performed on specific hosts (mainly tobacco) or focused on specific localized geographic areas.

Pathogenicity assays conducted on tobacco have been very useful to investigate the physiological diversity of the pathogen and permitted the identification of four different races (0,1,2 and 3). Initially, race 0 was the most common in South Eastern USA (Lucas, 1975). However, the advent of single-gene resistant cultivars of tobacco caused the emersion of race 1 which is now the predominant race, especially in Georgia and North Carolina (Csinos, 2005; Sullivan et al., 2005).

In the last two decades, DNA marker technologies have greatly contributed to the study of plant pathogens since they offer the possibility of faster and accurate identification, characterization and detection of target species (Bridge et al., 2003; Benali et al., 2011). A number of specific molecular detection methods based on conventional and real-time PCR have been developed to detect *P. nicotianae* in soil, water and on roots (Ippolito et al., 2002;

2004; Kong et al., 2003) while other molecular approaches , such as Random Amplified Polymorphisms DNA- (RAPD), Amplified Fragment length Polymorphisms (AFLP), and Single Nucleotide Polymorphism (SNP)NPs) have been used to investigate intraspecific variability.

The RAPD technique was 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). AFLP was utilized by Lamour et al. (2003) to study a population from different floricultural hosts and production sites, enabled the identification of six clonal lineages. In another study Sullivan and co-workers (2010) characterized isolates of *P. nicotianae* from tobacco to monitor changes in the genetic diversity of pathogen races 0 and 1 according to the host genotype grown. 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).

Recently mitochondrial and nuclear single nucleotide polymorphisms (SNPs) were utilized to study genetic diversity in a worldwide collection of *P. nicotianae* isolates (Mammella et al., 2011; 2013). Both mitochondrial and nuclear markers revealed a high level of dispersal of isolates and an inconsistent geographic structuring of populations although the utilization of only three nuclear markers did not allow to fully assess the variation throughout the genome. Nonetheless, 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. Results of these studies also suggested 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. However, the utilization of only three nuclear markers did not allow to assess the variation throughout the genome.

The aim of the present study was to deepen currently available knowledge about the population structure of *P. nicotianae* by using a more powerful molecular tool (SSRs analysis) and a broader collection of isolates as compared to those utilized by Mammella et al (2013). To this aim 9 validated microsatellite markers specifically designed for the nuclear genome(Cfr. Chaper II) were utilized to characterize a of total 268 isolates of *P. nicotianae* collected all over the world.

3.2 Materials and methods

3.2.1 Isolates of Phytophthora nicotianae and DNA extraction

Two hundred sixty-nine isolates of *P. nicotianae* recovered from different geographic regions (Fig. 1) and various hosts (ornamentals, citrus, tobacco and horticultural) were used in this study (Table 2). Isolates were representative of the six continents although most of them were sourced in Italy (197), Vietnam (37), USA (16) and Australia (6). Isolates were mainly obtained from *Citrus* species (103), and several horticultural and ornamental cultures (167), mainly from myrtle (37), lavender (22) and tobacco (22).

Isolates obtained in the present study were obtained from soil, roots and basal stem of plants showing variable symptoms of decline or apparently healthy, using a selective medium (BNPRAH) and standard procedures (Masago et al.,1977). All isolates were preliminary

identified by means of morphological criteria and using the internal transcribed spacer (ITS) region of the rDNA as a barcoding gene (Schoch et al., 2012). Isolates were also analyzed to assess their mating type by pairing each isolate with known A1 and A2 strains on V8 juice agar medium as described by Erwin and Ribeiro (1996) and DNA was extracted according to the procedure described by Ippolito et al. (2002). Fifteen isolates were obtained from the World Phytophthora Collection at the University of California, Riverside (http://phytophthora.ucr.edu) and DNA extracted as described by Blair et al. (2008).

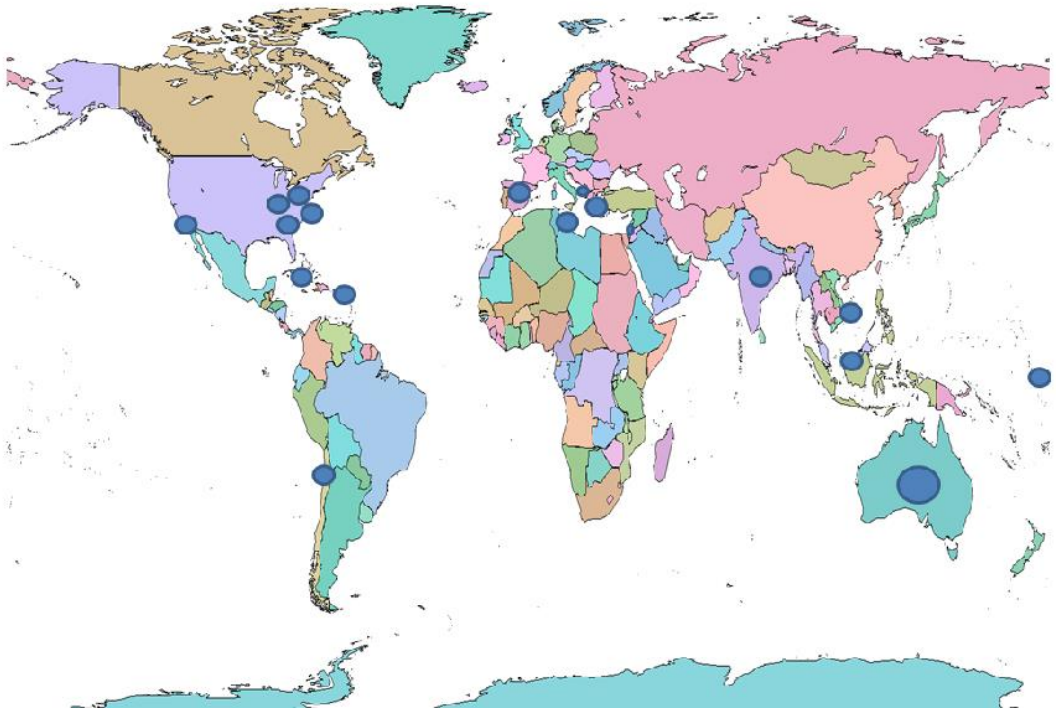

Figure 1: Geographic origin of the isolates used for this study

3.2.2 Analysis of SSR genotypes

Nine validated polymorphic SSR markers were utilized to genotype the complete panel of isolates (Cfr. Chapter II). 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 out of the nine forward primers were labeled with two different fluorophores (HEX and 6-FAM) and assembled into 4

multiplex PCR sets in order to be analyzed simultaneously (Table 1). Reaction mixtures and amplification conditions were as described in Chapter II.

The data were collected using the software Data Collection v.2.0 (Applied Biosystems), while Gene Mapper v. 4.1 (Applied Biosystems) was used to derive the size of the labeled DNA-fragments using the known fragment lengths of the LIZ-labeled marker peaks.

Table 1: Panel of the SSR primers used for the massive analysis along with fluorophores used for labeling and SSR motifs. Equal colors on the primer code indicate the primer pairs coupled for multiplex amplifications.

Primers code	Primer sequence	Dye	SSR motif
P5	F-CAAGCCCGCTGAGGTTGAA R-CTCCGAGGTCCAAATGTGAT	HEX	TGTC
P15	F-AGCTTCTGCAGTAACGGTAA R-CGATCAAAGATTACTGCAACT	FAM	TAC
P17	F-GTCCTCAGGGATCAGCACAT R-TGGATATCGTTCCCGTTGTT	FAM	AAC
P643	F-TTTCAATCGTTTGACCATGC R-CAAGTCCAAACCGTCCTGTC	HEX	GT
P788	F-GATGGCAAACCGCCCGACTT R-CGAGAAGCAGCAGAAGAAGC	FAM	GA
P1129	F-TTCGTTTATGACAGCCTCCA R-TGTTAGGGGTCTCCAACTGC	HEX	GTA
P1509	F-GTACGCACGTTATGCCATTG R-CCAGCTTGACGCCGGGATTA	FAM	GT
P2039	F-GCAGTCGGTTGGATTGATCA R- TGAACCTTGTCCAGATTATTG	FAM	CGA
P2040	F-ACGAGTTTGGGCATCGTTTA R-ATTTTCGCACGGAGGAGAT	HEX	AGT

3.2.3 Data analysis

For the analysis of the complete data set obtained with the Genetic Analyzer, a software R-package called POPPR (Kamvar et al., 2013) was used. POPPR is a software with convenient functions for analysis of genetic data with mixed modes of reproduction. This software is also designed for the analysis of haploid and diploid dominant/co-dominant marker data including microsatellites; besides it allows to define multiple population

hierarchies and subset data sets. Furthermore, the software is able to calculate some basics functions useful for the population analysis, such as the index of association (I_a), which quantifies the degree of recombination within the loci through the formula $I_a = V_o/V_e-1$ (V_o= Variation observed ; V_e = Variation expected) and the Shannon's diversity index , which gives the frequency of the multilocus genotype (MLG) in the sample. The MLG represents the composite genotype over all loci analyses and it is an estimation of the diversity of the samples analyzed.

The Bruvo's distance was utilized to measure the genetic distance among isolates. This function calculates the minimum distance across all combinations of possible pairs of alleles at a single locus and then averaging that distance across all loci (Bruvo et al., 2004). This function calculates the distance between two individuals at one microsatellite locus and allows the analysis also in the cases of different levels of ploidy. Minimum spanning networks (MSN) were constructed using the Bruvo's distance to graphically show the possible evolutionary relationships among MLGs. MSN contains a set of pairwise distances that describe the degree of dissimilarity among individuals. An MSN represents a set of edges (connections) that link together nodes (MLGs) by the shortest possible distance (Salipante and Hall, 2011). This graphic is obtained from simple arithmetic distance matrices and allows to infer population structure such as phylogenetic analysis (Ronquist and Huelsenbeck, 2003). Bruvo's method takes repeat number into account and considers distance of 0.1 equivalent to one mutational step (one repeat), but larger distances do not strictly correspond to a given number of mutation steps. A matrix is created containing all differences between the alleles of two individuals at one locus and these values are then geometrically transformed to achieve the probabilities of mutation among the alleles. Bruvo's method is particularly advised for microsatellites data, since the allele copy number is frequently unknown in polyploid SSRs data.

3.3 Results

3.3.1 SSR fragment analysis

Microsatellite regions were amplified and analyzed from almost the complete panel of isolates (Table 3). Considering the possible combinations isolate/marker investigated only 6 out of 2412 reactions did not yield a positive amplification (Table 2). Different levels of intraspecific polymorphism were observed. The automated analysis of fragments with Genetic Analyzer produced a total of 129 MLGs within the 268 isolates, meaning that most genotypes were detected only once or very few times (Table 2). The number of alleles for each locus varied from a minimum of 5 (locus 2039) and a maximum of 25 (locus 1509) (Table 2). The size of the amplified fragments ranged from 21 bp (locus 2039) and 80 bp (locus P5) (Table 2).

The MLG75 was the most frequently observed genotype within the population analyzed, (shared by 38 isolates). All the isolates sharing this MGL were collected from *Citrus* species, excluding isolates from *C. maxima* (pommelo), in different geographic areas (Table 2). In particular, 6 isolates were recovered from Vietnam (specifically from three different districts), 1 from Philippines and 27 from regions in Italy (Sicily, 27; Calabria, 3 and Puglia, 1). Interestingly, MLG70, the second most numerous MGL (19 isolates), was shared by isolates from the same host (*C. maxima*) and geographic origin (three different districts of Vietnam). A single isolate with this MGL was obtained from *Citrus reticulata*. Isolates with MLG97 and MLG92 contained 12 and 11 isolates, respectively, and were recovered from the same nursery and host (myrtle) in Sardinia, Italy. Similarly, isolates with MLG123 (10 isolates) were obtained from a common nursery and host (*Correa reflexa*) in Sicily, Southern Italy. It is important to highlight that data about the frequency of different MLGs must be considered with precautions because they are greatly influenced by the number of isolated collected from different localities and/or hosts.

Table 2: Product size and number of alleles of the primers used for the analysis

Primers code	**Number of alleles**	**Product size (bp)**
P5	16	186-266
P15	15	66-123
P17	18	102-165
P643	18	148-202
P788	10	121-143
P1129	8	133-166
P1509	25	116-176
P2039	5	99-120
P2040	10	143-170

Table 3: Isolates of *Phytophthora nicotianae* included in the study, their designation, host of recovery, geographic origin, mating type and nuclear multilocus genotypes (MLG). For each marker, different letters indicates a different genotype

Isolate	***Origin***		***MT***	***MLG***	***Genotype***								
	Host	**Country and Region**			**ssr1509**	**ssr15**	**ssr788**	**ssr643**	**ssr2039**	**ssr1129**	**ssr17**	**ssr5**	**ssr2040**
P1495	*Nicotiana tabacum*	Australia	A2	**1**	a*	**k**	**o**	**k**	**h**	**a**	**s**	**f**	**n**
irf8	*Anemone americana*	Italy (Liguria)	A2	**2**	a*	**j**	**j**	**x**	**h**	**h**	**m**	**i**	g
m1f1h	*Myrtus communis*	Italy (Sardinia)		**3**	a*	**f**	**l**	**z**	**i**	**k**	**i**	**c**	**p**
phvib5	*Viburnum tinus*	Italy (Sicily)		**4**	a*	**h**	**q**	**l**	**h**	**r**	**r**	**y**	h
44000	*Cyclamen spp*	Italy (Liguria)	A2	**5**	**b**	**h**	**q**	**l**	**h**	**m**	**r**	**y**	h
phvib1	*Viburnum tinus*	Italy (Sicily)		**5**	**b**	**h**	**q**	**l**	**h**	**m**	**r**	**y**	h
24C	*Lavandula spp*	Italy (Sicily)		**6**	**b**	**u**	**i**	**y**	**c**	**n**	**l**	**o**	j
23RA	*Lavandula spp*	Italy (Sicily)		**6**	**b**	**u**	**i**	**y**	**c**	**n**	**l**	**o**	j
23C	*Lavandula spp*	Italy (Sicily)		**6**	**b**	**u**	**i**	**y**	**c**	**n**	**l**	**o**	j
22SA	*Lavandula spp*	Italy (Sicily)		**6**	**b**	**u**	**i**	**y**	**c**	**n**	**l**	**o**	j
22SB	*Lavandula spp*	Italy (Sicily)		**7**	**b**	**u**	**i**	**y**	**h**	**n**	**l**	**o**	j
Ceanothus	*Ceanothus spp*	Italy (Sicily)	A2	**8**	**b**	**ae**	**i**	**s**	**h**	**k**	**x**	**k**	k
cham5	*Chamaleucium uncinatum*	Italy (Apulia)		**9**	**b**	**s**	**l**	**ag**	**i**	**n**	**s**	**h**	j
HibiscusB	*Hibiscus rosa-chinensis*	Italy (Calabria)	A2	**9**	**b**	**s**	**l**	**ag**	**i**	**n**	**s**	**h**	j
HibiscusA	*Hibiscus rosa-chinensis*	Italy (Calabria)	A2	**9**	**b**	**s**	**l**	**ag**	**i**	**n**	**s**	**h**	j
CM 2d	*Convolvulus mauritanicus*	Italy (Apulia)		**10**	**b**	**h**	**l**	**z**	**d**	**k**	**v**	**o**	**p**
CM 1f	*Convolvulus mauritanicus*	Italy (Apulia)		**10**	**b**	**h**	**l**	**z**	**d**	**k**	**v**	**o**	**p**
CM 1a	*Convolvulus mauritanicus*	Italy (Apulia)		**10**	**b**	**h**	**l**	**z**	**d**	**k**	**v**	**o**	**p**
CM5A	*Convolvulus mauritanicus*	Italy (Apulia)		**11**	**b**	**s**	**r**	**ai**	**h**	**h**	**r**	**p**	**o**
CM 5c	*Convolvulus mauritanicus*	Italy (Apulia)		**12**	**b**	**s**	**k**	**ai**	**h**	**h**	**r**	**p**	**o**
corba	*Arbutus unedo*	Italy (Calabria)		**13**	**b**	**af**	**l**	**y**	**i**	**f**	**aa**	**d**	j
gl3	*Grevillea lanigera*	Italy (Apulia)		**14**	**b**	**h**	**l**	**z**	**a***	**k**	**j**	**o**	**p**
HVMRA	*Hebe Veronica myrtifolia*	Italy (Sicily)		**16**	**b**	**h**	**e**	**q**	**i**	**e**	**r**	**o**	f
lavb1a	*Lavandula spp*	Italy (Apulia)		**17**	**b**	**r**	**l**	**j**	**h**	**n**	**f**	**f**	**q**
lavb2b	*Lavandula spp*	Italy (Apulia)		**18**	**b**	**v**	**j**	**n**	**i**	**n**	**l**	**l**	b
m1r2f	*Myrtus communis*	Italy (Sardinia)		**19**	**b**	**f**	**l**	**z**	**h**	**k**	**i**	**c**	**p**
m1f1d	*Myrtus communis*	Italy (Sardinia)		**19**	**b**	**f**	**l**	**z**	**h**	**k**	**i**	**c**	**p**
m1r2c	*Myrtus communis*	Italy (Sardinia)		**20**	**b**	**f**	**l**	**z**	**h**	**k**	**i**	**o**	**p**
pcham2	*Chamaleucium uncinatum*	Italy (Sicily)		**21**	**b**	**p**	**i**	**ac**	**i**	**o**	**a**	**v**	i
2C	*Citrus aurantium*	Italy (Sicily)		**22**	**f**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**

RGRS24	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RGRS23	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RGRS21	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RGRS16	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RGRS14	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RCGS23	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RCGS22	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RCGS21	*Ruta Graveolens*	Italy (Sicily)		**23**	**b**	**t**	**j**	**o**	**c**	**i**	**l**	**f**	g
RGRS15	*Ruta Graveolens*	Italy (Sicily)		**24**	**b**	**t**	**j**	**o**	**d**	**i**	**l**	**f**	g
scrp469	*Durio zibethinus*	Malaysia	A2	**25**	**b**	**u**	**l**	**y**	**h**	**n**	**s**	**e**	i
647B03	*Phormium tenax*	Italy (Liguria)	A2	**26**	**c**	**j**	**e**	**ae**	**i**	**k**	**i**	**z**	**p**
ph242	*Lycopersicon esculentum*	n.d.	A2	**27**	**c**	**j**	**e**	**x**	**h**	**h**	**i**	**j**	e
RGRS22	*Ruta Graveolens*	Italy (Sicily)		**27**	**c**	**j**	**e**	**x**	**h**	**h**	**i**	**j**	e
A178	*Lycopersicon esculentum*	Italy (Lazio)		**27**	**c**	**j**	**e**	**x**	**h**	**h**	**i**	**j**	e
A203	*Lycopersicon esculentum*	Italy (Lazio)		**28**	**c**	**j**	**j**	**x**	**h**	**h**	**i**	**j**	e
F16	*Lycopersicon esculentum*	Italy (Lazio)		**29**	**c**	**t**	**j**	**ac**	**c**	**i**	**l**	**f**	g
IRF25	*Phoenix spp*	Italy (Liguria)	A2	**30**	**c**	**j**	**e**	**aa**	**h**	**k**	**k**	**i**	**p**
NICLAV3	*Lavandula spp*	Italy (Liguria)		**31**	**c**	**j**	**e**	**x**	**e**	**f**	**i**	**s**	**s**
niclav1	*Lavandula spp*	Italy (Sicily)	A2	**31**	**c**	**j**	**e**	**x**	**e**	**f**	**i**	**s**	**s**
NICLAV4	*Lavandula spp*	Italy (Liguria)	A2	**32**	**c**	**j**	**e**	**x**	**e**	**j**	**i**	**s**	**s**
PN475	*Capsicum annum*	Spain		**33**	**d**	a*	**j**	**ac**	**i**	**b**	**x**	**l**	**n**
pf1l	*Citrus maxima*	Vietnam (MoCay)		**34**	**e**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1i	*Citrus maxima*	Vietnam (MoCay)		**34**	**e**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
1C3	*Citrus aurantium*	Italy (Sicily)		**35**	**f**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	k
1B3	*Citrus aurantium*	Italy (Sicily)		**35**	**f**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	k
1A2	*Citrus aurantium*	Italy (Sicily)		**35**	**f**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	k
1B41	*Citrus aurantium*	Italy (Sicily)		**36**	**f**	**c**	**c**	**d**	**g**	**b**	**r**	**e**	k
4A2	*Citrus aurantium*	Italy (Sicily)		**37**	**f**	**c**	**c**	**d**	**f**	**b**	**x**	**e**	k
4B4	*Citrus aurantium*	Italy (Sicily)		**38**	**f**	**w**	**n**	**d**	**g**	**b**	**x**	**e**	**p**
4C1	*Citrus aurantium*	Italy (Sicily)		**39**	**f**	**b**	**i**	**d**	**g**	**b**	**x**	**e**	k
PH168	*Citrus spp*	Tunisia	A1	**40**	**f**	**e**	**c**	**c**	**g**	**c**	**p**	**h**	**p**
2B4	*Citrus aurantium*	Italy (Sicily)		**41**	**g**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cicl1A	*Cyclamen spp*	Italy (Sicily)	A1	**42**	**h**	**af**	**p**	**j**	**i**	**n**	**d**	**w**	m
IRF5	*Polygala myrtifolia*	Italy (Liguria)		**43**	**h**	**u**	**i**	**u**	**h**	**n**	**c**	**p**	j
A364	*Theobroma cacao*	Cuba	A2	**44**	**i**	**g**	**j**	**q**	**i**	**g**	**r**	**e**	a

lavb2a	*Lavandula spp*	Italy (Apulia)		**45**	**i**	**v**	**j**	**n**	**i**	**n**	**l**	**l**	b
kvb	*Howea spp*	Italy (Sicily)	A2	**46**	**j**	**h**	**q**	**l**	**i**	**r**	**r**	**ad**	h
MP5RAD	*Myrtus communis*	Italy (Sicily)	A2	**47**	**k**	**q**	**f**	**ae**	**e**	**n**	**i**	**f**	**n**
A95607	*Lavandula spp.*	Italy (Sicily)		**48**	**l**	**ab**	**a**	**ab**	**b**	**j**	**f**	**x**	**n**
CM2A	*Convolvulus mauritanicus*	Italy (Apulia)		**49**	**l**	**u**	**j**	**ac**	**e**	**i**	**b**	**u**	i
CM4B	*Convolvulus mauritanicus*	Italy (Apulia)		**50**	**l**	**u**	**j**	**ac**	**e**	**i**	**b**	**p**	i
cm4a	*Convolvulus mauritanicus*	Italy (Apulia)		**50**	**l**	**u**	**j**	**ac**	**e**	**i**	**b**	**p**	i
cm2c	*Convolvulus mauritanicus*	Italy (Apulia)		**50**	**l**	**u**	**j**	**ac**	**e**	**i**	**b**	**p**	i
A149	*Lycopersicon esculentum*	Spain	A2	**51**	**m**	**j**	**j**	**i**	**e**	**b**	**i**	**f**	g
A65303	*Choisya ternata*	Italy (Liguria)	A2	**51**	**m**	**j**	**j**	**i**	**e**	**b**	**i**	**f**	g
F10	*Lycopersicon esculentum*	Italy (Lazio)		**52**	**m**	**l**	**h**	**p**	**h**	**h**	**m**	**i**	g
F11B	*Lycopersicon esculentum*	Italy (Lazio)		**53**	**m**	**m**	**c**	**d**	**h**	**h**	**m**	**i**	g
nicmel1	*Solanum melongena*	Italy (Sicily)		**54**	**m**	**j**	**j**	**i**	**i**	**b**	**k**	**e**	n
nicpom	*Lycopersicon esculentum*	Italy (Sicily)		**55**	**m**	**j**	**j**	**x**	**i**	**b**	**m**	**e**	g
P1	*Lycopersicon esculentum*	Italy (Lazio)		**56**	**n**	**l**	**j**	**x**	**h**	**h**	**m**	**i**	g
P1577	*Citrus spp*	California	A1	**57**	**o**	**c**	**i**	**x**	**e**	**f**	**i**	**h**	**r**
A34203	*Limonium sinensis*	Italy (Liguria)	A2	**58**	**p**	**ae**	**c**	**r**	**b**	**j**	**i**	**r**	h
tl8v	*Lavandula spp*	Italy (Piedmont)	A2	**59**	**q**	**u**	**s**	**y**	**c**	**j**	**i**	**q**	**s**
lav8v	*Lavandula spp*	Italy (Sicily)	A2	**59**	**q**	**u**	**s**	**y**	**c**	**j**	**i**	**q**	**s**
c88	*Simmosia chinensis*	Italy (Apulia)	A2	**59**	**q**	**u**	**s**	**y**	**c**	**j**	**i**	**q**	**s**
A28300	nd	Italy (Liguria)		**59**	**q**	**u**	**s**	**y**	**c**	**j**	**i**	**q**	**s**
24STA	*Rhamnus alathernus*	italy (Sicily)	A2	**60**	**r**	**n**	**b**	**i**	**i**	**f**	**o**	**l**	e
c301	*Myrtus communis*	Italy (Sicily)	A2	**61**	**r**	**o**	**i**	**h**	**i**	**h**	**z**	**e**	g
H02	*Vanilla spp*	FrenchPolynesia		**62**	**r**	**t**	**f**	**ad**	**g**	**b**	**g**	**g**	b
irf27	*Agapanthus spp*	Italy (Liguria)	A2	**63**	**r**	**j**	**i**	**x**	**h**	**h**	**m**	**i**	g
nicmel	*Solanum melongena*	Italy (Sicily)	A2	**64**	**r**	**j**	**i**	a*	**e**	**b**	**k**	**f**	**n**
PEPGJ	*Capsicum annuum*	Italy (Calabria)	A1	**65**	**r**	**o**	**i**	**h**	**i**	**h**	**z**	**e**	g
scrp465	*Lycopersicon esculentum*	Chile		**66**	**r**	**j**	**i**	**i**	**e**	**b**	**i**	**f**	**n**
MagniRa	*Aeonium arboreum*	Italy (Sicily)		**67**	**s**	**u**	**r**	**x**	**h**	**h**	**f**	**s**	i
peprc	*Capsicum annuum*	Italy (Calabria)	A2	**68**	**s**	**o**	**j**	**h**	**i**	**h**	**z**	**e**	g
anthur2	*Anthurium spp*	Italy (Sicily)		**69**	**t**	**ad**	**t**	**q**	**h**	**d**	**w**	**a**	j
anthur	*Anthurium spp*	Italy (Sicily)	A1	**69**	**t**	**ad**	**t**	**q**	**h**	**d**	**w**	**a**	j
pandorea2c	*Pandoreajasminoides*	Italy (Sicily)	A2	**69**	**t**	**ad**	**t**	**q**	**h**	**d**	**w**	**a**	j
pf2g	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf2f	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**

pf2e	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf2d	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf2c	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf2b	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf2a	*Citrus maxima*	Vietnam (ThotNot)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1p	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1o	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1m	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1h	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1f	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1e	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1d	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1c	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1b	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
pf1a	*Citrus maxima*	Vietnam (MoCay)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
CH230	*Citrus maxima*	Vietnam (DongNai)	A2	**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
CH229	*Citrus reticulata*	Vietnam (DongNai)		**70**	**t**	**c**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
P1569	*Citrus spp*	California	A1	**71**	**t**	**c**	**c**	**f**	**b**	**b**	**y**	**e**	**p**
pf1g	*Citrus maxima*	Vietnam (MoCay)		**72**	**t**	**c**	**i**	**g**	**b**	**b**	**x**	**e**	**p**
pf1n	*Citrus maxima*	Vietnam (MoCay)		**73**	**t**	**d**	**c**	**g**	**b**	**b**	**x**	**e**	**p**
CH281	*Citrus aurantifolia*	Vietnam (CaoPhong)	A1	**74**	**t**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
CH280	*Citrus aurantium*	Vietnam (CaoPhong)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
CH237	*Citrus maxima*	Vietnam (Bihuong)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
CH236	*Citrus maxima*	Vietnam (Bihuong)	A2	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
CH233	*Citrus maxima*	Vietnam (DongNai)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
CH231	*Citrus maxima*	Vietnam (DongNai)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
d929	*Citrus jambhiri*	Philippines	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ph3	*Citrus clementina*	n.d.	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
serrav4	*Citrus aurantium*	Italy (Sicily)	A2	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
serrav2	*Citrus aurantium*	Italy (Sicily)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
serrav1	*Citrus aurantium*	Italy (Sicily)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
RADSIJ	*Citrus aurantium*	Italy (Calabria)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
phtast2	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ph87	*Citrus clementina*	Italy (Calabria)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ferrar6	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**

ferrar5	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ferrar3	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ferrar11	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ferrar10	*Citrus aurantium*	Italy (Sicily)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
ferrar1	*Citrus aurantium*	Italy (Sicily)	A1	**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
e2at	*Citrus clementina*	Italy (Calabria)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr8a2	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr8a1	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr8a	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr7d	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr6c	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr3b	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr1c	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
cedr10a	*Citrus medica*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
4B1	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
4A4	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
4A1	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
3B	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
2B3	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
2A4	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
2A	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
1C1	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
1B2	*Citrus aurantium*	Italy (Sicily)		**75**	**u**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
3A1	*Citrus aurantium*	Italy (Sicily)		**76**	**u**	**d**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
CH228	*Citrus maxima*	Vietnam (DongNai)	A2	**77**	**u**	**c**	**c**	**e**	**g**	**b**	**x**	**e**	**o**
CH235	*Citrus maxima*	Vietnam (DongNai)		**78**	**u**	**c**	**c**	**d**	**g**	**b**	**w**	**e**	**p**
CH232	*Citrus maxima*	Vietnam (DongNai)	A2	**78**	**u**	**c**	**c**	**d**	**g**	**b**	**w**	**e**	**p**
P1452	*Citrus spp*	California	A1	**79**	**u**	**c**	**i**	**b**	**b**	**b**	**x**	**e**	k
m1r1c	*Myrtus communis*	Italy (Sardinia)		**80**	**u**	**f**	**l**	**z**	**i**	**k**	**i**	**c**	**p**
PH142	*Poncirus trifoliata*	Albania	A1	**81**	**u**	**c**	**d**	**d**	**g**	**b**	**ab**	**e**	**p**
PH195	*Citrus spp*	Syria	A1	**82**	**u**	**c**	**e**	**i**	**g**	**b**	**x**	**e**	**p**
ph5	*Citrus spp*	Italy (Apulia)	A1	**83**	**u**	**c**	**c**	**x**	**g**	**b**	**x**	**e**	**p**
ph9	*Citrus spp*	Italy (Apulia)	A1	**84**	**u**	**c**	**i**	**d**	**g**	**b**	**x**	**e**	**p**
serrav3	*Citrus aurantium*	Italy (Sicily)	A2	**85**	**u**	**c**	**n**	**d**	**g**	**b**	**x**	**e**	**p**
VinICa	nd	Italy (Sicily)		**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l

LAVCB	*Lavandula spp.*	Italy (Sicily)	A1	**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l
LAVCA	*Lavandula spp.*	Italy (Sicily)	A1	**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l
LAV5	*Lavandula spp.*	Italy (Sicily)		**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l
lav2Ra	*Lavandula spp.*	Italy (Sicily)		**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l
IRF4	*Polygala myrtifolia*	Italy (Liguria)	A2	**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l
IRF3	*Polygala myrtifolia*	Italy (Liguria)	A2	**86**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**d**	l
irf26	nd	Italy (Liguria)		**86**	**v**	**r**	**s**	**o**	**i**	**o**	**f**	**d**	l
irf26p1	nd	Italy (Liguria)		**87**	**v**	**r**	**r**	**o**	**i**	**o**	**h**	**a**	l
LAV6	*Lavandula spp.*	Italy (Sicily)		**88**	**v**	**r**	**r**	**o**	**i**	**o**	**f**	**u**	l
3A	*Citrus aurantium*	Italy (Sicily)		**89**	**w**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
2B2	*Citrus aurantium*	Italy (Sicily)		**89**	**w**	**c**	**c**	**d**	**g**	**b**	**x**	**e**	**p**
LAV4	*Lavandula spp.*	Italy (Sicily)		**90**	**w**	**p**	**j**	**ac**	**i**	**o**	**a**	**v**	i
lav3c	*Lavandula spp.*	Italy (Sicily)		**90**	**w**	**p**	**j**	**ac**	**i**	**o**	**a**	**v**	i
LAV3	*Lavandula spp.*	Italy (Sicily)		**90**	**w**	**p**	**j**	**ac**	**i**	**o**	**a**	**v**	i
LAV1	*Lavandula spp.*	Italy (Sicily)		**90**	**w**	**p**	**j**	**ac**	**i**	**o**	**a**	**v**	i
HVBCA2	*Hebe Veronica buxifolia*	Italy (Sicily)		**90**	**w**	**p**	**j**	**ac**	**i**	**o**	**a**	**v**	i
HVBCA1	*Hebe Veronica buxifolia*	Italy (Sicily)		**90**	**w**	**p**	**j**	**ac**	**i**	**o**	**a**	**v**	i
scrp462	*FragrariaXananassa*	India India	A1	**91**	**w**	**o**	**t**	**j**	**i**	**h**	**ac**	**b**	n
m1r2a	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f2f	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f2c	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f2b	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f2a	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f1g	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f1e	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f1c	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f1b	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m1f1a	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m13r1a	*Myrtus communis*	Italy (Sardinia)		**92**	**x**	**q**	**s**	**w**	**e**	**f**	**i**	**s**	**s**
m13r1b	*Myrtus communis*	Italy (Sardinia)		**93**	**x**	**f**	**l**	**z**	**h**	**k**	**i**	**c**	**q**
m1f1f	*Myrtus communis*	Italy (Sardinia)		**94**	**x**	**q**	**i**	**w**	**e**	**f**	**i**	**s**	**s**
m1r2d	*Myrtus communis*	Italy (Sardinia)		**95**	**x**	**f**	**l**	**z**	**h**	**k**	**i**	**c**	**p**
m1r1e	*Myrtus communis*	Italy (Sardinia)		**95**	**x**	**f**	**l**	**z**	**h**	**k**	**i**	**c**	**p**
m1r1a	*Myrtus communis*	Italy (Sardinia)		**95**	**x**	**f**	**l**	**z**	**h**	**k**	**i**	**c**	**p**
m1r1b	*Myrtus communis*	Italy (Sardinia)		**96**	**x**	**f**	**l**	**z**	**h**	**g**	**i**	**c**	**p**

m6r1	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r4f	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r4e	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r4c	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r4b	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r4a	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r2b	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r1d	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r1c	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m5r1b	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m1r2b	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m1r1d	*Myrtus communis*	Italy (Sardinia)		**97**	**x**	**q**	**s**	**w**	**e**	**j**	**i**	**s**	**s**
m1r2e	*Myrtus communis*	Italy (Sardinia)		**98**	**x**	**q**	**s**	**w**	**i**	**f**	**i**	**s**	**s**
ROSM5B	*Rosmarinus spp*	Italy (Sicily)		**99**	**x**	**p**	**s**	**af**	**h**	**h**	**u**	**r**	**n**
LAV2	*Lavandula spp.*	Italy (Sicily)		**100**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**m**	**n**
chamca	*Chamaleucium uncinatum*	Italy (Sicily)	A2	**100**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**m**	**n**
albic9	*Prunus armeniaca*	Italy (Sicily)	A2	**100**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**m**	**n**
dodrad4	*Dodonaea viscosa purpurea*	Italy (Sicily)	A2	**101**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**n**	**n**
dodrad1	*Dodonaea viscosa purpurea*	Italy (Sicily)	A2	**101**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**n**	**n**
dodcoll1	*Dodonaea viscosa purpurea*	Italy (Sicily)	A2	**101**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**n**	**n**
dod5	*Dodonaea viscosa purpurea*	Italy (Sicily)	A2	**101**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**n**	**n**
dod2	*Dodonaea viscosa purpurea*	Italy (Sicily)	A2	**101**	**y**	**ac**	**f**	**ad**	**b**	**k**	**q**	**n**	**n**
P0582	*Nicotiana tabacum*	Kentucky	A2	**102**	**z**	**i**	**g**	**t**	**e**	**j**	**t**	**e**	j
P1335	*Nicotiana tabacum*	Virginia	A2	**103**	**aa**	**i**	**g**	**v**	**e**	**j**	**t**	**e**	j
P1334	*Nicotiana tabacum*	Virginia	A2	**103**	**aa**	**i**	**g**	**v**	**e**	**j**	**t**	**e**	j
P1333	*Nicotiana tabacum*	Virginia	A2	**103**	**aa**	**i**	**g**	**v**	**e**	**j**	**t**	**e**	j
A310	*Nicotiana tabacum*	Australia	A1	**104**	**ab**	**z**	**h**	**p**	**h**	**q**	**l**	**ac**	f
P1752	*Nicotiana tabacum*	Australia	A1	**105**	**ab**	**aa**	**h**	**p**	**h**	**l**	**l**	**aa**	f
P1751	*Nicotiana tabacum*	Australia	A1	**106**	**ab**	**z**	**h**	**p**	**h**	**l**	**l**	**aa**	f
P1753	*Nicotiana tabacum*	Australia	A1	**107**	**ab**	**z**	**h**	**p**	**h**	**q**	**l**	**ab**	f
P1494	*Nicotiana tabacum*	Australia	A2	**108**	**ab**	**z**	**b**	**p**	**h**	**l**	**l**	**aa**	f
PH121	*Nicotiana tabacum*	Virginia	A1	**109**	**ac**	**i**	**c**	**t**	**e**	**j**	**t**	**e**	j
PH122	*Nicotiana tabacum*	Virginia	A2	**110**	**ac**	**i**	**c**	**t**	**b**	**j**	**t**	**e**	j
PH125	*Nicotiana tabacum*	Virginia	A2	**111**	**ac**	**i**	**g**	**t**	**e**	**j**	**t**	**e**	j
PH124	*Nicotiana tabacum*	Virginia	A2	**111**	**ac**	**i**	**g**	**t**	**e**	**j**	**t**	**e**	j

arm8b	*Armeria spp*	Italy (Apulia)		**112**	**ad**	**ae**	**i**	**r**	**e**	**p**	**x**	**q**	f
irf26p2	*Impatiens spp*	Italy (Liguria)	A2	**113**	**ad**	**h**	**j**	**l**	**h**	**m**	**r**	**x**	h
pittos	*Pittosporum spp*	Italy (Sicily)	A1	**114**	**ae**	**q**	**u**	**aa**	**e**	**f**	**i**	**t**	**s**
rosmISa	*Rosmarinus spp*	Italy (Sicily)		**115**	**ae**	**af**	**j**	**ab**	**i**	**h**	**a**	**h**	i
P0583	*Nicotiana tabacum*	Kentucky	A2	**116**	**af**	**x**	**q**	**al**	**e**	**j**	**e**	**e**	d
PN23	*Nicotiana tabacum*	GEORGIA		**117**	**ag**	**x**	**o**	**am**	**e**	**j**	**e**	**e**	c
scrp468	*Citrus spp*	Trinidad&Tobago	A1	**118**	**ah**	**j**	**e**	**ah**	**g**	**f**	**q**	**e**	n
P1350	*Nicotiana tabacum*	NorthCarolina	A1	**119**	**ai**	**x**	**o**	**am**	**e**	**j**	**e**	**e**	c
pn26	*Nicotiana tabacum*	GEORGIA		**120**	**ai**	**y**	**o**	**an**	**e**	**j**	**e**	**e**	c
A329	*Nicotiana tabacum*	Greece	A2	**121**	**al**	**x**	**j**	**ac**	**e**	**j**	**e**	**e**	c
PH123	*Nicotiana tabacum*	Virginia	A1	**122**	**am**	**x**	**o**	**am**	**e**	**j**	**e**	**e**	c
correa9	*Correa reflexa*	Italy (Sicily)	A2	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa8	*Correa reflexa*	Italy (Sicily)	A1	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa5	*Correa reflexa*	Italy (Sicily)	A1	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa4	*Correa reflexa*	Italy (Sicily)	A1	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa3	*Correa reflexa*	Italy (Sicily)	A1	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa2	*Correa reflexa*	Italy (Sicily)		**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa12	*Correa reflexa*	Italy (Sicily)	A1	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa11	*Correa reflexa*	Italy (Sicily)	A1	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa10	*Correa reflexa*	Italy (Sicily)	A2	**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa1	*Correa reflexa*	Italy (Sicily)		**123**	**an**	**f**	**j**	**m**	**h**	**h**	**l**	**e**	n
correa6	*Correa reflexa*	Italy (Sicily)	A1	**124**	**an**	**f**	**j**	**m**	**h**	**n**	**l**	**e**	n
ROSM6A	*Rosmarinus spp*	Italy (Sicily)		**125**	**ao**	**ae**	**m**	**ac**	**c**	**n**	**x**	**f**	**j**
ROSM4A	*Rosmarinus spp*	Italy (Sicily)		**125**	**ao**	**ae**	**m**	**ac**	**c**	**n**	**x**	**f**	**j**
CH270	*Citrus aurantium*	Vietnam (HamYen)	A2	**126**	**ap**	**c**	**c**	**d**	**g**	**b**	**n**	**e**	**p**
CH271	*Citrus aurantium*	Vietnam (HamYen)	A2	**127**	**ap**	**c**	**v**	**d**	**g**	**b**	**n**	**e**	**p**
CH275	*Citrus reticulata*	Vietnam (CaoPhong)	A1	**128**	**ap**	**c**	**h**	**d**	**g**	**b**	**n**	**e**	**p**
CH272	*Citrus aurantium*	Vietnam (HamYen)	A1	**128**	**ap**	**c**	**h**	**d**	**g**	**b**	**n**	**e**	**p**
1B42	*Citrus aurantium*	Italy (Sicily)		**129**	**aq**	**d**	**c**	**d**	**g**	**b**	**x**	**e**	**p**

Interestingly, some of the isolates analyzed showed a condition of "mixed ploidy", with more than two alleles detected at one or more SSR loci (Table 4). In particular, locus 1129 exhibited three different alleles for 5 isolates: the genotype "d" (139/148/154) was found for three different isolates (Anthurium, Anthurium2 and Pandorea 2C) of the MLG69, all recovered from ornamental plants in Sicily. Locus 788 exhibited different levels of ploidy for two isolates, CM5C and Ph142. The latter isolate showed a condition of tetraploidy (127/129/137/139), which was also revealed for the locus 17 (123/135/139/144). Condition of tetraploidy (105/129/132/144) was also discovered for the isolate GL3, while isolate H02 exhibited a condition of triploidy. Only 1 isolate exhibited triploidy for the locus 2039 (4A2), while 4 different triploid genotypes were discovered for the locus 15, where the genotype "d" was present for three different isolates (PF1N, 3A1 and 1B42), all collected from *Citrus* species. Finally, locus 1509 showed 6 different triploid genotypes, and the genotype "q" was revealed for 4 different isolates (283/00, TL8V, Lav8v, C88), all collected in Italy from different ornamental plants. Among the different loci with a condition of different ploidy, the isolate Ph168 exhibited three triploid loci (P15, P1129 and P17).

Table 4: Complete list of detected nuclear multilocus genotypes (MLG) and locus-specific genotypes with specific fragments detected for each MLG.

MLG	ssr1509	code	ssr15	code	ssr788	code	ssr643	code	ssr2039	code	ssr1129	code	ssr17	code	ssr5	code	ssr2040	code
1	0	a	75/81	k	131/135	o	155/178	k	99/120	h	133/139/154	a	126/144	s	190/194	f	158/158	n
2	0	a	75/78	j	129/135	j	164/164	x	99/120	h	139/154	h	105/159	m	190/210	i	152/158	g
3	0	a	66/78	f	129/137	l	164/168	z	99/99	i	151/154	k	105/129	i	186/194	c	158/164	p
4	0	a	66/93	h	135/135	q	158/160	l	99/120	h	163/163	r	126/129	r	210/226	y	152/164	h
5	116/116	b	66/93	h	135/135	q	158/160	l	99/120	h	151/163	m	126/129	r	210/226	y	152/164	h
6	116/116	b	78/96	u	129/129	i	164/166	y	111/120	c	154/154	n	105/144	l	194/194	o	155/158	j
7	116/116	b	78/96	u	129/129	i	164/166	y	99/120	h	154/154	n	105/144	l	194/194	o	155/158	j
8	116/116	b	93/96	ae	129/129	i	160/168	s	99/120	h	151/154	k	129/144	x	190/226	k	155/164	k
9	116/116	b	78/90	s	129/137	l	166/190	ag	99/99	i	154/154	n	126/144	s	190/202	h	155/158	j
10	116/116	b	66/93	h	129/137	l	164/168	z	120/120	d	151/154	k	129/132	v	194/194	o	158/164	p
11	116/116	b	78/90	s	135/137	r	168/174	ai	99/120	h	139/154	h	126/129	r	194/202	p	158/161	o
12	116/116	b	78/90	s	129/135/137	k	168/174	ai	99/120	h	139/154	h	126/129	r	194/202	p	158/161	o
13	116/116	b	96/96	af	129/137	l	164/166	y	99/99	i	139/151	f	132/144	aa	186/202	d	155/158	j
14	116/116	b	66/93	h	129/137	l	164/168	z	0	a	151/154	k	105/129/132/144	j	194/194	o	158/164	p
15	116/116	b	78/90	s	129/137	l	166/190	ag	99/99	i	154/154	n	126/144	s	190/202	h	155/158	j
16	116/116	b	66/93	h	127/135	e	160/164	q	99/99	i	139/148/163	e	126/129	r	194/194	o	152/155	f
17	116/116	b	78/78	r	129/137	l	155/164	j	99/120	h	154/154	n	105/105	f	190/194	f	158/167	q
18	116/116	b	78/99	v	129/135	j	158/164	n	99/99	i	154/154	n	105/144	l	190/230	l	149/155	b
19	116/116	b	66/78	f	129/137	l	164/168	z	99/120	h	151/154	k	105/129	i	186/194	c	158/164	p
20	116/116	b	66/78	f	129/137	l	164/168	z	99/120	h	151/154	k	105/129	i	194/194	o	158/164	p
21	116/116	b	75/96	p	129/129	i	166/168	ac	99/99	i	154/160	o	102/105	a	202/230	v	155/155	i
22	116/116	b	66/93	h	135/135	q	158/160	l	99/120	h	151/163	m	126/129	r	210/226	y	152/164	h
23	116/116	b	78/93	t	129/135	j	158/166	o	111/120	c	139/163	i	105/144	l	190/194	f	152/158	g
24	116/116	b	78/93	t	129/135	j	158/166	o	120/120	d	139/163	i	105/144	l	190/194	f	152/158	g
25	116/116	b	78/96	u	129/137	l	164/166	y	99/120	h	154/154	n	126/144	s	190/190	e	155/155	i
26	116/118	c	75/78	j	127/135	e	166/178	ae	99/99	i	151/154	k	105/129	i	210/230	z	158/164	p

MLG	ssr1509	code	ssr15	code	ssr788	code	ssr643	code	ssr2039	code	ssr1129	code	ssr17	code	ssr5	code	ssr2040	code
27	116/118	c	75/78	j	127/135	e	164/164	x	99/120	h	139/154	h	105/129	i	190/214	j	152/152	e
28	116/118	c	75/78	j	129/135	j	164/164	x	99/120	h	139/154	h	105/129	i	190/214	j	152/152	e
29	116/118	c	78/93	t	129/135	j	166/168	ac	111/120	c	139/163	i	105/144	l	190/194	f	152/158	g
30	116/118	c	75/78	j	127/135	e	164/178	aa	99/120	h	151/154	k	105/132	k	190/210	i	158/164	p
31	116/118	c	75/78	j	127/135	e	164/164	x	99/111	e	139/151	f	105/129	i	194/230	s	164/164	s
32	116/118	c	75/78	j	127/135	e	164/164	x	99/111	e	151/151	j	105/129	i	194/230	s	164/164	s
33	116/120	d	0	a	129/135	j	166/168	ac	99/99	i	139/139	b	129/144	x	190/230	l	158/158	n
34	116/122	e	66/75	c	127/129	c	148/172	g	111/111	b	139/139	b	129/144	x	190/190	e	158/164	p
35	116/124	f	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
36	116/124	f	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	126/129	r	190/190	e	155/164	k
37	116/124	f	66/75	c	127/129	c	148/162	d	99/111/114	f	139/139	b	129/144	x	190/190	e	155/164	k
38	116/124	f	84/84	w	129/143	n	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
39	116/124	f	66/66	b	129/129	i	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	155/164	k
40	116/124	f	66/75/93	e	127/129	c	148/160	c	99/114	g	139/148/151	c	123/135/139/144	p	190/202	h	158/164	p
41	116/124/126	g	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
42	116/126/144	h	96/96	af	133/137	p	155/164	j	99/99	i	154/154	n	102/144	d	202/246	w	155/170	m
43	116/126/144	h	78/96	u	129/129	i	162/166	u	99/120	h	154/154	n	102/129	c	194/202	p	155/158	j
44	116/130	i	66/84/96	g	129/135	j	160/164	q	99/99	i	139/151/154	g	126/129	r	190/190	e	143/155	a
45	116/130	i	78/99	v	129/135	j	158/164	n	99/99	i	154/154	n	105/144	l	190/230	l	149/155	b
46	116/132	j	66/93	h	135/135	q	158/160	l	99/99	i	163/163	r	126/129	r	226/226	ad	152/164	h
47	116/144	k	78/108	q	127/137	f	166/178	ae	99/111	e	154/154	n	105/129	i	190/194	f	158/158	n
48	118/118	l	90/90	ab	121/135	a	166/166	ab	111/111	b	151/151	j	105/105	f	210/210	x	158/158	n
49	118/118	l	78/96	u	129/135	j	166/168	ac	99/111	e	139/163	i	102/126	b	202/202	u	155/155	i
50	118/118	l	78/96	u	129/135	j	166/168	ac	99/111	e	139/163	i	102/126	b	194/202	p	155/155	i
51	118/120	m	75/78	j	129/135	j	152/168	i	99/111	e	139/139	b	105/129	i	190/194	f	152/158	g
52	118/120	m	75/84	l	127/143	h	158/168	p	99/120	h	139/154	h	105/159	m	190/210	i	152/158	g
53	118/120	m	75/84/96	m	127/129	c	148/162	d	99/120	h	139/154	h	105/159	m	190/210	i	152/158	g
54	118/120	m	75/78	j	129/135	j	152/168	i	99/99	i	139/139	b	105/132	k	190/190	e	158/158	n

MLG	ssr1509	code	ssr15	code	ssr788	code	ssr643	code	ssr2039	code	ssr1129	code	ssr17	code	ssr5	code	ssr2040	code
55	118/120	m	75/78	j	129/135	j	164/164	x	99/99	i	139/139	b	105/159	m	190/190	e	152/158	g
56	118/122	n	75/84	l	129/135	j	164/164	x	99/120	h	139/154	h	105/159	m	190/210	i	152/158	g
57	118/130	o	66/75	c	129/129	i	164/164	x	99/111	e	139/151	f	105/129	i	190/202	h	161/164	r
58	118/142	p	93/96	ae	127/129	c	160/166	r	111/111	b	151/151	j	105/129	i	194/226	r	152/164	h
59	118/168/170	q	78/96	u	135/139	s	164/166	y	111/120	c	151/151	j	105/129	i	194/210	q	164/164	s
60	120/120	r	75/90	n	127/127	b	152/168	i	99/99	i	139/151	f	114/159	o	190/230	l	152/152	e
61	120/120	r	75/93	o	129/129	i	152/164	h	99/99	i	139/154	h	129/159	z	190/190	e	152/158	g
62	120/120	r	78/93	t	127/137	f	166/174	ad	99/114	g	139/139	b	105/120/129	g	190/198	g	149/155	b
63	120/120	r	75/78	j	129/129	i	164/164	x	99/120	h	139/154	h	105/159	m	190/210	i	152/158	g
64	120/120	r	75/78	j	129/129	i	0	a	99/111	e	139/139	b	105/132	k	190/194	f	158/158	n
65	120/120	r	75/93	o	129/129	i	152/164	h	99/99	i	139/154	h	129/159	z	190/190	e	152/158	g
66	120/120	r	75/78	j	129/129	i	152/168	i	99/111	e	139/139	b	105/129	i	190/194	f	158/158	n
67	120/126	s	78/96	u	135/137	r	164/164	x	99/120	h	139/154	h	105/105	f	194/230	s	155/155	i
68	120/126	s	75/93	o	129/135	j	152/164	h	99/99	i	139/154	h	129/159	z	190/190	e	152/158	g
69	122/122	t	93/93	ad	137/137	t	160/164	q	99/120	h	139/148/154	d	129/141	w	186/186	a	155/158	j
70	122/122	t	66/75	c	127/129	c	148/172	g	111/111	b	139/139	b	129/144	x	190/190	e	158/164	p
71	122/122	t	66/75	c	127/129	c	148/170	f	111/111	b	139/139	b	129/147	y	190/190	e	158/164	p
72	122/122	t	66/75	c	129/129	i	148/172	g	111/111	b	139/139	b	129/144	x	190/190	e	158/164	p
73	122/122	t	66/75/84	d	127/129	c	148/172	g	111/111	b	139/139	b	129/144	x	190/190	e	158/164	p
74	122/122	t	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
75	124/124	u	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
76	124/124	u	66/75/84	d	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
77	124/124	u	66/75	c	127/129	c	148/164	e	99/114	g	139/139	b	129/144	x	190/190	e	158/161	o
78	124/124	u	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	129/141	w	190/190	e	158/164	p
79	124/124	u	66/75	c	129/129	i	148/148	b	111/111	b	139/139	b	129/144	x	190/190	e	155/164	k
80	124/124	u	66/78	f	129/137	l	164/168	z	99/99	i	151/154	k	105/129	i	186/194	c	158/164	p
81	124/124	u	66/75	c	127/129/137/139	d	148/162	d	99/114	g	139/139	b	135/144	ab	190/190	e	158/164	p
82	124/124	u	66/75	c	127/135	e	152/168	i	99/114	g	139/139	b	129/144	x	190/190	e	158/164	P

MLG	ssr1509	code	ssr15	code	ssr788	code	ssr643	code	ssr2039	code	ssr1129	code	ssr17	code	ssr5	code	ssr2040	code
83	124/124	u	66/75	c	127/129	c	164/164	x	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
84	124/124	u	66/75	c	129/129	i	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
85	124/124	u	66/75	c	129/143	n	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
86	124/126	v	78/78	r	135/137	s	158/166	o	99/99	i	154/160	o	105/105	f	186/202	d	155/167	l
87	124/126	v	78/78	r	135/137	r	158/166	o	99/99	i	154/160	o	105/126	h	186/186	a	155/167	l
88	124/126	v	78/78	r	135/137	r	158/166	o	99/99	i	154/160	o	105/105	f	202/202	u	155/167	l
89	126/126	w	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p
90	126/126	w	75/96	p	129/135	j	166/168	ac	99/99	i	154/160	o	102/105	a	202/230	v	155/155	i
91	126/126	w	75/93	o	137/137	t	155/164	j	99/99	i	139/154	h	141/147	ac	186/190	b	158/158	n
92	126/144	x	78/108	q	135/139	s	162/178	w	99/111	e	139/151	f	105/129	i	194/230	s	164/164	s
93	126/144	x	66/78	f	129/137	l	164/168	z	99/120	h	151/154	k	105/129	i	186/194	c	158/167	q
94	126/144	x	78/108	q	129/129	i	162/178	w	99/111	e	139/151	f	105/129	i	194/230	s	164/164	s
95	126/144	x	66/78	f	129/137	l	164/168	z	99/120	h	151/154	k	105/129	i	186/194	c	158/164	p
96	126/144	x	66/78	f	129/137	l	164/168	z	99/120	h	139/151/154	g	105/129	i	186/194	c	158/164	p
97	126/144	x	78/108	q	135/139	s	162/178	w	99/111	e	151/151	j	105/129	i	194/230	s	164/164	s
98	126/144	x	78/108	q	135/139	s	162/178	w	99/99	i	139/151	f	105/129	i	194/230	s	164/164	s
99	126/144	x	75/96	p	135/139	s	166/182	af	99/120	h	139/154	h	129/129	u	194/226	r	158/158	n
100	132/132	y	93/111	ac	127/137	f	166/174	ad	111/111	b	151/154	k	126/126	q	190/234	m	158/158	n
101	132/132	y	93/111	ac	127/137	f	166/174	ad	111/111	b	151/154	k	126/126	q	190/238	n	158/158	n
102	136/142	z	75/75	i	127/139	g	160/170	t	99/111	e	151/151	j	126/165	t	190/190	e	155/158	j
103	136/144	aa	75/75	i	127/139	g	162/170	v	99/111	e	151/151	j	126/165	t	190/190	e	155/158	j
104	140/140	ab	90/120	z	127/143	h	158/168	p	99/120	h	160/160	q	105/144	l	218/268	ac	152/155	f
105	140/140	ab	90/120	z	127/143	h	158/168	p	99/120	h	151/160	l	105/144	l	214/238	aa	152/155	f
106	140/140	ab	90/123	aa	127/143	h	158/168	p	99/120	h	151/160	l	105/144	l	214/238	aa	152/155	f
107	140/140	ab	90/120	z	127/143	h	158/168	p	99/120	h	160/160	q	105/144	l	214/266	ab	152/155	f
108	140/140	ab	90/120	z	127/127	b	158/168	p	99/120	h	151/160	l	105/144	l	214/238	aa	152/155	f
109	140/142	ac	75/75	i	127/129	c	160/170	t	99/111	e	151/151	j	126/165	t	190/190	e	155/158	j
110	140/142	ac	75/75	i	127/129	c	160/170	t	111/111	b	151/151	j	126/165	t	190/190	e	155/158	J

MLG	ssr1509	code	ssr15	code	ssr788	code	ssr643	code	ssr2039	code	ssr1129	code	ssr17	code	ssr5	code	ssr2040	code
111	140/142	ac	75/75	i	127/139	g	160/170	t	99/111	e	151/151	j	126/165	t	190/190	e	155/158	j
112	142/142	ad	93/96	ae	129/129	i	160/166	r	99/111	e	154/163	p	129/144	x	194/210	q	152/155	f
113	142/142	ad	66/93	h	129/135	j	158/160	l	99/120	h	151/163	m	126/129	r	210/210	x	152/164	h
114	144/144	ae	78/108	q	139/139	u	164/178	aa	99/111	e	139/151	f	105/129	i	194/234	t	164/164	s
115	144/144	ae	96/96	af	129/135	j	166/166	ab	99/99	i	139/154	h	102/105	a	190/202	h	155/155	i
116	144/146	af	87/90	x	135/135	q	168/196	al	99/111	e	151/151	j	102/147	e	190/190	e	149/161	d
117	144/148/150	ag	87/90	x	131/135	o	172/172	am	99/111	e	151/151	j	102/147	e	190/190	e	149/158	c
118	148/148	ah	75/78	j	127/135	e	166/202	ah	99/114	g	139/151	f	126/126	q	190/190	e	158/158	n
119	148/156	ai	87/90	x	131/135	o	172/172	am	99/111	e	151/151	j	102/147	e	190/190	e	149/158	c
120	148/156	ai	87/99	y	131/135	o	174/174	an	99/111	e	151/151	j	102/147	e	190/190	e	149/158	c
121	148/160/162	al	87/90	x	129/135	j	166/168	ac	99/111	e	151/151	j	102/147	e	190/190	e	149/158	c
122	150/152	am	87/90	x	131/135	o	172/172	am	99/111	e	151/151	j	102/147	e	190/190	e	149/158	c
123	160/160	an	66/78	f	129/135	j	158/162	m	99/120	h	139/154	h	105/144	l	190/190	e	158/158	n
124	160/160	an	66/78	f	129/135	j	158/162	m	99/120	h	154/154	n	105/144	l	190/190	e	158/158	n
125	168/170	ao	93/96	ae	129/139	m	166/168	ac	111/120	c	154/154	n	129/144	x	190/194	f	155/158	j
126	174/176	ap	66/75	c	127/129	c	148/162	d	99/114	g	139/139	b	108/144	n	190/190	e	158/164	p
127	174/176	ap	66/75	c	143/143	v	148/162	d	99/114	g	139/139	b	108/144	n	190/190	e	158/164	p
128	174/176	ap	66/75	c	127/143	h	148/162	d	99/114	g	139/139	b	108/144	n	190/190	e	158/164	p
129	116/124	aq	66/75/84	d	127/129	c	148/162	d	99/114	g	139/139	b	129/144	x	190/190	e	158/164	p

3.3.2 Data analysis

In a first analysis, the 268 isolates were combined into 8 putative populations that were made up according to the host of isolation. The eight groups represented isolates from Tobacco, *Citrus (*except pommelo*)*, *Solanaceae*, *Lamiaceae*, Myrtle, *Rutaceae*, Pommelo and others species with 20, 61, 15, 28, 37, 23, 29, and 55 isolates, respectively. Specifically, the group "others species" included isolates recovered from a number of different ornamental plants. The analysis of these 8 populations with the software "POPPR" (Kamvar et al., 2013) produced the results summarized in Table 5.

Table 5: Population diversity measures for the nine populations considered

	Analised populations	N	MLG	G	Hexp	E_5	Ia
1	Tobacco	20	17	14.286	0.979	0.896	2.928
2	Citrus	61	25	3.119	0.691	0.315	2.010
3	Solanaceae	15	14	13.235	0.990	0.965	0.750
4	Others	55	36	25.420	0.978	0.826	1.547
5	Lamiaceae	28	16	11.200	0.944	0.828	2.909
6	Myrthle	37	14	5.127	0.827	0.606	4.371
7	Rutaceae	23	8	3.130	0.711	0.687	5.098
8	Pommelo	29	8	2.396	0.603	0.533	1.738
	Total	268	129	27.731	0.968	0.415	2.550

N = number of the samples

MLG = multilocus genotype

G = Stoddart and Taylor's Index of MLG diversity

Hexp= heterozygosity expected

E_5 = Measure how the genotypes are distributed on a given population

I_A = Index of association, that quantifies the degree of recombination of the loci

This preliminary approach revealed a prevalence of clonal reproduction among isolates examined. In particular, the index of association (Ia) was higher than 0.5 in all populations indicating clonality was prevalent, althogh significant differences were revealed among populations. The Stoddart and Taylor's Index (G) was used to estimate the genotypic diversity. The G index ranged between 2.396 (for Pommelo) and 25.42 (for "Others).

Although the detected number can be also influenced by the number of isolates, this data clearly indicate a higher diversity among isolates from different ornamental species. The index E_5 indicates how genotypes were distributed within a sample by scaling a diversity index (such as Shannon-Wiener or Stoddart and Taylor's Index) by the maximum number of expected genotypes. The value of E_5 could range between 0 and 1: if the measure tends to be 0, the parameter indicates that the population is composed of a single or very few predominant genotypes, while if the measure tends to 1, it means that different genotypes occur at a similar frequency. Obtained data indicated the presence of several genotypes with a similar frequency in the *Citrus* population (E_5 0.315) followed by the Pommelo population (E_5 0.533). In all other populations there was a sharp prevalence of one or few genotypes (Table 5).

To assess the possible evolutionary relationships among MLGs obtained for each isolate, minimum spanning networks (MSN) were constructed using genetic distances calculated with the method described by Bruvo et al. (2004) (Figure 2). In this figure the size of the nodes is proportional to the number of isolates represented by each MLG while line thickness and colors are inversely proportional to Bruvo's distance (the larger the distance among two nodes, the thinner and less intense is the line that connects them). Graphic results suggests a significant level of association among hosts and genotypes which was particularly evident for isolates from citrus, pommelo, myrtle and *Rutaceae*. As expected isolates from "other species" were scattered within the network. However the orange node (correlated to Citrus' MLGs) and the purple node (correlated to Pommelo's MLGs) clearly showed an excessive size which prevented the analyses of minor MLGs (appearing as smaller nodes within the larger ones).

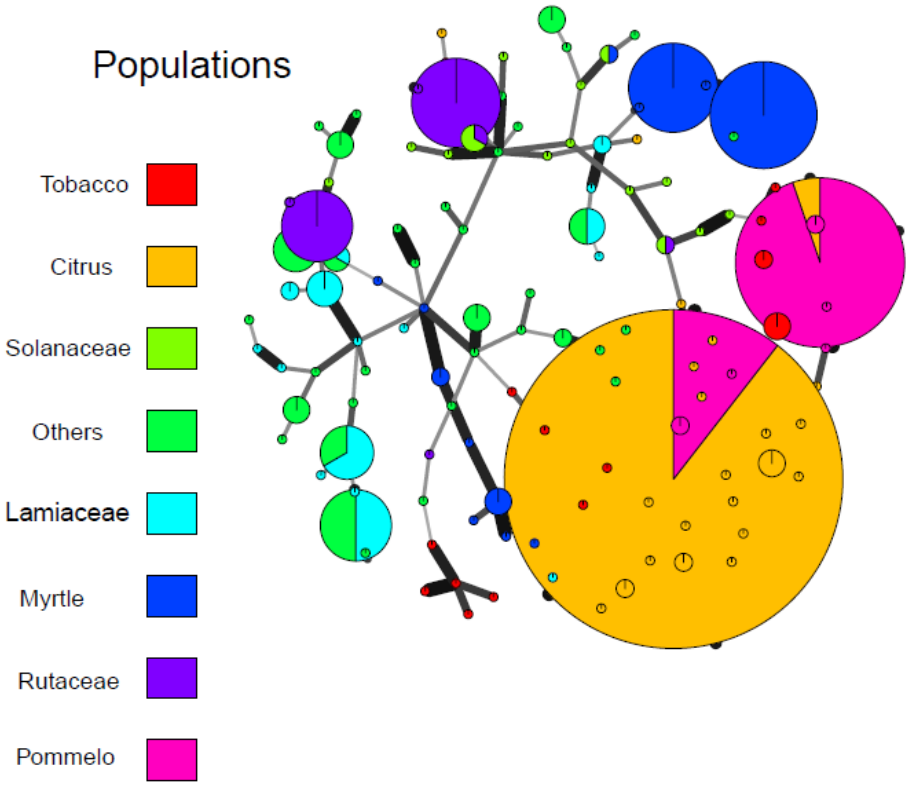

Figure 2 : Minimum spanning network of the complete panel of *Phytophthora nicotinae* isolates describing relationships among all MLGs.

To overcome the above mentioned graphical problems, 97 isolates belonging to 77 different MLGs were selected as representative of the entire population and utilized to construct a MSN in which connections among different MLGs were better visualized (Figure 3). According to this MSN most isolates from *Citrus* spp. clustered together, indicating a strong correlation among MLGs. This figure also highlight a clear distinction between isolates recovered from *Citrus* spp. and Pommelo, although a small part of isolates from the latter population were gathered with isolates from Citrus and *vice versa*. Isolates collected from Rutaceae (such as *Correa reflexa* and *Ruta Graveolens*) resulted to be quite scattered in the graph (red nodes), as well as the ones collected from ornamental plants (blue and blue black nodes, thus including also the Lamiaceae's group), while isolates recovered from *Solanaceae*

(green nodes) showed to be pretty closely related each other (they were separated from thick branches).

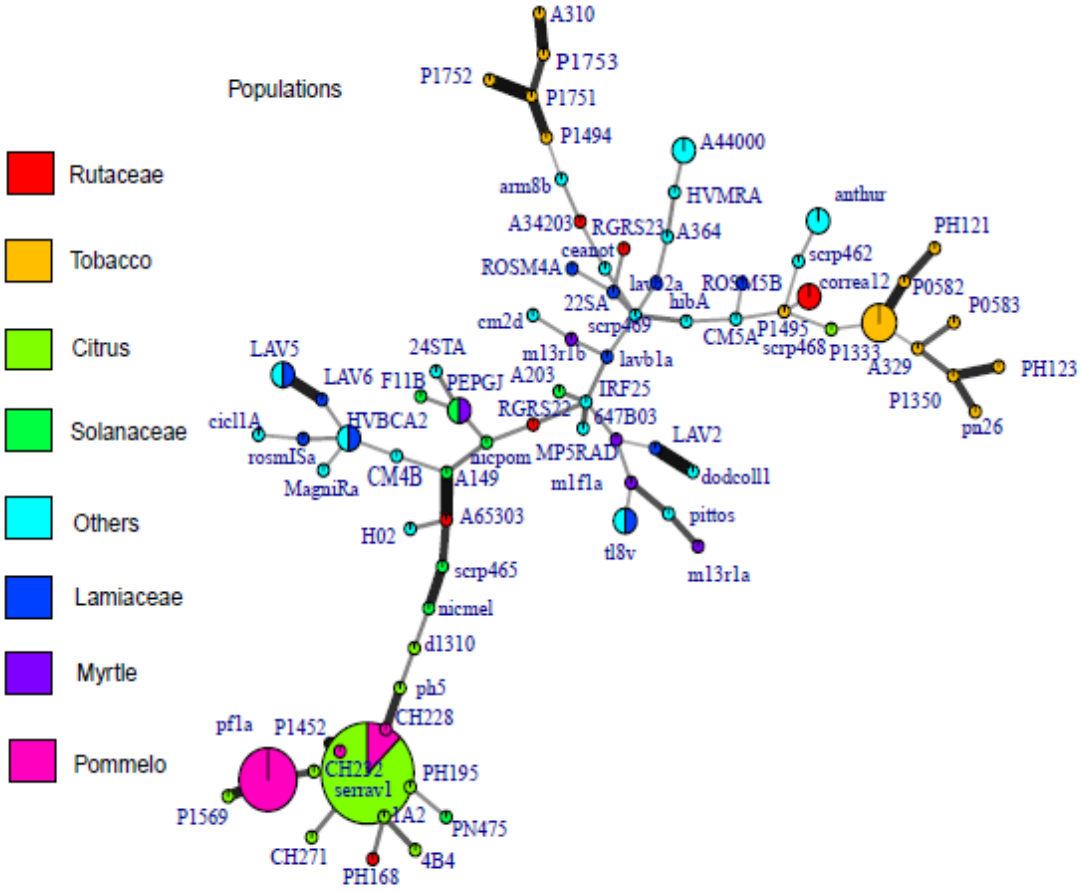

Figure 3: Minimum spanning network showing the relationships among the MLGs observed in a representative dataset of 97 isolates. Different nodes were labeled with the name of a representative isolate for each MLG.

Interestingly, isolates from Tobacco (orange nodes) were separated into two different areas. In particular, all the Australian isolates grouped in the same region, and they appeared phylogenetically distant from the group of isolates from United States. This latter group can be separated into two subgroups since separated by a thin branch, thus representing two distinct clusters.

According to the results obtained with the MSN using a subset of 97 isolates, the relationship between the MLGs from Pommelo and those obtained from other Citrus species were specifically analyzed. In particular, 89 isolates, 60 collected from *Citrus* spp. and 29 from Pommelo were assessed for studying their phylogenetic correlations (Figure 4). The number of alleles and MLGs referred to these two groups are listed in Table 6.

Table 6: Alleles detected per each locus and multi locus genotypes (MLGs) detected within "Citrus" and "Pommelo" isolates

	Locus									MLG
	P15	P788	P643	P2039	P1129	P17	P5	P1509	P2040	
Number of alleles	6	5	11	4	4	11	3	10	5	30

The MSN containing the subset of data from *Citrus* spp. and Pommelo confirmed the existence of relevant differences within these isolates, although both main groups contained few isolates from the other group (Figure 4). In fact, the most common MLG of citrus (MGL75) contained also 4 isolates collected from Pommelo, and two other isolates from this species (CH235 and CH232) appeared genotypically very close to the Citrus' group. On the other hand, the most common MLG of Pommelo (MLG70) contained 1 isolate (CH229) from *Citrus reticulata*, sourced in the same orchard along with some isolates from Pommelo.

A specific analysis was also conducted to analyzed isolates from three different nurseries and an orchard located in different Italian regions. Analyzed isolates were from a citrus orchard called "Serravalle" located in Sicily (27 isolates), a nursery called Tecnopiante located in "Apulia" (15 isolates), a nursery called "Cubeda" located in "Sicily" (24 isolates) and a nursery called "Sardegna1" located in Sardinia (35 isolates).

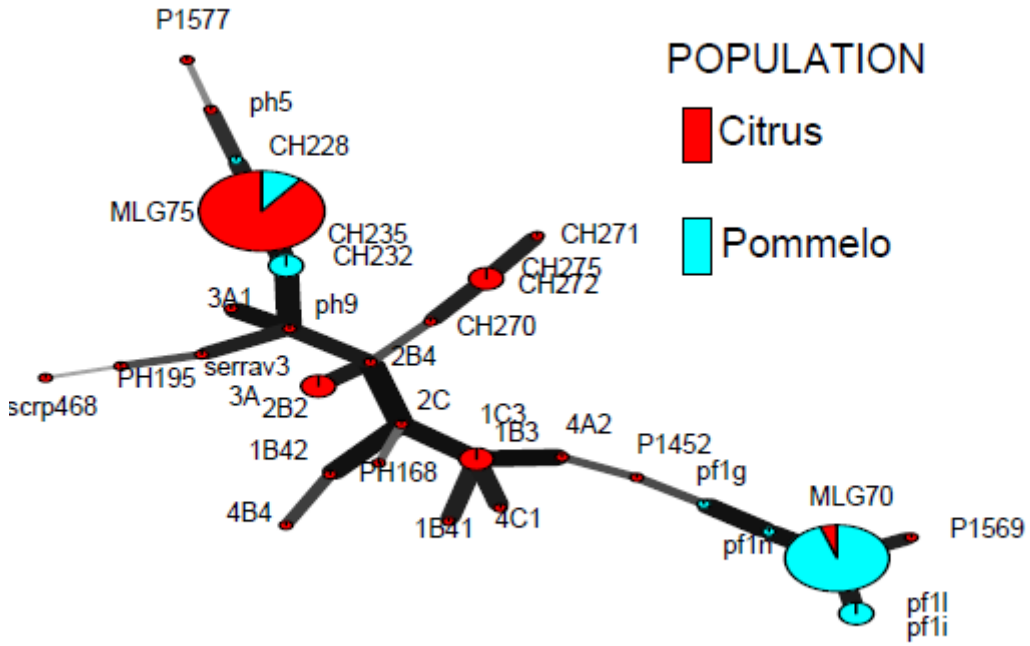

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Figure 4: Minimum spanning network showing the relationship existing among isolates obtained from pommelo and from other citrus species. The different nodes were labelled with the name of a representative isolate of the MLG.

Isolates from the nurseries "Tecnopiante" and "Cubeda" were obtained from ornamental plants, mainly *Convolvolus mauritanicus, Lavandula spp., Chamaleucium uncinatum, Ruta Graveolens* and *Rosmarinus spp.,* while only isolates from *Myrtus communis* and *Citrus spp.* were collected from the nursery "Sardegna1" and the orchard "Serravalle", respectively.

Results of the MSN revealed a close correlation among isolates from the orchard Serravalle with a sharply prevalent MLG and several related minor MLGs (Figure 5). A much higher level of genetic variability was detected within the nurseries Tecnopiante (green nodes) and Cubeda (blue nodes). In fact, isolates were dispersed in different groups and MLGs were pretty distant from each other (branches that connect them were very thin). Interestingly, MLGs of the two nurseries were not exactly clustered according to their provenience. . As an

example, MLG50 (Tecnopiante) was found to be more strictly related to MLG23 (Cubeda). Isolates of the nursery Sardegna 1 clustered together but were divided into two genetically distant groups separated by a very thin branch (Figure 5). The first group was mainly composed of two prevalent MLGs (92 and 97), while the second group comprised of several closely related MLGs. Interestingly the second group of Sardegna 1 isolates also comprised a MLGs representative of an isolate from the nursery Cubeda.

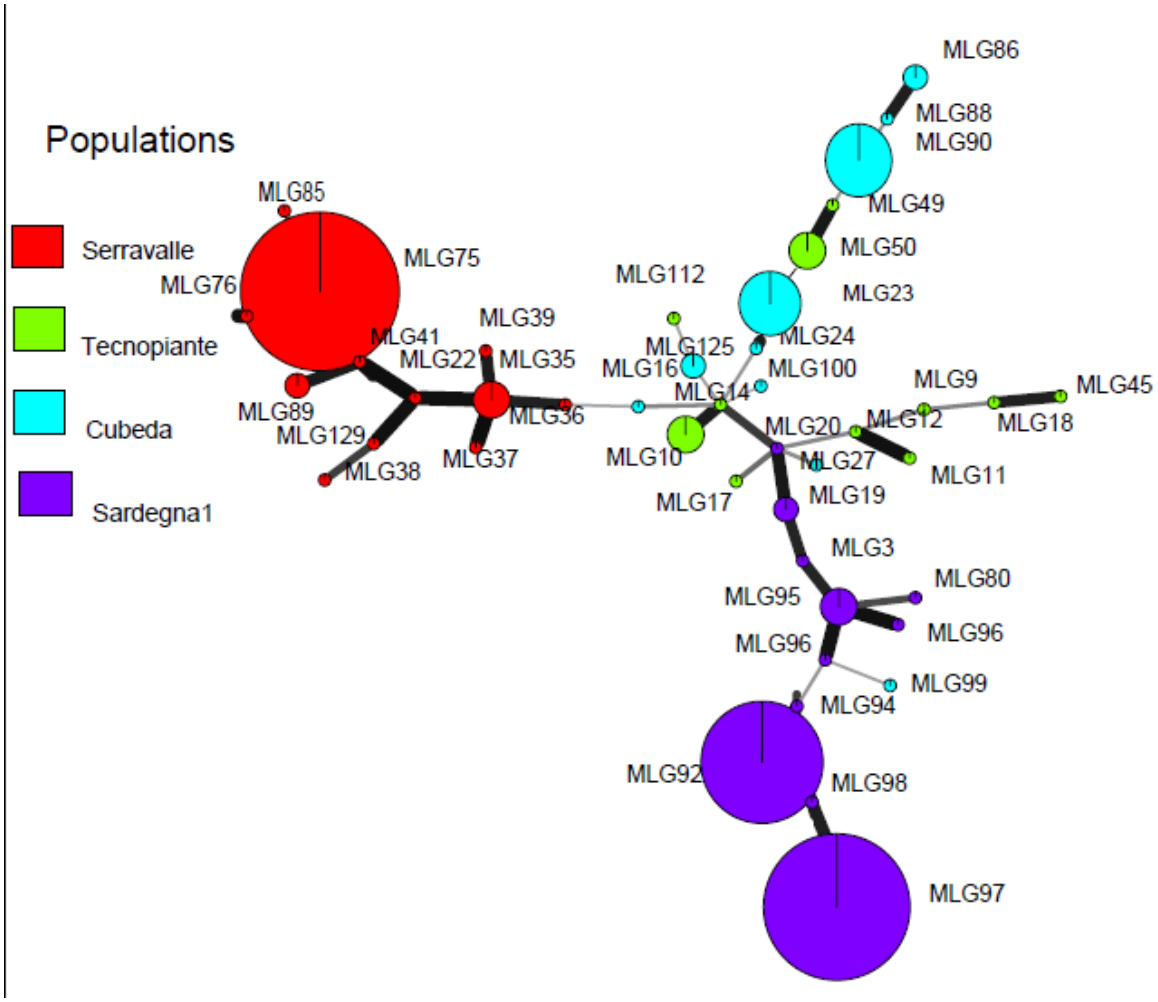

Figure 5: Minimum spinning network showing the relationship among MLGs obtained from three different nurseries (Tecnopiante, Cubeda and Sardegna 1) and an citrus orchard (Serravalle).

3.4 Discussion

Nine polymorphic microsatellites markers were used to reveal genetic diversity in 268 isolates of *P. nicotianae* representing a wide host range and geographic location. To accommodate fast, accurate and cost-effective genotyping, eight of the labelled primers were coamplified using two different fluorophores (HEX and FAM), and thus 5 reactions (4 duplex and 1 uniplex) were developed for high-throughput screening of the complete panel of *P. nicotianae* isolates. Selected markers proved to be very consistent with a percentage of amplification very close to 100%, since only 6 out of the 2412 reactions did not produce a positive amplification. Significantly higher levels of failures in producing results with SSR loci have been reported for other *Phytophthora* species. Brurberg et al. (2011) noticed that 2 out of the 9 loci previously selected for the assessment of the variability of 200 *Phytophthora infestans* isolates did not give any results for about 30% of the isolates. The positive result achieved in the present study is a consequence of the accurate evaluation of primers utilized. As described in Chapter II, selected SSRs were consistent within the genome of six different isolate of *P. nicotianae* and were experimentally evaluated using 5 isolates representative of the genetic diversity within the species (Mammella et al., 2011; 2013).

On the whole, the nine markers showed a high level of polymorphism in the population assessed, although significant differences were revealed among markers. In particular, the number of detected alleles ranged from 5 (P2039) to 25 (P1509). Length changes in microsatellite loci generally arise from replication slippage, that is the transient dissociation of the replicating DNA strands followed by misaligned reassociation (Ellegren, 2004). Quantitative experiments showed that the *Taq* polymerase slippage rate increases with the number of repeat units and is inversely correlated with the length of the repeat unit (Shinde et al,2003). Interestingly, the allelic variability achieved in the present study was not strictly correlated to the size of the SSR motif. As an example, the 4 bp motif analyzed in the

present study (locus P5) yielded 16 different alleles and was more polytrophic than other 4 loci with 3 bp motif, characterized. Nonetheless, it is worth mentioning that the markers with the highest number of alleles were dinucleotide (P1509 and P643 with 25 and 18 alleles, respectively).

The combined data sets of isolates analyzed with the 9 different SSR markers allowed the identification of 129 multilocus genotypes (MLGs) within the complete panel of isolates. This mean that most isolates had a distinct genetic fingerprint. The presence of an unexpected genetic framework for a diploid organism like *P. nicotianae* was also revealed. In particular conditions of triploidy were found for the locus P1129 (5 MLGs), P788 (2 MLGs), P17 (1 MLG), P2039 (1 MLG), P15 (4 MLGs), P1509 (6 MLGs), while four different alleles were observed for locus 788 (1 MLG) and 17 (2 MLG). Interestingly, isolate Ph168 exhibited three triploid loci (P15, P1129 and P17). The presence of polyploidy in *P. nicotianae* has been recently reported by Mammella and co-workers (2012) using a SNP approach and unusual recombination patterns were also observed by Förster and Coffey (1990) using a RFLP approach to investigate sexual recombination during oospore formation. Furthermore, polyploidy has been observed in other *Phytophthora* species. Using SSRs markers, this phenomenon was described for the first time by Dobrowolski et al. (2002) in *P. cinnamomi.* A large proportion of non-Mendelian inheritance was observed across all loci for all four microsatellite markers used. This aberrant inheritance was best explained by nondisjunction at meiosis in the trisomic parents, since aneuploidy progeny was also identified. Ivors et al. (2006) revealed conditions of trisomy for some isolates of *P. ramorum*. Initially, they hypothesized that multiple alleles resulted from horizontal gene transfer occurred after introgression of genes from other *Phytophthora* species, 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* (Van der Lee et al.,

2001; Lees et al., 2006; Brurberg et al., 2011). Lees et al. (2006) demonstrated the presence of trisomic linkage groups in 10-16% of progeny from two individual crosses of *P. infestans.* Different meiotic rearrangements were also observed in the related genus *Pythium* in crosses of *Pythium sylvaticum* (Martin, 1995). By analyzing the progeny from a sexual outcross with RFLP and RAPDs, he found unexpected patterns of marker inheritance at a karyotypic level most likely due to translocation and aneuploidy events. This level of variation among karyotypes was also found in the field, so it is possible that these unusual meiotic rearrangements could also be present in the offspring in nature.

In the present study the Bruvo's distances (Bruvo et al., 2004) calculated with the R package software (Kamvar et al., 2013) was utilized to construct MSNs and graphically evaluate phylogenetic correlations among isolates. This function enables the measuring of genetic distance without considering the ploidy of the locus, and permits the management of data comprising mixtures of isolates with different ploidies. Nonetheless, it is important to create virtual alleles so that all the genotypes are of the same length. This method allows to overcome the challenges of most methods currently utilized for population genetic analysis, since they are designed for examining diploid or haploid populations (Cooke et al., 2011).

Preliminary analyses using the Bruvo's distances revealed that all citrus isolates clustered together regardless of their geographic origin. However, within the main citrus group two separate groups representing isolates from pommelo and other citrus species were visualized. Based on this result, analyses were performed by separating pommelo isolates from those obtained from other citrus hosts. Among the 129 MLGs found, MLG75 resulted to be the most prevalent within the population analyzed. This genotype included 38 isolates recovered from *Citrus* spp. in different geographic areas including Vietnam (6), Philippines (1), and three different Italian regions including Sicily (27), Calabria (3), and Apulia (1). The second numerous MLG (MLG70) was composed of 19 isolates, all collected from pommelo.

Analyzing 89 isolates (60 from Citrus, 28 from Pommelo) a total of 30 MLGs shared between the two populations were identified with two predominant groups (pommelo and other citrus species) exhibiting an evident phylogenetic distance each other. In particular, the differences were due to three loci, P643, P2039 and P1509. Our results are in agreement with those recently reported by Mammella et al. (2013) based on the analysis of SNPs. 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. Authors speculated that considerable evidence supports host preference by some isolates of *P. nicotianae* (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). In general, although the degree of susceptibility among hosts is not clear it seems that the greatest degree of virulence would be commonly shown by isolates on their own host.

In agreement with previous reports (Mammella et al., 2011; 2013) 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. 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.

Previous hypothesis is in agreement with results obtained with isolates from pommelo. Since this species is a native plant of Vietnam and plant material was not introduced from

other countries, this finding would suggest a specific co-evolution of *P. nicotianae* and pommelo in this country. Understanding of plant pathogen co-evolution in natural systems could allow to unravel the molecular basis of interactions between individual host and their pathogens, developing a comprehensive picture of how life history traits of both players interact with the environment to shape evolutionary trajectories (Burdon and Thrall, 2009).

The complete panel of isolates was divided into 8 different population based on their host of origin and basics statistics were calculate to assess the rate of clonality. In particular, the E_5 index (Kamvar et al., 2013) was utilized to relate the number of genotypes with the number of isolates per each population, with a range between 0 and 1. The lowest index value was determined for the *Citrus* (0.315) and seem to be a further confirmation that the movement of isolates worldwide on planting stock have contributed to a homologation of isolates. Indeed the E5 index for other populations ranged from 0.533 of Pommelo to 0.965 of Solanaceae.

The MSN of the Bruvo's distances for 97 selected isolates revealed the presence of a panmittic population of isolates sourced in three Italian nurseries. By focusing the analysis in three different nurseries and on an orchard, it was possible to notice a very high variability in term of MLGs for ornamental plants. This result was expected considering the very large number of species that can be simultaneously cultivated in the same nursery, often obtained from different geographic regions. Isolates from these plants can potentially interact each other and increase genetic recombination of the pathogens. Interesting, several examples of isolates with different genotype, although recovered from the same host and from the same nursery, were revealed. As an example, isolates collected from *M. communis* in a nursery located in Sardinia accounted for 11 different MLGs grouping into two distinct phylogenetic groups. It can be speculated that this behavior is the result of the presence of other plant

species in the same nursery that can favor the migration of pathogenic species from one host to another. Similar findings were also found for other hosts and nurseries.

The higher genotypic diversity in nurseries is likely to be the result of the repeated exchange of pathogen genotypes through the trade of infected plant material and/or by strong selection pressure selecting new genotypes created through recombination or mutation. The genetic diversity in nursery populations was investigated by using microsatellites for *P. ramorum* (Ivors et al., 2006; Vercauteren et al., 2010) and *P. plurivora* (Schoebel et al., in press), and it was concluded that the nursery trade plays an important role in the spread of the diseases.

A marked association among molecular groups and host of recovery was found for isolates causing black shank in tobacco. Furthermore, the MSN enabled the visualization of a relevant geographical structuring for isolates from tobacco with two phylogenetic groups: a clade with only isolates from Australia, and another clade (composed by two sub-clades) containing isolates recovered from several US states. A single isolate (d1836) did not cluster with other tobacco isolates. As discussed above for citrus, there appears to be a preferential virulence association between tobacco and *P. nicotianae*. Indeed, a specific association among molecular groups and host of recovery was reported for isolates causing black shank in tobacco that were differentiated from other *P. nicotianae* isolates (Colas et al., 1998). As regards to the revealed geographical structuring of tobacco isolates it could be the result of the cultural system utilized for this species. In fact, tobacco is propagated by seeds which do not contribute to the spread of the pathogen and plantlets are very rarely transplanted in areas different from those in which have been produced.

With the exception of isolates from tobacco, the distance analysis of the complete dataset did not show a clear differentiation of isolates according to their geographic origin, confirming a panmitic distribution of genotypes (Mammella et al., 2013). The conspicuous

number of MLGs found in the nurseries as compared to those isolated from open fields (mainly citrus and tobacco) suggests a higher incidence of sexual reproduction in nurseries. The presence of several host species should facilitate the meeting of different mating types from different populations., also considering the wider geographic areas that plants were sent from.

In conclusion the present study provided important advancements on the ecology and epidemiology of *P. nicotianae* confirming the powerful of SSR analysis to study *P. nicotianae* populations as already done for other *Phytophthora* species. However, the present study represent only a limited application of the SSR markers herein described (Cfr. chapter II) since a more hierarchical approach in collecting isolates can surely further increase the potentiality of this tool. For example a specific sampling to obtain a large population with several subpopulations each corresponding to a smaller area such as country, region, province, and so on would be useful to accurately investigate the spatial behavior of the species. Furthermore, monitoring of isolates through multiple sampling in several years would be useful to evaluate the preferential reproduction system within a field or in a limited geographical area.

3.4 Literature cited

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Appendix 1

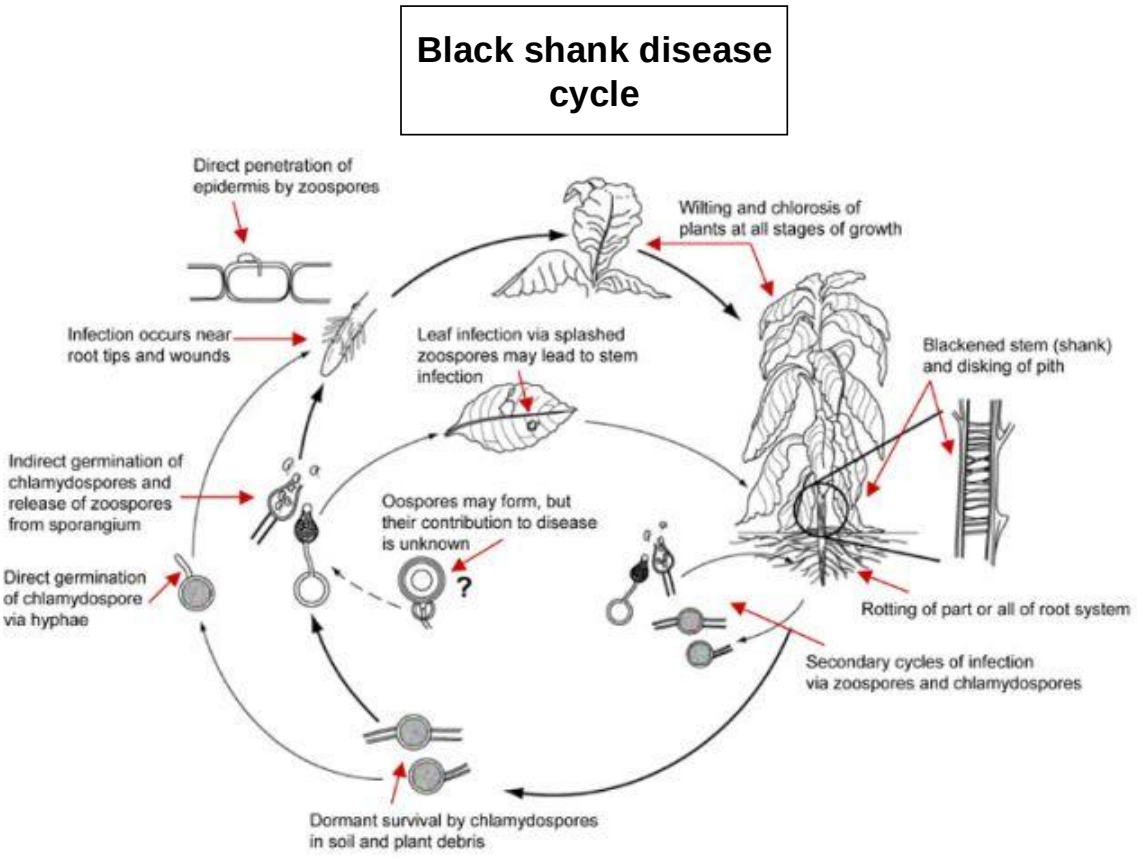

Fig.1 - Disease and life cycle of black shank pathogen *Phytophthora nicotianae*. Image from apsnet.org (Gallup et al., 2006).

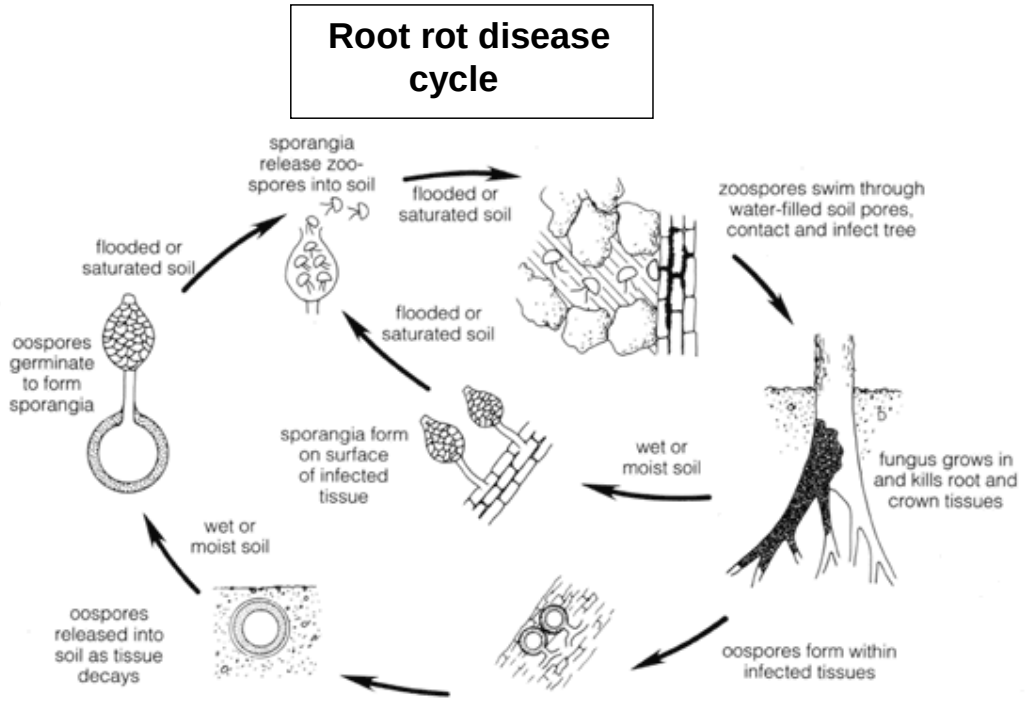

Fig.2 - Disease and life cycle of root rot and crown rot *Phytophthora* pathogen. Image adapted from Agrios, 1988.

Appendix 2

Protocol for DNA extraction (adapted from Ippolito et al., 2002) of the 51 *Phytophthora nicotianae* isolates (utilized in chapter II and III) from Department of "Gestione dei Sistemi Agrari e Forestali", Mediterranean University, Italy.

- Isolates were grown in PDB broth at room temperature for ten days;
- 100-200 mg of mycelia were rinsed with ultra pure water;
- 100-200 mg of dry mycelia were suspended in 800 µl of breaking buffer (200 mM Tri–HCl [pH8], 250 mM NaCl, 25 mM EDTA, 0.5% SDS) with 200 µl of phenol and 200 µl of chloroform/isoamyl alcohol (24:1) and added of a small quantity of glass beads (Sigma - Aldrich);
- DNA extracted using FastPrep FP120 Instrument (Qbiogene, Inc. Cedex, France) at the maximum speed for 1 minute and centrifuged at 14,000 rpm for 10 minutes;
- The upper phase was extracted twice with 200 µl of phenol and 200 µl of chloroform/isoamyl alcohol (25:24:1) and once with 200 µl of chloroform/isoamyl alcohol (24:1), respectively;
- DNA was precipitated with an equal volume of isopropanol and 20 µl of sodium acetate 3 M pH 5,2 for 1 h at -20°C;
- DNA was centrifuged for 20 min at max speed;
- DNA was washed with 70% cold ethanol (−20 °C) and centrifuged for 5 min at max speed;
- DNA was dried and stored in ultra pure water at −20 °C.

Protocol for DNA extraction (Blair et al., 2008) of 45 *P. nicotianae* isolates (utilized in chapter III) from the World Phytophthora Collection at Riverside, CA, USA.

- Actively growth mycelia were produced in clarified V8 broth after incubation at room temperature for ten days;
- 200 mg of mycelia were rinsed with ultra pure water;
- DNA extracted with FastDNA kit (MP Biomedicals Inc., Irvine, CA) using FastPrep FP 120 instrument according to the manufacturer's instructions, with modifications using 1 ml of CLS-VF cell lysis solution and omitting the PPS protein precipitation solution;
- All DNA samples were stored in ultrapure water at - 80 °C.