DOTTORATO DI RICERCA IN

“GESTIONE FITOSANITARIA ECO-COMPATIBILE IN AMBIENTI AGRO-FORESTALI E URBANI”

XXII Ciclo (S.S.D. AGR/12)

UNIVERSITÀ DEGLI STUDI DI PALERMO
Dipartimento DEMETRA

Sede consorziata
UNIVERSITÀ “MEDITERRANEA” DI REGGIO CALABRIA
Dipartimento GESAF

Intraspecific variability in the Oomycete plant pathogen *Phytophthora nicotianae*

Dottorando
Dott. Marco Antonio Mammella

Coordinatore
Prof. Stefano Colazza

Tutor
Prof. Leonardo Schena

Co-tutor
Dott. Frank Martin
Prof.ssa Antonella Pane

Contents

Preface

Late blight of potato, which occurred in Ireland in 1845, brought the attention of the scientific community to the genus Phytophthora. Phytophthora infestans (De Bary, 1876) in particular caused the loss of entire potato fields in Ireland that caused poverty, starvation and emigration of millions of people. The impact of the genus Phytophthora on worldwide agriculture is still enormous. The member of this genus are able to cause disease with remarkable economic losses in a large number of crop, as well as environmental damage in natural ecosystems. For example P. nicotianae and P. cinnamomi have a wide host range of approximately 900-1000 plant species and P. cinnamomi in particular is known for the epidemics that is causing the death of Jarrah forests in Australia. Phytophthora ramorum is another important species responsible for the Ramorum dieback epidemics that recently rose in California and Oregon coasts and in nursery, as well as forest ecosystems in Europe. In the last decade the number of newly described Phytophthora species has increased considerably through the development and application of molecular techniques that overcome the morphology-based methods. The molecular studies are more and more fundamental to reveal differences between very close species and to determine changing in the taxonomic classification within the genus. The increasing of sequence data available has helped with the identification of new species, thinking that in the last decade 39 new Phytophthora species and two species hybrids have been formally described (1) and the total number raised at more than 100 species. Furthermore, has been suggested, that a greater number of species (until 500 species) still undiscovered (2).

1) Ersek T, and Ribeiro K, 2010. *Acta Phytopathologica et Entomologica Hungarica* 45 (2), pp. 251–266.

2) Brasier C, 2009. *Phytophthora* biodiversity: how many *Phytophthora* species are there? In: E. M. Goheen and S. J. Frankel (tech coords): *Phytophthoras* in Forests and Natural Ecosystems. Proc., 4thMeeting of IUFRO Working Party 07.02.09, August 26–31, 2007, Monterey, CA. Gen. Tech. Rep. PSW-GTR-221, Albany,CA, pp. 101–115.

General Abstract

Phytophthora nicotianae is an Oomycete plant pathogen affecting a broad range of plant species comprising more than 250 genera. It causes several economically relevant diseases and is particularly known as the causal agent of black shank of tobacco and root rot, stem rot and gummosis of citrus. Despite the relevance of this pathogen, information on the field reproductive strategy, population structure and global distribution of genetic variability is not completely known. This is because available studies have been conducted to characterize local populations (mainly from tobacco) or have been based on physiological aspects such as resistance to fungicides and pathogenicity on differential hosts.

In the present study, variable mitochondrial and nuclear loci were explored to evaluate genetic intraspecific variability of *P. nicotianae* on global scale using a collection of isolates from different geographic regions and hosts. A mixed approach of comparative genomics and sequencing of known and anonymous loci was utilised to identify suitable regions for population studies. High mitochondrial and nuclear genetic diversity and a panmictic distribution of haplotypes and genotypes were observed using these markers. This work offers useful tools to analyze the population genetic structure and migrations of *P. nicotianae*.

Chapter I

General introduction

I.1 Introduction to Oomycetes and *Phytophthora*.

The Oomycetes and the genus *Phytophthora* fall within the kingdom Stramenopila (= Chromista), which also includes golden brown algae, diatoms, and brown algae such as kelp (Baldauf et al., 2000). These organisms, known also as "water molds," include a various group of species that have a similar ecology and biology as Fungi since they parasitize plants using similar mechanisms and grow by polarized hyphal extension, produce spores, use an absorptive mode of nutrition and are present in different environments.

Until a couple of decades ago these organisms were included in the kingdom Fungi (Eumycota) and considered as "lower fungi". Lately, accurate biological, morphological, biochemical and phylogenetic analyses have revealed that these organisms are closely related with heterokont algae in the Chromalveolate super kingdom (Cavalier-Smith and Chao, 2006; Tsui et al., 2009). Oomycetous organisms differ from the true fungi because of their diploid nature; the morphology of mitochondrial cristae; the flagellar heterokont apparatus of the zoospores; the biochemistry of the cell wall, which contain a cellulose microfibril skeleton and β-1,3-glucans amorphous material instead of chitin; their lack of epoxidation of squalene to sterols; their different metabolic pathways and their unique molecular biology system (Erwin and Ribeiro, 1996).

The phylum Oomycete comprises saprophytes, facultative parasites and obligate parasites (biotrophes) that are able to live in both terrestrial and aquatic environments. The Oomycetes can be pathogen of plants, animals, seaweed, crustaceans, fishes, amphibians and also humans. Despite their great diversity (Fig. 1) more than 60% of Oomycetes are plant pathogen (Thines and Kamoun, 2010). *Phytophthora* belongs to this percentage of plant pathogens.

The genus *Phytophthora* is located in the family Peronosporaceae and order Peronosporales (Cannon and Stalpers, 2008) that include obligate biotrophic pathogens,

hemibiotrophic and saprophytic organism. For nearly all of their life *Phytophthora* are diploid whereas the higher fungi are haploid (Erwin and Ribeiro, 1996).

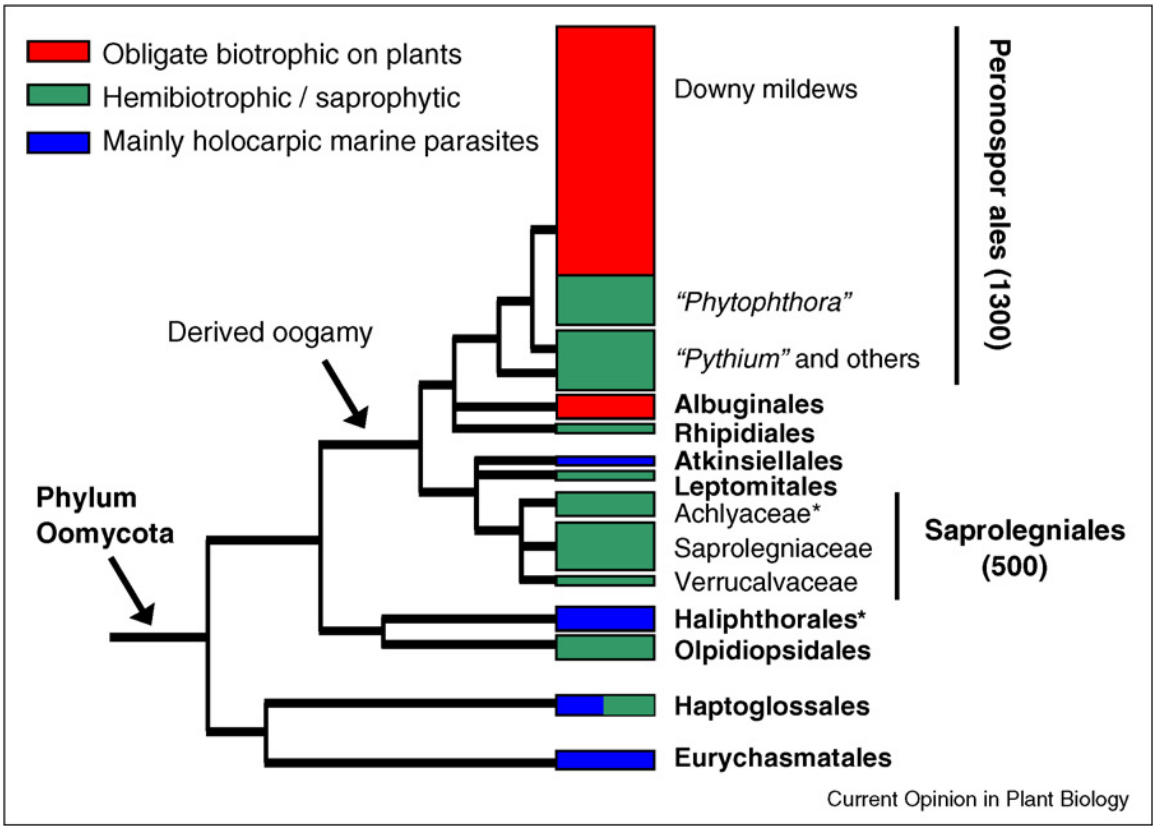

Fig. 1 - Phylogenetic tree of the phylum Oomycota from Thines and Kamoun, 2010. Only the number of species for the Saprolegniales and Peronosporales are present because of their large number of described species.

I.1.1 *Biology and genetics of* Phytophthora nicotianae

Phytophthora nicotianae produces the typical structures characteristic of the genus. The mycelium is composed of hyaline, branched, coenocytic filaments, except in old cultures where septa can sometime be seen. It reproduces asexually by forming sporangium papillate and ovoid from which are discharged biflagellate zoospores which enable the movement of the pathogen in water for short distances.

Sexual reproduction is characterized by the production of thick walled structure called oospores. This spherical structure (average diameter 20 µm) originates from the union of two gametangia, an amphigynous antheridia (paternal) and a spherical oogonia (maternal). The

haploid nuclei of the antheridia pass into the maternal gametangia and fuse with the haploid nuclei of oogonia to generate the diploid oospore. Antheridia and oogonia represent the only haploid stage in the life cycle of *Phytophthora* species.

Phytophthora nicotianae requires two opposite mating types, A1 and A2, for oospore production and like all other heterothallic species, it is bisexual and self-incompatible (Erwin and Ribeiro, 1996). This means that isolates of both mating types (A1 and A2) can be either the maternal or paternal parent during sexual crossing.

Phytophthora nicotianae is known also for the production of abundant asexual structure called chlamydospores. These structures are globose and have thick walls that allow the pathogen to persist in soil and plant tissue in unfavorable conditions for long periods (Mircetich and Zentmyer 1966). Usually, the chlamydospores are dark but can be also hyaline and can be formed intercalary or terminal at the tip of the hyphae. These structures germinate and infect host plants when environmental conditions are favorable.

I.2 *Phytophthora nicotianae* diseases

Phytophthora nicotianae (syn. *P. parasitica*) is a soilborne pathogen of a wide range of plant species. It is the most important soilborne pathogen in citrus production areas in the world (Matheron et al., 1997; Graham and Menge, 1999; Magnano di San Lio et al., 2002) as well as for tobacco (Erwin and Ribeiro, 1996; Lucas, 1975) and ornamental plants (Lamour et al., 2003; Pane et al., 2001). This pathogen can cause foliar and fruit diseases (blight and brown rot of citrus) however root rot is the most common disease manifestation in both herbaceous and trees plants. The pathogen is favored by temperatures ranging from 10 to 35 °C, high humidity and rainfall (Erwin and Ribeiro, 1996). Black shank of tobacco and root rot of citrus are among the most important *P. nicotianae* diseases.

I.2.1 *Black shank of tobacco*

Since its description in 1931 by Tucker in North Carolina, black shank of tobacco has been considered one of the most destructive disease on this plant with annual losses of millions of dollars. The tobacco plant is susceptible at any stage of the growth, from seedbed to mature plant in the field where root necrosis, wilting, chlorosis, stem lesions, and stunting occur (Lucas, 1975). The spread of the disease is favored by transplants from infested seed beds (Lucas, 1975).

To control the disease an integrated approach consisting of cultural practices, fungicide applications and host resistance is needed (Melton et al., 2003). It is important to reduce the passage of water, soil and contaminated materials from infested to non-infested soil to limit the spread of the pathogen. Because the pathogen can persist in soil as oospores and chlamydospores for more than five years, long-term rotations (more than 5 years) with non susceptible crops such as peanut or cotton are useful to reduce the population of the pathogen (Lucas, 1965). Chemical control gives better results when applied in combination with other methods, but it is not highly effective when a susceptible cultivar is grown. Chemicals commonly used are metalaxyl and its derivative, mefenoxam. Dosage and time of application vary based on the type of host resistance and incidence of the disease in the specific field (Gallup et al., 2006).

However, the most widely used and effective method of control for black shank is the use of host resistance (Sullivan et al., 2005a). Four physiological races of *P. nicotianae* (0, 1, 2 and 3) have been identified even though 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. The first variety available for the growers was the cigar wrapper cultivar Florida 301 (Fla. 301), which gives partial resistance against different races of *P.*

nicotianae (Carlson et al., 1997). However, resistance derived from Florida 301 provides only partial protection from disease, requiring supplemental soil fungicide applications and crop rotation for growers to meet their production goals (Parkunan et al., 2010). Resistant varieties come from intraspecific and interspecific crosses using three different genetic sources: *N. tabacum* (cultivar Fla. 301), *N. plumbaginifolia* and *N. longiflora*. The latter two species are the source of single dominant resistance genes (Ph_l and Ph_p) that provide complete resistance against race 0 but does not provide any resistance against race 1 (Apple, 1967). Since the deployment of these single gene resistant cultivars the frequency of the most virulent race 1 has increased in the South Eastern area of the US (Apple, 1967). Because of this problem, integrated pest management approaches that involves the use of metalaxyl or mefenoxam and resistant crop rotation scheme are suggested to the growers by different scientists to reduce the development of the disease by the different races of black shank.

I.2.2 *Root rot of citrus*

Root rot caused by *Phytophthora* spp., together with damping-off and gummosis, are among the most economically important fungal diseases in citrus, occurring in nearly all production regions (Leoni and Ghini, 2004). *Phytophthora.nicotianae* and *P. citrophthora* are the most common species in citrus production areas worldwide. *Phytophthora nicotianae* growths at higher temperatures (28-30°C with max of 35-38°C) than *P. citrophthora* (25°C with max 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). Root rot of citrus cause mortality of newly planted trees and a slow decline and yield loss of mature trees. The management of the disease is based on an integrated approach that includes the use of tolerant rootstocks, cultural practices and fungicides. Several biocontrol agents also have been tested for the control of this disease but they are still not commercially available (Colburn and Graham, 2007). The severity of the disease is related to the use of specific susceptible

rootstock. Furthermore, a top graft with highly susceptible species or cultivars, such as the clementine or nucellari clones of sweet orange, reduces the resistance of the rootstock (Cacciola and Magnano, 2008). Another aspect to consider is that even when resistant rootstocks are utilized, hypoxia resulting from water saturation of the soil makes the roots more susceptible to infection by *Phytophthora* and inhibits the growth of new roots.

The systemic fungicides metalaxyl, mefenoxam and fosetyl-Al can be used to control the disease according to the physiological state of the plant and the population of the pathogen. In the case of root rot caused by *P. nicotianae* the best time to apply the chemical is immediately before the roots start to grow when the first flush of spring vegetation has reached about three-quarters of its maximum development (Cacciola and Magnano, 2008). Treatment with systemic products can only be justified if the inoculums density reach the 10-15 propagules/cm^3 because their high costs (Timmer et al., 1988; Sandler et al., 1989). For the first two years after planting the treatment against root rot should be done routinely because the young citrus seedlings are very susceptible to this disease.

Fig. 2 - (a) Characteristic wilting of leaves due to the black shank in flue-cured tobacco (picture by apsnet.org, Gallup et al., 2006,). (b) Little roots appearing filiform due to the disintegration of the cortex in citrus plant (picture by Marco Mammella).

I.3 Population genetics of *Phytophthora*

The study of the population genetics of a plant pathogen is important because the genetic structure reflects the evolutionary potential of the pathogen (McDonald and Linde, 2002).

Several factors are involved in shaping the populations structure of pathogens like *Phytophthora* spp. and include natural selection, reproductive system of the organism, mutation rate, gene flow, migration and genetic drift (Goodwin, 1997). Plant pathogens are immersed in an environment where there are several factors that can cause strong directional selection and changes in the viability of the pathogen, in its aggressiveness and therefore in the occurrence of the disease. Resistant cultivars, different environmental conditions, crop rotation, fertilizers, application of specific fungicides or presence of antagonists in the soil are some of the factors that can lead to changes and require continuous adaptation by the pathogen. These aspects have been accurately analyzed and reviewed for different *Phytophthora* species (Flier et al., 2003; Goodwin, 1997; Grunwald and Flier, 2005; Grunwald et al., 2008).

I.3.1 Forces acting on natural populations

I.3.1.1 *Selection*

Natural selection is the process driving the evolution of populations of all organisms on earth. The main requirement for selection to take place is the presence of genetic variation in the population. Favorable genetic variation in a particular environment will be maintained and its frequency in the population will increase compared with unfavorable variations that will decrease. In *Phytophthora*, one of the most obvious causes of selection is the changing in host resistance genes (Goodwin, 1997). The spread of resistant plants in a specific geographic area can result in monoculture systems characterized by high genetic uniformity and could stimulate selective pressure for specific mutants, recombinants or introduced genotypes of the pathogen that can overcome resistance (McDonald and Linde, 2002). The survival of portions

of populations following the use of chemical agents also is the result of selection of genotypes with a higher fitness. For example, the metalaxyl sensitive US-1 clonal lineage of *P. infestans* was replaced by the new resistant genotype US-8 from Mexico as a major component of the US population following metalaxyl applications (Goodwin, 1997). Similarly, the US-1 genotype was no longer able to compete with new introduced genotypes in Europe, Asia and South America and was progressively replaced by other genotypes (Drenth et al., 1994; Koh et al., 1994; Fry et al., 1992).

I.3.1.2 *Reproductive system*

Sexual reproduction is certainly a process that leads to genetic variations that may provide the opportunity for faster changes in the genetic structure of populations. The contribution of new genotypes derived from meiotic recombination is the basis for changing the genetic structure of a population. Different *Phytophthora* species presented clear evidence of the sexual cycle as a constant element of their life cycle in nature, which influences the biology of the organism such as self/non self recognition, intercellular communication, cell-type differentiation, and evolution (Judelson, 2009). The sexual cycle creating genetic diversity can improve fitness of pathogen population and this, obviously, can affect population genetics and can negatively influence control strategies to prevent the spread of diseases. There are different examples showing that isolates of a *Phytophthora* spp. from populations in regions where the sexual cycle has been active are more aggressive than isolate from clonal population (Fry, 2008). Sexual reproduction may also promote survival when unfavorable environmental conditions are present and in the absence of the host since oospores can survive for a long time in soil. A key factor that can enhance or negatively affect the possibility of sexual recombination in a population is the environment that allows contact between new genotypes and opposite mating type (in case of heterothallic species as *P. nicotianae*) or avoid it with geographical barriers leading in particular cases to vicariant

speciation. For *P. infestans* it has already been demonstrated that there is an unequal distribution of mating type in case where recombination does not occur (Fry, 2008). In fact, the geographic separation between opposite mating type limits the possibility for sexual recombination in many areas of the world where both mating types are present (Goodwin et al; 1998).

Clonality in natural *Phytophthora* populations is widely documented (Dobrowolsky et al., 2003; Grunwald et al., 2008; Lamour et al., 2003). In this case, genetic variation is unidirectionally transmitted from the genotype of the parent to the new generation. Obviously, it is possible to assume that such populations exhibit lower variability and thus lead to a slower process of evolution comparing with sexually reproducing populations. The reduced level of genetic variations present in a clonal population might make these individuals more susceptible to unfavorable environmental changes or new control strategies. Furthermore, in clonal populations, there are no mechanisms for avoiding inheritance of deleterious genes from the parent to the new generation.

I.3.1.3 *Mutation*

Mutation is one of the most important sources of genetic variation and is basic for natural selection of populations and can lead to future evolutionary divergence. Pathogens with fast mutation rates represent a major concern compared to pathogens with low mutation rates (Mc Donald and Linde, 2002). Mutations have an evident impact when new variants present in a population are able to overcome specific resistance genes of plants. A typical example of the effect of mutation and selection is the continuous origin of new pathovars in *P. sojae* and *P. infestans* that are able to overcome resistance genes of newly released cultivars. Similarly, mutations can also allow the pathogen to resist specific fungicides. Metalaxyl resistant genotypes for the US-1 clonal lineage of *P. infestans* were observed in Philippines (Koh et al., 1994). This was unusual if think that in Philippines there were only US-1 lineage of *P.*

infestans that was known to have an extreme sensitivity to metalaxyl. This change in a resistant genotype of the US-1 lineage was clearly due to mutations. In fact one of the first metalaxyl resistant genotype was discovered in Ireland in 1980 (Carter et al., 1982) and was shown that it was a mutated genotype of the US-1 lineage (Goodwin et al., 1996).

I.3.1.4 *Gene flow and migration*

Gene flow and migration contribute to the fluctuation and exchange of alleles and genotypes among geographically separated population. These events of genetic transfer can lead to change the allele frequencies in a population. Both these processes are strongly influenced by human activities such as nursery trade or agricultural practices that facilitate the spreading of pathogens in specific areas. For example, dispersion trhough potato tuber distribution allows movement for *P. infestans*, *P. erythroseptica* and *P. nicotianae* (Goodwin et al., 1997). The mobility of the pathogens is another important factor influencing gene flow and migration.

Pathogens that develop asexual structures easily transportable by wind or rain (sporangia and zoospores) have a better mobility than pathogens that don't differentiate these kinds of structures. Classical example of migration for *Phytophthora* was the spread of the US-1 *P. infestans* genotype from US (where was found for the first time in 1843) to Europe, around 1845. This event caused the great Irish famine (O'Neill, 2009). Migration vents have significantly changed the population structure of *P. infestans* in Europe, South America, North America, north of Mexico and Asia (Fry et al., 2009). Migrations occurring in regions where no previous populations of the pathogen were present are defined as "founder effects".

I.3.1.5 *Genetic drift*

Genetic drift is the process that drives changes in allele frequencies of a population by random sampling of alleles from the parents to the offspring. The main consequence of genetic drift is the loss of genetic variation in a population. This effect is larger in small population, which

can easily reach genetic uniformity. In addiction to random mating other factors can determine genetic drift. Environmental conditions unfavorable for a certain pathogen can heavily reduce the number of these individuals in a population and significantly lower the allele frequencies of their genes (Goodwin et al., 1997). In the next generation the frequency of a pathogen forming survival structures such as oospores or chlamydospores will be higher compared to a pathogen without resting structures that will be subjected to a substantial reduction of its population. For example, a treatment with metalaxyl in a field that has a genetically variable population of *P. nicotianae* will favor resistant genotypes that will survive and contribute to the offspring. This condition will change the allele frequencies of specific genes in the next generation.

I.3.2 Genetic structure of population in the genus *Phytophthora* spp.

The population genetic structure has been widely investigated in some important *Phytophthora* species such as *P. infestans*, *P. ramorum* and *P. cinnamomi*, while only a few studies are available for *P. nicotianae,* and the majority of them focused on isolates from tobacco (Zhang et al., 2001; Zhang et al., 2003; Sullivan et al., 2010). Many studies on population structure of *P. nicotianae* are based on the analysis of physiological traits such as resistance to fungicides (metalaxyl and mefenoxam) and differential aggressiveness on tobacco cultivars. *Phytophthora nicotianae* is a cosmopolitan pathogen most likely widespread by trading of infected plant material, in particular of ornamental plants. However, there is no existing information about migration and gene flow of *P. nicotianae* among different geographical regions.

I.3.2.1 Phytophthora infestans

Events of migration have completely changed the population structure of this pathogen in different regions of the world (Fry et al., 2008). The migration of different genotypes and

opposite mating types has led to the emergence of new and aggressive strains that displaced the existing populations in Europe and North America (Drenth et al., 1994; Fry and Goodwin, 1997). Over the last decade, the analysis of the global population structure of *P. infestans* has seen areas with sexual reproduction compared to other regions dominated by asexual reproduction. Initially, evidence of sexual reproduction for *P. infestans* was found only in Mexico, which most likely represented the center of origin of this species in constant co-evolution with native *Solanum* hosts (Grunwald and Frier, 2005); however, a new hypothesis based on an higher genetic diversity identified, assign Peru and Andean region as center of origin of this pathogen (Gomez-Alpizar et al., 2007).

With the spread of new genotypes and the A2 mating type in Europe it was believed that sexual recombining populations were present in these regions. First of all, the migration in the 1980's of the A2 mating type in Europe laid the foundations for the emergence of new genotypes by sexual recombination with the existing isolates of A1 mating type. Furthermore, a high genetic diversity and similar level of both mating types supported sexual recombination for populations in Netherlands (Drenth et al., 1994), Poland (Sujkowski et al., 1994), Norway and Finland (Bruberg et al., 1999; Lehtinen et al., 2008).

In North America migrations from Mexico has contributed to modeling the population structure. Before events of migrations in the 1990s, the US-1 was the first and widest spread genotype in United States (Fry et al., 2009; Goodwin et al., 1998). Subsequently different genotypes were found in the US originating from Mexico, changing the genetic background and characteristics of the previous populations. In particular, new genotypes US-7, US-8 and US-17 were resistant to metalaxyl in comparison with the US-1 genotype that was susceptible (Goodwin et al., 1998). However, with both mating types in the US asexual reproduction was widespread and geographic separation between the opposite mating types played an important role in preventing the emergence of a stable sexual recombining populations in this region (Fry et al., 2009).

I.3.2.2 Phytophthora ramorum

Unlike *P. infestans,* where its population structure in different continents is known, for *P. ramorum* population studies are limited only to Europe and North America. The populations are characterized by three distinct clonal lineages EU1, NA1, NA2 (Ivors et al., 2004, 2006; Grunwald et al., 2009).

These three clonal lineages were identified by AFLP analysis, SSR (Ivors et al., 2004; Ivors et al., 2006; Prospero et al., 2007) and sequencing analysis of 13 nuclear loci (Bilodeau, 2008). Furthermore, these three lineages were confirmed also by sequencing analysis of 8 mitochondrial loci (Martin et al., 2008). EU1 is the first and only lineage identified in nurseries in Europe as well as in natural ecosystem, considering also the two A2 mating type isolates discovered in Belgium (Werres and De Merlier, 2003) that were defined as EU1-A2 lineages (Vercauteren et al., 2010a). While the other two clonal lineages NA1 and NA2 are present in US where two genotypes of the EU1 lineage have also been reported (Hansen et al., 2003; Ivors et al., 2006, Mascheretti et al., 2008). The NA1 lineage (Ivors et al., 2006; Prospero et al., 2007) is the principal cause of disease in forest in Oregon and California. The NA1 lineage is characterized by different intraspecific genotypes, in contrast, the NA2 lineage has a low level of genotype diversity with only two microsatellite multilocus genotypes identified in nurseries in North America (Ivors et al., 2006). North American population of NA1 and NA2 lineages analyzed to date were of A2 mating type, while in Europe the EU1 genotype identified were all of A1 genotype except for three isolates found in 2002 and 2003 in Belgium that were A2 (Werres and De Merlier, 2003). The presence of both mating types in particular for the European situation suggest that recombination may be possible but recent studies indicates that sexual recombining population are no present yet, as well as in US. In fact, no evidence for sexual recombination was identified by microsatellites analysis in the US forest population, European nurseries or in Belgium where both mating types were present (Ivors et al., 2006; Vercauteren et al., 2010b). However, the analysis of the nuclear genome of

P. ramorum revealed the presence of heterozygous sites consistent with a sexual recombining species (Tyler et al., 2006). In fact, a phylogenetic approach inferred using haplotypes of each clonal lineage derived from nuclear genes showed that haplotypes from the same clonal lineage did not tend to cluster together and genealogical relationships among the lineages varied with each gene (Goss et al., 2009). The fact that alleles within the same lineages exhibited different origins suggested an ancient origin of these clonal lineages through sexual recombination (Grunwald et al., 2009).

Genotypic diversity has been observed in the European (EU1) and North American (NA1) lineages, although the EU1 lineage had a major level of diversity with a total of 30 distinct genotypes observed (Vercauteren et al., 2010b). Rare genotypes were found in different regions in Europe and this could be explained by nursery shipments of *Viburnum* and *Rhododendron* (Vercauteren et al., 2010b; Werres and De Merlier, 2003). Shipment of infected plant material could explain also the presence of the two EU1 genotypes in Oregon and Washington nurseries (Ivors et al., 2006). Different genotypes within each lineage were very similar and this kind of population structure can be due to introgression of closely related genotypes followed by the creation of new genotypes via mutation or mitotic recombination (Ivors et al., 2006). In fact, this could be hypothesized for the typical EU1 lineage from the nursery that had a higher genotypic diversity than the other two lineages. In nurseries, the repeated exchange of pathogen genotypes through the trade of infected plant material and strong selection pressure for new genotypes are all factors contributing to the development of genetic variability (Ivors et al., 2006).

I.3.2.3 Phytophthora cinnamomi

Phytophthora cinnamomi, as with *P. infestans* and *P. nicotianae*, is a heterothallic species wide spread in the world. Similarly to *P. nicotianae,* this *Phytophthora* sp. causes diseases in a large number of plant species, in particular woody plants (Zentmyer, 1980). *Phytophthora*

cinnamomi is known to cause epidemic diseases with significant impact on natural ecosystem, as exemplified by the dieback of jarrah and eucalyptus forest in Australia (Dubrowsky et al., 2003; Weste, 1994). Despite the importance of the pathogen and its presence in different countries around the world, detailed information about the population structure at global level are not available. Most of the studies available to date were performed using populations from Australia, South Africa and Papua New Guinea (Old et al., 1984; Linde et al., 1997; Linde et al., 1999; Dobrowolsky et al., 2003, 2008). This is probably because the two mating types are globally distributed (Zentmyer, 1980), but only in the three above mentioned countries the two mating types were found together (Linde et al., 1997; Pratt and Heather, 1973; Arentz and Simpson, 1986). Furthermore, even in these countries studies conducted up to now have not revealed specific evidences of sexually recombining populations. In fact, low level of gene and genotypes diversity was identified using isozyme analysis in South Africa (Linde et al., 1997). Similarly restriction fragment length polymorphisms (RFLP) analyses have shown that South African and Australian population has a similar low level of diversity and shared some RFLP alleles (Linde et al., 1999). A high level of genetic diversity supported by RFLP analysis (Linde et al., 1999) and isozyme multilocus genotype (Old et al., 1984) was only found among *P. cinnamomi* isolates from Papua New Guinea, suggesting this region may be the center of origin of the species (Old et al., 1984; Linde et al., 1999). On the whole, a low level of heterozigosity, low differences between Australia and South Africa populations, significant deviation from Hardy-Weinberg equilibrium of the loci tested and RAPD profiles suggested that the populations in these regions consist of clonal lines. Furthermore the limited number of alleles observed in South African and Australian populations implied an introduced origin of *P. cinnamomi* (Old et al., 1984; Linde et al., 1997; Linde et al., 1999). The absence of recombination for *P. cinnamomi* populations was confirmed in Australia where three clonal lineages, commonly present in other regions in the world, were identified using four dinucleotide microsatellite markers (Dubrowsky et al., 2003). Loss of heterozigosity

identified in the Australian clonal lineages has shown the possibility that mitotic crossing over has been involved in the development of new genetic variability, even though sexual recombination has not been found (Dubrowsky et al., 2003).

I.4 Marker for population studies

Molecular markers have received much attention as useful tools to investigate intraspecific diversity of organisms and geographical distribution of genotypes within a species. Specific mitochondrial and nuclear markers may be used to follow and understand the changes in the genetic structure of populations. As reported above, there are different causes that can lead to a change in the genetic structure of a population. These causes can be identified through the analysis of appropriate markers within a representative number of samples (appropriate sampling technique) followed by accurate statistical analyses. In particular, markers can be used to determine the sources of introduction into new areas and migration events between different populations. Several markers have been used to analyze genetic diversity and population structure starting with isozymes, nuclear and mitochondrial fingerprinting, microsatellite and markers-based on sequencing approaches such as SNPs (single nucleotide polymorphisms).

It is important to mention that phylogenetic analysis at the intraspecific level can be influenced by specific 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; Templeton, 1998; Posada and Crandall, 2001).

I.4.1 Mitochondrial DNA

Mitochondrial DNA has been the marker of choice for microevolutionary and genealogical studies at the intraspecific level for many organisms, in particular for higher animal taxa (Avise, 2004). Because mitochondrial DNA is present in multiple copies it can be easily amplified and is a good target for PCR reactions (McDonald, 1997). Mitochondrial markers are independently inherited from the nuclear genome and in a uniparental way from the maternal parent (Forster and Coffey, 1990). They can be used to determine lines of descent or paths of gene flow (McDonald, 1997, Avise, 2004, Zink and Barrowclough, 2008).

The use of the mitochondrial genome is favored by a higher rate of mutation (evolutionary rate) compared to the nuclear genome and the absence of interference from recombination events (Avise, 2004; Zhang and Hewitt, 2003), which has been rarely documented in fungi (Seville et al., 1998; Anderson et al., 2001). The hypothesis of higher evolutionary rates in animal mtDNA enhanced its use in genealogical and phylogenetic studies. The rate of evolution of the mtDNA in plants was found to be lower compared to nuclear and chloroplast DNA (Palmer and Herbon, 1988) and therefore the latter was preferentially chosen as cytoplasmic marker (Wolfe et al., 1987).

Several factors were hypothesized as the cause of the faster evolution of animal mitochondrial DNA (Wilson et al., 1985; Gillespie, 1986): i) less functional constraints because mtDNA does not codify for protein involved in its transcription and translation; ii) high mutation rates due to less accurate mechanism of DNA repairing, iii) to the particular oxidative mitochondrial environment and fast turnover within cell lineages; iv) mitochondrial DNA is not protected by histones, and this leads to a lower functional constraints. Mitochondrial markers have been widely utilized also to study phytopathogenic fungi and oomycetes. They were applied to assess intraspecific variability in different *Phytophthora* species (Griffith and Shaw et al., 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* (F. Martin, unpublished). Furthermore, intraspecific mt DNA variation in fungi has been useful for testing hypothesis on the evolutionary origins of *P. infestans* (Ristaino et al., 2001; Alpizar et al., 2007) and for providing evidence of recombination in mitochondrial genome in fungi (Anderson et al., 2001; Saville et al., 1998).

The "haploid genome" of mtDNA represents a great advantage in the estimation of haplotypes that characterize a population and in the genealogical and phylogenetic analysis since it gives the maternal history of the population. This characteristic highly biased phylogenetic analyses because the mitochondrial DNA can not entirely represent the history of a population. Furthermore, the mtDNA is characterized by a faster lineage sorting and a higher allele extinction rate, since it has only a quarter of the population size of the nuclear DNA (in a diploid organisms, 1 mitochondrial locus vs 4 nuclear alleles). Another important aspect is that for the higher lineage sorting and alleles extinction of the mitochondrial DNA is very likely to lose some genealogical connection during a pedigree analysis (Zhang and Hewitt et al., 2003). Although, the mitochondrial DNA remains in some respects the marker of choice for phylogeographic analyses, in some cases, particularly for studying the genealogy and the genetic structure in species in which the reproductive system is not exclusively clonal reproduction, nuclear markers are necessary to follow the evolutionary processes and genealogy in sexual recombining populations.

I.4.2 Nuclear marker

I.4.2.1 *Random amplified polymorphic DNA (RAPD)*

This technique is based on the amplification of random DNA regions using a single short primer (commonly 10 bp decamer) (Williams et al., 1990). A major advantage of RAPDs is that it does not require specific previous knowledge of the genome of the target individual. This technique has been widely utilized to study the genetic diversity of different organisms

for its easy application and interpretation of results and also for its low cost. In fact, polymorphisms are identified as the presence or absence of a particular DNA amplicon. The technique is informative since different independent loci in the genome of an organism can be amplified and screened and a limited quantity of DNA is required. RAPDs are dominant markers and this characteristic does not allow the differentiation of the homozygote from the heterozygote genotype, which could be an issue in diploid organisms such as Oomycetes (McDonald et al., 1997).

A major drawback of RAPD is the low reproducibility of the technique since amplification patterns can be influenced by a number of factors including the quality of target DNA, PCR reagents, Thermal cycler, manual skill of the operators, etc. This is partially due to the low stringency that characterizes the technique (annealing temperature from 40-50°C) that may also allow the amplification of non-specific or partially-specific targets.

I.4.2.2 *Restriction fragment length polymorphisms (RFLP)*

This technique has been widely applied for the analysis of polymorphisms in both nuclear and mitochondrial genomes and specifically in plant pathology to study intraspecific variability. The analysis is based on the identification of differences in the molecular size of DNA fragments after enzymatic digestion. Individuals with different positions for the restriction cutting sites will produce fragments of different lengths. The results of this technique are easily interpretable since it consists in looking at the presence of bands of different lengths. Furthermore, this type of marker is codominant and allows for the identification of homozygous and heterozygous individuals. An advantage compared to RAPD analyses is the reproducibility of results since the same results can be obtained by using the same battery of probes and restriction enzymes, allowing the comparison of data from different scientists, time periods and laboratories (McDonald et al., 1997). However this method may not accurately discriminate closely related strains, requires a relatively high quantity of DNA

(around 5-10 μg) and is quite laborious and costly. An alternative to RFLP is PCR-RFLP that avoids the slow hybridization step with the probe. This technique is performed by digesting locus-specific PCR amplicons with one or more restriction enzyme, followed by separation of the digested DNA on agarose or polyacrylamide gels (Konieczny and Ausubel, 1993). The method is simple, relatively inexpensive, and utilizes the ubiquitous technologies of PCR, restriction digestion and agarose gel analysis (Agarwal et al., 2008).

I.4.2.3 *Amplified fragment length polymorphisms (AFLP)*

AFLP is based on the analysis of whole genome restriction fragments which are selectively amplified by PCR after ligation with specific adapters to the cohesive ends produced by the restriction enzyme (Vos et al., 1995). The fragments produced by amplification reactions are visualized on denaturing polyacrylamide gels either through autoradiography or using fluorescent labeled-primers with automated DNA sequencing methods. This technique shares some characteristics with RAPDs; it does not require specific knowledge of the target genome, allows for the identification of many polymorphic loci within the genome and only dominant markers can be analyzed. A significant advantage of this technique compared to RAPDs is the more accurate discrimination of closely related strains since it produces a much more complex polymorphic patterns with a very high number of fragments. Furthermore, the use of primers specific for the sequences of the adaptors and for the restriction sites enable a higher reproducibility of results, although the comparison of data from different laboratories and time period still remain challenging. Disadvantages of AFLPs are the complexity of the technique, the higher cost of reagents and facilities needed for the analyses and the more complex interpretation of data.

I.4.2.4 *Microsatellites*

Microsatellite, or simple sequence repeat (SSR), are genomic regions composed of tandem repeats usually ranging from 1 to 6 nucleotides. The repeated unit can be present different times (e.g. from 10 to 100 copies) in several individuals resulting in a polymorphism. Several features make these markers the most currently used and also preferred to mitochondrial markers (Zhang and Hewitt, 2003). First of all these markers are widely distributed in the genome in coding and non-coding regions, are easily amplifiable by PCR and give an high level of alleles polymorphisms due to high mutation rate of order of 10^{-4}. Although SSRs are widely used, little is know about microsatellite modes of evolution. Understanding the evolutionary mechanism of microsatellites is needed not only to realize how the genome is organized but also to better interpret and correctly use microsatellites data in population genetic studies (Ellegren, 2004). Two models of evolution are primarily considered: slipped-strand mispairing during DNA replication (Tachida and Iizuka, 1992) and unequal recombination between DNA strands (Harding et al., 1992). Apparently microsatellites are considered as neutral marker (Schlötterer & Wiehe 1999), even though there is evidence of structural and functional constraints. The hypothesis that microsatellites are neutral markers is supported by the fact that different loci were observed conserved among long evolutionary distance (Martin et al., 2002). However, multiple studies reveal the functional importance of several microsatellite loci. It was shown how microsatellites could be involved in DNA structure, in particular in telomeric and centromeric regions (Canapa et al., 2002). Furthermore it has been hypothesized that in DNA recombination dinucleotide motifs can act as hot spots for recombination due to their higher affinity for recombination enzymes (Biet et al., 1999). Finally it has been also seen that a different number of motif repetitions can be involved in differential expression of the nucleolin promoter gene (*Ncl*) in rat (Rothenburg et al., 2001); in the promoter of the *Escherichia coli lacZ* gene, the motif $(GAA)_{12}$ allows the

gene expression, whereas different repetition of the motif $(GAA)_{14-16}$, $(GAA)_{5-11}$ does not allow this gene to be expressed (Liu et al., 2000).

Several studies in the genus *Phytophthora* have shown the appropriateness of microsatellites for analysis of genetic structure and population dynamics and, of course, for analysis of genetic variability at intraspecific and interspecific level. For example, microsatellites have been used to distinguish three different populations of *P. ramorum* in Europe (EU1) and North America (NA1, NA2) (Ivors et al., 2006) and to study the dynamics of this pathogen population in Oregon (Prospero et al., 2007) and California (Mascheretti et al., 2008). In the study of *P. cinnamomi* SSRs were useful to detect the presence of three different clonal populations in Australia (Dobrowolski et al., 2003).

One of the major drawbacks for the use of microsatellites is the high cost for their development when specific primers are not available for a particular species. Furthermore, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring (Jarne and Lagoda, 1996). Null alleles may result in a biased estimate of the allelic and genotypic frequencies and an underestimation of heterozygosity (Dakin and Avise, 2004). Size homoplasy may occur at microsatellite loci due to the high mutation rates that generate forward and backward mutations, which may cause underestimation of genetic divergence or wrong assumption where a simply convergent or parallel evolution could be mistaken with descent.

I.4.2.5 *Single nucleotide polymorphisms (SNPs)*

The rapid growth of available data from genome projects has increasingly justified the use of SNPs as genetic markers in the evolutionary studies of population. Initially, SNPs were used in whole-genome linkage and association studies, but their high occurrence through the genomes, level of variation and easy screening suggested them as useful markers for studying

the evolutionary history of specific populations (Brumfield et al., 2003). Not always genomic data are not always available for a species of interest, therefore different approaches need to be considered to discover SNPs. Comparative genomic approaches can be used to design primer in conserved flanking regions of related species which genomes are already available, or sequencing of anonymous nuclear loci (Karl and Avise, 1993). A panel of mitochondrial and nuclear SNPs were discovered for a number of *Phytophthora* species representative of the entire genus using a similar approach (Schena and Cooke, 2006; Schena et al., 2008). A bias that may arise from the use of this method is the need for an initial screening for discovery of polymorphisms in a limited panel of isolates that include enough genetic variability. The use of a representative panel of individuals, limited in size and composition as compared with the target samples can save considerable time and money (Wakeley et al., 2001). Furthermore non-sequence methods such as melt curve analysis and alleles specific oligonucleotide real time PCR can be used to reduce the costs of the analyses. For example, an alleles-specific oligonucleotide (ASO) method was used to differentiate the European from the North American lineage of *P. ramorum* (Bilodeau et al., 2007).

The SNPs are easier to screen as compared with microsatellites and their being widespread in the genome make them ideal for evolutionary studies considering that several unlinked nuclear loci are required to estimate population genetic parameters with statistical confidence (Brumfield et al., 2003). SNPs have a lower mutation rates (10^{-8} - 10^{-9}) as compared to microsatellites, but because multiple mutations events are very improbable on the same site, most SNPs are bi-allelic and therefore appropriate for high-throughput genotyping (Brumfield et al., 2003).

I.4.2.5.1 *Challenges using nuclear sequence markers*

Nuclear sequence markers offer a great opportunity for studying population genetic structure and mechanisms of evolution but different analytical and/or biological issues need to be

addressed during SNPs analyses. They include difficulties during sequencing of PCR products, insertion and deletion polymorphisms, heterozigosity, recombination, selection and so on. In diploid organisms such as *Phytophthora* spp., as well as in haploid heterokaryotic organisms, one of the most important problems is the haplotype (allele) determination. Diploid organisms are often characterized by the presence of heterozygous loci which "hide" two different alleles (haplotypes). In some cases haploid tissues (i.e. antheridium or oogonium in *Phytophthora* spp.) or specific sex related chromosomes such as the Y chromosome in humans the haploid status of the gene can be known. Alternatively, there are several empirical and computational-statistical methods that allow the determination of haplotypes. Among the experimental methods, cloning of PCR products is the most used method. However the application of this technique on a wide scale is challenging, expensive and time-consuming. The analysis of different clones in needed to avoid the risk of losing genetic diversity (alleles) and reducing replication errors of Taq polymerase and recombination artifacts created during the cloning process (Zhang and Hewitt, 2003). Other experimental techniques that can be used are: i) allele-specific amplification; ii) haplotype separation by SSCP (Single-strand conformation polymorphism) or DGGE (Denaturating gradient gel electrophoresis); iii) allele dropout effect which consists of a substantial dilution of genomic DNA until a single molecule is present; and iv) haplotype resolution by denaturating high performance liquid chromatography (Taberlet et al., 1996).

Using computer software that implement specific algorithms it is possible to infer the frequency of specific haplotypes. The software PHASE based on Bayesian statistical method, assigns probabilities of haplotypes to individual sites within a specific haplotypes (Stephens et al., 2001). In a specific study to compare the efficiency of the software PHASE with the standard cloning procedure to reveal haplotypes in nuclear loci, the software was able to determine haplotypes correctly (in a comparable manner with cloning procedure) and significantly reduce costs and speeding up the analysis (Harrigan et al., 2008). Furthermore,

haplotypes inference performed well considering the evidence of recombination and homoplasy (Hurrigan et al., 2008). Another program for haplotype determination based on Bayesian approach is HAPLOTYPER (Niu et al., 2002).

I.5 Literature cited

Agarwal M, Shrivastava N, Padh H, 2008. Advances in molecular marker techniques and their applications in plant sciences. *Plant Cell Reports* **27**: 617-631.

Anderson JB, Wickens C, Khan M, Cowen LE, Federspiel N, Jones T, Kohn LM, 2001. Infrequent Genetic Exchange and Recombination in the Mitochondrial Genome of *Candida albicans*. *Journal of Bacteriology* **183**: 865-872.

Apple JL, 1967. Occurrence of race 1 of *Phytophthora parasitica* var. *nicotianae* in North Carolina and its implications in breeding for disease resistance. *Tob*acco *Sci*ence **11**.

Arentz F, and Simpson JA, 1986. Distribution of *Phytophthora cinnamomi* in Papua NewGuinea and notes on its origin. *Transactions of the British Mycological Society* **87**: 289-295.

Avise JC, 2004. Molecular Markers, Natural History, and Evolution, Second Edition Sinauer Associates, Inc. Publishers Sunderland Massachusetts.

Bandelt HJ, Forster P, Röhl A, 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* **16**: 37-48.

Biet E, Sun J-S, Dutreix M, 1999. Conserved sequence preference in DNA binding among recombination proteins: an effect of ssDNA secondary structure. *Nucleic Acids Research* **27**: 596-600.

Bilodeau G, 2008. Détection et génomique de *Phytophthora ramorum* agent causal de la mort subite du chêne (l'encre des chênes rouges) Chapitre IV : SNP Discovery and Multilocus Strain Genotyping in *Phytophthora ramorum*. PhD thesis.

Bilodeau G, Levesque CA, de Cock A, Briere SC, and Hamelin RC, 2007. Differentiation of European and North American genotypes of Phytophthora ramorum by real-time polymerase chain reaction primer extension. *Canadian Journal of Plant Pathology* **29**: 408-420.

Bruberg MB, Hannukkala A, Hermansen A, 1999. Genetic variability of *Phytophthora infestans* in Norway and Finland as revealed by mating type and fingerprint probe RG57. *Mycological research* **103**: 1609-1615.

Brumfield RT, Beerli P, Nickerson DA, Edwards SV, 2003. The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution* **18**: 249-256.

Cacciola SO, Magnano di San Lio G, 1998. Management of citrus diseases caused by *Phytophthora* spp. In: Ciancio A, Mukerji KG (eds), *Integrated management of diseases caused by fungi, phytoplasma and bacteria*. Springer Science+Business Media, pp. 61-84.

Canapa A, Cerioni PN, Barucca M, Olmo E, Caputo V, 2002. A centromeric satellite DNA may be involved in heterochromatin compactness in gobiid fishes. *Chromosome Research* **10**: 297-304.

Carlson SR, Wolff MF, Shew HD, and Wernsman, EA, 1997. Inheritance of resistance to race 0 of *Phytophthora parasitica var. nicotianae* from the flue-cured tobacco cultivar Coker 371-gold. *Plant Dis*. **81**:1269-1274.

Carter GA, Smith RM, and Brent, KJ, 1982. Sensitivity to metalaxyl of *Phytophthora infestans* populations in potato crops in south-west England in 1980 and 1981. *Annals of Applied Biol*ogy **100**:433-441.

Cavalier-Smith T, Chao E, 2006. Phylogeny and Megasystematics of Phagotrophic Heterokonts (Kingdom Chromista). *Journal of Molecular Evolution* **62**: 388-420.

Colburn and JH, Graham, 2007. Protection of Citrus rootstocks against *Phytophthora* spp. with a Hypovirulent isolate of *Phytophthora nicotianae*. *Phytopathology* **97**: 958-963.

Dakin EE, Avise JC, 2004. Microsatellite null alleles in parentage analysis. *Heredity* **93**: 504-509.

Dobrowolski MP, Tommerup IC, Shearer BL, O'Brien PA, 2003. Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology* **93**: 695-704.

Dobrowolski MP, Shearer BL, Colquhoun IJ, O'Brien PA, Hardy GES, 2008. Selection for decreased sensitivity to phosphite in *Phytophthora cinnamomi* with prolonged use of fungicide. *Plant Pathology* **57**: 928-936.

Drenth A, Tas ICQ, Govers F, 1994. DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. *European Journal of Plant Pathology* **100**:97-107.

Ellegren H, 2004. Microsatellites: simple sequences with complex evolution. *Nature Review Genet*ics **5**: 435-445.

Erwin DC, Ribeiro OK, 1996. Phytophthora *diseases worldwide*. St. Paul, MN, APS Press.

Forster H, and Coffey MD, 1990. Mating behaviour of *Phytophthora parasitica*: evidence for sexual recombination in oospores using DNA restriction fragment length polymorphisms as genetic markers. *Experimental Mycology* **14**:351-359.

Flier WG, Grunwald NJ, Kroon LPNM, Sturbaum AK, van den Bosch TBM, Garay-Serrano E, Lozoya-Salda H, Fry WE, Turkensteen LJ, 2003. The Population Structure of *Phytophthora infestans* from the Toluca Valley of Central Mexico Suggests Genetic

Differentiation Between Populations from Cultivated Potato and Wild Solanum spp. *Phytopathology* **93**: 382-390.

Fry W, 2008. *Phytophthora infestans*: the plant (and R gene) destroyer. *Molecular Plant Pathology* **9**: 385-402.

Fry WE, Grunwald NJ, Cooke DEL, McLeod A, Forbes GA, Cao K, 2009. Population Genetics and Population Diversity of *Phytophthora infestans*. John Wiley & Sons, Inc.

Fry WE, Goodwin SB, Matuszak JM, Spielman LJ, Milgroom MG, and Drenth A, 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annual Review of Phytopathology* **30**:107-129.

Gallup CA, MJ Sullivan, and HD, Shew, 2006. Black Shank of Tobacco. The Plant Health Instructor. DOI: 10.1094/PHI-I-2006-0717-01.

Gillespie JH, 1986. Variability of evolutionary rates of DNA. *Genetics* **113**: 1077-1091.

Gomez-Alpizar L, Carbone I, Ristaino JB, 2007. An Andean origin of *Phytophthora infestans* inferred from mitochondrial and nuclear gene genealogies. *Proceedings of the National Academy of Sciences* **104**: 3306-3311.

Goodwin SB, Sujkowski LS, and Fry, WE, 1996. Widespread distribution and probable origin of resistance to metalaxyl in clonal genotypes of *Phytophthora infestans* in the United States and western Canada. *Phytopathology* **86**:793-800.

Goodwin SB, 1997. The Population Genetics of *Phytophthora*. *Phytopathology* **87**: 462-473.

Goodwin SB, Smart CD, Sandrock RW, Deahl KL, Punja ZK, Fry WE, 1998. Genetic Change Within Populations of *Phytophthora infestans* in the United States and Canada During 1994 to 1996: Role of Migration and Recombination. *Phytopathology* **88**: 939-949.

Goss EM, Carbone I, Grunwald NJ, 2009. Ancient isolation and independent evolution of the three clonal lineages of the exotic sudden oak death pathogen *Phytophthora ramorum*. *Molecular Ecology* **18**: 1161-1174.

Graham JH, and Menge JA, 1999. Root health: Fungal diseases. Pages 126-135 in: Citrus Health Management. L. W. Timmer and L. W. Duncan, eds. American Phytopathological Society, St. Paul, MN.

Griffith GW, Shaw DS, 1998. Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. *Applied and Environmental Microbiology* **64**: 4007-4014.

Grunwald NJ, Flier WG, 2005. The Biology of *Phytophthora infestans* at Its Center of Origin. *Annual Review of Phytopathology* **43**: 171-190.

Grunwald NJ, Goss EM, Press CM, 2008. *Phytophthora ramorum*: a pathogen with a remarkably wide host range causing sudden oak death on oaks and ramorum blight on woody ornamentals. *Molecular Plant Pathology* **9**:729-740.

Grunwald NJ, Goss EM, Ivors K, Garbelotto M, Martin FN, Prospero S, Hansen E, Bonants PJ, Hamelin RC, Chastagner G, Werres S, Rizzo DM, Abad G, Beales P, Bilodeau GJ, Blomquist CL, Brasier C, Briere SC, Chandelier A, Davidson JM, Denman S, Elliott M, Frankel SJ, Goheen EM, de Gruyter H, Heungens K, James D, Kanaskie A, McWilliams MG, Man In 't Veld W, Moralejo E, Osterbauer NK, Palm ME, Parke JL, Sierra AM, Shamoun SF, Shishkoff N, Tooley PW, Vettraino AM, Webber J, Widmer TL, 2009. Standardizing the Nomenclature for Clonal Lineages of the Sudden Oak Death Pathogen, *Phytophthora ramorum*. *Phytopathology* **99**: 792-795.

Hansen EM, Reeser PW, Sutton W, Winton LM, Osterbauer N 2003. First report of A1 mating type of *Phytophthora ramorum* in North America. *Plant Disease* **87**: 1267.

Harding RM, Boyce AJ, Clegg JB, 1992. The Evolution of Tandemly Repetitive DNA: Recombination Rules. *Genetics* **132**: 847-859.

Harrigan RJ, Mazza ME, Sorenson MD, 2008. Computation vs. cloning: evaluation of two methods for haplotype determination. *Molecular Ecology Resources* **8**: 1239-1248.

Ivors K, Garbelotto M, Vries ID, Ruyter-Spira C, Te Hekkert B, Rosenzweig N, Bonants P, 2006. Microsatellite markers identify three lineages of *Phytophthora ramorum* in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology* **15**: 1493-1505.

Ivors K, Hayden KJ, Bonants PJ, Rizzo DM, Garbelotto M, 2004. AFLP and phylogenetic analyses of North American and European populations of *Phytophthora ramorum*. *Mycological Research* **108**: 378-392.

Jarne P, Lagoda PJL, 1996. Microsatellites, from molecules to populations and back. *Trends in Ecology and Evolution* **11**: 424-429.

Judelson HS, 2009. Sexual Reproduction in Oomycetes: Biology, Diversity, and Contributions to Fitness. John Wiley & Sons, Inc.

Karl SA, and Avise JC, 1993. PCR-based assays of Mendelian polymorphisms from anonymous single-copy nuclear DNA: techniques and applications for population genetics. *Molecular Biology and Evolution* **10**: 342–361

Kirk PM, Cannon PF, Minter DW, Stalpers JA, 2001. Dictionary of the fungi (10th Ed.) CABI, UK, CBS, The Netherlands.

Koh YJ, Goodwin SB, Dyer AT, Cohen BA, Ogoshi A, Sato N, and Fry WE, 1994. Migrations and displacements of *Phytophthora infestans* populations in east Asian countries. *Phytopathology* **84**: 922-927.

Konieczny A, Ausubel FM, 1993. Procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. *Plant Journal* **4**:403-410.

Kurdyla TM, Guthrie PAI, McDonald BA, Appel DN, 1995. RFLPs in mitochondrial and nuclear DNA indicate low levels of genetic diversity in the oak wilt pathogen Ceratocystis *fagacearum*. *Current Genetics* **27**: 373-378.

Lamour KH, Daughtrey ML, Benson DM, Hwang J, Hausbeck MK, 2003. Etiology of *Phytophthora drechsleri* and *P. nicotianae* (=*P. parasitica*) diseases affecting floriculture crops. *Plant Disease* **87**: 854-858.

Leoni C, Ghini R, 2006. Sewage sludge effect on management of *Phytophthora nicotianae* in citrus. *Crop Protection* **25**: 10-22.

Lehtinen A, Hannukkala A, Andersson B, Hermansen A, Le VH, Nærstad R, Brurberg MB, Nielsen BJ, Hansen JG, Yuen J, 2008. Phenotypic variation in Nordic populations of *Phytophthora infestans* in 2003. *Plant Pathology* **57**: 227-234.

Linde C, Drenth A, Kemp GHJ, Wingfield MJ, and von Broembsen SL, 1997. Population structure of *Phytophthora cinnamomi* in South Africa. *Phytopathology* **87**: 822-827

Linde C, Drenth A, Wingfield MJ, 1999. Gene and genotypic diversity of *Phytophthora cinnamomi* in South Africa and Australia revealed by DNA polymorphisms. *European Journal of Plant Pathology* **105:** 667-680.

Liu L, Dybvig K, Panangala VS, van Santen VL, French CT, 2000. GAA trinucleotide repeat region regulates M9/pMGA gene expression in *Mycoplasma gallisepticum*. *Infection and Immunity* **68**: 871-876.

Lucas, G. B. 1975. Diseases of Tobacco, 3rd ed. Biological Consulting Associates, Raleigh, NC.

Magnano di San Lio, G., Cacciola, SO, Marziano F, Pane A, & Raudino F, 2002. Protezione integrata dell'agrumeto dalle malattie fungine. *Informatore fitopatologico – La difesa delle piante* **52**: 13-23.

Martin FN, 2008. Mitochondrial haplotype determination in the oomycete plant pathogen *Phytophthora ramorum*. *Current Genetics* **54**: 23–34.

Martin FN, 2010. Mitochondrial Haplotype Analysis as a Tool for Differentiating Isolates of *Verticillium dahliae*. *Phytopathology* **100**: 1231-1239.

Mascheretti S, Croucher PJP, Vettraino A, Prospero S, Garbelotto M, 2008. Reconstruction of the Sudden Oak Death epidemic in California through microsatellite analysis of the pathogen *Phytophthora ramorum*. *Molecular Ecology* **17**: 2755–2768.

Matheron ME, Porchas M, and Matejka JC, 1997. Distribution and seasonal population dynamics of *Phytophthora citrophthora* and *P. parasitica* in Arizona citrus orchards and effect of fungicides on tree health. *Plant Disease* **81**:1384-1390.

Martin AP, Pardini AT, Noble LR, Jones CS, 2002. Conservation of a dinucleotide simple sequence repeat locus in sharks. *Molecular Phylogenetics and Evolution* **23**: 205-213.

McDonald BA, Linde C, 2002. Pathogen Population Genetics, Evolutionary Potential, and Durable Resistance. *Annual Review of Phytopathology* **40**: 349-379.

McDonald BA, 1997. The Population Genetics of Fungi: Tools and Techniques. *Phytopathology* **87**: 448-453.

Melton TA, and Broadwell A, 2003. Disease management. Pages 142-166 in: 2004 Flue-Cured Tobacco Information. N. C. Coop. Ext. Serv. Bull. AG-187 (revised), North Carolina State University, Raleigh.

Mircetich SM, and Zentmyer GA, 1966. Production of oospores and chlamydospores of *Phytophthora cinnamomi* in roots and soil. *Phytopathology* **56**: 1076.

Niu T, Qin ZS, Xu X, Liu JS, 2002. Bayesian Haplotype Inference for Multiple Linked Single-Nucleotide Polymorphisms. *American journal of human genetics* **70**: 157-169.

Old KM, Moran GF and Bell JC, 1984. Isozyme variability among isolates of *Phytophthora cinnamomi* from Australia and Papua New Guinea. *Canadian Journal of Botany* **62**: 2016-2022

O'Neill JR, 2009. The Irish Potato Famine, ABDO Publishing, ISBN 9781604535143.

Palmer J, Hebron LA, 1988. Plant mitochondrial DNA evolves rapidly in structure but slowly in sequence. *Journal Molecular Evolution* **28**: 87-97.

Pane A, Martini P, Chimento A, Rapetti S, Savona S, Grasso FM, Cacciola SO, 2005. *Phytophthora* species on ornamental plants in Italy. *Journal Of Plant Pathology* **87**: 301 (Abstract).

Parkunan V, Johnson CS, Bowman BC, Hong CX, 2010. Population Structure, Mating Type, and Mefenoxam Sensitivity of Phytophthora nicotianae in Virginia Tobacco Fields. *Plant Disease* **94**: 1361-1365.

Posada D, Crandall KA, 2001. Intraspecific gene genealogies: trees grafting into networks. *Trends in Ecology & Evolution* **16**: 37-45.

Pratt BH, and Heather WA, 1973. The origin and distribution of *Phytophthora cinnamomi* Rands in Australian native plant communities and the significance of its association with particular plant species. *Australian Journal of Biological Sciences* **26**: 575–582

Prospero S, Hansen EM, Grünwald NJ, Winton LM, 2007. Population dynamics of the sudden oak death pathogen *Phytophthora ramorum* in Oregon from 2001 to 2004. *Molecular Ecology* **16**: 2958-2973.

Ristaino JB, Groves CT, Parra GR, 2001. PCR amplification of the Irish potato famine pathogen from historic specimens. *Nature* **411**: 695-697.

Rothenburg S, Koch-Nolte F, Rich A, Haag F, 2001. A polymorphic dinucleotide repeat in the rat nucleolin gene forms Z-DNA and inhibits promoter activity. *Proceedings of the National Academy of Sciences USA* **98**: 8985-8990.

Sandler HA, Timmer LW, Graham JH, and Zitko SE, 1989. Effect of fungicide applications on populations of Phytophthora parasitica, and on feeder root densities and fruit yields of citrus trees. *Plant Disease* **73**:902-906.

Saville BJ, Kohli Y, and Anderson JB, 1998. mtDNA recombination in a natural population. *Proceedings of the National Academy of Sciences*. **95**:1331-1335.

Schena L, Cardle L, Cooke DEL, 2008. Use of genome sequence data in the design and testing of SSR markers for *Phytophthora* species. *BMC Genomics* **9**: 620.

Schena L, Cooke DEL, 2006. Assessing the potential of regions of the nuclear and mitochondrial genome to develop a "molecular tool box" for the detection and characterization of *Phytophthora* species. *Journal of Microbiological Methods* **67**: 70-85.

Schlötterer C, Wiehe T, 1999. Microsatellites, a neutral marker to infer selective sweeps. In: Microsatellites: Evolution and Applications (eds Goldstein DB, Schlštterer C), pp. 238-247. Oxford University Press, Oxford.

Sommerhalder RJ, McDonald BA, Zhan J, 2007. Concordant evolution of mitochondrial and nuclear genomes in the wheat pathogen *Phaeosphaeria nodorum*. *Fungal Genetics and Biology* **44**: 764-772.

Stephens M, Smith NJ, Donnelly P, 2001. A New Statistical Method for Haplotype Reconstruction from Population Data. *American journal of human genetics* **68**: 978-989.

Sujkowski LS, Goodwin SB, Dyer AT, and Fry WE, 1994. Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. *Phytopathology* **84**:201-207.

Sullivan MJ, Melton TA, and Shew HD, 2005. Managing the race structure of *Phytophthora parasitica* var. nicotianae with cultivar rotation. *Plant Disease* **89**:1285-1294.

Sullivan MJ, Parks EJ, Cubeta MA, Gallup CA, Melton TA, Moyer JW, Shew HD, 2010. An assessment of the genetic diversity in a field population of *Phytophthora nicotianae* with a changing race structure. *Plant Disease* **94**: 455-460.

Taberlet P, Griffin S, Goossens Bt, Questiau S, Manceau Vr, Escaravage N, Waits LP, Bouvet J, 1996. Reliable Genotyping of Samples with Very Low DNA Quantities Using PCR. *Nucleic Acids Research* **24**: 3189-3194.

Templeton AR, 1998. Nested clade analyses of phylogeographic data: testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**: 381–397.

Thines M, Kamoun S, Oomycete-plant coevolution: recent advances and future prospects. *Current Opinion in Plant Biology* **13**: 427-433.

Timmer LW, Sandler HA, Graham JH, and Zitko SE, 1988. Sampling citrus orchards in Florida to estimate populations of *Phytophthora parasitica*. *Phytopathology* **78**:940-944.

Tsui CKM, Marshall W, Yokoyama R, Honda D, Lippmeier JC, Craven KD, Peterson PD, Berbee ML, 2009. Labyrinthulomycetes phylogeny and its implications for the evolutionary loss of chloroplasts and gain of ectoplasmic gliding. *Molecular Phylogenetics and Evolution* **50**:129-140.

Tyler BM, Tripathy S, Zhang X, Dehal P, Jiang RHY, Aerts A, Arredondo FD, Baxter L,

Bensasson D, Beynon JL, Chapman J, Damasceno CMB, Dorrance AE, Dou D, Dickerman AW, Dubchak IL, Garbelotto M, Gijzen M, Gordon SG, Govers F, Grunwald NJ, Huang W, Ivors KL, Jones RW, Kamoun S, Krampis K, Lamour KH, Lee M-K, McDonald WH, Medina M, Meijer HJG, Nordberg EK, Maclean DJ, Ospina-Giraldo MD, Morris PF, Phuntumart V, Putnam NH, Rash S, Rose JKC, Sakihama Y, Salamov AA, Savidor A, Scheuring CF, Smith BM, Sobral BWS, Terry A, Torto-Alalibo TA, Win J, Xu Z, Zhang H, Grigoriev IV, Rokhsar DS, Boore JL, 2006. *Phytophthora* Genome Sequences Uncover Evolutionary Origins and Mechanisms of Pathogenesis. *Science* **313**: 1261-1266.

Vercauteren A, De Dobbelaere I, Van Bockstaele E, Maes M, Heungens K, 2010 (a). Genotypic and phenotypic characterization of the European A2 isolates of *Phytophthora ramorum*. *European journal of plant pathology* 10.1007/s10658-010-9727-5.

Vercauteren A, De Dobbelaere I, Grunwald NJ, Bonants P, Van Bockstaele E, Maes M, Heungens K, 2010 (b).Clonal expansion of the Belgian *Phytophthora ramorum* populations based on new microsatellite markers. *Molecular Ecology* **19**: 92-107.

Vos P, Hogers R, Bleeker M, Reijans M, Lee Tvd, Hornes M, Friters A, Pot J, Paleman J, Kuiper M, Zabeau M, 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research* **23**: 4407-4414.

Wakeley J, Nielsen R, Liu-Cordero SN, Ardlie K, 2001. The Discovery of Single-Nucleotide Polymorphisms and Inferences about Human Demographic History. *American journal of human genetics* **69**: 1332-1347.

Werres S, De Merlier D, 2003. First Detection of *Phytophthora ramorum* Mating Type A2 in Europe. *Plant Disease* **87**: 1266-1266.

Weste G, 1994. Impact of *Phytophthora* species on native vegetation of Australia and Papua New Guinea. *Australasian Plant Pathology* **23**: 190–209.

Wilson AC, Cann RL, Carr SM, George M, Gyllensten UB, Helm-Bychowski KM, Higuchi RG, Palumbi SR, Prager EM, Sage RD, Stoneking M, 1985. Mitochondrial DNA and two perspectives on evolutionary genetics. *Biological Journal of the Linnean Society* **26**: 375-400.

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV, 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**: 6531-6535.

Wolfe KH, Li WH, Sharp PM, 1987. Rates of nucleotide substitution vary greatly among plant mitochondrial, chloroplast, and nuclear DNAs. *Proceedings of the National Academy of Sciences of the United States of America* **84**: 9054-9058.

Zentmyer GA, 1980. *Phytophthora cinnamomi* and the diseases it causes. The American Phytopathological Society, St. Paul, MN.

Zhang DX, Hewitt GM, 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology* **12**: 563-584.

Zhang XG, Sun WX, Guo L, Yu JF, Chang CJ, 2003. Genetic and pathogenic variation among tobacco black shank strains of *Phytophthora parasitica* var. *nicotianae* from the main tobacco growing in china. *Journal of Phytopathology* **151**: 259-266.

Zhang XG, Zheng GS, Han HY, Han W, Shi CK, Chang CJ, 2001. RAPD-PCR for diagnosis of *Phytophthora parasitica* var. *nicotianae* isolates which cause black shank on tobacco. *Journal of Phytopathology* **149**: 569-574.

Zink RM, Barrowclough GF, 2008. Mitochondrial DNA under siege in avian phylogeography. *Molecular Ecology*, **17**: 2107-2121.

Chapter II

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

II.1 ABSTRACT

A new method based on the analysis of mitochondrial intergenic regions characterized by intraspecific variation in DNA sequences was developed and applied to the study of the plant pathogen *Phytophthora nicotianae*. Two regions flanked by genes *trnY* and *rns* and *trnW* and *cox2* were identified by comparing the whole mitochondrial genomes of *P. infestans*, *P. ramorum* and *P. sojae* and amplified using primers designed from the flanking conserved genes. These regions were sequenced from 51 isolates of *P. nicotianae* of both A1 and A2 mating type recovered from different hosts and geographic regions. Amplicon length varied from 429 to 443 bp (*trnY/rns*) and 322 to 373 bp (*trnW*/*cox2*) with intraspecific variation due to single nucleotide polymorphisms and indels. Seventeen, 7 and 20 different haplotypes were detected by individually analyzing regions *trny-rns*, *trnw-cox2* and the combined data set of sequences from both regions, respectively. Phylogenetic analysis inferred with 3 different methods enabled the grouping of isolates in 5 clades, each containing different mitochondrial haplotypes and revealed diversity in the mitochondrial genome of *P. nicotianae*. The majority of isolates from citrus grouped in a single clade indicating either movement of isolates on planting stock or an association of particular isolates with this host. Phylogenetic groups were not correlated with the radial growth rate of the isolates or the rapidity of apple flesh colonization. The method developed in the present study represents an innovative molecular tool for the characterization of natural populations of *P. nicotianae* and should be easily expanded to other species of *Phytophthora* as well as other plant pathogens. It can be used to track specific haplotypes and, thanks to its high genetic resolution, it could be standardized and applied in a DNA barcoding like strategy for the precise identification of sub-specific taxa. Compared to alternative molecular methods, a major advantage is that results are unbiased (a list of nucleotides) and highly reproducible, thus enabling the comparison of data from different laboratories and time

periods. Furthermore, the method could be further enhanced by the identification of additional variable mitochondrial and/or nuclear genomic regions.

II.2 Introduction

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

Apart from *N. tabacum* and *Citrus* species, *P. nicotianae* is responsible for heavy losses on a number of other economically important species including fruit trees and herbaceous hosts (Erwin and Ribeiro, 1996). Recent surveys have revealed that this species is one of the most common pathogens on ornamental plants, the cultivation and sale of which has been recognized as a principal pathway for the introduction and

spread of invasive plant pathogens (Cacciola et al., 1997, 2001; Reichard and White, 2001; Pane et al., 2005; Moralejo et al., 2009). Commonly, ornamental nurseries are intensive farming systems in which many plant species are simultaneously and repeatedly cultivated with limited crop rotation. In such conditions multiple generations of the pathogen can occur and different genotypes, including opposite mating types, can come in contact thereby increasing the potential for sexual outcrossing and evolutionary divergence.

Despite the relevance of *P. nicotianae*, specific studies to evaluate intraspecific genetic variability and to establish the possible pathways by which the pathogen has been introduced and distributed to new areas are quite limited and mainly restricted to populations from tobacco. Random Amplified Polymorphic DNAs (RAPDs) were utilized to study the variability among seven populations of *P. nicotianae* from different tobacco fields (Zhang et al., 2003). Populations were genotypically and phenotypically variable, but no distinct genotypic differences were identified among populations from the seven locations. The same technique was applied to differentiate isolates causing black shank (Zhang et al., 2001) and to identify markers linked to the dominant black shank resistance gene (Johnson et al., 2002). Amplified Fragment Length Polymorphism (AFLP), utilized by Lamour et al. (2003) to study a population from different floricultural hosts and production sites, enabled the identification of 6 clonal lineages. Although RAPD-PCR and AFLP have proved valuable within a particular study, results obtained with such fingerprinting tools are not always easily reproducible in different laboratories (Cooke and Lees, 2004).

Several alternative molecular approaches have been proposed to study intraspecific variability of *Phytophthora* species (Cooke et al., 2007). Microsatellites or simple sequence repeats (SSRs) have been recognized as one of the most powerful choices, but their main limitation is the need for knowledge of the DNA sequence of the

SSR flanking regions in order to design specific primers. As a consequence, microsatellites have been widely utilized for those species whose genome has been partially or completely sequenced (Dobrowolski et al., 2003; Ivors et al., 2006; Prospero et al., 2007; Weng et al., 2007; Widmark et al., 2007), but their application to other *Phytophthora* species remains challenging despite recent attempts to create a comprehensive dataset of candidate SSRs for a range of species (Schena et al., 2008).

Accurate analysis of mitochondrial and nuclear DNA has elucidated the phylogenetic relationships within the genus *Phytophthora* with a grouping of 10 genetically related clades (Cooke et al., 2000; Kroon et al., 2004; Blair et al., 2008; Martin and Tooley., 2003) However, these studies were based on genes commonly conserved within a species and therefore unsuitable to characterize intraspecific variability. Recently, the analysis of different intergenic regions of the mitochondrial DNA (mt-IGS) from 31 *Phytophthora* species, representing the breadth of diversity in the genus, revealed the existence of regions too variable to be used for broad scale phylogenetic analyses. However, it was suggested these markers were suitable for the examination of intraspecific variation and the analysis of closely related species (Schena and Cooke, 2006). Intraspecific polymorphisms in mitochondrial DNA have been useful for characterizing populations by mitochondrial haplotypes for *P. infestans* (Griffith and Shaw, 1998) and the recent classification of mitochondrial haplotypes in *P. ramorum* should be useful for this species as well (Martin, 2008).

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

II.3 Materials and methods

II.3.1 Phytophthora nicotianae *isolates*

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

II.3.2 *Identification of easily amplifiable and variable mtDNA intergenic regions*

The complete mitochondrial genome from 4 different haplotypes of *P. infestans* (accession numbers NC_002387, AY894835, AY898627, AY898628), *P. ramorum* (accession number DQ832718) and *P. sojae* (accession number DQ832717) were aligned using ClustalX (http://www.clustal.org/) and manually analyzed to select variable intergenic regions flanked by conserved sequences on both sides. The latter condition was necessary to design primers suitable to amplify selected intergenic regions from other *Phytophthora* spp., including *P. nicotianae*. Two intergenic regions appeared to be of particular interest, one was flanked by genes *trnY* and *rns* (*trnY/rns*) and had been previously sequenced from a number of other *Phytophthora* species using primers Mt2F-Mt5R (Schena and Cooke, 2006). A second region, flanked by genes *trnW* and *cox2* (*trnW*/*cox2*) was amplified with two primers (Mt17F AAATACTTTTTAACAAAAGGGAATTTA and Mt12R TGGAGTTGCTGGATCTTGAA) selected among six candidates during preliminary tests to identify the best primer combinations and amplification conditions. All primers were designed using the Primer3 Software (Rozen and Skaletsky, 2000).

II.3.3 *DNA amplification and sequencing*

Total DNA was extracted from all isolates according to the procedure described by Ippolito et al. (2002). Amplification conditions consisted of 1 cycle of 94°C for 3

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

II.3.4 *Sequence analysis*

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

II.3.5 *Haplotype analysis and networks*

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

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

II.3.6 *Phylogenetic analysis*

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

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

II.3.7 *Biological tests*

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

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

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

Isolate code	Origin			Mating Type	*trnY-rns* region			*trnW-cox2* region			*trnY-rns + trnW-cox2*	
	Host	Region and Country	Year		Amplicon size (bp)	Accession number	Haplotype	Amplicon size (bp)	Accession number	Haplotype	Haplotype	Phylogenetic group
Albicocco9	*Prunus armeniaca*	Calabria (Southern Italy)	2005	A2	430	GU938492	1	373	GU938586	1	H1	N1
IMI 268688	*Citrus* sp.	Trinidad-Tobago	1982	A1	431	GU938493	16	373	GU938585	1	H2	N1
Dodonea Col1	*Dodonaea viscosa*	Sicily (Southern Italy)	2005	A2	429	GU938495	8	373	GU938591	1	H3	N1
Dodonea Rad1	*Dodonaea viscosa*	Sicily (Southern Italy)	2005	A2	429	GU938494	8	373	GU938592	1	H3	N1
Correa5	*Correa reflexa*	Sicily (Southern Italy)	2004	A1	431	GU938498	7	373	GU938590	1	H4	N1
Correa3	*Correa reflexa*	Sicily (Southern Italy)	2004	A1	432	GU938496	6	373	GU938588	1	H5	N1
Correa8	*Correa reflexa*	Sicily (Southern Italy)	2004	A1	431	GU938497	6	373	GU938589	1	H5	N1
Ciclamino1	*Cyclamen* sp.	Sicily (Southern Italy)	2004	A1	432	GU938499	5	373	GU938580	5	H6	N1
Ph168	*Citrus* sp. (root)	Tunisia	2003	A1	443	GU938542	4	373	GU938593	1	H7	N2
STA24	*Rhamnus alaternus*	Sicily (Southern Italy)	2000	A2	443	GU938540	4	373	GU938587	1	H7	N2
Ph440/00	*Cyclamen* sp.	Liguria (Northern Italy)	2004	A2	443	GU938539	4	373	GU938584	4	H8	N2
KVB	*Howea* sp.	Sicily (Southern Italy)	2000	A2	443	GU938541	4	373	GU938582	4	H8	N2
IRF26/2	*Impatiens wallerana*	Liguria (Northern Italy)	2007	A2	443	GU938512	4	373	GU938583	4	H8	N2
Ceanothus	*Ceanothus sp.*	Sicily (Southern Italy)	2002	A2	443	GU938538	4	373	GU938581	4	H8	N2
Ph3	*Citrus clementina* (fruit)	n.d.	2001	A1	431	GU938534	9	373	GU938551	6	H9	N3
Ph87	*Citrus clementina* (root)	Apulia (Southern Italy)	2000	A1	431	GU938531	9	373	GU938550	6	H9	N3
Ph195	*Citrus* sp.	Tartaus (Syria)	2003	A1	431	GU938535	9	373	GU938543	6	H9	N3
Ferrara R11	*Citrus* sp. (root)	Sicily (Southern Italy)	2004	A1	431	GU938529	9	373	GU938547	6	H9	N3
Ferrara R3	*Citrus* sp. (root)	Sicily (Southern Italy)	2004	A1	431	GU938533	9	373	GU938549	6	H9	N3
Ferrara R8	*Citrus* sp. (root)	Sicily (Southern Italy)	2004	A1	431	GU938536	9	373	GU938548	6	H9	N3
Serravalle 1	*Citrus* sp. (root)	Sicily (Southern Italy)	2004	A1	431	GU938530	9	373	GU938544	6	H9	N3
Ph342/03	*Limonium sinensis*	Liguria (Northern Italy)	2004	A2	431	GU938532	9	373	GU938545	6	H9	N3

Ph9	*Citrus* sp. *(soil)*	Apulia (Southern Italy)	n.d.	A1	432	GU938528	10	373	GU938552	6	H10	N3
Ph142	*Citrus trifoliata*	Valona (Albania)	2001	A1	432	GU938527	10	373	GU938553	6	H10	N3
Serravalle 3	*Citrus* sp. (root)	Sicily (Southern Italy)	2004	A1	430	GU938537	11	373	GU938546	6	H11	N3
SCRP462	*Fragaria x ananassa*	India	1998	A1	431	GU938522	15	366	GU938563	7	H12	N4
Ph653/03	*Choisya ternata*	Liguria (Northern Italy)	2004	A2	431	GU938518	15	373	GU938570	3	H13	N4
Ph647/b03	*Phormium tenax*	Liguria (Northern Italy)	2004	A2	431	GU938519	15	373	GU938568	3	H13	N4
Ph5	*Citrus* sp. (root)	Basilicata (Southern Italy)	2000	A1	431	GU938520	15	373	GU938572	3	H13	N4
Lavanda4	*Lavandula angustifolia*	Sicily (Southern Italy)	2002	A2	431	GU938516	15	373	GU938569	3	H13	N4
IMI 379626	*Lycopersicum esculentum*	Chile	n.d.	A1	431	GU938521	15	373	GU938571	3	H13	N4
Melanzana1	*Solanum melongena*	Sicily (Southern Italy)	1998	A2	431	GU938517	15	373	GU938564	3	H13	N4
Lavanda1	*Lavandula angustifolia*	Sicily (Southern Italy)	2002	A2	432	GU938514	14	373	GU938574	3	H14	N4
Mirtus3	*Myrtus communis*	Sicily (Southern Italy)	2002	A1	432	GU938515	14	373	GU938566	3	H14	N4
IRF5	*Polygala myrtifolia*	Liguria (Northern Italy)	2007	A2	433	GU938526	13	373	GU938573	3	H15	N4
TL8VP	*Lavandula angustifolia*	Piedimont (Northern Italy)	2000	A2	433	GU938524	3	373	GU938575	3	H16	N4
Nic8Vasi	n.d.	Sicily (Southern Italy)	2000	A2	433	GU938525	3	373	GU938576	3	H16	N4
C88	*Simmondsia chinensis*	Apulia (Southern Italy)	1984	A2	433	GU938523	3	373	GU938567	3	H16	N4
IMI 207770	*Durio zibethinus*	Malaysia	1976	A2	431	GU938508	12	373	GU938577	3	H17	N5
IRF3	*Polygala myrtifolia*	Liguria (Northern Italy)	2007	A2	431	GU938502	12	373	GU938565	3	H17	N5
C301	*Myrtus communis*	Sicily (Southern Italy)	1991	A2	430	GU938500	17	322	GU938557	2	H18	N5
IRF27	*Agapanthus* sp.	Liguria (Northern Italy)	2007	A2	431	GU938501	12	322	GU938561	2	H19	N5
IRF8	*Anemone americana*	Liguria (Northern Italy)	2007	A2	431	GU938503	12	322	GU938578	2	H19	N5
Peperone GJ	*Capsicum annuum*	Calabria (Southern Italy)	2000	A1	431	GU938506	12	322	GU938558	2	H19	N5
Peperone RC	*Capsicum annuum*	Calabria (Southern Italy)	2000	A2	431	GU938507	12	322	GU938560	2	H19	N5
Pomodoro	*Lycopersicum esculentum*	Sicily (Southern Italy)	2000	n.d.	431	GU938504	12	322	GU938555	2	H19	N5
Mirto p5	*Myrtus communis*	Sicily (Southern Italy)	2007	A2	431	GU938509	12	322	GU938579	2	H19	N5
Pittosporo	*Pittosporum* sp.	Sicily (Southern Italy)	1996	A1	431	GU938505	12	322	GU938559	2	H19	N5
Anthurium	*Anthurium* sp.	Sicily (Southern Italy)	2005	A1	432	GU938510	2	322	GU938554	2	H20	N5
Hybiscus B	*Hybiscus* sp.	Calabria (Southern Italy)	2004	A2	432	GU938511	2	322	GU938556	2	H20	N5
Pandorea2C	*Pandorea jasminoides*	Sicily (Southern Italy)	2005	A2	432	GU938513	2	322	GU938562	2	H20	N5

II.4 Results

II.4.1 *Characterization of polymorphic mtDNA regions*

Two different primer pairs were utilized to amplify mitochondrial regions *trny/rns* and *trnw*/*cox2* from 51 isolates of *P. nicotianae* from different hosts and geographic origins (Table 1). Amplicons exhibited variable length ranging from 429 to 443 bp (*trnY/rns*) and 322 to 373 bp (*trnW*/*cox2*) (Table 1). Intraspecific variability was observed as single nucleotide polymorphisms (SNPs), short indels (1-4 bp), long indels (a 19 bp indel and two 7 and 51 bp indels were revealed in the *trny/rns* and *trnW/cox2* regions, respectively) (Fig. 1) and length variations in a homopolimeric T region in the *trnY-rns* spacer (Fig 2). For the *trny-rns* region 9 SNPs were identified; 3 were transversions and 6 were transitions with 8 of these parsimony informative. When combined with data for indels 17 different haplotypes were observed. The *trnW-cox2* region was less polymorphic with 4 SNPs (3 transversions and 1 transition) and a single parsimony informative site; when combined with data for indels 7 haplotypes were present. Combining DNA sequences from the two intergenic regions identified 20 different haplotypes (Table 1).

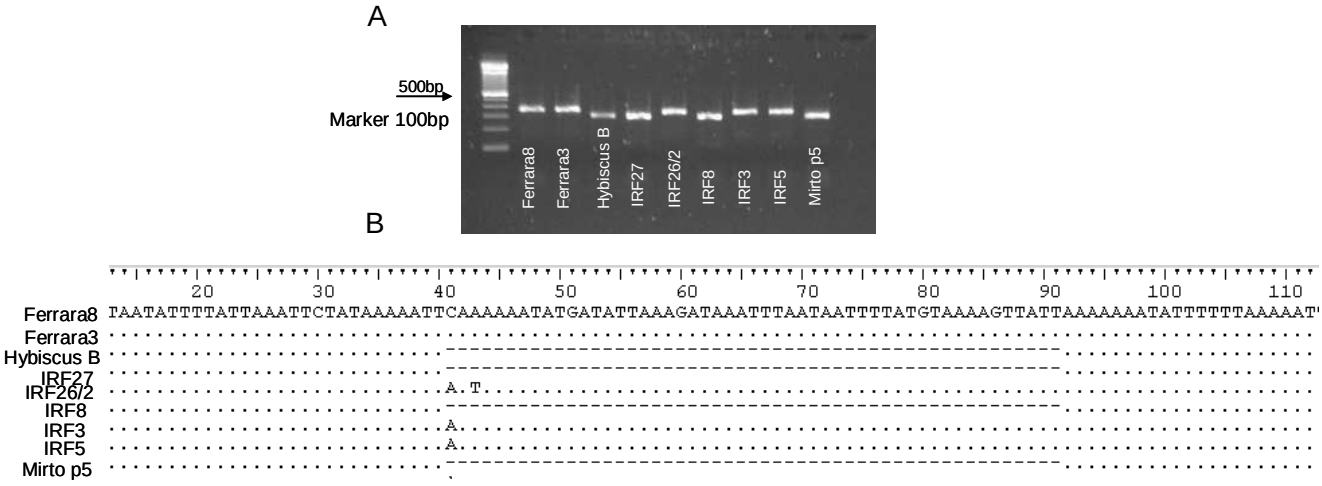

Fig. 1 - Electrophoretic gel containing PCR products of the mitochondrial intergenic region *trnW-cox2* of 9 *Phytophthora nicotianae* isolates (A). Differences in the length of fragments is due to a deletion of 51 bp (B).

The *P. nicotianae trnY/rns* region had a length comparable to that of the homologous region from *P. ramorum* (353 bp; DQ832718), but was significantly shorter compared to homologous regions in *P. sojae* (952 bp; DQ832717) and *P. infestans* haplotype IIa (2785 bp; AY898627), IIb (2729 bp; AY898628), Ia (904 bp; AY894835) and Ib (904 bp; NC_002387). Similarly, the *trnW/cox2* region in *P. nicotianae* was shorter compared to homologous sequences from *P. ramorum* (846 bp; DQ832718), *P. sojae* (725 bp; DQ832717) and *P. infestans* haplotype IIa (1007 bp; AY898627), IIb (849 bp; AY898628) and Ia (814 bp; AY894835) and Ib (850 bp; NC_002387).

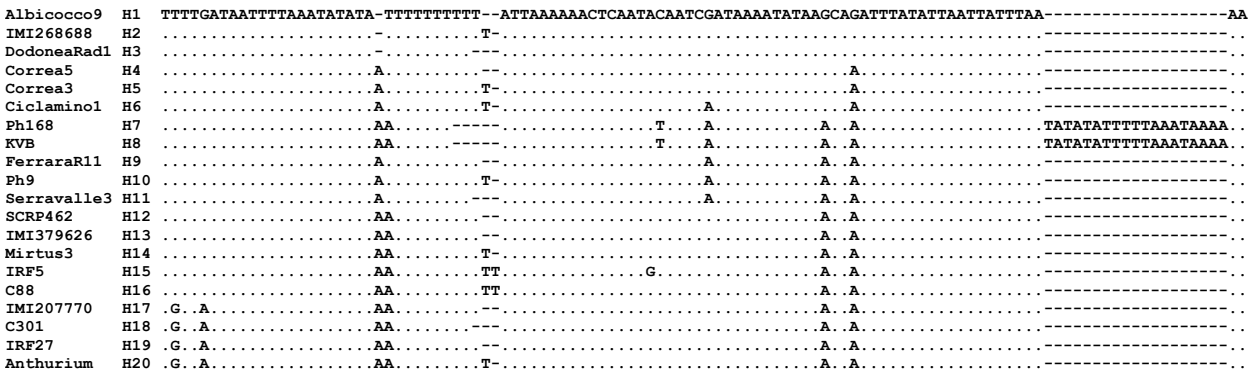

Fig. 2 - Sequence alignment of portion of the *trnY-rns* region of twenty representative *Phytophthora nicotianae* haplotypes.

II.4.2 *Haplotype analysis and networks*

Seventeen and 7 haplotypes were identified by individually analyzing regions *trny/rns* and *trnw/cox2*, respectively; the combined data set of sequences revealed 20 different haplotypes (Table 1). The haplotype network (Fig 3) of the combined data set confirmed the presence of five different genetic groups defined by the phylogenetic analyses (Fig 4). The N2 group was more distant compared to the other groups while the N3 group, which was almost exclusively represented by citrus isolates, presented the most frequent haplotype in the network. No patterns of geographic association were revealed among the most frequent haplotypes. The network has shown cases where identical haplotypes were present within isolates with opposite mating types. In particular, the haplotypes

H14, H19 and H20 had opposite mating types in the same geographic location (Table 1). Cases of homoplasy were observed in the combined data set network (Fig 3). This homoplasy was also confirmed in the analysis of the network for the two intergenic region considered individually (data not shown).

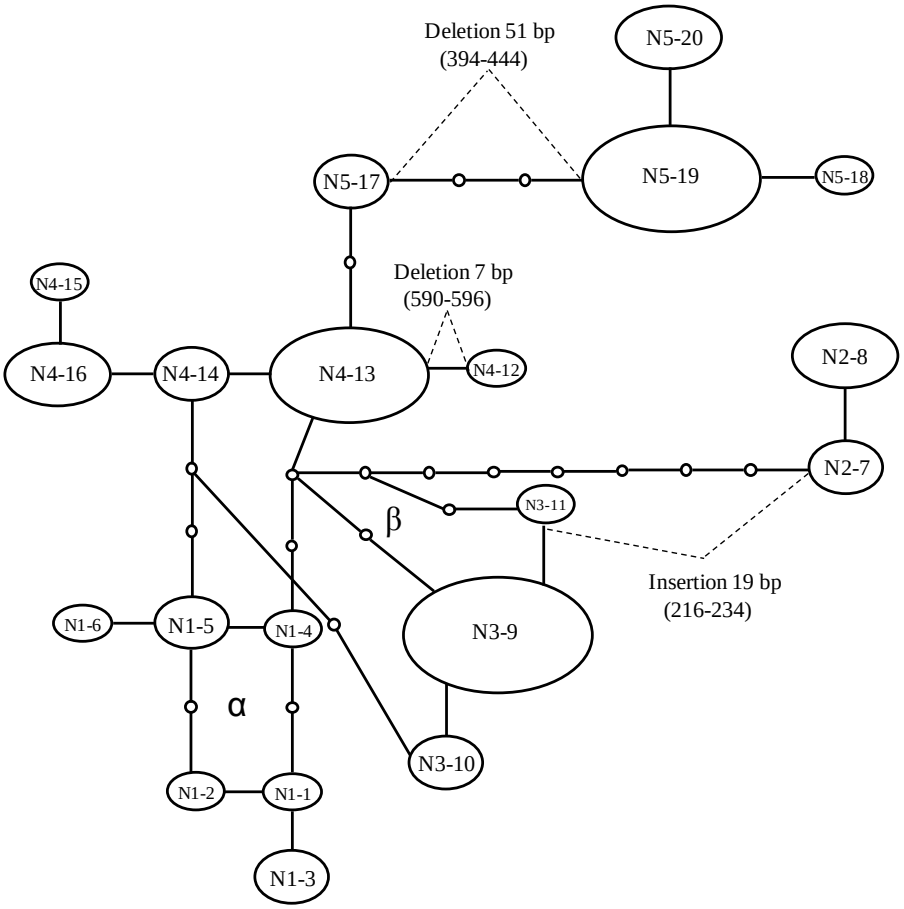

Fig. 3 - Parsimony haplotype network of *Phytophthora nicotianae* isolates constructed using combined sequence data from *trnW-cox2* and *trnY-rns* regions. The letter codes identify the single or the groups of haplotypes with N referring to the phylogenetic clade in Fig 3 and the number after the "-"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

II.4.3 *Phylogenetic analyses*

In the partition homogeneity test a significant congruence (p = 0.74) between the two mitochondrial regions was observed so the concatenated file of the two regions was used for analysis with the three different phylogenetic methods (maximum parsimony, maximum likelihood and Bayesian analysis). A tree with the same five major clades was observed with each type of analysis (Fig 4), a grouping that was also observed when the two regions were analyzed separately (although a less accurate discrimination among isolates was possible, data not shown).

The first clade (N1) was comprised of seven isolates from Southern Italy (6 from Sicily and 1 from Calabria) and an isolate (IMI 268688) obtained from citrus in Trinidad (Table 1; Fig 4). This clade contained six mitochondrial haplotypes distributed in three branches. Isolates of this clade shared differences that clearly distinguished them from the other clades such as a SNP in position 193 of the *trnY-rns* region. The isolate Ciclamino1 constituted an individual haplotype branch within this group. No mating type association within this clade was revealed. Six out of the eight isolates were recovered from host plants of the *Sapindales* order (*Correa reflexa*, *Dodonea viscosa* and *Citrus* sp.).

Clade N2 was well differentiated from other clades with two distinct mitochondrial haplotypes clustered in this group (Fig 4). Except for the citrus isolate Ph168, all isolates were mating type A2 (Table 1). Isolates of this clade had a 19 bp insertion in the *trnY-rns* region that differentiated them from all the other isolates analyzed in this work.

Clade N3 was almost exclusively represented by isolates from citrus recovered from different regions of southern Italy, Syria (isolate Ph195) and Albania (isolate Ph142). This clade also contained an isolate from *Limonium sinensis* that, unlike all other isolates, was an A2 mating type (Table 1; Fig 4). Three different mitochondrial

haplotypes clustered in this group that differed in base numbers in a homopolymeric thymine region in the *trnY-rns* intergenic region (Fig 2).

Five mitochondrial haplotypes clustered in clade N4 (Fig 4). This group of isolates was heterogeneous for geographic origin, host and mating type. Only two SNPs (pos. 148 and 394) differentiate this group of isolates from the N3 clade of isolates from citrus. Except for the isolate IRF5 from *Polygala myrtifolia* that constituted an individual haplotype branch within this group (SNP in position 175 in *trnY-rns* region) and the isolate SCRP462 that had a 7 bp deletion in the *trnW-cox2* region, the other isolates within this group were distinguished by differences in length variation in the homopolymeric thymine region in the *trny-rns* intergenic spacer.

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

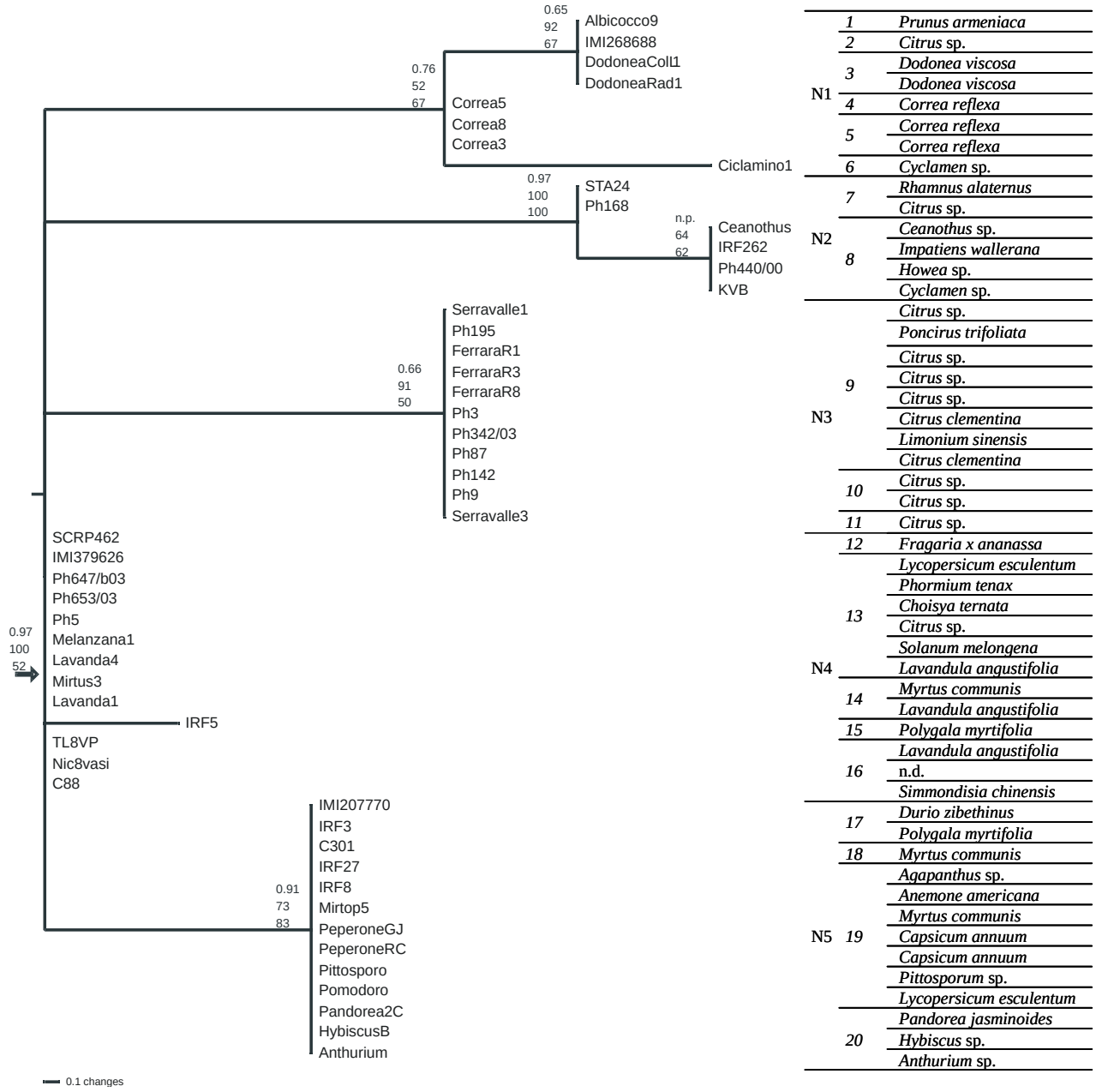

Fig. 4 - Phylogenetic relationships between *Phytophthora nicotianae* isolates based on the combined data set of sequences from the two mitochondrial intergenic region *trnY-rns* and *trnW-cox*2. The tree was midpoint rooted. Numbers on nodes represent the statistical support for the Bayesian method (posterior probabilities, top number), maximum likelihood (100 replicates, middle number) and maximum parsimony (1000 bootstrap replicates, bottom number). Table on the right of the tree reports phylogenetic clades (first column; N followed by a number) distinctive haplotypes (second column; number only) and plant host from which isolates were obtained (third column).

II.4.4 *Biological tests*

The characterization of 51 isolates of *P. nicotianae* from different geographic regions and hosts showed that 23 and 27 isolates were A1 and A2 mating type, respectively (Table 1). Among these, all isolates from citrus were of mating type A1.

One isolate from tomato did not form oospores when paired with the two reference mating types.

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

II.5 Discussion

Fifty-one isolates of *P. nicotianae* from different hosts and geographic origins were characterised using two variable intergenic regions of the mitochondrial DNA to evaluate mitochondrial haplotypes and their phylogenetic relationships. The analysis of haplotypes exhibited a different level of variation between the mitochondrial regions used. The *trnY-rns* intergenic region was more variable with 9 SNPs, 3 indels (29 bp) and length variations in a homopolymeric thymine region that differentiated 17 haplotypes. In contrast, for the *trnW-cox2* region 4 SNPs and 2 indels (58 bp) differentiated isolates into 7 mitochondrial haplotypes. The combined data set for both regions revealed a total of 20 mitochondrial haplotypes.

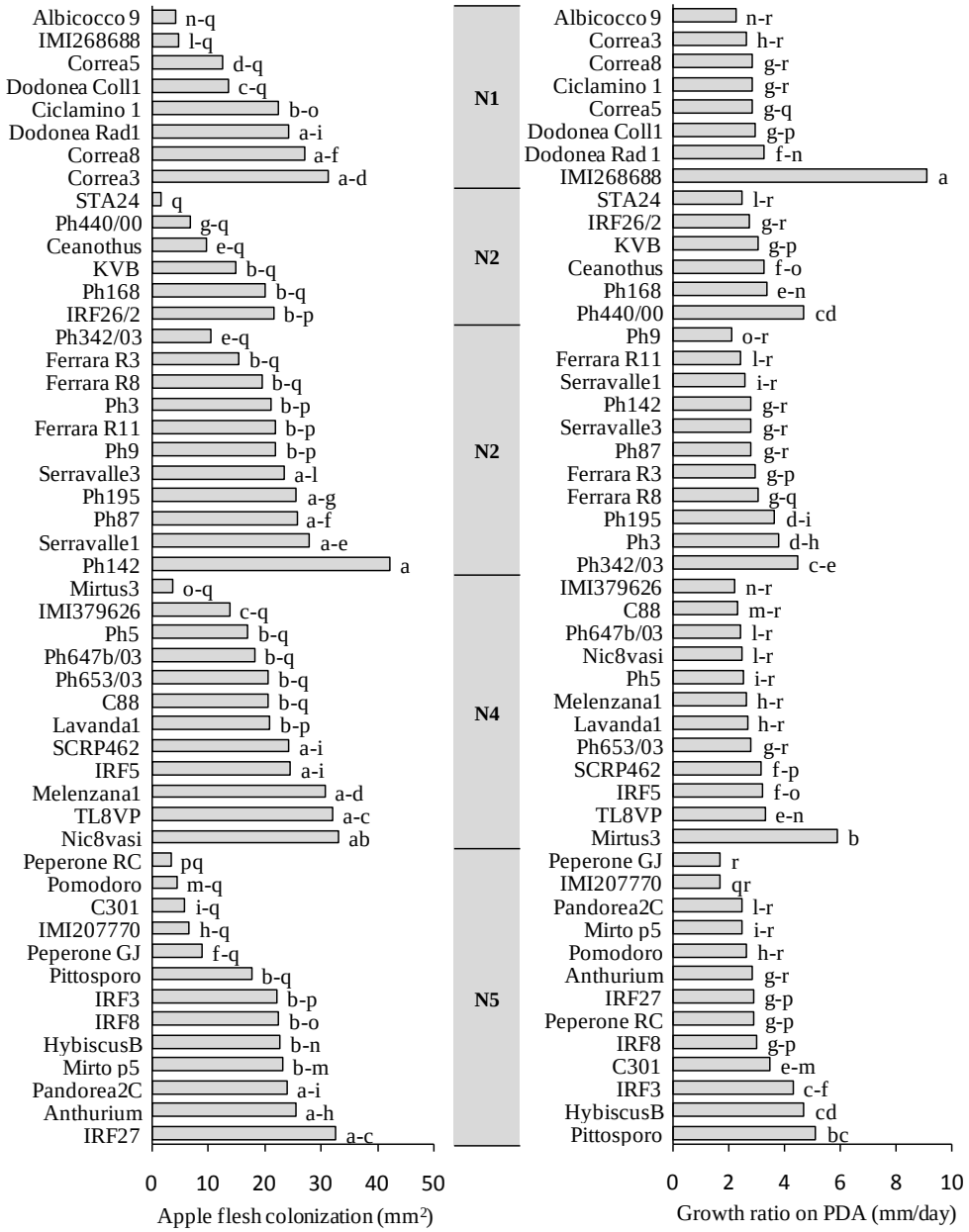

Fig. 5 - Comparison between apple flesh colonization after 5 days of incubation (left) and growth rate on PDA (right) of *Phytophthora nicotianae* isolates grouped according to phylogenetic clades (center). In both charts isolates were primarily listed accordingly to their phylogenetic groups (Fig 2)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

No consistent association of haplotypes with the geographic location of isolation or host from which the isolates were recovered was observed. The majority of isolates from citrus had similar haplotypes (H9, H10, H11) and differed only in the number of bases in a homopolymeric thymine region (haplotypes with 9, 10 or 11 thymine bases).

Similar length variations in homopolymeric stretches of "T" were also identified in other groupings of haplotypes (H13, H14, H16 and H18, H19, H20; Fig 2). It is likely these differences were generated by slippage events during DNA replication, which is known as source of length polymorphisms in sequence stretches in human populations (Schlötterer and Tautz, 1992). The fact that isolates from citrus recovered from different geographic regions had the same mitochondrial haplotype (or very similar) could be due to the nursery trade distributing infected plant material, which could also explain the presence of identical haplotypes for isolates from ornamental and horticultural plants from different production areas (H8, H13, H17, H19).

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

The use of mitochondrial markers, which are maternally inherited, would be useful to study clonally reproducing populations of the pathogen. Clonal populations of *P. nicotianae* were identified as a component of field populations from the same tobacco field in 4 consecutive years (Sullivan et al., 2010) and for isolates from different ornamental plants and production sites (Lamour et al., 2003). It was suggested that isolates within the same ornamental nurseries spread by asexually generated propagules, such as hyphal fragments, sporangia, chlamydospores or zoospores (Lamour et al., 2003). Furthermore, it was concluded that, at least in citrus orchards in Italy, *P. nicotianae* reproduced primarily asexually since in the majority of citrus orchards examined only the A1 mating type was found (Cacciola and Magnano di San Lio, 2008). However, the heterothallic mating behaviour of this pathogen can generate a sexual recombining population when both mating types are present and therefore nuclear markers should be included when analyzing field populations in much the same way as observed for *P. infestans* (Flier et al., 2007; Widmark et al., 2007). The observations that opposite mating types were found within the same mitochondrial haplotype (H7, H9, H13, H14, H19, H20 with isolates from the last three haplotypes having opposite mating types in the same geographic region) suggests that sexual recombination has occurred in the past.

These two mitochondrial intergenic regions were used also to infer phylogenetic analysis and highlight evolutionary divergence at mitochondrial genome level. The phylogenetic analysis of the combined sequences identified 5 phylogenetic clades, a result that was consistent with three different methods of analysis (maximum parsimony, maximum likelihood and Bayesian analysis).

Different haplotypes were distributed in each of the 5 clades and did not exhibit a consistent clustering based on geographic origin, mating type or host. However, the majority of haplotypes representing citrus isolates (10/13) grouped together in clade N3,

reinforcing the idea that these haplotypes came from the same ancestral mitochondrial genome and indicating that the differences found at level of homopolymeric T region (as for some isolates of N4 and N5 clades), were meaningless in a phylogenetic sense. A divergent evolutionary pattern was shown in particular for clade N2 as shown also in the parsimony haplotype network (Fig 3). Two haplotypes (H7, H8) were identified in the N2 clade by a different number of polymorphisms. In particular, 3 SNPs that were parsimony informative (SNPs specific of this clade), a 19 bp insertion and 4 bp deletions differentiated this clade from the others. In contrast, the lower amount of genetic variation between the N3 clade and the N4, clade N5 and N4, N1 and N4 suggested a more recent evolutionary divergence among these groups of haplotypes. In particular, only 2 SNPs that were parsimony informative differentiated the clade N5 (haplotype H17 of clade N5, Fig 4) from the clade N4 (haplotype H13 of clade N4); 3 SNPs that were parsimony informative discriminated clade N1 (haplotype H5, Fig 4) and the citrus clade N3 from clade N4 (from haplotypes H14 and H13 of clade N4). Furthermore, as shown in the haplotype network analysis, the homoplasy revealed between haplotypes could explain the reduction of evolutionary resolution, in particular, between the citrus clade N3 and clade N4. This not to exclude the possibility that events of inter molecular recombination between different mitochondrial DNA can occur leading to homoplasies, although given the uniparental inheritance of mitochondrial genomes in sexual outcrossing this would be unlikely (Forester and Coffey, 1990). Taking into consideration that the majority of these isolates come from nurseries, the conjunct effect of rare mutation in mitochondrial DNA and possible drift of haplotypes due to different trade pathways could be involved in establishing new divergent haplotypes populations.

Whereas mitochondrial DNA was one of the markers more available in the studies of population evolution, we need to bear in mind that phylogenetic analysis

based solely on mitochondrial markers could have some important limitations. The main thing to consider is that mitochondrial DNA represents the historical map of the maternal lineage, ignoring part of the genetic history present in the population (Zhang and Hewitt, 2003). Therefore, a phylogenetic analysis based only on this cytoplasmatic marker may only partially help to answer questions on the evolution of populations and resulting evolutionary relationships may be biased. However, these two intergenic regions give an idea, even partly, of the evolutionary relationships between the haplotypes identified in this study. A larger number of isolates representing the range of diversity of *P. nicotianae* is required to verify the usefulness of these mitochondrial intergenic regions to highlight different evolutionary pathways.

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

A key step for the development of the present method was the identification of genetic regions variable enough to differentiate closely related strains of *P. nicotianae*. The *trny-rns* region had been previously amplified and sequenced from a number of *Phytophthora* species, but not *P. nicotianae* (Schena and Cooke, 2006). In their report the Authors concluded this region was too variable to align accurately when amplified

from different species and suggested its possible use for the examination of intraspecific variation and analysis of closely related species. Unlike the *trny-rns* region, the *trnW-cox2* region had not been amplified from other *Phytophthora* species but also appears to be useful for evaluation of intraspecific variation. Since both regions were amplified using primers designed using conserved regions from whole mitochondrial genome comparisons of *P. infestans*, *P. ramorum* and *P. sojae* it is likely will also be useful for the intraspecific characterization of a number of other *Phytophthora* species (this has been independently confirmed by F. Martin, unpublished).

In conclusion, a new molecular approach to characterize intraspecific variability in *P. nicotianae* is proposed. This method may be further improved by the identification of new target regions and could be easily extended to other species of the genus *Phytophthora* as well as other plant pathogens. Compared to alternative molecular methods, such as RAPD-PCR and AFLP, a major advantage of this approach is that results are objective (a list of nucleotides) and can be highly reproducible because the results are not affected by a number of factors such as the purity of target DNA, amplification reagents, thermocyclers, etc. This aspect is particularly important since it would enable the comparison of data from different research groups or time periods and could be implemented as soon as a molecular database is available from submission of sequences to GenBank. Application of this method could also be used in standardized protocols to develop a DNA barcoding like strategy for the precise identification of sub-specific taxa in *P. nicotianae* as well as in other species of *Phytophthora*. In fact, although more variable than the loci commonly used for barcode analysis of *Phytophthora* spp. (ITS, *cox1*; C. A. Levesque, personal communication), the regions used in this experimentation possess many of the important characteristics of a desirable locus for DNA barcoding since they are present in most of the taxa of interest, can be amplified without species-specific PCR primers and are short enough to be easily

sequenced with current technology (Kress and Erickson, 2008). A limitation to the wide exploitation of the method proposed in the present study could be represented by the cost of sequencing multiple target genes from a large number of isolates. However, the increasing development of high throughput sequencing equipment and the consequent reduction of sequencing costs recorded in recent years are encouraging.

II.6 Literature cited

Allagui MB, Lepoivre P, 2000. Molecular and pathogenicity characteristics of *Phytophthora nicotianae r*esponsible for root necrosis and wilting of pepper (*Capsicum annuum* L.) in Tunisia. *European Journal of Plant Pathology* **106**: 887-894.

Apple JL, 1957. Pathogenic, cultural and physiological variation within *Phytophthora parasitica* var. *nicotianae*. *Phytopathology* **47**: 733-739.

Blair JE, Coffey MD, Park SY, Geiser DM, Kang S, 2008. A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology* **45**: 266-277.

Bonnet P, Maia N, Tello-Marquina J, Venard P, 1978. Pathogenesis of *Phytophtora parasitica*: variability and relative host specificity. *Annual Review of Phytopathology* **12**: 109-118.

Breda de Haan J van, 1896. De bibitziekte in de Deli tabak veroorzaakt door *Phytophthora nicotianae* [The root disease in Deli-tobacco caused by *Phytophthora nicotianae*]. *Meded. S. Lands Plantentuin* 15.

Cacciola SO, Agosteo GE, Pennisi AM, Pane A, Pappalardo P, 2001. *Phytophthora* species in nurseries of ornamental plants in Southern Italy. *Journal of Plant Pathology* **83**: 231.

Cacciola SO, Pane A, Magnano di San Lio G, 1997. Identification and quantitative determination of *Phytophthora* species infecting ornamental plants in nurseries. In: Dehne HW, Adam G, Diekman M, Frahm J, Mauler-Machnik A, van Halteren P, (eds), *Diagnosis and identification of plant pathogens*. Kluwer Academic Publishers, Dordrecht, NL, pp. 483-485.

Cacciola SO, Magnano di San Lio G, 1998. Management of citrus diseases caused by *Phytophthora* spp. In: Ciancio A, Mukerji KG (eds), *Integrated management of diseases caused by fungi, phytoplasma and bacteria*. Springer Science+Business Media, pp. 61-84.

Clement M, Posada D, Crandall KA, 2000. TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**: 1657-1660.

Cline ET, Farr DF, Rossman AY, 2008. Synopsis of *Phytophthora* with accurate scientific names, host range, and geographic distribution. *Plant Health Progress* doi:10.1094/PHP-2008-0318-01-RS.

Colas V, Lacourt I, Ricci P, Vanlerberghe-Masutti F, Venard P, Poupet A, Panabières F, 1997. Diversity of virulence in *Phytophthora parasitica* on tobacco, as reflected by nuclear RFLPs. *Phytopathology* **88**: 205-212.

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM, 2000. A molecular phylogeny of *Phytophthora* and related *Oomycetes*. *Fungal Genetics and Biology* **30**: 17-32.

Cooke DEL, Lees AK, 2004. Markers, old and new, for examining *Phytophthora infestans* diversity. *Plant Pathology* **53**: 692-704.

Cooke DEL, Schena L, Cacciola SO, 2007. Tools to detect, identify and monitor *Phytophthora* species in natural ecosystems. *Journal of Plant Pathology* **89**: 13-28.

Dobrowolski MP, Tommerup IC, Shearer BL, O'Brien PA, 2003. Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology* **93**: 695-704.

Erwin DC, 1964. A strain of *Phytophthora parasitica* from Okra and its sexual compatibility with isolates from citrus. *Phytopathology* **54**: 114-115.

Erwin DC, Ribeiro OK, 1996. Phytophthora *diseases worldwide*. St. Paul, MN, APS Press.

Farris JS, Kallersjo M, Kluge AG, Bult C, 1995. Testing significance of incongruence. *Cladistics* **10:** 315–319.

Flier WG, Kroon LPNM, Hermansen A, van Raaij HMG, Speiser B, Tamm L, Fuchs JG, Lambion J, Razzaghian J, Andrivon D, Wilcockson S, Leifert C, 2007. Genetic structure and pathogenicity of populations of *Phytophthora* infestans from organic potato crops in France, Norway, Switzerland and the United Kingdom. *Plant Pathology* **56**: 562-572.

Forster H, and Coffey MD, 1990. Mating behaviour of *Phytophthora parasitica*: evidence for sexual recombination in oospores using DNA restriction fragment length polymorphisms as genetic markers. *Experimental Mycology* **14**:351-359.

Griffith GW, Shaw DS, 1998. Polymorphisms in *Phytophthora infestans*: four mitochondrial haplotypes are detected after PCR amplification of DNA from pure cultures or from host lesions. *Applied and Environmental Microbiology* **64**: 4007-4014.

Guindon S, Gascuel O, 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696-704.

Hall TA, 1999. Bioedit a user-friendly biological sequence alignment editor and analysis program for Windows 95/98NT. *Nucleic Acids Symposium Series* **41**: 95-98.

Hasegawa M, Kishino K, Yano T, 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**: 160-174.

Hickman CJ, 1958. *Phytophthora* - Plant destroyer. *Transactions of the British Mycological Society* **41**: 1-13.

Ippolito A, Schena L, Nigro F, 2002. Detection of *Phytophthora nicotianae* and *P. citrophthora* in citrus roots and soils by nested PCR. *European Journal of Plant Pathology* **108**: 855-868.

Ivors K, Garbelotto M, Vries ID, Ruyter-Spira C, Te Hekkert B, Rosenzweig N, Bonants P, 2006. Microsatellite markers identify three lineages of Phytophthora ramorum in US nurseries, yet single lineages in US forest and European nursery populations. *Molecular Ecology* **15**: 1493-1505.

Johnson ES, Wolff MF, Wernsman EA, Rufty RC, 2002. Marker-assisted selection for resistance to black shank disease in tobacco. *Plant Diseae* **86**: 1303-1309.

Kress WJ, Erickson DL, 2008. DNA barcodes: genes, genomics, and bioinformatics. *Proceedings of the National Academy of Sciences* **105**: 2761-2762.

Kroon LP, Bakker FT, van den Bosch GB, Bonants PJ, Flier WG, 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* **41**: 766-782.

Lamour KH, Daughtrey ML, Benson DM, Hwang J, Hausbeck MK, 2003. Etiology of *Phytophthora drechsleri* and *P. nicotianae* (=*P. parasitica*) diseases affecting floriculture crops. *Plant Disease* **87**: 854-858.

Librado P, Rozas J, 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452

Martin F, 2008. Mitochondrial haplotype determination in the oomycete plant pathogen *Phytophthora ramorum*. *Current Genetics* **54**: 23-34.

Martin FN, Tooley PW, 2003. Phylogenetic relationships among Phytophthora species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia* **95**: 269-284.

Matheron ME, Matejka JC, 1990. Differential virulence of *Phytophthora parasitica* recovered from *citrus* and other plants to rough lemon and tomato. *Plant Disease* **74**: 138-140.

Menge JA, Nemec S, 1997. Citrus. In: Hillcocks RJ, Waller JM (eds), *Soilborne Diseases of Tropical Crops*, Oxon, CAB, pp.185-227.

Milne I, Lindner D, Bayer M, Husmeier D, McGuire G, Marshall DF, Wright F, 2009. TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics*. 25, 126-127.

Moralejo E, Pérez-Sierra AM, Álvarez LA, Belbahri L, Lefort F, Descals E, 2009. Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain. *Plant Pathology* **58**: 100-110.

Pane A, Martini P, Chimento A, Rapetti S, Savona S, Grasso F.M, Cacciola S.O., 2005. *Phytophthora* species on ornamental plants in Italy. *Journal Of Plant Pathology* **87**: 301 (Abstract).

Philips DJ, Baker R, 1962. *Phytophthora* crown rot of petunia. *Plant Disease Report* **46**: 506-508.

Posada D, 2008. jModelTest: Phylogenetic model aAveraging. *Molecular Biology and Evolution* **25**: 1253-1256.

Powers HR, Lucas CB, 1952, Variation within *Phytophthora parasitica* var. *nicotianae*. *Plant Disease Report* **36**: 335-336.

Prospero S, Hansen EM, Grünwald NJ, Winton LM, 2007. Population dynamics of the sudden oak death pathogen *Phytophthora ramorum* in Oregon from 2001 to 2004. *Molecular Ecology* **16**: 2958-2973.

Reichard SH, White P, 2001. Horticulture as a pathway of invasive plant introductions in the United States. *BioScience* **51**: 103-113.

Rozen S, Skaletsky HJ, 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods in Molecular Biology* **132**: 365-386.

Schena L, Cardle L, Cooke DEL, 2008. Use of genome sequence data in the design and testing of SSR markers for *Phytophthora* species. *BMC Genomics* **9**: 620.

Schena L, Cooke DEL, 2006. Assessing the potential of regions of the nuclear and mitochondrial genome to develop a "molecular tool box" for the detection and characterization of *Phytophthora* species. *Journal of Microbiological Methods* **67**: 70-85.

Schlötterer C, Tautz D, 1992. Slippage synthesis of simple sequence of DNA. *Nucleic Acids Research* **20**: 211 -215.

Shew HD, 1987. Effect of host resistance on spread of *Phytophthora parasitica* var. *nicotianae* and subsequent development of tobacco black shank under field conditions. *Phytopathology* **77**: 1090-1093.

Sullivan MJ, Parks EJ, Cubeta MA, Gallup CA, Melton TA, Moyer JW, Shew HD, 2010. An assessment of the genetic diversity in a field population of *Phytophthora nicotianae* with a changing race structure. *Plant Disease* **94**: 455-460.

Tamura K, Nei M, 1993. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution* **10**: 512–526.

Templeton AR, Crandall KA, Sing CF, 1992. A cladistic analysis of the phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNAsequence data. III. Cladogram estimation. *Genetics* **132**: 619–633.

Weng C, Yu K, Anderson T, Poysa V, 2007. A quantitative trait locus influencing tolerance to *Phytophthora* root rot in the soybean cultivar 'Conrad'. *Euphytica* **158**: 81-86.

Widmark AK, Andersson B, Cassel-Lundhagen A, Sandström M, Yuen JE, 2007. *Phytophthora infestans* in a single field in southwest Sweden early in spring: symptoms, spatial distribution and genotypic variation. *Plant Pathology* **56**: 573-579.

Zhang DX, Hewitt GM, 2003. Nuclear DNA analyses in genetic studies of populations: practice, problems and prospects. *Molecular Ecology* **12**: 563-584.

Zhang XG, Sun WX, Guo L, Yu JF, Chang CJ, 2003. Genetic and pathogenic variation among tobacco black shank strains of *Phytophthora parasitica* var. *nicotianae* from the main tobacco growing in china. *Journal of Phytopathology* **151**: 259-266.

Zhang XG, Zheng GS, Han HY, Han W, Shi CK, Chang CJ, 2001. RAPD-PCR for diagnosis of *Phytophthora parasitica* var. *nicotianae* isolates which cause black shank on tobacco. *Journal of Phytopathology* **149**: 569-574.

Chapter III

Mitochondrial and nuclear DNA analyses suggests a global panmictic population structure of *Phytophthora nicotianae.*

III.1 ABSTRACT

Genetic variations in mitochondrial and nuclear DNA of the cosmopolitan plant pathogen *Phytophthora nicotianae* were determined by characterizing four mitochondrial regions corresponding to the 10% of the genome and three nuclear loci of 96 isolates from a wide range of hosts and geographic locations. Fifty-two SNPs (1 every 58 bp) and 313 sites with gaps enabled the identification of 50 different mitochondrial haplotypes. Similarly, 24 SNPs (1 every 69 bp), with heterozigosity observed at each locus, differentiate the isolates in 40 nuclear multilocus genotypes. The analyses of both mitochondrial and nuclear data sets showed a typical panmictic distribution of *P. nicotianae* isolates since no specific associations between geographic distribution and mitochondrial haplotypes or nuclear genotypes were revealed. The most frequent haplotypes (H5, H19) and genotype (g1) were shared among isolates from different worldwide geographic locations suggesting a high level of dispersal of this pathogen. A specific association was found among mitochondrial and nuclear groups and some hosts of origin. In particular, the majority of citrus isolates from different geographic locations clustered in the same mitochondrial group and had the same or very similar nuclear multilocus genotype. Although less evident a specific association was also reveled for isolates from tobacco. On the whole the combined analysis of both mitochondrial and nuclear markers showed interesting congruent groupings of isolates in relation to both geographic origin and host of provenience. Both markers clearly indicated diffused events of migration for this pathogen. In species like *P. nicotianae* where there is no clear evidence of the favorite mechanism of reproduction, the association of pattern from different markers could address questions related to the genetic structure and migration.

III.2 Introduction

Phytophthora nicotianae can affect a variety of plants and trees causing foliar diseases and root rot and crown rot diseases in annual herbaceous and in perennial host plants (Erwin & Ribeiro, 1996; Benson & von Broembsen, 2001). On perennial woody plants a single cycle of the disease commonly occur, while more cycles per year can be performed on herbaceous hosts, especially in nursery where repeated growing of annual crops can favor the pathogen (Hu et al., 2008). Despite the great economic importance of this pathogen, studies on the genetic structure of population are quite limited. Intraspecific variability has been mainly studied in China (Zhang et al., 2001, 2003) and North Carolina (Sullivan et al., 2010) with isolates from tobacco fields using RAPD and AFLP techniques. AFLP has been also utilized to analyze the genetic variability of isolates from ornamental plants in different nurseries in Tennessee (Lamour et al., 2003). In this study, different clonal lines specific for each nursery were identified. A comprehensive statewide survey conducted throughout major tobacco-growing areas of Virginia to determine population structure and mating type suggested that it is unlikely that sexual recombination serves as a major mechanism enhancing the genetic diversity of the pathogen in this country (Parkunan et al., 2010). Similar results have been also obtained by analyzing isolates from citrus fields (Cacciola and Magnano di San Lio, 2008).

All these studies were designed to analyze variability and genetic structure in specific fields or within the same geographic region, while there are not specific studies about the global distribution of genetic variability of this pathogen in relation to geographic origin and the hosts. Furthermore, the populations structure of *P. nicotianae* has been mainly assessed by studying physiological aspects such as resistance to fungicides (mainly

mefenoxam and metalaxyl) and differential pathogenicity on tobacco cultivars which has been utilized to identify four different races (0, 1, 2 and 3) of the pathogen (Sullivan et al., 2005a, 2005b; Hu et al., 2008; Parkunan et al., 2010). It has been demonstrated that the aggressiveness of the pathogen changes depending on the races structure in the specific fields (Sullivan et al., 2005). Since *P. nicotianae* was introduced in the tobacco production area in South Eastern US in the 1931, race 0 was the most common in these regions (Lucas, 1975). Single-gene resistant cultivar with a complete resistance against this race of the pathogen were obtained by crossing the cultivated *Nicotianae tabacum* with the wild species such as *Nicotianae plumbaginifolia* and *Nicotianae longiflora* (Apple, 1967; Valleau et al., 1960). However, these single-gene resistant cultivars were susceptible to race 1 of *P. nicotianae* and while this initially did not represent a problem given the lower population of this race compared to race 0 (Johnson et al., 2002) with the widespread deployment of single-gene resistant hybrid flue-cured tobacco cultivars in the 1990s, the frequency of race 1 has increased dramatically in Georgia and North Carolina (Csinos et al., 1994, 2005). It has been demonstrated that the population structure of the pathogen in a field varies according to differently resistant tobacco variety (Sullivan et al., 2005a; 2005b). Population genetic structure and evolutionary history has rarely been analyzed in plant pathogens using both mitochondrial and nuclear genomes (Sommerhalder et al., 2007; Gomez-Alpizar et al., 2007). Because of different biological processes at the basis of their inheritance and the different model of evolution, the genetic component of variability that is found with the two types of markers could be totally different. This aspect has been demonstrated in the phytopathogenic fungus *Mycosphaerella graminicola* (McDonald et al., 1995) as well as in other eukaryotes (Bensch et al., 2006). However, because mitochondrial DNA is uniparentally inherited and is much smaller compared to nuclear

DNA, some evolutionary processes can be explained only through the analyses of nuclear markers (Godinho et al., 2008). In particular, mitochondrial analyses conducted with species that are not exclusively clonally reproducing may produce contradictory results (Zhang and Hewitt, 2003). However, conjunct analysis of nuclear and mitochondrial genomes can provide more comprehensive insights into the evolutionary forces acting on natural populations (Asmussen and Basten, 1994).

In the present study both mitochondrial and nuclear markers were utilized to study distribution and genetic variability in isolates of *P. nicotianae* from different hosts and geographic origins. Four mitochondrial markers and three nuclear regions containing single nucleotide polymorphisms (SNPs) were analyzed. SNP analysis have been rarely applied to study the populations of plant pathogens since other techniques such as microsatellites are considered more appropriate markers for this purpose. However SNPs are widely distributed in all the genomes and can be quite easily identified in a population through sequencing of specific loci or using non-sequence method such as melting curve analysis or allele-specific oligonucleotides real time PCR. The analysis of patterns of intraspecific variation can be used to study the evolution of a species. In particular, the analysis of the distribution of the genetic diversity may reveal information about the center of origin of a particular species (Gomez-Alpizar et al., 2007; Grunwald and Frier 2005), patterns of migration (Stukenbrock et al., 2006, Atallah et al., 2010) and obviously population structure (Ivors et al., 2006; Dobrowolski et al., 2003). Similar approaches proved valuable for testing phylogenetic and evolutionary divergence at intraspecific level in different fungal species (Banke et al., 2004; Stukenbrock et al., 2007; Ceresini 2007). The genealogical history of *P. infestans* that assigned an hypothetical South American origin for this species was studied using nucleotide differences in two nuclear genes and in four

mitochondrial regions (Gomez-Alpizar et al., 2007). Similarly, the analysis of five nuclear genes applied in coalescent based genealogies, suggested a major role of recombination events in modeling the population structure of *P. ramorum*, until the actual different three known lineages (EU1, NA1, NA2; Ivors et al., 2006; Grunwald et al., 2009) indicating that the three lineages may have a sexual recombining ancestor population (Goss et al., 2009). Rather than looking for SNPs in nuclear genes, Abbott et al (2010), developed a method for discovering SNPs in microsatellite flanking regions (MFRs) because of their high rate of mutation. Using this method, they were able to identify and genotype 25 different isolates of *P. infestans* on a total of 32 representative of an international panel (Abbott et al., 2010).

In the present work mitochondrial and nuclear patterns were utilized to study isolates of *P. nicotianae* from a wide range of hosts and geographic locations. The main objectives of this work were: (1) the screening of several potential mitochondrial and nuclear marker appropriate for population genetic structure studies, (2) the assessment of the appropriateness of mitochondrial markers for phylogenetic studies and (3) the comparison of the pattern of genetic variation from mitochondrial and nuclear markers.

III.3 Material and Methods

III.3.1 *Strains of* Phytophthora nicotianae *and DNA extraction*

Ninety-six isolates of *Phytophthora nicotianae* representing different geographic regions in 6 continents (Fig. 1) and various hosts (ornamentals, citrus, tobacco and horticultural) were used in this study (Table 1). Forty-five 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. (Blair et al., 2008). The remaining 51 isolates were from the collection of the Department of "Gestione dei Sistemi Agrari e Forestali",

Mediterranean University, Italy and DNA was extracted according to the procedure described by Ippolito et al. (2002).

Fig. 1 - Geographic origins of the isolates of *Phytophthora nicotianae* used in this study.

III.3.2 *Mitochondrial and nuclear markers analyzed*

To amplify different regions of nuclear and mitochondrial DNA a large number of different primers were selected either from the literature or designed on the bases of published DNA sequences. Furthermore the complete mitochondrial genome of *P. nicotiane* was provided by Frank Martin at the USDA-ARS, Salinas, CA (unpublished data).

To design mitochondrial primers the genome of *P. nicotianae* was compared with other mitochondrial genomes available in GenBank for *P. infestans* (NC_002387, AY894835, AY898627, AY898628), *P. ramorum* (DQ832718) and *P. sojae* (DQ832717) in order to

identify conserved regions flanking variable intergenic regions. Primers were designed on conserved regions to facilitate the amplification of the targets from a broader range of isolates. Preliminary screenings conducted with a restricted number of representative isolates of *P. nicotianae* enabled the identification of 4 variable mitochondrial regions representing the 10% of the whole genome of *P. nicotianae* (*trnG-rns, rns-cox2, cox2-spacer, atp1-nad5*) (Fig. 2). Three of these regions (*trnG-rns, rns-cox2, cox2-spacer*) were already developed and tested for analysis of intraspecific variability in other *Phytophthora* species (Martin et al., 2008; F. Martin, unpublished).

Similarly 3 nuclear regions were selected during preliminary investigations since they had a quite high level of intraspecific variability. A hypothetical conserved protein (*hyp*) was amplified using primers I11F-I12R developed to amplified a SSR marker from a panel of 15 *Phytophthora* species (which not include *P. nicotianae*; Schena et al., 2008). The Scp-like protein (*scp*) marker was amplified using primers designed on an expressed sequence tag (EST) from a cDNA library generated during the appressorium formation of *P. nicotianae* (Kebdani et al., 2008). Finally a couple of *β-tubulin* (*βtub*) gene primers (Blair et al., 2008) were modified to perfectly match the sequence of this gene in *P. nicotianae* (Fig. 2).

Amplifications with selected primers (Table 2) were run in a total volume of 25 µl containing 1 to 10 ng of genomic DNA, 1X PCR buffer, 0.1 mM dNTPs, 1 unit Taq polymerase (Applied Biosystem, USA) and 0.5 µM for each primer. The concentration of $MgCl_2$ and the annealing temperature were optimized for each primer pair (Table 2). Amplification conditions were 95°C for 3 min, 40 cycles of 95°C for 1min, annealing temperature for 1 min, and 72°C for 1 min, with a final extension at 72°C for 5 min.

PCR products were purified using ExoSap (USB, Cleveland, OH, USA) in accordance with manufacturer's instructions and sent to the Nucleic Acid Sequencing Facility at the Penn State University (University Park, Pennsylvania) for sequencing.

When the quality of the sequences was not fully satisfactory, additional nested sequencing primer specific for *P. nicotianae* were designed and target regions (mainly *cox2-spacer*, *atp1-nad5* region and *β tubulin* gene) were re-amplified and sequenced.

Other methods for the identification of intraspecific variability were also investigated but preliminary results were not positive. Primers with dinucleotide or trinucleotide repeats were used as internal simple sequence repeat method (ISSR) to amplify regions between microsatellite repeats, but problems with reproducibility and interpretability of the results were encountered. Also, primers amplifying microsatellite regions obtained from a Genome Sequence Survey (GSS) in *P. nicotianae* (J. Blair unpublished) were tested for five isolates from different geographic regions, hosts and with a different genetic background (mitochondrial and nuclear) but differences in the repeated regions were not identified.

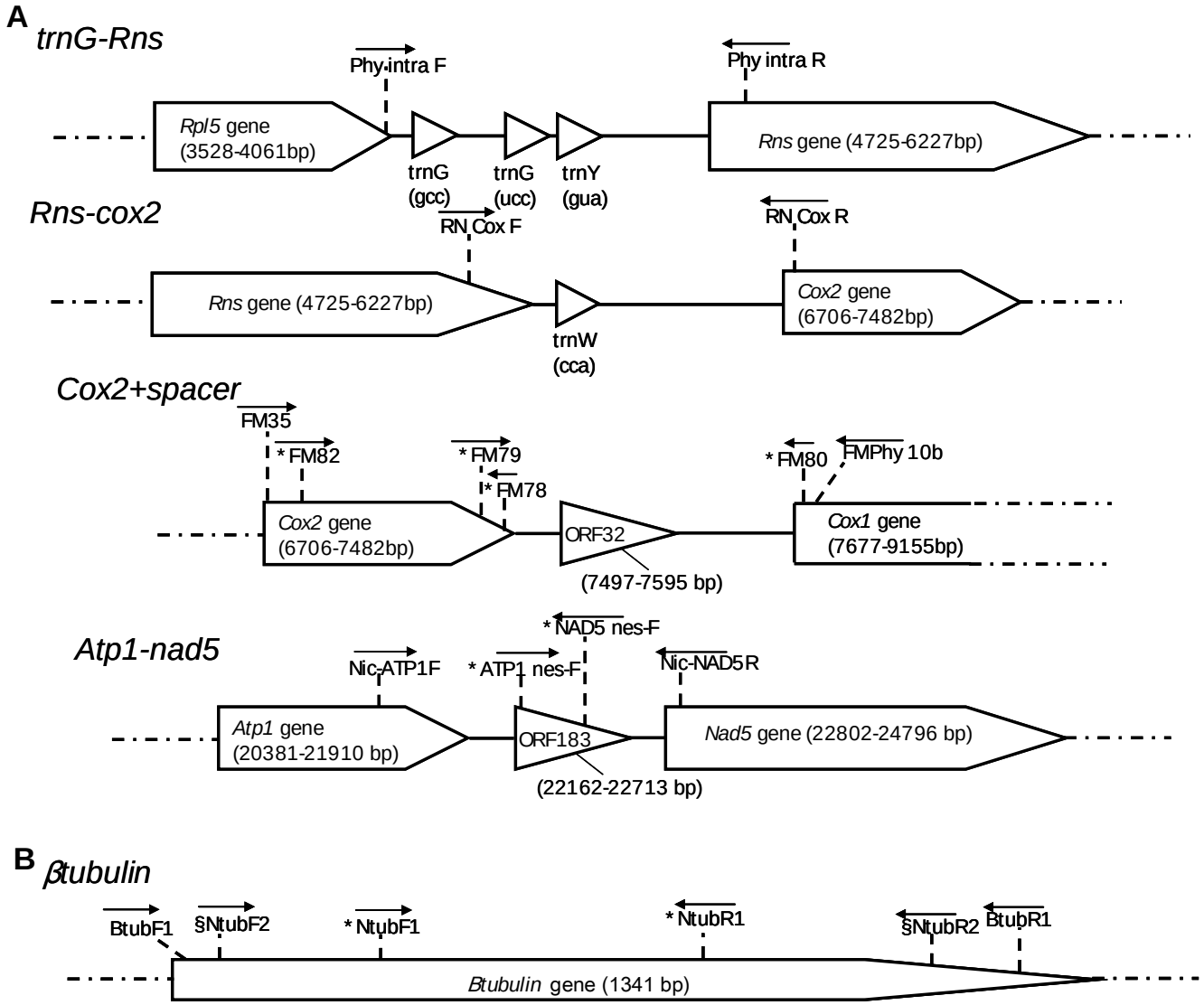

Fig. 2 - Schematic representation of mitochondrial regions (A) and *βtubulin* nuclear gene (B) examined in this study with location and orientation of selected primers. Symbol (*) indicates sequencing primer and additional sequencing nested primers. Symbol (§) indicates additional amplification primers. The reference sequence used for the *βtubulin* nuclear gene is obtained from the deposited sequence of *P. nicotianae* (EU080504.1) (Blair et al., 2008).

Table 1. Isolates of *Phytophthora nicotianae* included in the study, their designation, host of recovery, geographic origin, mating type mitochondrial haplotype and nuclear multilocus genotype.

Isolates	Host	Origin	Mating type	*trnG-rns*		*rns-cox2*		*cox2+* spacer		*atp1-nad5*		Final haplotype[c]	Multilocus nuclear genotype[d]
				Haplotype	Size (bp)[b]	Haplotype	Size (bp)	Haplotype	Size (bp)	Haplotype	Size (bp)		
P10802 (A144)[a]	*Dianthus caryophyllus*	Japan	A2	1	607	1	577	1	910	1	893	H1	g4
N035 (IMI 207770)	*Durio zibethinus*	Malaysia	A1	1	607	1	577	1	910	1	893	H1	g4
P10297 (A550)	*Dieffenbachia maculata*	Florida		2	607	2	464	2	910	2	882	H2	g17
P6915 (A658)	*Dieffenbachia maculata*	Germany		2	607	2	464	2	910	2	882	H2	g17
N001 (Albic9)	*Prunus armeniaca*	Calabria, Italy	A2	3	606	3	577	3	910	3	876	H3	g10
N002 (Anthurium)	*Anthurium sp.*	Sicily, Italy	A1	4	608	4	526	1	910	4	893	H4	g14
N027 (Pandorea2c)	*Pandorea jasminoides*	Sicily, Italy	A2	4	608	4	526	1	910	4	893	H4	g14
P1494 (D1835)	*Nicotianae tabacco*	Australia		5	619	3	577	4	910	5	890	H5	g5
P7665 (C1327)	*Leucodendron sp.*	Australia (WA)		5	619	3	577	4	910	5	890	H5	g5
P7387 (C1333)	*Hippeastrum sp.*	Netherlands		5	619	3	577	4	910	5	890	H5	g19
P7330 (C1345)	*Lavandula sp.*	Australia (WA)		5	619	3	577	4	910	5	890	H5	g5
P1751 (D166)	*Nicotianae tabacco*	Australia (Q)	A1	5	619	3	577	4	910	5	890	H5	g18
P1753 (D1832)	*Nicotianae tabacco*	Australia (Q)	A1	5	619	3	577	4	910	5	890	H5	g5
P1752 (D1831)	*Nicotianae tabacco*	Australia(Q)	A1	5	619	3	577	4	910	5	890	H5	g5
N038 (Sta24)	*Rhamnus alaternus*	Sicily, Italy	A2	5	619	3	577	4	910	5	890	H5	g19
P7346 (C1328)	*Choisya ternata*	UK		1	607	4	526	1	910	6	893	H6	g8
P6113 (C1339)	*Lilium sp.*	Japan	A2	6	619	5	577	4	910	7	898	H7	g16
P7522 (C1346)	*Catharanthus roseus*	California	A2	7	607	6	577	1	910	8	893	H8	g34
P0700 (C1828)	*Solanum lycopersicum*	Ponape	A2	4	607	1	577	1	910	1	893	H9	g4
N003 (C301)	*Mirtus communis*	Sicily, Italy	A2	8	606	4	526	1	910	4	893	H10	g2
P3815 (D206)	*Rosa cv. Sonia*	USA		8	606	4	526	1	910	4	893	H10	g31
N004 (C88)	*Simmondsia chinensis*	Puglia, Italy		10	609	1	577	1	910	10	881	H12	g11
N026 (nic8vasi)	*Lavandula angustifolia*	Sicily, Italy	A2	10	609	1	577	1	910	10	881	H12	g11
N039 (Tl8vp)	*Lavandula angustifolia*	Sicily, Italy	A2	10	609	1	577	1	910	10	881	H12	g11
N005 (Ceanothus)	*Ceanothus sp.*	Sicily, Italy	A2	11	619	7	577	5	910	11	891	H13	g6
N020 (KVB)	*Howea sp.*	Sicily, Italy	A2	11	619	7	577	5	910	11	891	H13	g6
N016 (irf26/2)	*Impatiens sp.*	North Italy	A2	11	619	7	577	5	910	11	891	H13	g6
N044 (Ph440/00)	*Cyclamen sp.*	North Italy	A2	11	619	7	577	5	910	11	891	H13	g6
N006 (Ciclamino1)	*Cyclamen sp.*	Sicily, Italy	A1	12	608	8	577	6	910	12	788	H14	g20
N007 (correa3)	*Correa reflexa*	Sicily, Italy	A1	13	608	3	577	6	910	13	785	H15	g7

N009 (correa8)	*Correa reflexa*	Sicily, Italy	A1	13	608	3	577	6	910	13	785	H15	g7
N008 (correa5)	*Correa reflexa*	Sicily, Italy	A1	14	607	3	577	6	910	14	785	H16	g7
P1577 (D1310)	*Citrus sp.*	California	A1	4	608	6	577	7	912	15	894	H17	g13
P6115 (D149)	*Gypsophila sp.*	Japan	A2	15	608	8	577	6	910	16	788	H18	g32
P1955 (D215)	*Nicotianae tabacco*	South Africa	A1	15	608	8	577	6	910	16	788	H18	g28
N028 (PeperoneGJ)	*Capsicum annum*	Calabria, Italy	A1	1	607	4	526	1	910	4	893	H19	g15
N029 (PeperoneRC)	*Capsicum annum*	Calabria, Italy	A2	1	607	4	526	1	910	4	893	H19	g35
N031 (Pomodoro)	*Lycopersicum esculentum*	Sicily, Italy	n.d.	1	607	4	526	1	910	4	893	H19	g8
P7622 (D161)	*Gypsophila sp.*	South Africa		1	607	4	526	1	910	4	893	H19	X
N017 (Irf27)	*Agapanthus sp.*	North Italy	A2	1	607	4	526	1	910	4	893	H19	g8
N040 (Irf8)	*Anemone americana*	North Italy	A2	1	607	4	526	1	910	4	893	H19	g8
P16870 (D2044)	*Solanum lycopersicum*	Spain		1	607	4	526	1	910	4	893	H19	g15
P16883 (D2047)	*Solanum lycopersicum*	Spain		1	607	4	526	1	910	4	893	H19	g21
P1083 (D169)	*Gypsophila sp.*	California	A1	1	607	4	526	1	910	17	893	H20	g25
P1452 (D1725)	*Citrus sp.*	California	A1	16	607	6	577	1	910	9	892	H21	g12
P16824 (D1955)	*Catharanthus roseus*	Japan	A1	5	619	3	577	4	910	18	890	H22	g18
P3813 (D173)	*Vinca sp.*	California		5	619	3	577	4	910	18	890	H22	g30
P1350 (D1834)	*Nicotiana tabacum*	North Carolina	A1	10	609	1	577	1	910	19	880	H23	g3
P1495 (D1836)	*Nicotiana tabacum*	Australia	A2	17	607	8	577	6	910	16	788	H24	g26
P0583 (D1855)	*Nicotiana tabacum*	Kentucky	A2	18	608	1	577	8	876	19	880	H26	g24
P1333 (D1857)	*Nicotiana tabacum*	Virginia	A2	19	617	3	577	9	910	21	894	H27	g3
P1334 (D1860)	*Nicotiana tabacum*	Virginia	A2	19	617	3	577	9	910	21	894	H27	g3
P1335 (D1870)	*Nicotiana tabacum*	Virginia	A2	19	617	3	577	9	910	21	894	H27	g3
P0582 (D1913)	*Nicotiana tabacum*	Kentucky	A2	19	617	3	577	9	910	21	894	H27	g3
P16823 (D1961)	*Karankoe sp.*	Japan	A2	20	609	4	526	1	910	4	893	H28	g27
P3234 (D223)	*Hibiscus sp.*	China		1	607	1	577	1	910	6	893	H29	g4
P6832 (D244)	*Cyclamen sp.*	Greece		10	609	1	577	1	910	23	881	H30	g13
P3549 (D245)	*Aphelandra sp.*	Florida		8	606	1	577	1	910	24	893	H31	g7
P3456 (D246)	*Hibiscus sp.*	Pakistan		21	607	1	577	1	910	6	893	H32	g4
P7449 (D256)	*Chrysanthemum sp.*	India		15	608	8	577	6	910	12	788	H33	g33
P3461 (D273)	*Solanum lycopersicum*	UK	A2	2	607	1	577	1	910	10	881	H34	g2
P3118 (D303)	*Solanum lycopersicum*	Australia	A2	2	607	1	577	1	910	10	881	H34	g2
Ph5	*Citrus sp.*	Basilicata, Italy	A1	2	607	1	577	1	910	10	881	H34	g37
N041 (Ph653/03)	*Choisia Ternata*	North Italy	A2	2	607	1	577	1	910	10	881	H34	g2
N042 (Ph647b/03)	*Phormium tenax*	North Italy	A2	2	607	1	577	1	910	10	881	H34	g9

N033 (IMI 379626)	*Lycopersicum esculentum*	Chile	A1	2	607	1	577	1	910	10	881	H34	g2
N023 (melanzana1)	*Solanum melongena*	Sicily, Italy	A2	2	607	1	577	1	910	10	881	H34	g2
P7561 (D929)	*Citrus (rough lemon)*	Philippines	A1	9	608	6	577	1	910	25	891	H35	g1
N011 (Dodrad1)	*Dodonea viscosa*	Sicily, Italy	A2	22	605	3	577	6	910	3	876	H36	g10
N010 (Dodcoll1)	*Dodonea viscosa*	Sicily, Italy	A2	22	605	3	577	6	910	3	876	H36	g10
P1569 (D1837)	*Citrus sp.*	California	A1	16	607	6	577	1	910	20	893	H25	g12
Ph3	*Citrus sp.*	Italy	A1	16	607	6	577	1	910	20	893	H25	g1
Ph195	*Citrus sp.*	Syria	A1	16	607	6	577	1	910	20	893	H25	g1
P1325 (C376)	*Citrus sp.*	California	A2	9	608	6	577	1	910	9	892	H11	g12
N013 (Ferrara3)	*Citrus sp.*	Sicily, Italy	A1	16	607	6	577	1	910	26	893	H37	g1
N014 (Ferrara8)	*Citrus sp.*	Sicily, Italy	A1	16	607	6	577	1	910	26	893	H37	g1
N012 (Ferrara11)	*Citrus sp.*	Sicily, Italy	A1	16	607	6	577	1	910	26	893	H37	g1
N036 (Serravalle1)	*Citrus sp.*	Sicily, Italy	A1	16	607	6	577	1	910	26	893	H37	g1
Ph87	*Citrus sp.*	Puglia, Italy	A1	16	607	6	577	1	910	26	893	H37	g1
N043 (Ph342/03)	*Limonium sinensis*	Liguria, Italy	A2	16	607	6	577	1	910	26	893	H37	g36
Ph142	*Citrus sp.*	Albania	A1	9	608	6	577	1	910	26	893	H44	g1
Ph9	*Citrus sp.*	Puglia, Italy	A1	9	608	6	577	1	910	26	893	H44	g1
Pn17	*Citrus sp.*	Florida		25	609	6	577	1	910	26	893	H46	g39
N037 (Serravalle3)	*Citrus sp.*	Sicily, Italy	A1	27	606	6	577	1	910	26	893	H49	g1
N015 (Hybiscus b)	*Hybiscus sp.*	Calabria, Italy	A2	4	608	4	526	11	910	27	892	H38	g6
N018 (irf3)	*Polygala myrtifolia*	North Italy	A2	1	607	1	577	12	910	6	893	H39	g16
N019 (irf5)	*Polygala myrtifolia*	North Italy	A2	23	609	1	577	1	910	23	881	H40	g22
N021 (lavanda1)	*Lavandula angustifolia*	Sicily, Italy	A2	18	608	1	577	1	910	10	881	H41	g9
N025 (mirtus3)	*Myrtus communis*	Sicily, Italy	A1	18	609	1	577	1	910	10	881	H41	g23
N022 (lavanda4)	*Lavandula angustifolia*	Sicily, Italy	A2	24	606	1	577	1	910	10	881	H42	g9
N024 (mirtop5)	*Myrtus communis*	Sicily, Italy	A2	1	607	4	526	1	910	28	893	H43	g9
N030 (Pittosporo)	*Pittosporum sp.*	Sicily, Italy	A1	1	607	4	526	1	910	28	893	H43	g38
Ph168	*Citrus sp.*	Tunisia	A1	11	619	3	577	9	910	29	894	H45	g13
N032 (scrp462)	*Fragaria x ananassa*	India	A1	2	607	9	570	1	910	30	895	H47	g2
N034 (IMI 268688)	*Citrus sp.*	Trinidad	A1	26	607	3	577	13	910	31	876	H48	g40
P3458 (D212)	*Carthamus tinctorius*	Venezuela		28	620	3	577	10	910	22	890	H50	g29

[a] In parenthesis (in the first column) are reported the original denomination of isolates as reported in chapter 2 or dilution number for the isolates of the World *Phytophthora* Collection (Mike Coffey). [b] Size of the fragments obtained by sequencing analysis. [c] Final haplotypes are indicated for the combined mitochondrial data set. [d] Nuclear multilocus genotypes are indicated in the last column for the combined data set of the three nuclear regions (*hyp-scp-ß tub*).

Table 2. Primers and PCR conditions used for the mitochondrial and nuclear loci.

Mitochondrial DNA	Rpl5-Rns	[a]Phy intra-F1 GGTAGAGTATAACCTTGC [a]Phy intra-R1 ATAGCATTTATTCTGAGCCA	3 mM Mg, 57ºC T
	Rns-Cox2	[a]RN-CoxF GATGAAGTCGTAACAAGGTA [a]RN-CoxR AAACCTAATTGCCAAGGTTC	3 mM Mg, 64ºC T
	Cox2-spacer	[b]FM35 CAGAACCTTGGCAATTAGG [b]FMphy-10b GCAAAAGCACTAAAAATTAAATATAA [c]FM78 ACAAATTTCACTACATTGTCC* [c]FM79 GGACAATGTAGTGAAATTTGT* [c]FM 82 dTTGGCAATTAGGTTTTCAAGATCC* [c]FM 80 dAATATCTTTATGATTTGTTGAAA*	3 mM Mg, 54ºC T
Nuclear DNA	*hyp*	[d]I11F TCGTCBGTGTCCTCBACGTC [d]I12R ACCAGCATCTTRTTCTGRGCAG	1 mM Mg, 55ºC T
	scp	NscpF TGTGCGGTGATGTCTGTGC NscpR TCACCACCTTTGCGAARCC	1 mM Mg, 60ºC T
	βtub	[e]BtubF1 GCCAAGTTCTGGGAGGTCATC [e]BtubR1 CCTGGTACTGCTGGTACTCAG NtubF1 ACGCTTCTTATCTCGAAGATT* NtubR1 CTTACGCAGGTCCGAGTTC* NtubF2 CTCGGACCTGCAGCTGGA[§] NtubR2 CGTAAACTGTTCGGACACAC[§]	2.5 mM Mg, 60ºC T

[a]Primers from F. Martin (Personal communication); [b]Martin, 2008; [c]Martin and Tooley, 2003.
[d]Primers from Schena et al., 2008.
[e]Primers from Blair et al., 2008
*Additional nested sequencing primer; § Additional amplification and sequencing primer.

III.3.3 *Analysis of sequences*

Both mitochondrial and nuclear sequence chromatograms were analyzed and consensus sequences generated in Sequencher 4.7 (Gene Codes, Ann Arbor, MI). Sequences were aligned manually and edited for the presence of single nucleotide polymorphisms (SNPs) and indels in MacClade ver 4.02 (Sinaur Associates, Sunderland, MA, USA). All polymorphic sites observed in the alignment were checked back in the chromatogram. Heterozygous SNPs identified in the nuclear coding regions by the presence of double peaks in both forward and reverse sequences were marked with standard degeneracy codes (e.g. W = T or A) (Fig. 3).

Representative SNPs from genotypes g1, g12, g14, g17, g22, g31, and g40 (Table 1) were also analyzed and confirmed by cloning the amplicons with TOPO easy vector kit

according to manufacturer's protocol (Invitrogen, USA). Approximately 10 different clones were sequenced per each cloned PCR product using standard T7 and T3 primers.

III.3.4 *Genetic diversity*

Genetic diversity was evaluated for each individual mitochondrial region and for the combined data set (Table 3). The number of polymorphic sites and haplotypes and the nucleotide diversity Pi (π, average number of differences per site between two sequence) were analyzed using DnaSP ver. 5.10.01 software (Librado and Rozas, 2009). The number of haplotypes and the haplotypes diversity were estimated with gaps included in the analysis. Tajima's D (Tajima 1989), Fu and Li's D* and Fu and Li's F* (Fu and Li 1993; Fu 1997) neutrality tests were performed to test the deviation from neutral evolution of the mutations for the *cox2* gene. In this latter analysis the sequenced portion (684 bp) of the cox2 gene was used.

Genetic diversity in nuclear coding region was assessed by determining the number of polymorphic sites and genotypes and by estimating synonymous and non-synonymous substitution. The number of multilocus genotypes was determined manually and using a SNP allele and position calling program (MJBv1.2Gba) edited in python (N. Feau and G. Bilodeau, unpublished). The positions of synonymous and non-synonymous substitution were inferred using the orthologue sequences of *P. infestans* retrieved from the genome database (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans) at Broad Institute for *hyp* (PITG_18320.2) and *scp* (PITG_10036.1) genes and a specific sequence of *P. nicotianae* (EU080504) for the *βtub* gene.

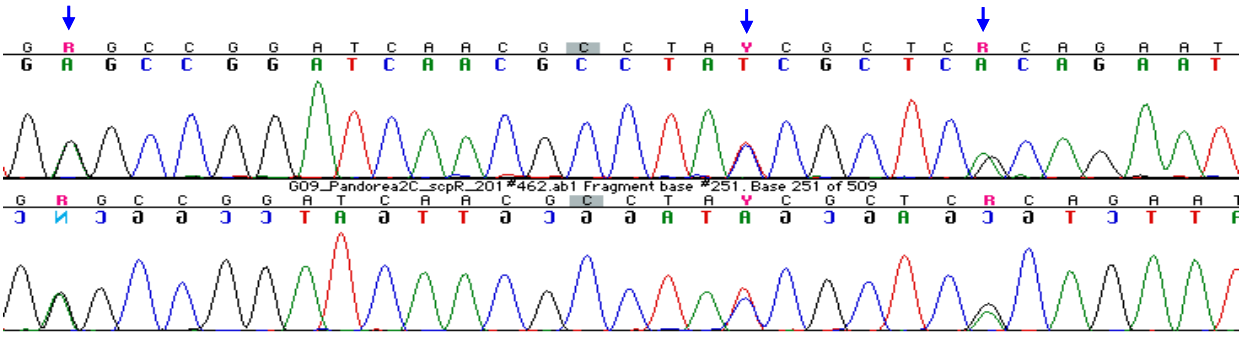

Fig. 3 - Example of hetorozygous SNP loci (arrows) identified in the SCP-like protein region. Forward sequence (top) and reverse sequence (bottom) are shown.

III.3.5 *Population structure analysis*

To analyze the population structure with regard of geographic origin and host of recovery of the isolates, a network of the combined mitochondrial data set of haplotypes was generated using the program NETWORK 4.2 (http://www.fluxus-engineering.com). Networks were calculated with Median Joining method (ε = 0) (Bandelt et al. 1995) and using successive maximum parsimony (MP) calculation (Polzin et al., 2003) to delete links in the networks not supported by the shortest trees.

Distance analysis of nuclear sequences in the concatenated data set was analyzed to see the relationships between different multilocus genotypes using a neighbour-joining tree. The neighbor-joining tree was constructed using absolute measure of distances and a random input order of sequences in PAUP v4.0b10 (Sinaur Associates, Sunderland, MA, USA).

III.3.6 *Phylogenetic analyses based on mitochondrial DNA*

A partition homogeneity test (PHT) (Farris et al 1995) was performed on the concatenated dataset using PAUP v4.0b10 to test the congruence of the combined data set using a heuristic search with 1000 replicates. All indels were manually recoded as a single mutation event. A maximum parsimony analysis was performed in PAUP using a heuristic search algorithm with random stepwise addition of taxa (10 replicates), tree bisection reconnection (TBR) branch swapping and multiple trees option. The statistical support was

determined by bootstrap values for 1000 replicates. TOPALi v2 (Milne et al., 2004) was used to determine the substitution model that best fit the data. The model HKY+I+G (Hasegawa et al., 1985) was selected for the Bayesian and maximum likelihood phylogenetic analysis using MrBayes ver 3.1.1 (Ronquist and Huelsenbeck, 2003) and PhyML ver 2.4.5 (Guindon and Gascuel, 2003) implemented in TOPALi. Bayesian analysis was performed with four runs conducted simultaneously for 500,000 generations with 10% sampling frequency and burn in of 30%. Maximum likelihood was performed with 100 bootstrap replicates.

Phylogenetic analyses for nuclear regions were not inferred because of the high level of heterozigosity and the consequent difficulties in the determination of haplotypes.

III.4 Results

III.4.1 *Amplification and sequencing*

Selected mitochondrial (*trnG-rns*, *rns-cox2*, *cox2-spacer*, *atp1-nad5*) and nuclear (*hyp*, *scp*, *βtub*) regions were amplified and sequenced from the complete panel of isolates (Table 1). Other mitochondrial regions (*secY*, *rps10* and *rpl5*) were discarded during preliminary screenings because did not show enough variations within the restricted panel of isolates analyzed. Similarly, additional nuclear regions among coding and intergenic regions were preliminarily screened (ISSR, SSR markers, elicitin, Ras-related protein ypt1, necrosis inducing protein pp1), but difficulties during amplification and/or sequencing, the presence of heterozygote indels or multiple copy targets or the limited level of polymorphisms, suggested their inappropriateness for the present investigation.

III.4.2 *Mitochondrial and nuclear genetic diversity*

Intraspecific polymorphisms were observed in each of the four mitochondrial loci (Table 3). The *rns-cox2* region (length variable from 464 to 577 bp) was the least variable region with a nucleotide diversity (π) of 0.00182. The analyzed region consisted of the entire intergenic

region and portions of the flanking coding regions of the (*rns*, 39 bp and *cox2*, 58 bp). No variation was observed in these coding regions and in the *trnW* (cca) tRNA that is localized within the intergenic region. The *cox2*+spacer region (length from 876 to 912 bp) consisted of almost the entire reading frame for the *cox2* gene (92 bases at the 5' end were not included) plus 197 bp of the spacer (including the putative *orf32*), and 31 bp of the *cox1* gene. In the *cox2* coding region 8 SNPs were identified (position 64, 114, 133, 206, 226, 547, 634, 680), six of these led to a non-synonymous change in the protein sequence (Table 4). In particular, two non-synonymous mutations in nucleotide position 64 (Val to Leu) and 547 (Glu to Lys) differentiated 2 isolates from *Dieffenbachia maculata* sourced in Florida and Germany from all other isolates. The *trnG-rns* intergenic region (length from 605 to 620 bp) contained 12 SNPs and 33 sites with gaps giving a nucleotide diversity of (π) 0.00510; no variation was present in the *trnG* and *trnY* genes encoded within the intergenic region. The *atp1-nad5* intergenic region (length from 785 to 895 bp) was the most variable region examined with 23 SNPs and 124 sites with gaps identified giving a nucleotide diversity (π) of 0.00770. Fifty-two SNPs (average of 1 SNP every 59 bp) and 313 sites with gaps were observed in the combined dataset for the 4 mitochondrial regions (3.023 bp). Neutrality tests for the intergenic regions and for the *cox2* gene were not significant, indicating that the variation followed the model of neutral evolution (Table 5).

Intraspecific variability was also detected in the three nuclear regions analyzed (Table 6). Three, 10 and 11 SNPs differentiated 7, 10 and 14 genotypes in the *hyp*, *scp, β-tub* nuclear loci, respectively. In the concatenated data set (1,654 bp, 24 SNPs total), 1 SNP was identified every 69 bp and heterozigosity was observed at each SNP locus (Table 7 and 8). In three genotypes (g2, g3, g4) was no observed heterozigosity at all (Table 7). Non synonymous substitutions were identified in the *hyp* locus, where 27 isolates had a SNP in position 101 that led to a change from Cys to Ser. For the *scp* locus there were 41 isolates with a non synonymous change in position 238 leading to a change from Ser to Gly as well as a unique

SNP in isolate P3815 from Rosa (Table 1) in position 397 of the gene leading to a change from Ala to Thr. Non-synonymous substitutions in the *β-tubulin* gene were not observed. A total of 40 genotypes were observed for the combined nuclear data set and 21 of them were unique. Cloned and sequenced PCR product for the *scp* region of two isolates N002 and N034 showed an unexpected genetic framework for a diploid organism like *P. nicotiane* since a different number of alleles were identified for these isolates (N002, 5 alleles) and (N034, 4 alleles).

Table 3. Number of characters analyzed, haplotypes, polymorphic sites (single nucleotide polymorphisms and gaps), parsimony informative sites and genetic diversity parameters for the four mitochondrial regions, the combined data set and the *cox2* gene without flanking spacer regions.

	4 region	*trnG-rns*	*rns-cox2*	*cox2*-spacer	*cox2* gene	*atp1-nad5*
Isolates	96	96	96	96	96	96
Region length (bp)	3023	629	577	912	684	905
Haplotype number	50	28	9	13	8	31
Haplotype diversity	0.9735	0.9325	0.8132	0.6243	0.5816	0.9414
Polymorphic sites excluding gaps	52	12	4	13	8	23
Site with gaps	313	33	120	36	0	124
Parsimony informative sites	39	9	3	9	6	18
Nucleotide diversity (π)	0.00431	0.00510	0.00182	0.00215	0.00144	0.00770

Table 4. Non synonymous substitutions identified in the *cox2* gene among the 96 isolates of *Phytophthora nicotianae* analyzed in the present study.

Nucleotidic position[a]	Codon	Isolates	Amino acid change
64	21	P10297, P6915	GTT (Val) to ATT (Ile)
114	38	N034	AAT (Asn) to AAA (Lys)
133	45	N018	GTA (Val) to ATA (Ile)
547	183	P10297, P6915	GAA (Glu) to AAA (Lys)
634	212	N005, N016, N020, N044	GAT (Asp) to AAT (Asn)
680	227	N034, N001, N006, N007, N008, N009, P6115, P1495, P1955, P7449, N010, N011	GCA (Ala) to GTA (Val)

[a] Considering a total length of the target region of 912 bp wich comprised 684 bp of *cox2* gene.

Table 5. Neutrality tests for the *cox2* gene.

Tajima's D	-0.90265
*Fu and Li's D**	-0.34159
*Fu and Li's F**	-0.63083

D, Tajima's statistic (Tajima 1989); Fs, Fu's statistic (Fu 1997); D* and F*, Fu and Li's statistics (Fu & Li 1993). Values are non-significant for P > 0.1.

Table 6. Number of single nucleotide polymorphisms, number and position in the nucleotide sequences of the three nuclear regions leading to synonymous and non synonymous substitution.

	Polymorphic sites (SNPs)	Synonymous substitution		Non synonymous substitution	
		Number	Position	Number	Position
hyp	3	2	85; 181	1	101
scp	10	8	90; 96; 210; 255; 261; 333; 450; 486	2	238; 397
βtub	10	10	29; 77; 101; 362; 443; 467; 590; 635; 773; 782; 803	-	-

Table 7. List of the 24 single nucleotide polymorphisms identified in the pool of 96 isolates of *Phytophthora nicotianae*. Order and nucleotide position of the loci are referred to the concatenated sequence data.

		Hypothetical protein (*hyp*)			SCP-like extra cellular protein (*scp*)										β tubulin										
Genotype	Frequency	Site 85	Site 101	Site 181	Site 331	Site 337	Site 451	Site 479	Site 496	Site 502	Site 574	Site 638	Site 691	Site 727	Site 814	Site 862	Site 886	Site 1147	Site 1228	Site 1252	Site 1375	Site 1420	Site 1558	Site 1567	Site 1588
g1	11	G	A	C	C	R	G	R	C	G	G	G	R	G	C	C	C	G	G	C	Y	G	G	T	G
g2	7	.	T	.	.	G	.	G	.	.	.	.	A	.	T	.	.	.	.	.	T	.	.	.	A
g3	5	.	.	.	.	A	.	A	.	.	.	.	G	.	T	.	.	.	.	.	T	.	.	.	A
g4	5	.	T	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.
g5	5	R	T	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
g6	5	.	T	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
g7	4	.	T	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
g8	4	.	T	.	.	G	.	G	.	.	.	.	A	.	.	Y	Y	.	.	.	C	.	.	.	.
g9	4	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R
g10	3	.	T	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	C	R	.	.	.
g11	3	.	T	.	.	G	.	G	.	.	.	.	A	.	Y	.	.	.	.	.	.	.	.	.	R
g12	3	.	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
g13	3	.	T	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	A
g14	2	.	T	.	Y	.	.	.	Y	R	R	.	.	K	Y	.	.	.	.	.	.	.	.	.	R
g15	2	.	T	.	.	.	.	.	.	.	.	.	A	.	Y	Y	Y	.	.	.	.	.	.	.	R
g16	2	.	T	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	.	R	.	.	R
g17	2	.	T	.	.	A	.	A	.	.	.	.	G	.	T	.	.	R	R	Y	.	.	R	Y	R
g18	2	R	T	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	T	.	.	.	.
g19	2	.	W	.	.	A	.	A	.	.	.	.	G	.	.	Y	Y	.	.	.	C	.	.	.	.
g20	1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.
g21	1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	Y	Y	.	.	.	.	.	.	.	.
g22	1	.	W	Y	.	.	.	A	.	.	.	.	G	.	Y	.	.	.	.	.	.	.	.	.	R
g23	1	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
g24	1	R	W	.	.	A	.	A	.	.	.	.	G	.	Y	.	.	.	.	.	.	.	.	.	R
g25	1	.	T	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.
g26	1	.	T	.	Y	.	.	.	Y	R	R	.	.	K	T	.	.	.	.	.	T	.	.	.	A
g27	1	.	T	.	.	.	.	.	.	.	.	.	.	.	Y	.	.	.	.	.	T	.	.	.	R
g28	1	.	T	.	.	G	.	G	.	.	.	.	A	.	Y	Y	Y	.	.	.	.	.	.	.	R
g29	1	A	T	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.
g30	1	R	T	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	C	.	.	.	.
g31	1	.	W	.	.	G	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.
g32	1	.	T	.	Y	.	.	.	Y	R	R	.	.	K	Y	.	.	.	.	.	.	R	.	.	R
g33	1	.	W	.	.	G	.	G	.	.	.	.	A	.	.	.	.	.	.	.	C	.	.	.	.
g34	1	R	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
g35	1	.	T	.	.	.	.	.	.	.	.	.	.	.	Y	Y	Y	.	.	.	.	.	.	.	R
g36	1	.	.	.	.	G	.	G	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.
g37	1	.	T	.	.	G	.	G	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.
g38	1	.	T	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	T	.	.	.	A
g39	1	.	.	.	.	A	.	A	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.
g40	1	.	T	.	.	.	R	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	R

Table 8. Values of observed heterozygosity (HO) for the 24 SNPs loci of the nuclear combined data set.

	Hypothetical protein (*hyp*)				SCP-like extra cellular protein (*scp*)									β tubulin									
	Site 85	Site 101	Site 181	Site 331	Site 337	Site 451	Site 479	Site 496	Site 502	Site 574	Site 638	Site 691	Site 727	Site 814	Site 862	Site 886	Site 1147	Site 1228	Site 1252	Site 1375	Site 1420	Site 1558	Site 1567	Site 1588
Observed heterozygosity	0.10	0.09	0.01	0.04	0.45	0.01	0.46	0.04	0.04	0.04	0.01	0.40	0.04	0.20	0.11	0.11	0.02	0.02	0.02	0.58	0.07	0.02	0.02	0.23

III.4.3 *Mitochondrial haplotype analysis*

The polymorphisms observed in the four mitochondrial regions partitioned the isolates in 28, 9, 13 and 31 haplotypes for *trnG-rns*, *rns-cox2*, *cox2*-spacer and *atp1-nad5,* respectively. Haplotypes diversity for the four mitochondrial regions ranged from 0.6243 to 0.9414. Eight haplotypes were also identified in the coding region of *cox2* gene among the 96 isolates of *P. nicotianae*. Maximum likelihood phylogenetic tree (substitution model selected GTR+G), performed using Mega version 4 (Tamura et al., 2007), of these eight haplotypes with the species of clade 1 of *Phytophthora* spp. (sequences taken from http://www.phytophthoradb.org) showed that the high intraspecific variation within the *cox2* gene does not cause any change in terms of phylogenetic placement; in fact the 8 haplotypes clustered together in clade 1 of *Phytophthora* spp. (Fig. 4).

Fifty haplotypes were identified for the combined dataset of the four mitochondrial regions and a high haplotype diversity was determined (0.9735) due to the large number of unique multilocus haplotypes (31 unique haplotypes). Haplotypes H5 and H19 were the most frequently encountered (Table 1). According to haplotypes and nucleotide diversity (π) the intergenic spacers *trnG-rns* and *atp1-nad5* are the most variable regions among those investigated in this study. In fact, 47 haplotypes were observed using only these two regions.

III.4.4 *Population structure*

Network analysis of haplotypes using the combined mitochondrial data set showed a typical panmictic distribution of *P. nicotianae* isolates (Fig. 5a). In fact, excepted for a few tobacco isolates from Australia (haplotype H5) and USA (haplotype H27), specific clusters reflective of geographical origins of the isolates were not identified in the network (Fig. 5a). For example the most frequent haplotype (H5) contained isolates from different geographic origins, including 6 sourced from Australia (4 from tobacco, 1 from *Lavandula* sp. and 1 from *Leucodendron* sp.) one from Italy (*Rhamnus alaternus*) and one from the Netherlands (*Hyppeastrum* sp.). Interestingly, some haplogroups (H1, H4, H5, H18, H19, H34, H37, H41) contained isolates of both mating types from different geographic locations (Table 1). Furthermore 4 isolates from Southern Italy (N028, N029 from Calabria and N021, N023 from Sicily) had the same mitochondrial haplotype but opposite mating types.

Although, there was not a clear population structure with regard to the host of origin the majority of isolates recovered from citrus plants grouped together even if recovered from different geographic regions (Italy, California, Florida, Philippines, Syria and Albania) (Fig. 5b). Taking into consideration all the isolates from citrus the haplotype diversity (Hd) was 0.9181 (nucleotide diversity 0.00219) and dropped to 0.8667 (nucleotide diversity 0.00022) when examining only these citrus isolates clustering together. Other examples where isolates from the same host but from different geographical regions shared the same mitochondrial haplotype include isolates from *Dieffenbachia maculata* from Florida and Germany (haplotype H2), isolates from *Vinca* from Japan and California (haplotype H22), and isolates from *Solanum lycopersicum* from UK and Australia (P3461, P3118, haplotype H34).

In accordance with the mitochondrial haplotype network a typical panmictic distribution of *P. nicotianae* isolates was also confirmed by the analysis of the combined nuclear data set (Fig. 6). In fact no significant clustering with regards to geographic location of recovery were determined by using a neighbour-joining tree. However, the majority of

isolates from Australia (4 from tobacco and 1 from *Leucodendron* sp.) clustered in the same group (Fig. 6). Analysis of distance of the combined nuclear data set focused on the host confirmed the existence of a group for the majority of the isolates recovered from citrus (Fig. 6).

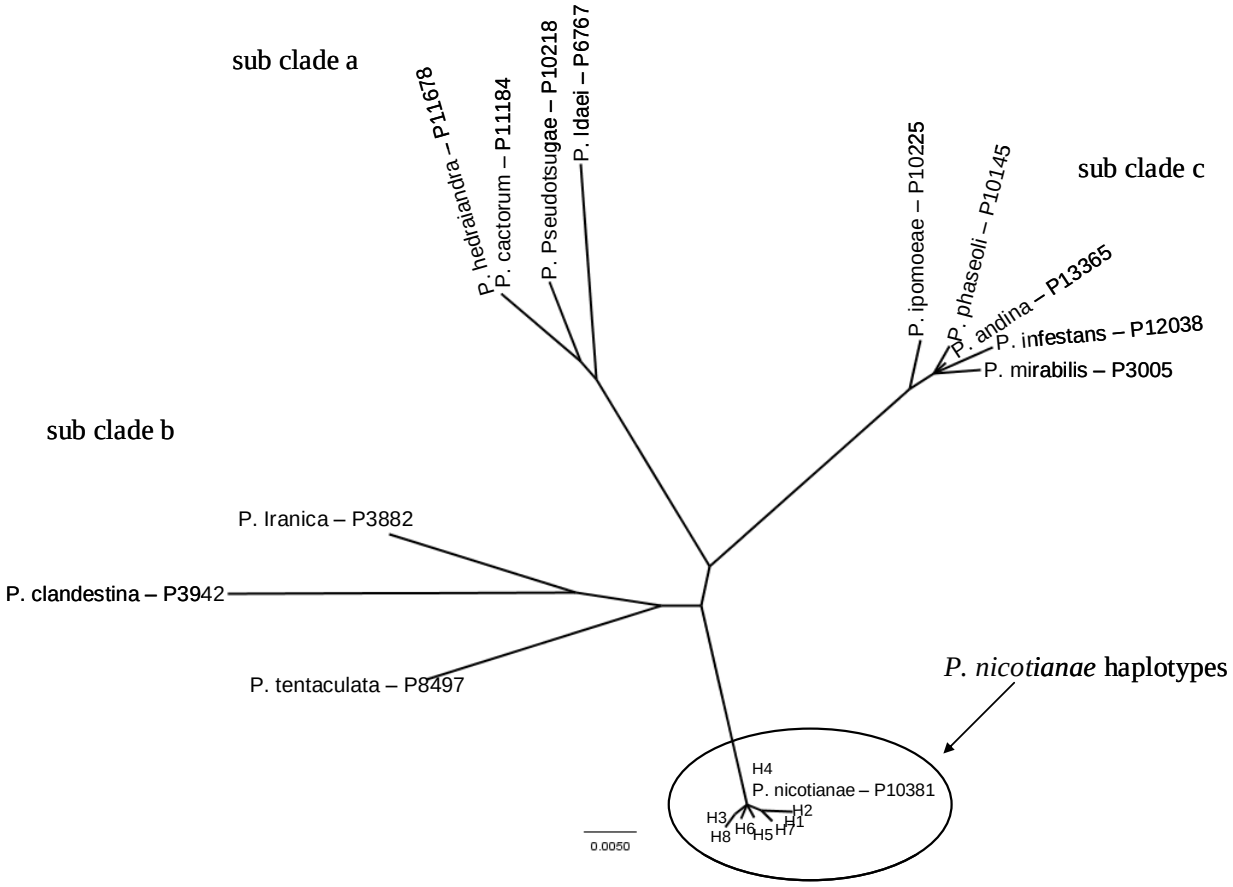

Fig. 4 - Maximum likelihood tree for the *cox2* gene of the eight haplotypes of *Phytophthora nicotianae* with the other clade 1 species (indicated with P collection number of the isolates from the World Phytophthora collection) described by Blair et al. (Blair et al., 2008). The eight haplotypes of *P. nicotianae* tend to cluster together, even assuming the high intraspecific variability in the *cox2* gene.

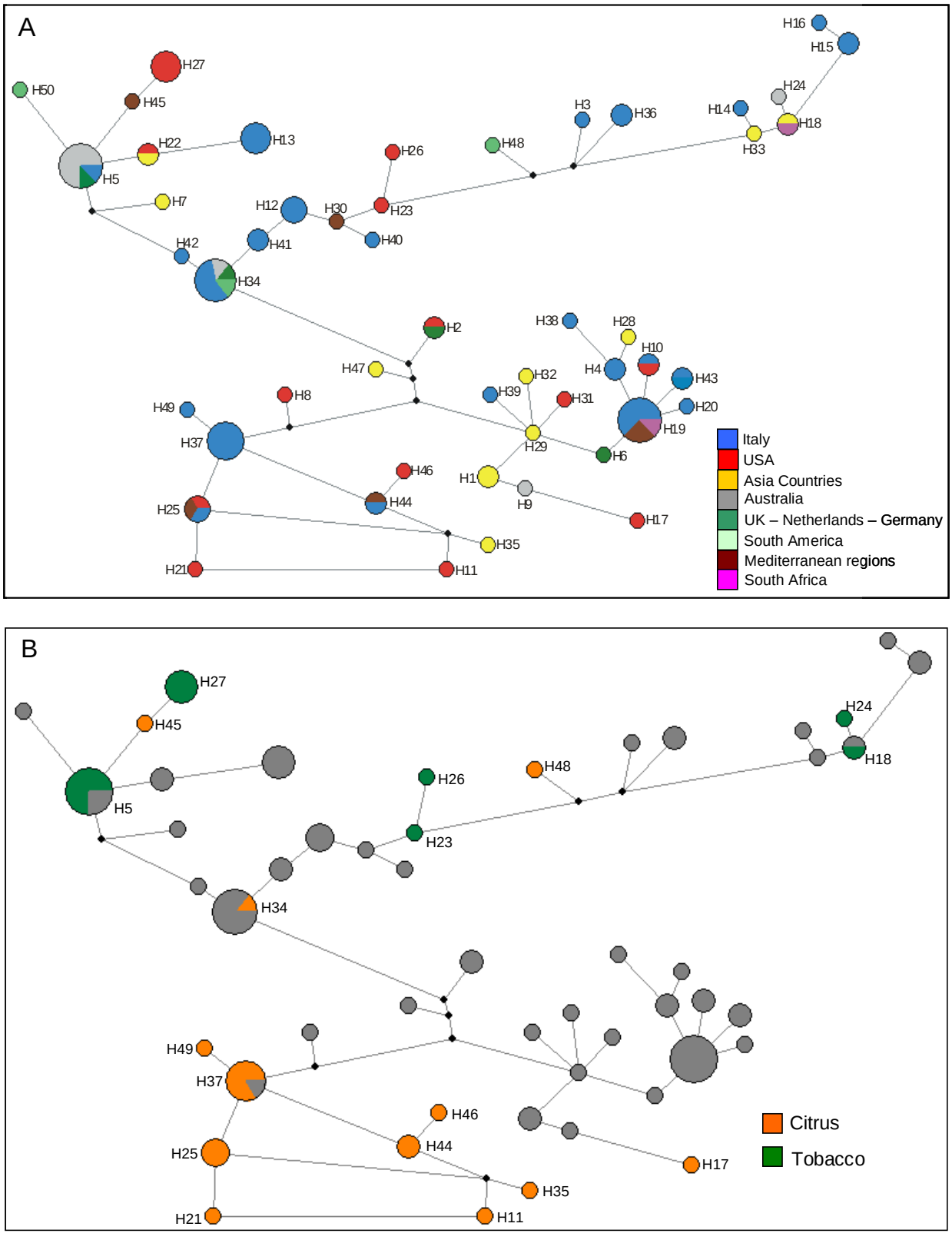

Fig. 5 - Median Joining network of haplotypes of *Phytophthora nicotianae* generated in NETWORK 4.2 (http://www.fluxus-engineering.com) using combined sequences of the 4 mitochondrial regions analyzed in this study (*trnG-rns*, *rns-cox2*, *cox2-spacer*, *atp1-nad5*). The diameter of the circle reflects the number of isolates corresponding to the specific haplotype. In (A) the color is indicative of the geographic origins while (B) isolates from citrus (orange) and tobacco (green) are differentiated from other isolates (grey).

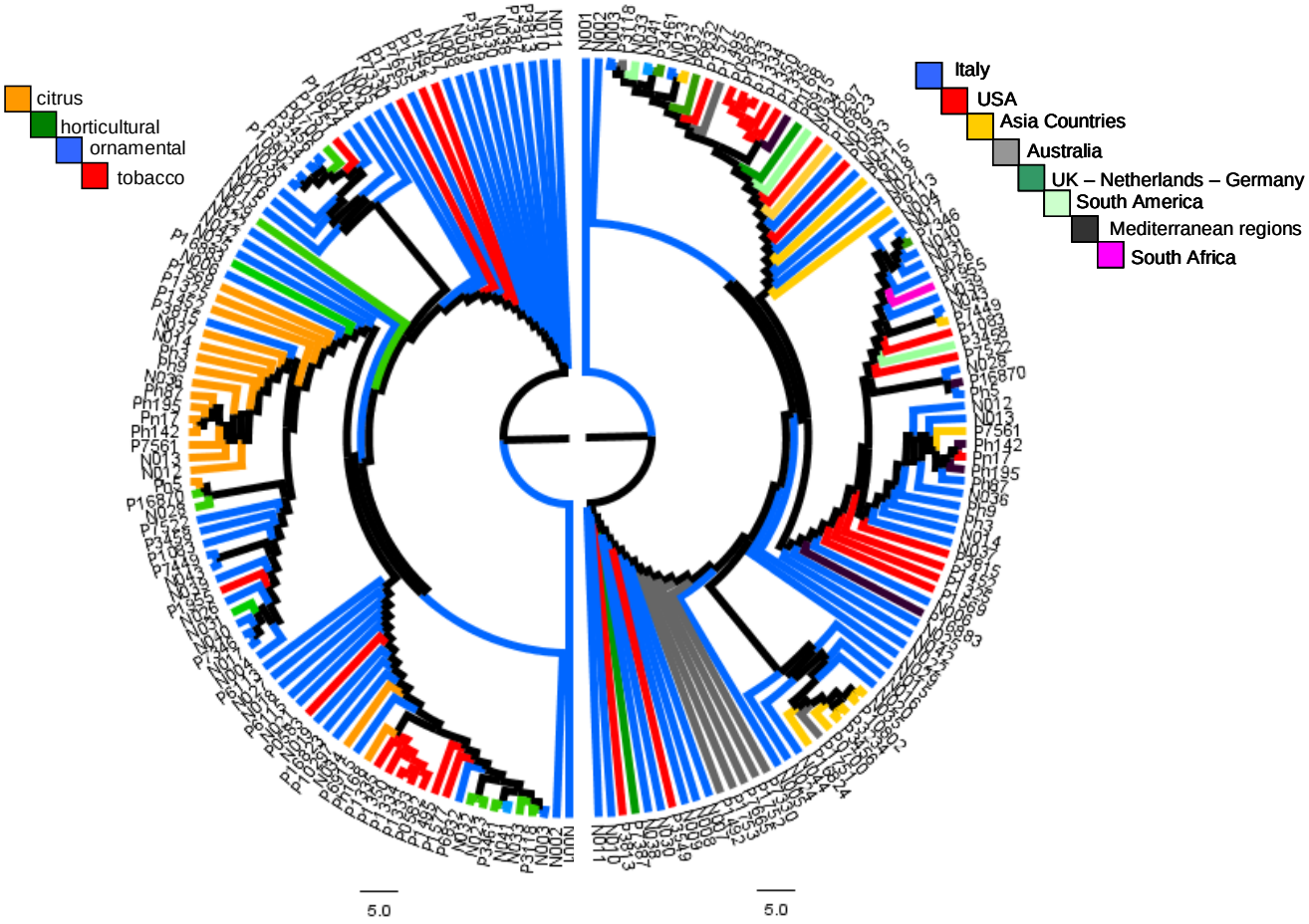

Fig. 6 - Neighbour-joining distance analysis of combined nuclear data set (*hyp-scp-ßtub*) (1654 bp). The isolates were analyzed according to their hosts (left side) or geographic (right side) origin. In the right hemisphere isolate N034 from Trinidad and Tobago and isolate P0700 from Ponape (Micronesia) were included with South American and Asian Countries respectively.

III.4.5 *Phylogenetic analysis*

Since the partition homogeneity test was not significant (p = 0.06) phylogenetic analyses were conducted using the concatenated data set of the four mitochondrial regions. The three methods of analysis (maximum parsimony, maximum likelihood and Bayesian) generated trees with a similar topology, however two clades (4 and 5) were not supported by the maximum parsimony and maximum likelihood methods. Clades were organized to be congruent as much as possible with those reported in Chapter II, which were generated using a smaller number of isolates and only two mitochondrial regions. In the present analysis's the 96 isolates (50 haplotypes) were distributed in 6 main clades; five of them corresponded to those reported in Chapter II while an additional clade (N6) was observed for two new isolates

from *Dieffenbachia maculata* recovered from Florida and Germany (Fig. 7). Clade one (N1) consisted of 9 haplotypes representing 12 isolates that can be separated in two sub-clades, 1a and 1b. Among isolates of this group there were 15 SNPs (12 parsimony informative) and 149 sites with gaps. Haplotype diversity within clade N1 was 0.9545. Sub clade 1a had 3 isolates from Southern Italy (N001, N010, N011) and 1 citrus isolate from Trinidad (N034). Sub-clade 1b had isolates from different geographic locations and hosts. Two different haplotypes for tobacco isolates from Australia (P1495) and South Africa (P1955) clustered in this group.

Clade N2 consisted of 7 haplotypes representing 21 isolates. Eighteen SNPs (10 parsimony informative) and 30 sites with gaps were observed in this clade with a haplotype diversity of 0.805. Isolates from various geographic locations and hosts clustered in this clade, including the majority of isolates from tobacco (8 of 12 isolates) from Australia and USA (Kentucky and Virginia). Seven SNPs differentiated tobacco isolates from Australia (sub clade 2a) from those recovered from the USA (sub-clade 2b) (Fig. 7).

In clade N3 there were 9 haplotypes (17 isolates) mainly recovered from citrus (15 isolates) in different geographic locations; two other isolates were from *Limonium sinensis* (N043) and *Catharanthus roseus* (P7522). Isolate N043 had the same mitochondrial haplotype (H37) as 5 isolates from citrus, but was the only isolate of this haplotype showing an A2 mating type. Isolate P7522 represented a unique haplotype and clustered in a single branch basal to the rest of the clade. A total of 5 SNPs (1 parsimony informative) differentiated isolates of this clade with a haplotype diversity of 0.8603. However, only 2 SNPs were observed (1 parsimony informative) in the *atp1-nad5* region and haplotypes diversity dropped to 0.8417 by excluding isolate P7522 from the analysis. Clade N4 had haplotype diversity of 0.8366 and 18 isolates (9 haplotypes) differed by 2 SNPs. This clade was heterogeneous for geographic origin, host and mating types. Clade N5 was consisted of 15 haplotypes representing 26 isolates and had a haplotype diversity of 0.9015. Haplotypes in this clade differed by 10 SNPs (2 SNPs parsimony informative) and 89 sites with gaps.

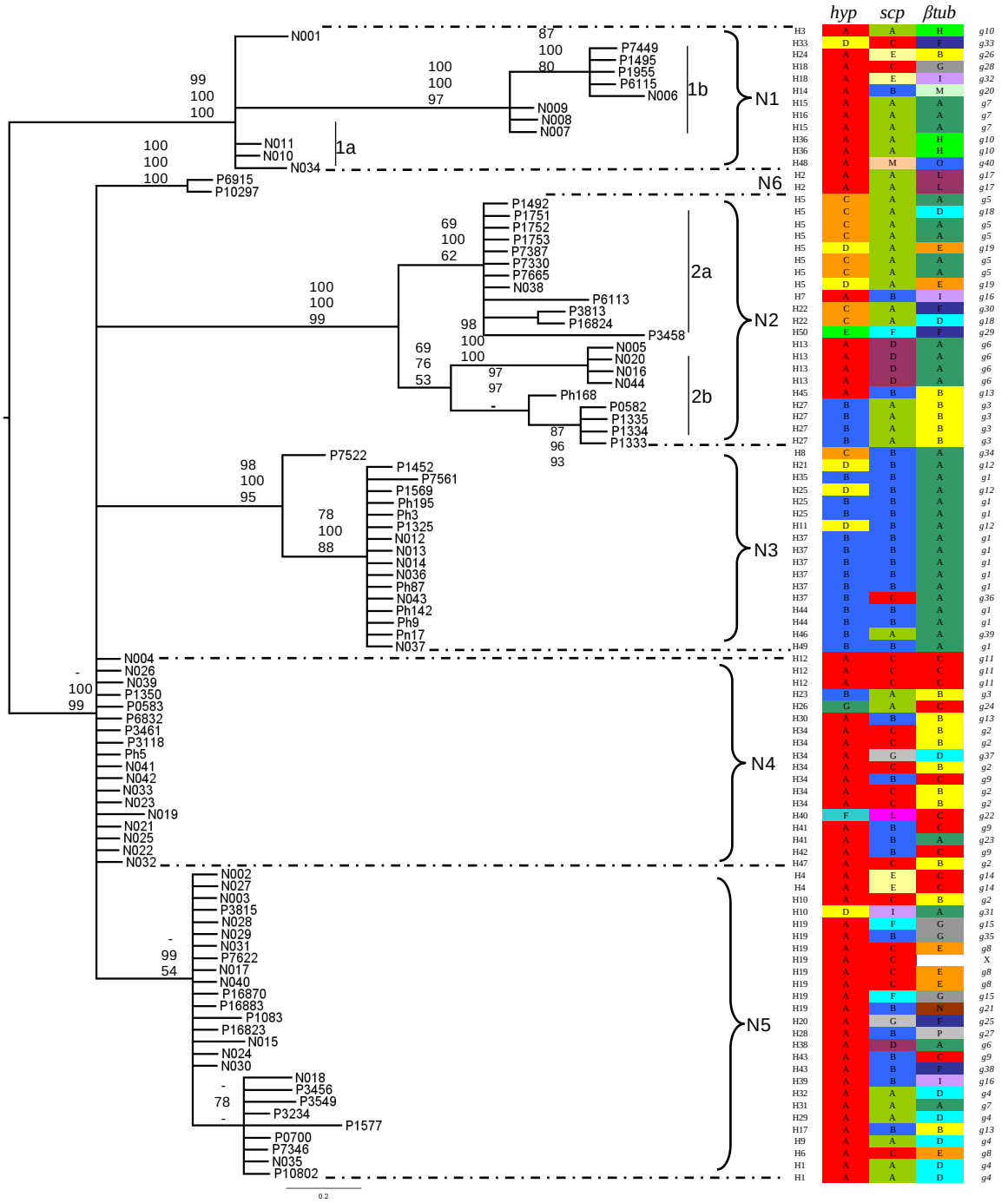

Fig. 7 - Phylogenetic relationships among 96 *Phytophthora nicotianae* isolates based on the combined data set of sequences of the four mitochondrial regions (*trnG-rns*, *rns-cox2*, *cox2-spacer*, *atp1-nad5*) (left) and their association with the nuclear multilocus genotype of the three nuclear regions (*hyp*, *scp*, *βtub*) (right). Numbers on nodes represent the statistical support for the maximum parsimony (1000 bootstrap replicates, top number), Bayesian method (posterior probabilities, middle number) and maximum likelihood (100 replicates, bottom number). Dash on nodes indicates branches not supported by the specific phylogenetic method. Dashed lines delimitate the clades and their specific nuclear multilocus genotype.

III.4.6 *Mitochondrial and nuclear markers association*

The comparison of mitochondrial haplotypes and nuclear multilocus genotypes exhibited the existence of several congruent groups, although mitochondrial DNA was more effective in differentiating closely related isolates.

The isolates from citrus that clustered together in the mitochondrial phylogenetic tree (clade N4) had nearly identical multilocus nuclear genotype and differed for a single SNP (pos. 101) in the *hyp* nuclear locus for 3 citrus isolates from California (P1325, P1569 and P1452) (Figs. 7 and 8) and 3 SNPs in the *scp* locus (pos. 96, 238, 450) for one isolate from Florida (Pn17). Similarly, American tobacco isolates P1333, P1334, P1335 and P0582 had an identical mitochondrial haplotype and multilocus genotype. Also the Australian tobacco isolates (with the exception for isolate P1751), had the same mitochondrial haplotype and the same nuclear multilocus genotype.

Interestingly, congruent data were also observed in relation to the geographic origin of isolates. For example identical mitochondrial haplotypes and multilocus genotypes were revealed for isolates P10297 and P6915 from *Deffenbachia maculata* from Florida and Germany, for isolates P3461 and P3118 from *Solanum lycopersicum* recovered from the UK and Australia and for 4 isolates (Ph5, N041, N042, N023) recovered from different regions of southern Italy. Furthermore, latter isolates that belonged to the A2 mating type shared identical mitochondrial and nuclear markers with an A1 isolates (N033) from *Lycopersicum esculentum* sourced in Chile.

Although less frequently, examples of non congruent data for mitochondrial and nuclear markers were also observed. Isolate P1350 from tobacco (North Carolina) had a different mitochondrial haplotype and grouped in a different phylogenetic clade from the other American tobacco isolates but exhibited the same nuclear multilocus genotype. Furthermore, there were cases where isolates having the same mating type showed different mitochondrial haplotypes and identical multilocus genotypes. For example, isolate N001

(mating type A2) from *Prunus Americana* (Calabria, Italy) had a different mitochondrial haplotype (H3) as compared to the A2 isolates N010 and N011 from *Dodonea viscosa* (H36, Sicily, Italy) but had the same nuclear multilocus genotype (*g*10).

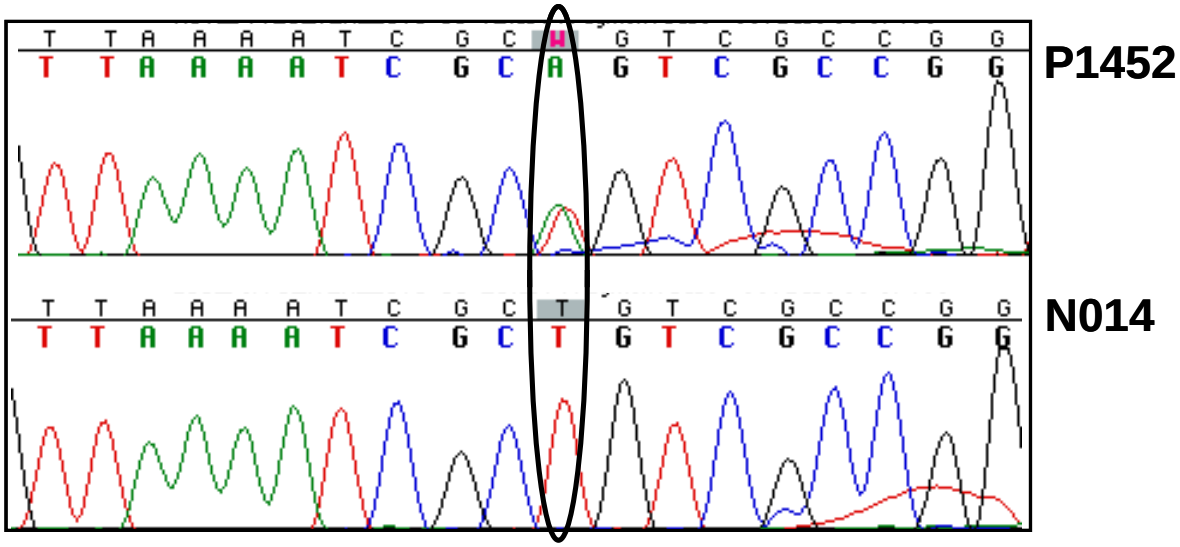

Fig. 8 - Aligned chromatograph showing a SNP in the *hyp* nuclear region (I11F-I12R) that differentiate nuclear multilocus genotype of citrus isolates from California (represented by isolate P1452) from the other citrus isolates recovered from different geographic regions (represented by N014).

III.5 Discussion

Four mitochondrial markers and three nuclear coding regions were used in this study to reveal patterns of genetic variation in 96 isolates of *Phytophthora nicotianae* representing a wide host range and geographic locations. Analysis of haplotypes showed a different level of discrimination among the isolates for the four mitochondrial regions.

The *rns-cox2* intergenic region includes a region (*trnW-cox2*) already analyzed in Chapter II but includes an additional 262 bases. The new sequenced portion, did not improve the haplotype identification in the 51 isolates discussed in the Chapter II. A new SNP was revealed in position 66 characterizing haplotype 7 wich was already identified with the shorter region. This region was the least variable with a total of 4 SNPs and 120 sites with gaps and enabled the identification of 9 haplotypes, which is 2 additional haplotypes compared to those

identified with the *trnW-cox2* region from 51 isolates. The *cox2*+spacer region identified of 13 haplotypes with 8 of them differentiated by 8 SNPs in the *cox2* gene (6 of these were non-synonymous substitutions). Intraspecific variability has been already identified for the *cox2* gene in *Phytophthora* species, including two isolates of *P. nicotianae* (Martin and Tooley, 2003). Nine out of 14 species with multiple isolates showed genetic variability at an intraspecific level for the *cox2* gene (Martin and Tooley, 2003). However, maximum likelihood analysis of the *cox2* gene haplotypes of *P. nicotianae* in comparison with other *Phytophthora* species of clade 1 revealed a uniform group for all isolates of *P. nicotianae* that can be clearly distinguished from other species of clade 1 (Fig. 4) according to previous reports (Kroon et al., 2004; Blair et al., 2008).

The *trnG-rns* region included a region (*trnY-rns*) already analyzed in Chapter II but was 275 bp longer. Three SNPs and 4 sites with gaps were identified and enabled the differentiation of 28 haplotypes. The *atp1-nad5* region was the most variable among the 4 mitochondrial regions analyzed in the present work and allowed the identification of 31 haplotypes that were differentiated by 23 SNPs, 124 sites with gaps and length variations in homopolymeric regions.

On the whole the combined data set for the 4 mitochondrial regions identified 50 haplotypes. The higher number of mitochondrial haplotypes observed in this study as compared with those of the previous study (Chapter II) reflects the larger portion of the mitochondrial genome of *P. nicotianae* examined and the broader panel of isolates analyzed. In an effort to reduce the amount of sequencing needed for the haplotype discovery, it was observed that two mitochondrial regions (*trnG-rns*, *atp1-nad5*) identify almost all the observed haplotypes for these isolates (47 haplotypes) and represent a valuable tool for the analysis of the worldwide distribution of *P. nicotianae* haplotypes.

Individual regions as well as the combined data set of mitochondrial regions did not revealed a specific association among grouping of isolates and their geographic provenience.

As an example, the most frequent haplotypes (H5, H19) were shared among isolates from different geographic origins and hosts. The absence of a specific geographic structure most probably reflects either a significant gene flow among isolates or migration of isolates of *P. nicotianae* that reduced geographic separation of haplotypes. In other words, the fact that haplotypes were shared among different geographic locations represents evidence of the panmictic distribution of haplotypes of *P. nicotianae* and could be the result of recurring events of migration favored by the ornamental plants trade. *Phytophthora nicotianae* is a polyphagous pathogen and, despite hardly ever giving rise to epidemic outbreaks, it is probably the species that, in general, causes the most damage in nurseries. The ornamental plant industry is particularly exposed to the risk of the emergence of new diseases as a consequence of their dynamism, the wide range of products, continual innovation in procedures or in products and the use of intensive cultivation techniques that characterize it. Other particular aspects of the nursery sector are the rapid substitution of varieties to adapt to market demand and the use of mono and oligogenic resistances to diseases, which favors the rise of new special forms and new families of pathogens. Finally, the sudden, and almost contemporary, appearance of ornamental plant diseases in different continents can be traced back to structural reasons. In this line of production, in fact, the propagation material is produced in just a few big nurseries, which in turn supply small nurseries in other regions or countries (Garibaldi et al., 2004). Numerous studies indicate that the critical sector, regarding both the outbreak and spread of new diseases in natural and forest ecosystems, is the nursery business. In nurseries, migration of pathogenic species from one host to another, contact between taxa (genotypes, families, species) and the potential threat of hybridization phenomena and the differentiation of new taxa that are sometimes particularly dangerous for crops and natural environments are inevitable (Magnano di San Lio e Cacciola, 2002; Brasier et al., 2004). The existence of interspecific hybrids has been demonstrated for a number of *Phytophthora* species including *P. nicotianae* (Man in't Veld et al 1998; Donahoo and

Lamour, 2008; Nirenberg et al., 2009). In recent years in Italy more than 20 *Phytophthora* species causing root rots have been reported in ornamental nurseries, the most common being *P. nicotianae* (Cacciola et al., 2008). Similarly, *P. nicotianae* was one of the most common *Phytophthora* species in a recent survey carried out in nurseries and garden centers in Spain (Moralejo et al., 2009). A panmictic distribution similar to that revealed for *P. nicotianae* has been reported for other pathogens such as the wheat pathogen *Phaeosphaeria nodorum* (Stuckenbrock et al., 2006) and the opportunistic coral reefs pathogen *Aspergillus sydowii* (Rypien et al., 2008).

Although data of the present study clearly indicate a panmictic distribution of *P. nicotianae*, specific associations were found among some genetic groups and hosts of provenience. The most interesting association was with citrus since the majority of isolates from this host (15 out of 19) clustered together, independent of their geographic origin (Philippines, Syria, Albania, California, Florida and Italy). These isolates were characterized by a low level of nucleotide diversity that was limited to two SNPs in the *atp1-nad5* region and length variation in some homopolimeric T regions. A similar host clustering was observed for 8 isolates from tobacco recovered from Australia and USA (Kentucky and Virginia) although a higher genetic variation (7 SNPs) differentiated these isolates. Similar results were also observed for different ornamental and horticultural host species, although a limited number of isolates were analyzed for each host. These results seem to be in contrast with the above mentioned polyphagy and panmictic distribution of *P. nicotianae*. However, as already speculated in Chapter II, by analyzing a small number of isolates it can be hypothesized that *P. nicotianae* isolates have been spread worldwide due to the nursery trade of infected plant materials and afterwards they have progressively diverged on specific hosts. A preferential association between subgroups of *P. nicotianae* and host specificity has been reported for several host species (Philips and Baker, 1962; Erwin and Ribeiro, 1996; Allagui and Lepoivre, 2000). It was demonstrated that isolates from *Citrus* spp. were more virulent on

roots of rough lemon than isolates from petunia, tomato, walnut, silk tree, jojoba, hibiscus and peach. Also, tomato plants exhibited high susceptibility to many isolates including citrus isolates (Matheron and Matejka, 1990). Furthermore, the analysis of both mitochondrial and nuclear DNA restriction fragments distinguished isolates causing black shank in tobacco from other *P. nicotianae* isolates (Colas et al., 1997).

The phylogenetic analysis of the combined mitochondrial data set inferred with maximum parsimony (MP), maximum likelihood (ML) and Bayesian analysis (B) revealed the existence of different evolutionary patterns and enabled the identification of six phylogenetic groups. The tree topology was consistent with the three different phylogenetic methods except for clade 5 that was supported only by the Bayesian method. Compared to Chapter II, the analysis of a significantly longer target region representing the 10% of the mitochondrial genome and a higher number of isolates enabled a more accurate definition of genetic groups and the identification of some homogeneous sub-clades. However, basic clades largely confirmed those reported in Chapter II (Fig. 7).

In agreement with the haplotype network none of these clades was associated to the geographic origin of isolates. Within each clade different haplotypes were identified, except for the clade 1, which consisted of two isolates from *Dieffenbachia maculata* (P10297, P6915) representing a single mitochondrial haplotype. Curiously, two haplotypes from tobacco recovered from Australia and USA (H5 and H27) represented by 8 isolates clustered in two different sub-clades of the main clade 2 (Fig. 7), indicating a potential common ancestral mitochondrial genome and a more recent evolutionary split. Similarly, 8 different haplotypes, with a low genetic diversity and representing 15 isolates from citrus from various geographic origins were grouped in clade 3, suggesting a common origin of these haplotypes, most probably due to the spread of the same or very similar haplotype in the major citrus production areas.

The analysis of the three nuclear loci led to the identification of 24 SNPs that divided the 96 isolates in 40 genotypes. Distance analysis of the combined nuclear data set did not show a differentiation of the isolates according to their geographic origin confirming a panmictic distribution of genotypes of *P. nicotianae* agreeing with the results of mitochondrial haplotype analyses. Heterozygosity was observed at each locus, which is compatible with the probabilities that some of the isolates used in this study were from a sexually reproducing population. According to the above remarks on nurseries it could be hypothesized their important role in favoring a sexual reproduction system in *P. nicotianae* by facilitating the meeting of different mating types from different populations. This hypothesis is supported the lower diversity revealed within isolates from citrus and tobacco as compared to isolates from ornamental plants. Eleven isolates recovered from citrus had identical nuclear multilocus genotype and another 4 citrus isolates (P1569, P1325, Pn17, P1452) clustered together with nearly similar genotypes (Fig. 6). The nuclear multilocus genotype g1 having 4 heterozygous SNPs was identified in 11 citrus isolates, 8 of which were from South Italy. The fact that isolates with this genotype were all mating type A1 and almost all had the same mitochondrial haplotype (H37) might suggest that these heterozygous loci were fixed in clonal populations. However, the analysis of a large numbers of isolates collected from specific citrus fields is still needed to confirm this conclusions. Fixed heterozygosity in clonal populations has already been detected in other heterothallic species of *Phytophthora*. For example, the analysis of 13 nuclear coding regions of *P. ramorum* identified heterozygous loci established in the European (EU1) and North American (NA1, NA2) clonal populations (Bilodeau, 2008). A similar result was observed for *P. capsici* where the screening of 6 loci identified heterozygous genotypes that were fixed in a clonal population in coastal Peru (Hurtado-Gonzales et al., 2008). These observations are in agreement with the tendency for heterothallic species to converge more often toward clonal reproduction, especially once the

selection favoring certain genotypes are spread and fixed in the population with asexual propagules.

Our data clearly indicate that SNP analysis is a valuable tool for the characterization of *P. nicotianae* populations, however it should be highlighted that heterozigosity represents a limitation since it makes haplotype determination challenging and reduces the amount of information that can be pulled out from the genetic data. Despite different empirical and computational methods that are available (Zhang and Hewitt, 2003; Stephens et al., 2001) cloning of PCR products is certainly the most reliable approach for the determination of haplotypes that constitute a particular heterozygous genotype. The main limit of this approach is the high cost when applied to a large number of samples. Furthermore, errors due to the activity of the *Taq* polymerase used in template amplification and *in vitro* recombination upon transformation of the bacterial cells are issues to consider for this approach (Zhang and Hewitt, 2003).

In the present study PCR products cloned and sequenced for the *scp* region showed an unexpected genetic framework for a diploid organism like *P. nicotianae* since a different number of alleles were identified for isolates N002 (5 alleles observed) and N034 (4 alleles). Although 2 genotypes represented by 49 out of 96 isolates were homozygous it can be hypothesized that the heterozygous genotypes were characterized by different meiotic rearrangements or gene duplication. Hypothesis of polyploidy, aneuploidy or gene duplication have been made for *P. ramorum* using microsatellites (Ivors et al., 2006) and sequencing of nuclear coding regions (Bilodeau, 2008). Similarly trisomy and multiple alleles for coding region were observed in *P. infestans* (Ospina-Giraldo and Jones 2003; van der Lee et al., 2004) and in *P. cinnamomi* (Dobrowolski et al., 2002). The possibility of different meiotic rearrangements has been demonstrated in *Pythium sylvaticum* (Martin, 1995). In this latter study the analysis of the chromosome size by pulse field gel electrophoresis and Southern hybridization of cDNA clones, in progeny derived from sexual crosses, revealed the presence

of a different electrophoretic karyotypes compared to the parents. It was proposed that translocation and aneuploidy events were the cause of these polymorphic karyotypes. Moreover, the evidence of polymorphic karyotypes in isolates collected in field, suggested that these meiotic rearrangements were not only due to sexual crossing in the laboratory but were also present in the offspring in nature (Martin, 1995).

The association of mitochondrial haplotypes with the nuclear genotypes revealed several interesting patterns that may reflect the above remarks on events of migration between different geographic regions. For example, the isolates from citrus clustering in clade 3 had a similar mitochondrial haplotype and multilocus genotype. Differences in the nuclear multilocus genotype were identified only as 1 SNP in 3 Californian isolates for the *hyp* region (Fig. 8) and 3 SNPs in the *scp* region for isolate Pn17 from Florida . The same mt haplotypes and nuclear genotypes were found for isolates within the same geographic region but also between different continents. Shared multilocus genotypes and mitochondrial haplotypes among isolates from different geographic regions was also identified for ornamental plants, confirming that the shipment of infected plant material from nurseries is the main reason of the spread of this cosmopolitan and polyphagous pathogen. For example, isolates from *Dieffenbachia maculata* from Germany shared the same haplotype and nuclear multilocus genotype with isolates from Florida. This was also observed for an isolate from *Hyppeastrum* sp. in Netherlands and from *Rhamnus alaternus* in Italy. Two isolates from *Solanum lycopersicum* from UK and Australia had the same haplotype and nuclear multilocus genotype of isolates from ornamental and horticultural plants from different regions in Italy.

Other data suggests that recombination has occurred for some isolates recovered from ornamentals in Sicily. In fact, isolates with opposite mating type but with the same mitochondrial haplotype and a different multilocus genotype have been identified (isolates N002, N0027, N021, N025, N024, N030). A similar observation was found for isolates recovered from tobacco; isolate P1751 from Australia had the same mitochondrial haplotype

as other Australian isolates from tobacco but a different nuclear multilocus genotype. An isolate from North Carolina from tobacco had a different mitochondrial haplotype of other tobacco isolates from Virginia and Kentucky but had the same nuclear multilocus genotype. All these examples give a clear evidence of the wide dispersal of this pathogen and a structure that is not geographically confined.

In conclusion, a combined analysis of mitochondrial and nuclear markers has been applied in the present study to characterize *P. nicotianae*. Using these approaches it has been possible to have an overview about intraspecific genetic variability within this species and it has been possible to formulate motivated hypothesis on the diffusion of gamic and agamic reproductive systems and on the role of nurseries and trading of propagating materials in favoring different reproductive system, the diffusion and the host specialization of *P. nicotianae*. Obviously much more can be done. Unlike other *Phytophthora* species such as *P. infestans*, *P. ramorum*, *P. sojae* and *P. capsici*, the nuclear genome of *P. nicotianae* has not been sequenced yet. This limits the ability to design additional nuclear markers, but new genomic sequencing projects are already progress for other *Phytophthora* species including *P. nicotianae* (http://pmgn.vbi.vt.edu/). Considering that approximately 1,600 bp of the nuclear genome of *P. nicotianae* has been screened in the present study, SNPs in the whole genome of *P. nicotianae* may provide an inexhaustible resource for marker development. After identifying new markers, the next step could consist in the selection of a larger number of isolates with an accurate sampling scheme from different geographic regions to have a clearer and more complete picture of the genetic structure and gene flow that characterizes *P. nicotianae* in individual populations. Furthermore, it would be interesting to monitor through multiple sampling in several years to evaluate if sexual or clonal reproduction is favored within a field (for example a citrus orchard) and follow the possible introduction of new genotypes that may determine the emergence of new variability in the population. It would also be useful to complement data from these markers with a panel of other markers, such as

SSR markers, that are still a primary choice for this type of analysis because of their wide distribution in the genome and their high polymorphism.

III.6 Literature cited

Abbott CL, Gilmore SR, Lewis CT, Chapados JT, Peters RD, Platt HW, Coffey MD, and Lévesque1 CA, 2010. Development of a SNP genetic marker system based on variation in microsatellite flanking regions of *Phytophthora infestans*. *Canadian Journal of Plant Pathology* **32**: 440-457.

Allagui MB, Lepoivre P, 2000. Molecular and pathogenicity characteristics of *Phytophthora nicotianae r*esponsible for root necrosis and wilting of pepper (*Capsicum annuum* L.) in Tunisia. *European Journal of Plant Pathology* **106**: 887-894.

Apple JL, 1967. Occurrence of race 1 of *Phytophthora parasitica* var. *nicotianae* in North Carolina and its implications in breeding for disease resistance. Tob. Sci. 11.

Asmussen MA, Basten JC, 1994. Sampling theory for cyto-nuclear disequilibria. *Genetics* **138**: 1351–1363.

Atallah ZK, Maruthachalam K, Toit Ld, Koike ST, Michael Davis R, Klosterman SJ, Hayes RJ, Subbarao KV, 2010. Population analyses of the vascular plant pathogen Verticillium dahliae detect recombination and transcontinental gene flow. *Fungal Genetics and Biology* **47**: 416-422.

Bandelt HJ, Forster P, Sykes BC, Richards MB, 1995. Mitochondrial Portraits of Human-Populations Using Median Networks. *Genetics* **141**:743-753.

Banke S, Peschon A, McDonald BA, 2004. Phylogenetic analysis of globally distributed *Mycosphaerella graminicola* populations based on three DNA sequence loci. *Fungal Genetics and Biology* **41**: 226-238.

Bensch S, Irwin DE, Irwin JH, Kvist L, ÅKesson S, 2006. Conflicting patterns of mitochondrial and nuclear DNA diversity in Phylloscopus warblers. *Molecular Ecology* **15**: 161-171.

Benson DM, von Broembsen S, 2001. Phytophthora root rot and dieback. In: Jones RK, Benson DM, eds. Diseases of Woody Ornamentals and Trees in Nurseries. St. Paul, MN, USA: APS Press, 52–6.

Bilodeau G, 2008. Détection et génomique de *Phytophthora ramorum* agent causal de la mort subite du chêne (l'encre des chênes rouges) PhD thesis.

Blair JE, Coffey MD, Park S-Y, Geiser DM, Kang S, 2008. A multi-locus phylogeny for *Phytophthora* utilizing markers derived from complete genome sequences. *Fungal Genetics and Biology* **45**: 266-277.

Brasier CM, Kirk SA, Delcan J, Cooke DEL, Jung T, Man In't Veld WA, 2004. *Phytophthora*

alni sp. nov. and its variants: designation of emerging heteroploid hybrid pathogens spreading on Alnus trees. *Mycological Research* **108**: 1172-1184.

Ceresini P, Shew HD, James T, Vilgalys R, Cubeta M, 2007. Phylogeography of the Solanaceae-infecting Basidiomycota fungus Rhizoctonia solani AG-3 based on sequence analysis of two nuclear DNA loci. *BMC Evolutionary Biology* **7**: 163.

Cacciola SO, Magnano di San Lio G, 2008. Management of citrus diseases caused by *Phytophthora* spp. In: Ciancio A, Mukerji KG (eds), *Integrated management of diseases caused by fungi, phytoplasma and bacteria*. Springer Science+Business Media, pp. 61-84.

Colas V, Lacourt I, Ricci P, Vanlerberghe-Masutti F, Venard P, Poupet A, Panabières F, 1997. Diversity of virulence in *Phytophthora parasitica* on tobacco, as reflected by nuclear RFLPs. *Phytopathology* **88**: 205-212.

Csinos AS, 2005. Relationship of isolate origin to pathogenicity of race 0 and 1 of *Phytophthora parasitica* var. nicotianae on tobacco cultivars. *Plant Dis*ease **89**:332-337.

Csinos AS, and Bertrand, P. F. 1994. Distribution of *Phytophthora parasitica* var. nicotianae races and their sensitivity to metalaxyl in Georgia. *Plant Dis*ease **78**: 471-474.

Dobrowolski MP, Tommerup IC, Shearer BL, O'Brien PA, 2003. Three clonal lineages of *Phytophthora cinnamomi* in Australia revealed by microsatellites. *Phytopathology* **93**: 695-704.

Donahoo RS, Lamour KH, 2008. Interspecific hybridization and apomixis between *Phytophthora capsici* and *Phytophthora tropicalis*. *Mycologia* **100**: 911-920.

Farris JS, Kallersjo M, Kluge AG, Bult C, 1995. Testing significance of incongruence. *Cladistics* **10**: 315–319.

Feau Nicholas and Bilodeau Guillaume, 2010. SNPs and alleles calling program (unpublished). For info contact: Nicolas.feau@bordeaux.inra.fr and Guillaume.Bilodeau@ars.usda.gov.

Forester H, and Coffey MD, 1990. Mating behaviour of *Phytophthora parasitica*: evidence for sexual recombination in oospores using DNA restriction fragment length polymorphisms as genetic markers. *Experimental Mycology* **14**:351-359.

Fu Y, Li W, 1993. Statistical tests of neutrality of mutations. *Genetics* **133**: 693 - 709.

Fu Y, 1997. Statistical tests of neutrality of mutations against population growth, hitchhiking and background selection. *Genetics* **147**: 915 - 925.

Garibaldi A, Magnano di San Lio G, Gullino ML, 2004. Alcune considerazioni sui problemi fitopatologici emergenti delle colture ornamentali. *Informatore fitopatologico – La difesa delle piante* **54** (7/8), 19-32.

Guindon S, Gascuel O, 2003. A simple, fast and accurate algorithm to estimate large phylogenies by maximum likelihood. *Systematic Biology* **52**: 696-704.

Godinho R, Crespo EG, Ferrand N, 2008. The limits of mtDNA phylogeography: complex patterns of population history in a highly structured Iberian lizard are only revealed by the use of nuclear markers. *Molecular Ecology* **17**: 4670-4683.

Gomez-Alpizar L, Carbone I, Ristaino JB, 2007. An Andean origin of *Phytophthora infestans* inferred from mitochondrial and nuclear gene genealogies. *Proceedings of the National Academy of Sciences* **104**: 3306-3311.

Goss EM, Carbone I, Grunwald NJ, 2009. Ancient isolation and independent evolution of the three clonal lineages of the exotic sudden oak death pathogen *Phytophthora ramorum*. *Molecular Ecology* **18**: 1161-1174.

Grunwald NJ, Flier WG, 2005. The Biology of *Phytophthora infestans* at Its Center of Origin. *Annual Review of Phytopathology* **43**: 171-190.

Hasegawa M, Kishino K, Yano T, 1985. Dating the human-ape splitting by a molecular clock of mitochondrial DNA. *Journal of Molecular Evolution* **22**: 160-174.

Hu JH, Hong CX, Stromberg EL, Moorman GW, 2008. Mefenoxam sensitivity and fitness analysis of *Phytophthora nicotianae* isolates from nurseries in Virginia, USA. *Plant Pathology* **57**: 728-736.

Hurtado-Gonzales O, Aragon-Caballero L, Apaza-Tapia W, Donahoo R, Lamour K, 2008. Survival and Spread of *Phytophthora capsici* in Coastal Peru. *Phytopathology* **98**: 688-694.

Ippolito A, Schena L, Nigro F, 2002. Detection of Phytophthora nicotianae and *P. citrophthora* in citrus roots and soils by nested PCR. *European Journal of Plant Pathology* **108**: 855-868.

Ivors K, Garbelotto M, Vries ID, Ruyter-Spira C, Te Hekkert B, Rosenzweig N, Bonants P, 2006. Microsatellite markers identify three lineages of Phytophthora ramorum in US nurseries, yet single lineages in US forest and European nurseries populations. *Molecular Ecology* **15**: 1493-1505.

Johnson ES, Wolff MF, Wernsman EA, Atchely WR, and Shew HD, 2002. Origin of the black shank resistance gene, Ph, in tobacco cultivar Coker 371-gold. *Plant Dis*ease **86**:1080-1084.

Kebdani N, Pieuchot L, Deleury E, Panabières F, Le Berre JY, Gourgues M, 2008. Cellular and molecular characterization of *Phytophthora parasitica* appressorium-mediated penetration. *New Phytologist* **185**: 248-257.

Kroon LP, Bakker FT, van den Bosch GB, Bonants PJ, Flier WG, 2004. Phylogenetic analysis of *Phytophthora* species based on mitochondrial and nuclear DNA sequences. *Fungal Genetics and Biology* **41**: 766-782.

Lamour KH, Daughtrey ML, Benson DM, Hwang J, Hausbeck MK, 2003. Etiology of *Phytophthora drechsleri* and *P. nicotianae* (=*P. parasitica*) diseases affecting floriculture crops. *Plant Disease* **87**: 854-858.

Librado P, Rozas J, 2009. DnaSP v5: A software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* **25**: 1451-1452

Lucas, G. B. 1975. Diseases of Tobacco, 3rd ed. Biological Consulting Associates, Raleigh, NC.

Magnano di San Lio G, and Cacciola SO, 2002. Diseases of tropical ornamental plants in Italy. *Informatore Fitopatologico* **52**: 39-46

Man In 't Veld WA, Veenbaas-Rijks WJ, Ilieva E, de Cock AW, Bonants PJ, Pieters R, 1998. Natural Hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* Demonstrated by Isozyme Analysis and Random Amplified Polymorphic DNA. *Phytopathology* **88**: 922-929.

Martin FN, Tooley PW, 2003. Phylogenetic relationships among *Phytophthora* species inferred from sequence analysis of mitochondrially encoded cytochrome oxidase I and II genes. *Mycologia* **95**: 269-284.

Martin F, 1995. Meiotic Instability of *Pythium sylvaticum* as Demonstrated by Inheritance of Nucler Markers and Karyotype Analysis. *Genetics* **139**: 1233-1246.

Matheron ME, Matejka JC, 1990. Differential virulence of *Phytophthora parasitica* recovered from *citrus* and other plants to rough lemon and tomato. *Plant Disease* **74**: 138-140.

McDonald BA, Pettway RE, Chen RS, Boeger JM, and Martinez JP, 1995. The population genetics of *Septoria tritici* (teleomorph *Mycosphaerella graminicola*). *Canadian Journal of Botany* **73**(Suppl.):S292-S301.

Milne I, Lindner D, Bayer M, Husmeier D, McGuire G, Marshall DF, Wright F, 2009. TOPALi v2: a rich graphical interface for evolutionary analyses of multiple alignments on HPC clusters and multi-core desktops. *Bioinformatics*. **25**: 126-127.

Moralejo E, Pérez-Sierra AM, Álvarez LA, Belbahri L, Lefort F, Descals E, 2009. Multiple alien *Phytophthora* taxa discovered on diseased ornamental plants in Spain. *Plant Pathology* **58**: 100-110.

Nirenberg HI, Gerlach WF, Grafenhan T, 2009. *Phytophthora x pelgrandis*, a new natural hybrid pathogenic to *Pelargonium grandiflorum* hort. *Mycologia* **101**: 220-231.

Ospina-Giraldo MD, Jones RW, 2003. Characterization of the glucose-6-phosphate isomerase gene in *Phytophthora infestans* reveals the presence of multiple alleles. *Fungal Genetics and Biology* **40**: 197-206.

Parkunan V, Johnson CS, Bowman BC, Hong CX, 2010. Population Structure, Mating Type, and Mefenoxam Sensitivity of *Phytophthora nicotianae* in Virginia Tobacco Fields. *Plant Disease* **94**: 1361-1365.

Philips DJ, Baker R, 1962. *Phytophthora* crown rot of petunia. *Plant Disease Report* **46**: 506-508.

Polzin T, Daneschmand S V, 2003. On Steiner trees and minimum spanning trees in hypergraphs. *Operations Research Letters* **31**:12-20.

Rypien KL, Andras JP, Harvell CD, 2008. Globally panmictic population structure in the opportunistic fungal pathogen *Aspergillus sydowii*. *Molecular Ecology* **17**: 4068-4078.

Ronquist F, Huelsenbeck JP, 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.

Schena L, Cardle L, Cooke D, 2008. Use of genome sequence data in the design and testing of SSR markers for *Phytophthora* species. *BMC Genomics* **9**: 620.

Sommerhalder RJ, McDonald BA, Zhan J, 2007. Concordant evolution of mitochondrial and nuclear genomes in the wheat pathogen *Phaeosphaeria nodorum*. *Fungal Genetics and Biology* **44**: 764-772.

Stephens M, Smith N, Donnelly P, 2001. A New Statistical Method for Haplotype Reconstruction from Population Data. *American journal of human genetics* **68**: 978-989.

Stukenbrock EH, Banke Sr, Javan-Nikkhah M, McDonald BA, 2007. Origin and Domestication of the Fungal Wheat Pathogen *Mycosphaerella graminicola* via Sympatric Speciation. *Molecular Biology and Evolution* **24**: 398-411.

Stukenbrock EH, Banke S, McDonald BA, 2006. Global migration patterns in the fungal wheat pathogen *Phaeosphaeria nodorum*. *Molecular Ecology* **15**: 2895-2904.

Sullivan, M. J., Melton, T. A., and Shew, H. D. 2005a. Managing the race structure of *Phytophthora parasitica* var. nicotianae with cultivar rotation. *Plant Disease* **89**:1285-1294.

Sullivan, M. J., Melton, T. A., and Shew, H. D. 2005b. Fitness of races 0 and 1 of *Phytophthora parasitica* var. nicotianae. *Plant Dis*ease **89**:1220-1228.

Sullivan MJ, Parks EJ, Cubeta MA, Gallup CA, Melton TA, Moyer JW, Shew HD, 2010. An assessment of the genetic diversity in a field population of *Phytophthora nicotianae* with a changing race structure. *Plant Disease* **94**: 455-460.

Tajima F, 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585 - 595.

Tamura K, Dudley J, Nei M, & Kumar S, 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution* **24**:1596-1599.

Valleau WD, Stokes GW, and Johnson, EM,1960. Nine years' experience with the *Nicotianae longiflora* factor for resistance to *Phytophthora parasitica* var. nicotianae in the control of black shank. Tob. Sci. **4**:92-94.

van der Lee T, Testa A, Robold A, van 't Klooster, J, Govers, F, 2004. High-Density Genetic Linkage Maps of *Phytophthora infestans* Reveal Trisomic Progeny and Chromosomal Rearrangements. Genetics **167**: 1643-1661.

Zhang DX, Hewitt GM, 2003. Nuclear DNA analyses in genetic studies of populations: practice problems and prospects. *Molecular Ecology* **12**: 563-584.

Zhang XG, Sun WX, Guo L, Yu JF, Chang CJ, 2003. Genetic and pathogenic variation among tobacco black shank strains of *Phytophthora parasitica* var. *nicotianae* from the main tobacco growing in China. *Journal of Phytopathology* **151**: 259-266.

Zhang XG, Zheng GS, Han HY, Han W, Shi CK, Chang CJ, 2001. RAPD-PCR for diagnosis of *Phytophthora parasitica* var. *nicotianae* isolates which cause black shank on tobacco. *Journal of Phytopathology* **149**: 569-574.

Concluding remarks and future perspectives

In the present study an alternative approach based on the analysis of polymorphic mitochondrial and nuclear DNA regions has been developed and utilised to study intraspecific variability in *P. nicotianae*. This method may be further improved by the identification of new mitochondrial and nuclear target regions taking advantage of the increasing number of available Oomycete complete genome sequences and could be easily extended to other species of the genus *Phytophthora* as well as other plant pathogens. Unlike other *Phytophthora* species such as *P. infestans, P. ramorum, P. sojae* and *P. capsici*, the nuclear genome of *P. nicotianae* has not been sequenced yet but this is currently in progress. A major advantage of the present method is that it enables the comparison of data from different research groups or time periods and could also be used in standardized protocols to develop a DNA barcoding like strategy for the precise identification of sub-specific taxa in *P. nicotianae*.

The application of the present method to characterize worldwide sourced isolates of *P. nicotianae* provided an overview about intraspecific genetic variability within this species and enabled the formulation of a hypothesis on the diffusion of gamic and agamic reproductive systems, the role of nurseries and trading of propagation materials in favouring different reproductive system, and the diffusion and the host specialization of *P. nicotianae*. Obviously much more can be done. A next step could consist of the selection of a larger number of isolates with an accurate sampling scheme from different geographic regions to have a clearer and more complete picture of the genetic structure and gene flow that characterizes *P. nicotianae* in an individual population and between populations. In fact, polymorphisms found in *P. nicotianae* could be employed to study population genetics and to advance knowledge in the evolutionary history of this pathogen as well as its potential to adapt to changing environments and migrations. Furthermore, it would be interesting to monitor multiple samplings of the same field over several years to evaluate if sexual or clonal reproduction is favoured within a field (for example a citrus orchard) and follow the possible introduction of new genotypes that may determine the emergence of new variability in the population. It would also be useful to complement data from these markers with a panel of other markers, such as SSR markers, that are still a primary choice for this type of analysis because of their wide distribution in the genome and their high level of polymorphism.

Appendix 1

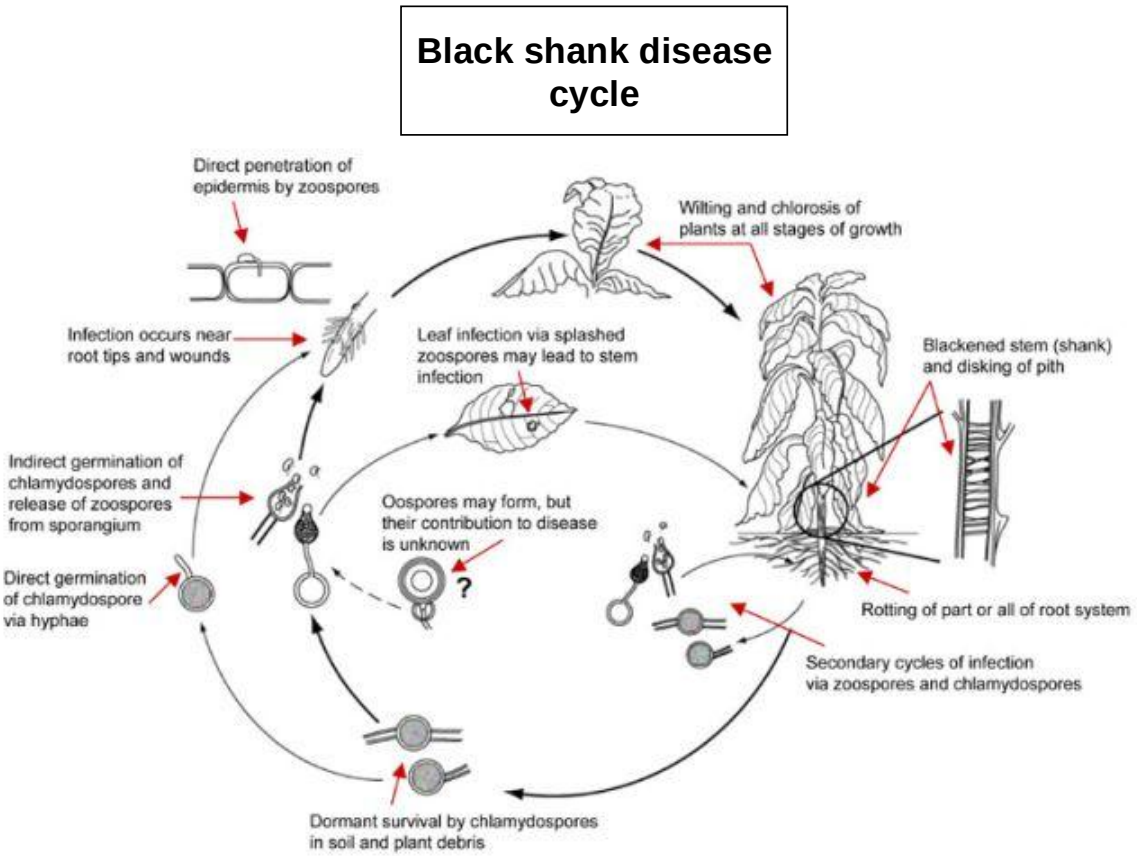

Fig. 1 - Life cycle of black shank pathogen *Phytophthora nicotianae*. Image from apsnet.org (Gallup et al., 2006).

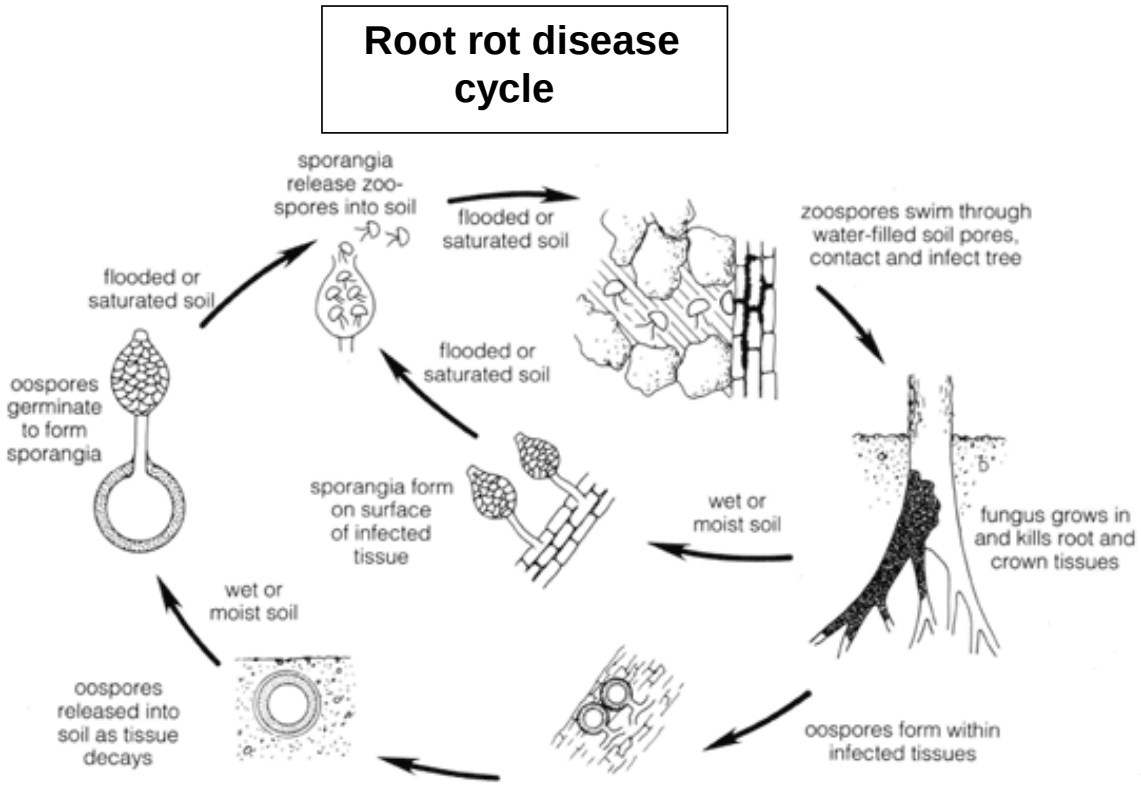

Fig. 2 - Life cycle of root rot and crown rot *Phytophthora* pathogen. Image adapted from Agrios, 1988.

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.

Acknowledgments

This work was carried out at the Dipartimento di Gestione dei Sistemi Agrari e Forestali of “Mediterranea” University of Reggio Calabria during the years 2008-2010. Part of the work was performed at the United States Department of Agriculture (USDA-ARS) of Salinas, California (USA).

I want to start by expressing my gratitude to my supervisor, Leonardo Schena, for his friendship, for the many encouragements and for helping me to reach a successful completion of my dissertation. Credit also goes to Prof. Gaetano Magnano di San Lio, for always being honest with me and being a great plant pathology mentor. It is impossible to acknowledge all of my lab mates in Reggio Calabria but, in particular, Saveria Mosca for always being kind and helpful. I want to thank Prof. Franco Nigro and Prof. Donato Gallitelli, who supported and encouraged me in the past to continue my studies.

I also need to thank the people and friends I met in the period I worked at the USDA-ARS station in Salinas, in the wonderful state of California. I don’t think I could have found a better place to do the work in a foreign country.

Dr. Frank Martin is a great plant pathologist and a great guide who taught me many things and gave me a lot of input and enthusiasm for this project. I am grateful also to my lab mates and friends in California with whom I shared many moments: Julia Schrandt, a wonderful person who helped and supported me, Guillaume Bilodeau, Zahi Atallah, Lorien Radmer, Amy Anchieta, Amy Atallah, Karun Marutachalam and Ravi Chitrampalam, who all gave me valuable advice and with whom I had a lot of interesting discussions concerning molecular biology, genetics and plant pathology subjects. I also thank Carolee Bull, Jim McCreight and Krishna Subbarao for their encouragement and all the other guys at USDA for being such nice people to share the station with.

A huge thanks to my father, my mother, my sister and the white little brother Piggy for the joy they give me every day.

Peace