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“Molecular analysis of genetic diversity of *Phytophthora citrophthora* using nuclear and mitochondrial markers”

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“Teach me the art of small steps”

Antoine de Saint-Exupery ‘The Little Prince’

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Chapter I: Introduction

I.1 Introduction to Oomycetes and *Phytophthora*

I.1.1 Oomycetes

The Oomycota (or Peronosporomycetes) are a group of eukaryotes including more than 800 species that may be saprobic or parasitic on terrestrial or aquatic plants and animals. The oomycetes have long been considered to be fungi because, like fungi, they obtain their nutrients via absorption and many of them produce filamentous hyphae characteristic of most fungi. However, molecular phylogenetic investigations (Cooke *et al.*, 2000; Kroon *et al.,* 2012) have found that the oomycetes are more closely related to the heterokont algae (i.e., the Phaeophyta [brown algae], Xanthophyta [yellow-green algae], Chrysophyta [golden algae], and Bacillariophyta [diatoms], and several smaller groups) than to the true Fungi (Chytridiomycota, Glomeromycota, Zygomycota, Ascomycota, and Basidiomycota). Figure I.1 shows a phylogenetic tree of the Oomycota.

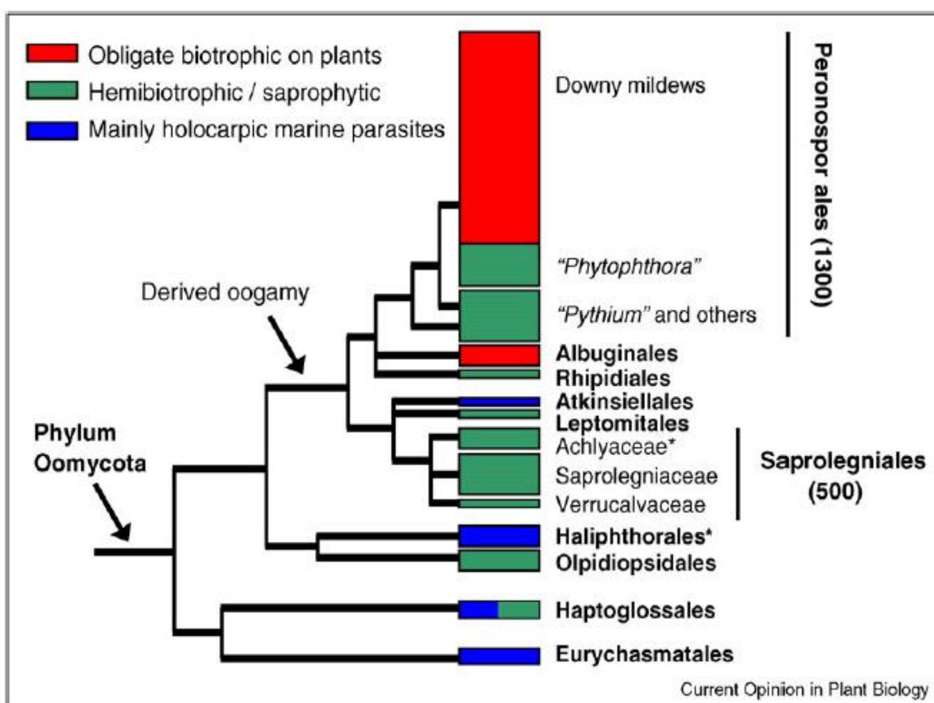

Fig.I.1 - Phylogenetic tree of the phylum Oomycota from Thines and Kamoun, 2010. For the Saprolegniales and Peronosporales only the number of species are given because of their large number of described species.

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 b-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 diseases caused by Oomycetes include seedling blights, damping-off, root rots, bark cankers, foliar blights and downy mildews (Heffer et al., 2002; Erwin and Ribeiro, 1996). Some notable diseases are the late blight of potato, downy mildew of grape vine, sudden oak death, and root and stem rot of soybean. More than 60% of Oomycetes are plant pathogens and *Phytophthora* belongs to this percentage of plant pathogens. (Thines and Kamoun, 2010).

I.1.2 *Phytophthora* species

The genus *Phytophthora* is located in the family of *Peronosporaceae* and order *Peronosporales* that includes obligate biotrophic pathogens, hemibiotrophic and saprophytic organisms (Cooke *et al.* 2000). The morphological properties and pathology of many of the described species are summarized in Erwin and Ribeiro (1996). In the traditional taxonomy, *Phytophthora* were discriminated mainly on the structure of the sporangial apex (Fig. I. 2) (nonpapillate, semipapillate, or papillate), the attachment of the antheridium to the oogonium (amphigynous or paragynous), and on whether the taxon is inbreeding (homothallic) or outbreeding with A1 and A2 sexual incompatibility or mating types (heterothallic) (Tucker, 1931; Waterhouse, 1963). Heterothallic taxa are exclusively amphigynous while homothallic taxa may be either amphigynous or paragynous or, in some cases, have antheridia of both types.

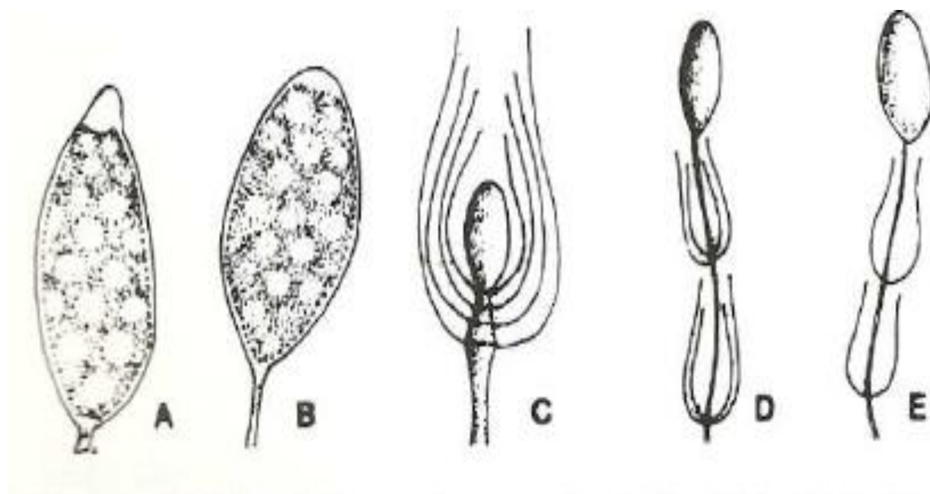

Fig. I.2 - Morphology of sporangia of *Phytophthora* spp. A: semipapillate(note the cospicous apical plug. B: semipapillate (note that although there is a protuberance at the tip of sporangium, the protoplasm extends nearly to the sporangium wall. C: Nested and extended proliferation of a nonpapillate sporangium. D: Extended proliferation of a nonpapillate sporangium (Drawn by A. Vaziri; Erwin and Ribeiro 1996)

The following description of the life cycle of *Phytophthora* species by Thomas Jung is taken from www.tree-diseases.com: "*Phytophthora* species are able to survive in unsuitable environmental conditions over several years as dormant resting spores (oospores or chlamydospores) in the soil or in infected root tissue. When environmental conditions become suitable (high soil moisture, soil temperature > c. 10 °C) the resting spores germinate by forming sporangia which release motile, biflagellate zoospores into the soil water. These zoospores are chemotactically attracted by young fine root tips. After penetrating the exoderm (or the periderm in suberized fine roots) *Phytophthora* is growing inter- and intracellular inside the fine root with typical coralloid to irregular, non-septate hyphae. After decomposition of the root by saprophytic fungi the resting spores are set free into the soil environment, and the cycle starts again. *Phytophthora* can increase and disseminate their inoculum from low, nearly undetectable levels during a relatively short time of favourable environmental conditions. On the other hand, the life cycle can run million times and it can take decades of inoculum build-up and progressive destruction of roots before that a mature tree begins to show visible symptoms (Tsao, 1990). Therefore, the epidemiology of *Phytophthora*-induced fine root diseases is considered to be multicyclic (Erwin & Ribeiro, 1996). Predisposing factors such as

waterlogging or planting of tree species which are not adapted to the site conditions as well as contributing factors, which either reduce the vitality of the tree (e.g. extreme droughts or defoliations) or favor the pathogen (e.g. excess soil moisture following heavy rain, flooding or irrigation) can accelerate the disease process or actually make it possible." Figure I. 3 shows the life cycle of soilborne *Phytophthora* species.

Approximately 100 species of *Phytophthora* have been described in the literature of which 58 were officially recognized (Kroon et al., 2012). This enormous increase is, on the one hand, due to the availability of more sophisticated tools for species delimitation and, on the other hand, large-scale surveys for the presence of novel *Phytophthora* species in natural and agricultural settings.

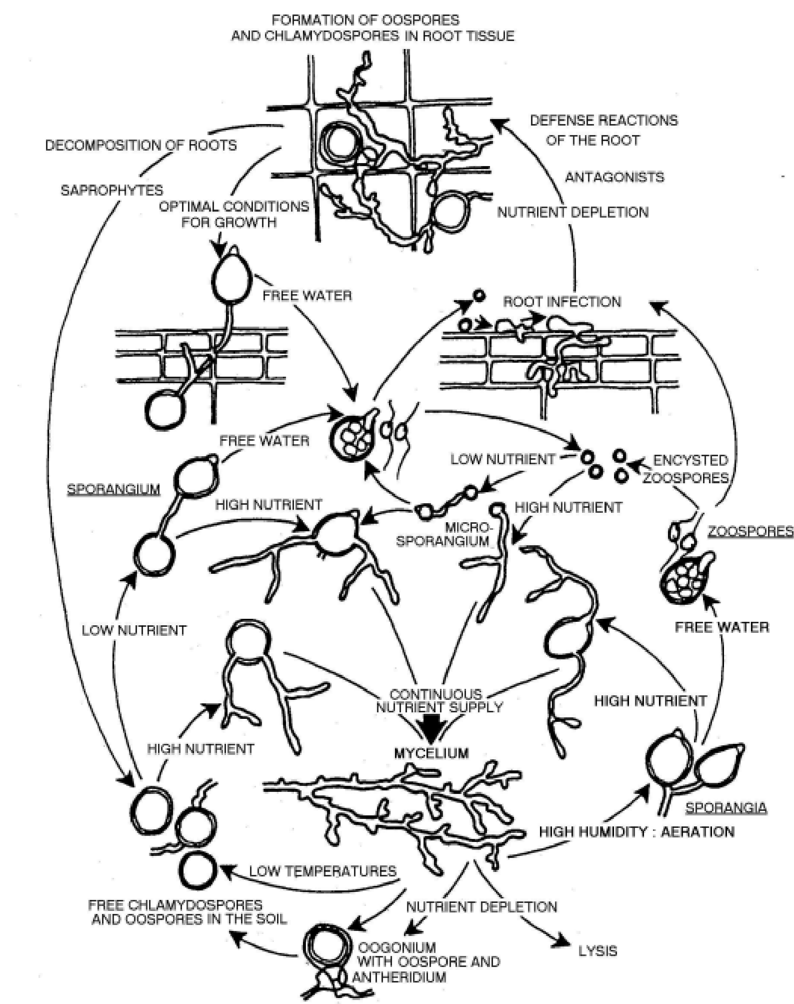

Fig. I.3 - Life cycle of soilborne *Phytophthora* species (from www.tree-diseases.com; adapted from Ribeiro 1978)

I.2 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 population structure of pathogens like *Phytophthora* spp. including 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, hence, in the expression 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; Grünwald and Flier, 2005; Grünwald et al., 2008).

I.2.1 Forces acting on natural populations

I.2.1.1 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 variants in a particular environment will be maintained and their frequency in the population will increase compared to unfavorable variants 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 parts of populations following

the use of chemical agents also is the result of selection of genotypes with a better performance. For example, the metalaxyl-sensitive US-1 clonal lineage of *P. infestans* was replaced by the new metalaxyl-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.2.1.2 Reproductive system

Sexual reproduction is 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. Various *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 affects 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 either increase or negatively affect the possibility of sexual recombination in a population is the environment that allows contact between new genotypes and opposite mating types (in case of heterothallic species like *P. nicotianae*) or prevents such contacts via 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 when recombination

does not occur (Fry, 2008). In fact, the geographic separation between opposite mating types limits the possibility for sexual recombination in many areas of the world (Goodwin et al; 1998).

In nature, clonality in *Phytophthora* populations s widely documented (eg. Dobrowolsky et al., 2003; Grünwald et al., 2008; Lamour et al., 2003). In this case, genetic variation is unidirectionally transmitted from the genotype of one parent to the next generation. It can be assumed that asexual populations exhibit lower genetic variability leading to a slower process of evolution compared to sexually reproducing populations. The reduced levels of genetic variation 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.2.1.3 Mutation

Mutation is one of the most important sources of genetic variation which is basic for natural selection of populations and can lead to future evolutionary divergence. Pathogens with fast mutation rates represent much higher potential threats than 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 (Mammella 2010). Similarly, mutations can also increase the resistance of the pathogen to specific fungicides. Metalaxyl-resistant genotypes of the US-1 clonal lineage of *P. infestans* were observed in the Philippines (Koh et al., 1994). This was unusual considering 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 genotypes which was discovered in Ireland in 1980 (Carter et al., 1982) was shown to be a mutated genotype of the US-1 lineage (Goodwin et al., 1996).

I.2.1.4 Gene flow and migration

Gene flow and migration contribute to the fluctuation and exchange of alleles and genotypes among geographically separated populations. 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 spread of pathogens in specific areas. For example, dispersion through 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 (eg. sporangia and zoospores) have a better mobility than pathogens that don't produce such structures. A classical example was the spread of *P. ramorum* via aerial sporangia in California causing the rapid epidemic Sudden-oak-death (Rizzo et al. 2002). An other example of migration for *Phytophthora* trought infected potato tubers 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.2.1.5 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 populations, which can easily reach genetic uniformity. In addition 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 (Mammella 2010). This condition will change the allele frequencies of specific genes in the next generation.

I.2.2 Genetic structure of populations 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).

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 coevolution with native *Solanum* hosts (Grünwald and Frier, 2005); however, a new hypothesis based on a higher genetic diversity identified, assign the Andean region of Peru as center of origin of this pathogen (Gomez-Alpizar et al., 2007).

Unlike *P. infestans,* of which the population structure in different continents is known, for *P. ramorum* population studies are limited only to Europe and North America. The populations are characterized mainly by three distinct clonal lineages EU1, NA1, NA2 (Ivors et al., 2004, 2006; Grunwald et al., 2009). EU2 was later added by Poucke et al. (2012) as the fourth clonal lineage

when an epidemic of sudden larch death occurred in the UK. The first 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). Until 2011, EU1 was the only lineage identified in nurseries in Europe and comprises mainly A1 mating type isolates plus a few A2 mating type isolates discovered in Belgium (Werres and De Merlier, 2003) that were designated as EU1-A2 lineages (Vercauteren et al., 2010a). However, in 2011 a fourth lineage was detected from dying Japanese larch trees in Northern Ireland and Scotland which was designated EU4 (Poucke *et al.*, 2012). While the two clonal lineages NA1 and NA2 are exclusively present in the US, 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 forests 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). 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 (Grünwald et al., 2009). 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).

For *P. cinnamomi*, a high level of genetic diversity supported by RFLP analysis (Linde et al., 1999) and isozyme multilocus genotyping (Old et al., 1984) was only found among isolates from Papua

New Guinea, suggesting this region may within the center of origin of this species (Old et al., 1984; Linde et al., 1999). For Australian and South African populations of P. cinnamomi, low levels of heterozygosity, significant deviation from Hardy-Weinberg equilibrium of the loci tested, RAPD profiles and low genetic differences suggested that the populations in these regions consist of clonal lineages. 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 (Dobrowolsky et al., 2003). Loss of heterozygosity 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 evidences for sexual recombination have not been found (Dobrowolsky et al., 2003).

I.2.2.1 Characterisation of *Phytophthora* major Clade 2

Currently, the genus *Phytophthora* is organised in 10 major clades (Fig. I. 4). The topology of the relationships between clades is depicted according to Blair *et al*. (2008). *Phytophthora citrophthora* is residing in Clade 2 which has become one of the largest clades in the *Phytophthora* phylogeny with 25 species and informally designated taxa in total and 18 new species described since 1996 (Erwin and Ribeiro, 1996; Kroon *et al.*, 2012; Ginetti *et al.*, 2014; Henricot *et al.*, 2014; Man In' t Veld *et al.*, 2014). Isolates of *P. mengei*, *P. capensis, P. elongata, P. multivora* and *P. plurivora* were previously identified as *P. citricola*. However, phylogenetic re-assessments showed that they constituted distinct subgroups within the so-called *P. citricola* complex leading to the description of multiple new species (Kroon *et al.*, 2012). One group of isolates was pathogenic on avocado trees, where they infected feeder roots and trunks. The pathogen responsible for this disease is now named *P. mengei.* It can be classified in clade 2 based on sequence homology with

P. capsici and *P. tropicalis,* but its morphological traits place it just outside clade 2b (Hong *et al.,* 2009). *Phytophthora multivora* was found in the rhizosphere of declining *Eucalyptus* trees in Australia. (Scott *et al.*, 2009). In Europe, large-scale surveys for soilborne *Phytophthora* species were conducted in more than a thousand forests, nurseries, and semi-natural stands showing devastating declines and diebacks of major forest tree species (Jung and Burgess, 2009). Based on morphological and physiological characters and the similarity of ITS DNA sequences, one of the major species involved in the declines was routinely identified as *P. citricola.* In a more detailed phylogenetic characterization based on additional DNA sequence data, it was however concluded that this species was similar to but distinct from *P. citricola*, and was named as *P. plurivora* (Jung and Burgess, 2009). *Phytophthora capensis*, a species from South Africa, has also recently been separated from *P. citricola* sensu stricto *(*Bezuidenhout *et al.*, 2010). The same study also described isolates of a related new taxon and provisionally named it as *P*. taxon *emzansi*, which has not yet been formally described as a new species. *P.* taxon *emzansi* can be distinguished from other members of the *P. citricola* complex by the production of amphigynous antheridia. *Phytophthora pini* was long considered to be a synonym of *P. citricola* but has recently been shown to be a valid separate species (Hong *et al.*, 2011). Two other new species from the *P. citricola* complex have recently been described. *Phytophthora acerina* causes bleeding stem cankers and mortality of mature *Acer pseudoplatanus* trees in Northern Italy (Ginetti *et al.*, 2014) whereas *P. pachypleura* is the cause of root rot of *Aucuba japonica* and multiple other ornamental species in the UK (Henricot *et al.*, 2014). Another new species in Clade 2 , *P. bisheria*, causes root rot on strawberry in the United States, roses in The Netherlands, and raspberry in Australia (Abad *et al*., 2008). It is related to several other new species in this clade including *P. multivesiculata (*Ilieva *et al.*,1998) and *P. elongata (*Rea *et al.*, 2010).

Soil and water stream monitoring studies revealed the presence of another homothallic Clade 2 species in Oregon named as *P. siskiyounensis. (*Reeser *et al.*, 2007). Another species, related to *P. citrophthora*, but separated from the latter species by its homotallic instead of a heterotallic mating

system, was found in Nepal. *Phytophthora himalsilva* was isolated from soil of natural forests by baiting but its host range is still unknown (Vettraino *et al.*, 2011). Recently, two other new homothallic species, P. occultans and P. terminalis, were described from nurseries in The Netherlands which like *P. himalsilva* are close relatives of *P. citrophthora* (Man In' t Veld et al., 2014). In Clade 2, *P. tropicalis* is a species that is pathogenic on several host including macadamia trees. Isolates of this species were initially described as *P. capsici*, but based on more detailed morphological analysis they were reclassified as *P. tropicalis* (Aragaki and Uchida, 2001) which is also supported by molecular data (Kroon *et al.*, 2012; Martin *et al.*, 2014).

Species in Clade 2 all have papillate or semipapillate sporangia, and the majority (19 out of 25) are homothallic. Although some morphological characters are specific to individual *Phytophthora* species, most characters have wide variations that overlap between species producing problems for classical identification of species. This underscores the importance of cataloging the genetic and phenotypic diversity of pathogens (Kang *et al.*, 2002). Variation among isolates, first indicated by morphological characters, is now confirmed by more recent studies of molecular techniques that have greatly enhanced species separation and identification (Chen *et al.*, 1992, Wang and White, 1997; Matsumoto *et al.*, 1999; Martin, 2000; Lévesque and de Cock, 2004; Kageyama *et al.*, 2005). In particular, the non-coding internal transcribed spacer (ITS) region evolves comparatively quickly and can, therefore, be used for phylogenetic analysis of closely related species and genera (Chen *et al.*, 1992, Wang and White, 1997; Kageyama *et al.*, 2005).

I. 3 Markers for population studies

Molecular markers have been extensively used 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 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).

As already discussed, currently the most common region of DNA being used for identification of oomycetes to the species level is the internal transcribed spacer (ITS) region of rDNA. The ITS region in oomycetes is easy to amplify for DNA sequencing in most species with the use of universal eukaryotic PCR (polymerase chain reaction) primers (White *et al.*, 1990; Ristaino *et al.*, 1998). Cooke *et al.* (2000) were the first to publish a database of ITS sequences that covered all the known and available species of an oomycete genus. ITS then became the de facto DNA barcode for identification of *Phytophthora* species and similar comprehensive databases for *Pythium* (Lévesque & de Cock, 2004) and downy mildews (Voglmayr, 2003) followed.

I. 3.1 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 due to its easy application and interpretation of results and also for its low costs. 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. 3.2 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 costs of reagents and facilities needed for the analyses and the more complex interpretation of data.

I.3.3 Mitochondrial markers

Mitochondrial DNA (mtDNA) 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) the particular oxidative mitochondrial environment and fast turnover within cell lineages; iv) mitochondrial DNA is not protected by histones which leads to lower functional constraints.

Mitochondrial markers have been widely utilized also to study phytopathogenic fungi and oomycetes. They were applied to assess intraspecific variability of 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) Furthermore, intraspecific mtDNA 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 the mitochondrial genome of 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 cannot 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 (1 mitochondrial locus vs 4 nuclear alleles in diploid organisms). 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).

The use of DNA for oomycete species identification is well established, but DNA barcoding with cytochrome c oxidase subunit I (COI) is a relatively new approach that has yet to be assessed over a significant sample of oomycete genera. COI is a mitochondrially encoded gene which is recognized as an extremely useful DNA barcode capable of accurate species identification in a very broad range of eukaryotic life forms (Hebert et al. 2004; Ward et al. 2005; Hajibabaei et al. 2006; Seifert et al. 2007). In a recent study of Robideau *et al.* 2011 they have sequenced COI, from 1205 isolates representing 23 genera. A comparison to internal transcribed spacer (ITS) sequences from the same isolates showed that COI identification is a practical option; complementary because it uses the mitochondrial genome instead of nuclear DNA. In some cases COI was more discriminative than ITS at the species level. This is in contrast to the large ribosomal subunit, which showed poor

species resolution when sequenced from a subset of the isolates used in the study. The results described in Robideau et al. (2011) indicate that COI sequencing and the dataset generated are a valuable addition to the currently available oomycete taxonomy resources. In fact, both COI, the default DNA barcode supported by GenBank, and ITS, the de facto barcode accepted by the oomycete and mycology community, are acceptable and complementary DNA barcodes to be used for identification of oomycetes.

COI has proven useful in phylogenetic studies of the oomycete genus *Phytophthora* (Martin & Tooley 2003; Kroon et al. 2004), and the success of COI barcoding in red algae (Saunders 2005) made it a very intriguing prospect for barcoding of all oomycetes due to their algal ancestry. Because COI is a protein-coding region, alignment of COI sequences is simple and devoid of gaps if introns are absent.

I.3.4 Nuclear markers

The mitochondrial DNA constitutes in some respects the marker of choice for phylogeographic analyses. However nuclear markers are necessary to follow the evolutionary processes, genetic structure and genealogy in species and populations that are at least partially sexually recombining.

I.3.4.1 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. These include difficulties during sequencing of PCR products, insertion and deletion polymorphisms, heterozygosity, recombination, selection and others. 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 is 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 methods assigns probabilities of haplotypes to individual sites within a specific haplotype (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, haplotype inference performed well considering the evidence of recombination and homoplasy (Hurrigan et al., 2008). Another program for haplotype determination based on a Bayesian approach is HAPLOTYPER (Niu et al., 2002).

I.3.5 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 of 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 labor-intense and expensive. 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 enzymes, 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.3.6 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 commonly used and also preferred to mitochondrial markers (Zhang and Hewitt, 2003). These markers are widely distributed in the genome in coding and non-coding

regions, are easily amplifiable by PCR and give a high level of allele polymorphisms due to high mutation rates in the magnitude of 10^{-4}. Although SSRs are widely used, little is known about microsatellite modes of evolution. Understanding the evolutionary mechanism of microsatellites is needed not only for realizing how the genome is organized, but also to better interpret and correctly use microsatellite 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 are evidences of structural and functional constraints. The hypothesis that microsatellites are neutral markers is supported by the fact that different loci were 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 noted that a different number of motif repetitions can be involved in differential expression of the nuclear promoter gene (*Ncl*) in rats (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 suitability 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 a study of *P. cinnamomi* SSRs were useful to detect the presence of three different clonal lineages in Australia (Dobrowolski et al., 2003).

One of the major drawbacks for the use of microsatellites is the high costs 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.3.7 Single Nucleotide Polymorphisms (SNPs)

The rapid growth of available data from genome projects has increasingly justified the use of SNPs as genetic markers in evolutionary studies of populations. Initially, SNPs were used in whole-genome linkage and association studies, but their frequent 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). Since genomic data are not always available for a species of interest, different approaches need to be considered to discover SNPs. Comparative genomic approaches can be used to design primers 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 set of isolates that include enough genetic variability. The use of a representative number 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 allele-specific oligonucleotide real time PCR can be used to reduce the costs of the analyses. For example, an allele-specific oligonucleotide (ASO) method was used to differentiate the European from the North American lineage of *P. ramorum* (Bilodeau et al., 2007).

SNPs are easier to screen than microsatellites and their widespread occurrence 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 lower mutation rates (10^{-8} -10^{-9}) than microsatellites, but because multiple mutation events are highly improbable to occur at the same site, most SNPs are bi-allelic and therefore appropriate for high-throughput genotyping (Brumfield et al., 2003).

I.3.8 Genetic characterization of *Phytophthora* species by the analysis of polymorphic regions of the mitochondrial DNA

Although some morphological characters are specific to individual *Phytophthora* species, most characters have wide variations that overlap between species producing problems for classical identification of species (Kang et al., 2002). Recently, population studies in 51 isolates of both A1 and A2 mating type of the heterothallic cosmopolitan plant pathogen *P. nicotianae* recovered from different hosts and geographic region have focused on the analysis of mitochondrial intergenic regions with an intraspecific variation in DNA sequences. It was demonstrated that specific primers designed in trnY–rns region are useful for the examination of intraspecific variation (Schena and Cooke, 2006; Mammella et al. 2011). In particular, two regions flanked by genes *trnY* and *rns* and *trnW* and *cox2* were identified by comparing the whole mitochondrial genome of *Phytophthora infestans*, *Phytophthora ramorum,* and *Phytopthora sojae* and amplified using primers designed from the flanking conserved genes.

Similarly, a panel of nuclear regions characterized by high intraspecific variation in different *Phytophthora* species was reported by Schena *et al.* (2008). This study was based on SSR markers, a Simple Sequence Repeats motif also known as microsatellites, from *P. infestans*, *P. sojae* and *P. ramorum* to analyze and identify useful loci, common to many *Phytophthora* species. Microsatellites have been recognized as one of the most efficient methods for population genetic studies; they are tandemly repeated motifs made of around six bases that occur frequently and randomly in all eukaryotic genomes although their frequency varies significantly among different organism (Selkoe et al., 2006). Schena et al. (2008) designed a total number of 62 different degenerate primers to investigate target regions in a panel of 15 species representative of the diversity within the genus *Phytophthora*.

I.4 Biology and genetics of *Phytophthora citrophthora*

The species now known as *Phytophthora citrophthora* was first described by Smith and Smith (1906) from *Citrus* in California. They assigned it to a new genus, *Pythiacystis*, as *Pythiacystis citrophthora*; Leonian (1925) recognized the similarity between *Pythiacystis* and *Phytophthora* and proposed the current name *Phytophthora citrophthora*.

Phytophthora citrophthora is classified in subclade a of Clade 2 (Kroon *el al*. 2012). This clade has become one of the most large in the *Phytopthora* phylogeny with 25 species in total (Table I.1).

Phytophthora citrophthora is morphologically primarily characterised by highly variable, non-caducous and mostly papillate sporangia (Erwin and Ribeiro, 1996). Although sporangia are described by Stamps *et al.* (1991) to be caducous in some isolates but not in others, Mchau and Coffey (1994) reported that all 77 isolates examined produced persistent sporangia. The shapes are variable, ranging from spherical, ovoid, obpyriform, obturbinate and ellipsoidal to highly distorted often bi- or tri-lobed shapes with more than one papilla (average 45 x 30 µm). Sporangia are occasionally laterally attached and some are intercalary (Fig. I.5). Sporangiophores are either

unbranched or irregularly branched, forming a loose sympodium with a swelling at branching node (Erwin and Ribeiro, 1996).

Colonies are dense cotton-like, uniform, rosaceous or stellate on clarified V8-agar (V8A) at 24°(Figure I.6). The minimum temperature for growth is 5°C, the optimum 24-28°C, and the maximum 32-33°C. Chlamydospores are only infrequently formed in some isolates and do usually not occur on citrus isolates. Mchau and Coffey (1994b) reported chlamydospores only on isolates from cacao in Brazil. Chlamydospores were 10^{-3} µm in diameter and hyaline and were produced both single and in clusters. In general, it has been assumed that the oospore stage is not a part of the life history of *P. citrophthora*. Sex organs do not occur in nature on citrus. Oospores have been shown to form when certain *P. citrophthora* isolates are paired on carrot agar. Four isolates from cocoa black pod in Brazil formed oospores when paired with an A2 isolate from citrus (P318 from Australia), and one cocoa isolate formed oospores when paired with P717 (A1), a *P. citrophthora* isolate (host unknown) from New Zealand. None of the cocoa isolates produced oospores when crossed among themselves, however, although some belonged to the A1 and another one to the A2 mating type. Kellam and Zentmyer (1986) concluded from these data that the compatibility or mating system was probably more complex than the usual A1/A2 system in *Phytophthora.*

Mchau and Coffey (1994b) reported that less than 30% of the 77 *P. citrophthora* isolates studied produced oospores when paired with an A2 mating type of *P. capsici.* By use of the polycarbonate membrane method of Ko (1978; see Chapter 3), *P. citrophthora* isolates acted only as inducers of oospores in the *P. capsici* partners (known as silent A1 mating type). Although oospores from pairing A1 and A2 isolates of *P. citrophthora* were reported by Savage et al. (1968), no evidence of oospores from pairing of *P. citrophthora* isolates was found. When isolates are paired with A1 or A2 mating types of other species, antheridia are amphigynous and oogonia are 26 to 36 µm in diameter and contain oospores 21 to 28 µm in diameter.

An isozyme pattern study of 32 isolates of *P. citrophthora* from various hosts including citrus, cocoa, *Actinidia deliciosa*, rubber, walnut, strawberry, pistachio, *Ficus elastica, Prunus domestica, Amygdalus communis, Ribes sanguineum,* and *Coptus japonica*, indicated that *P. citrophthora* was genetically related to *P. capsici,* and that amongst the *P. citrophthora* isolates studied two subgroups, CITR1 and CITR2, could be defined. Isolates from citrus resided in CITR1 and those from cocoa were assigned to CITR2 (Oudemans and Coffey 1991a). By use of polymerase chain reaction amplification of ITS rDNA, Lee and Taylor (1992) showed a close relationship between cocoa isolates of P. capsici and P. citrophthora. Using isozyme analysis, Mchau and Coffey (1994b) could differentiate three subgroups of *P. citrophthora* with a third subgroup, CTR3, which was distinct from *P. citrophthora* sensu stricto. Subgroup CITR1 contained isolates from a broad range of hosts, including citrus from various geographical areas. Subgroup CITR2 was composed exclusively of isolates from cacao grown in Brazil. Subgroup CITR3 included isolates from cacao grown in Indonesia. Isozyme and protein electrophoretic analysis have provided a bounty of information on genetic variability within and among *Phytophthora* species (Erselius and de Vallavieille, 1984; Old et al., 1984; Tooley et al., 1985, 1989; Hansen et al., 1986; Bielenin et al., 1988; Nygaard et al., 1989; Spielman et al., 1990; Oudemans and Coffey, 1991a, 1991b, 199lc).

Table I.1 - *Phytophthora* species in Clade 2 (adapted from Kroon *et al.*, 2012 and updated using data from Ginetti *et al.*, 2014; Henricot *et al.*, 2014 and Man In't Veld *et al.*, 2015)

Clade2 Phytophthora species							
Phytophthora sp	clade	host	infected issue	Sex	A/P	papillation	Source
P. bisheria	2	Multiple	Root	Ho	P	SP	Z.G.Abad et al., 2008
P. capensis	2	Multiple	Root	Ho	P	SP	Bezuidenhout et al., 2010
P. citricola	2	Multiple	Foliage/fruits/root	Ho	P	SP	Sawada 1927
P. elongata	2	Multiple	Root	Ho	P	SP	Rea et al., 2010
P. taxons emzansi	2	Unknow	Soil	Ho	A	SP	Bezuidenhout et al., 2010
P. frigida	2	*Eucalyptus smithii*	Root	He	A	P	Maseko at al., 2007
P. mengei	2	*Persea amiericana*	Root	Ho	P	SP	G.T.Browne et al., 2009
P. multivesiculata	2	*Cymbidium*	Foliage	Ho	A	NP/SP	IIeva et al., 2008
P. multivora	2	Multiple	Rhizosphere/foliage	Ho	P	SP	P.M. Scott & T. Jung, 2009
P. pini	2	Multiple	Root	Ho	P	SP	Leonian, 1925
P. plurivora	2	Multiple	Rhizosphere/foliage	Ho	P	SP	T. Jung & T.I. Burgess, 2009
P. siskiyouensis	2	*Umbellularia californica*	Rhizosphere	Ho	P	SP	Reeser & E.M. Hansen, 2008
P. botryosa	2a	*Hevea brasiliniensis*	Foliage	He	A	P	Chee ,1969
P. citrophthora	2a	Many hosts	Foliage/fruits/roots	He	A	P	(R.E. Smith and E.H. Smith) Leonian, 1925
P. colocasiae	2a	*Colocasia esculenta*	Foliage	He	A	SP	Raciborski, 1900
P. himalsilva	2a	Unknow	Soil	Ho	A/P	P	Vettraino, Brasier and Vannini, 2011
P. inflata	2a	*Hulmus americana*	Foliage	Ho	P	SP	Caroselli & Tucker, 1949
P. meadii	2a	*Hevea brasiliniensis*	Fruits/foliage	Ho	A	P	McRae, 1918
P. terminalis	2a	*Pachysandra terminalis*	Roots/stem base	Ho	A/P	P	Man In' t Veld et al. 2015
P. occultans	2a	*Buxus sempervirens*	Roots/stem base	Ho	A	P	Man In' t Veld et al. 2015
P. capsici	2b	Multiple	Foliage/fruits/roots	He	A	P	Leonian, 1922
P. glovera	2b	*Nicotiana* spp.	Root	Ho	A	SP	Z.G Abad & Shew, 2011
P. tropicalis	2b	Multiple	Foliage	He	A	P	Aragaki & J. Y. Uchida, 2001
P. acerina	2c	*Acer pseudoplatanus*	Roots/stem bark	Ho	P	SP	Ginetti et al. 2014
P. pachypleura	2c	Unknow	Roots/stem base	Ho	P(A)	SP	Henricot et al. 2014

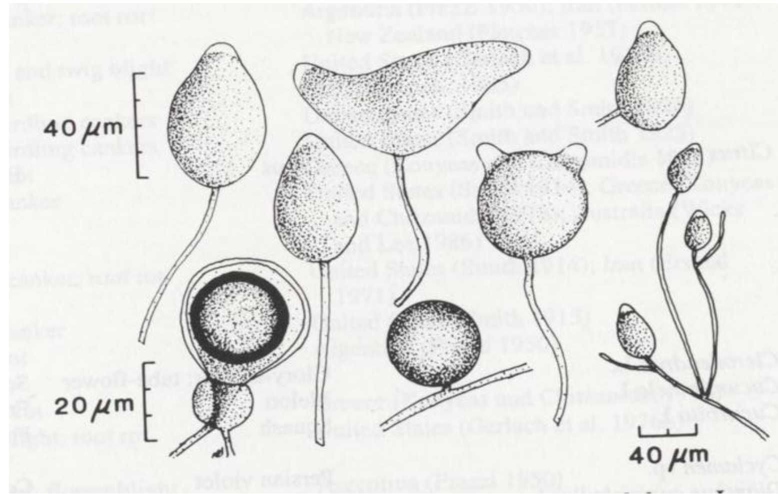

Fig. I.4 - Sporangia of *P. citrophthora* (Erwin and Ribeiro, 1996).

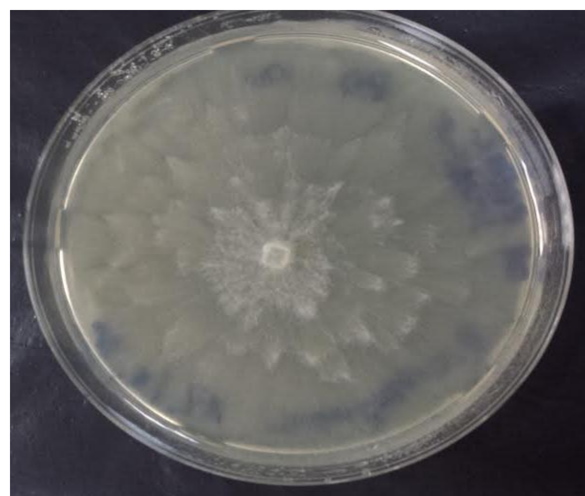

Fig. I.5 - Colony of *P. citrophthora* on clarified V8-agar.

I.5. Diseases caused by *Phytophthora citrophthora*

Phytophthora citrophthora is a serious pathogen of citrus worldwide, causing brown fruit rot, trunk gummosis, collar and root rot (Erwin and Ribeiro, 1996; Maseko and Coutinho, 2002; Dirac et al., 2003; Schutte and Botha, 2010; Alvarez et al., 2011), and leaf and shoot blight; symptoms vary with the host and the growing conditions. *Phytophthora citrophthora* is also reported to cause damping-off on nursery seedlings of a wide range of plant species including citrus, tomato and conifers (CMI, 1964).

On citrus, symptoms of foot (=crown) rot often begin near the soil line; dark water-soaked areas form in the bark and a sour smell may occur in wet conditions. Gum exudes from the affected parts, often copiously, and is particularly noticeable in dry weather. If the diseased bark is scraped off, brown necrotic areas can be see extending to the cambium and wood. Infections usually extend vertically more than laterally and may spread along the roots and stem for 50 cm or more. The bark remains firm but as it dries out it cracks and is shed leaving bare, dead areas. When the tree is partially girdled, decline starts. There may be a general appearance of unthriftiness in the whole tree, with the leaves turning pale, the foliage becoming sparse and the fruits small, but often the tree dies unevenly. Leaf infection itself is usually more serious in the lower part of the canopy, appearing first as spots which become necrotic. Young shoots are also attacked resulting in clusters of dead leaves. Defoliation can be severe. Small dark spots develop on fruits close to the ground which increase in size and turn in colour to various shades of greenish-brown. A soft rot (brown rot) eventually develops with a characteristic pungent smell. White mycelial growth can occur on the surface of fruits in storage (Fawcett, 1936; Holliday, 1995).

First reported in the Azores in 1834, foot rot quickly spread to Portugal (1845) and other Mediterranean countries. By 1863 it was destroying thousands of citrus trees in Italy and Sicily and had appeared as far away as Australia. Between 1834 and 1914 it had spread to virtually every citrus-producing region in the world. The study of plant diseases was still in its advent but it made

considerable progress by the time foot rot was ravaging the citrus orchards of the Mediterranean region. Several fungi and bacteria were erronously made responsible before in 1913 *Phytophthora citrophthora* was identified as the real cause of the disease. Two other species of *Phytophthora*, *P. parasitica* and *P. palmivora*, were later isolated from similar infections of citrus trees (Klotz and Child, 1953).

Phytophthora citrophthora is a soil-borne pathogen and, in fact, the soil is an important source of inoculum for infections. The inoculum density varies between seasons and appears to be related to soil temperature and soil water matric potential (Lio et al., 1988; Ippolito et al., 1992). The levels of inoculum can significantly increase following irrigation (Lio et al., 1990). Soils which are easily waterlogged predispose roots to infection and also cause a higher frequencies of fruit infection.

Phytophthora citrophthora is frequently recovered from effluent-collecting ponds in nurseries that practice the capture and recirculation of irrigation run-off water (MacDonald et al., 1994). Commercial citrus varieties vary considerably in their susceptibility to the pathogen, ranging from extreme susceptibility in lemons to marked resistance in Sampson tangelo and citrange (Broadbent et al., 1971). *Poncirus trifoliata* exhibits near immunity (Waterhouse and Waterston, 1964) and has been much used to serve as resistant rootstocks.

I.5.1 Major disease of Citrus caused by *Phytophthora citrophthora*

I.5.1.1 Brown rot of citrus fruits

Diagnosis, development, and control of fruit diseases, including brown rot, are discussed by Eckert and Brown (1986) and Whiteside *et al.* (1988). In the field, infection often starts on the bottom end of the fruit because inoculum in infested soil is splashed by rainfall from the ground onto the fruit. Several infected areas, which may develop on a single fruit, coalesce and eventually cover the whole fruit (Fig. I.6). The necrotic tissue is firm and leathery, even in advanced stages. After becoming mummified, fruits drop and *Phytophthora* can be isolated from the infected seeds.

During wet weather conditions, a white mycelium often appears on the surface of the diseased fruit. The characteristic pungent, rancid odor that emanates from rotted fruit tissue readily distinguishes this disease from fruit rots caused by other pathogens (Eckert and Brown, 1986). This is particularly obvious in storage facilities where picked fruit with incipient infections rot rapidly.

In areas of high rainfall, dark water-soaked areas develop on leaves, particularly at the tips and along the margins, and leaf tips die back. Severely affected leaves become black at advanced stages of disease and drop prematurely. Affected orange and mandarin trees can be completely defoliated. In Florida, United States, *P. nicotianae (*also known as *P. parasitica)* is more commonly found in soil and on roots than *P. citrophthora;* however *P. citrophthora* has a greater capability to cause brown rot on fruits than *P. nicotianae* (Whiteside 1970).

Fruit loss caused by brown rot is directly related to the total amount and frequency of rainfall. The susceptibility of fruits increases with maturity. Fruits that are infected prior to harvest may not show any external symptoms, but after the fruit is boxed for shipment the disease can develop and spread to healthy fruits. Approximately 3 days after harvest, spots develop on fruits stored at 25 °C. At 10° C the incubation period is ca 10 days. Under favorable conditions the entire fruit becomes brown within approximately 7 days (Klotz and Dewolfe, 1961).

Fruits infected with *Phytophthora* are readily colonized by *Penicillium italicum, P. digitalis* or *Geotrichum candidum,* all of which are wound pathogens (Eckert and Brown 1986). When orange or lemon fruits were maintained in packing boxes a 5°C, spread of disease did not occur, but at 10 °C and 14 °C *P. citrophthora* spread rapidly to cause fruit rot after 12 and 13 days, respectively.

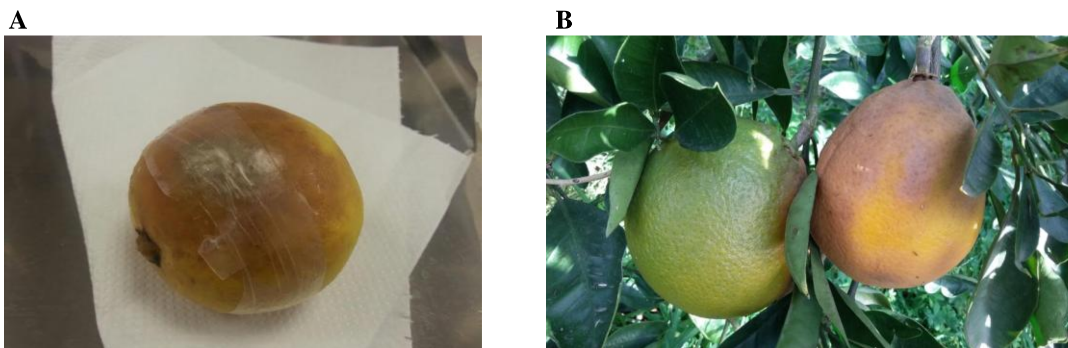

Fig. I.6 - A-B : Brown rot of citrus fruit.

I.5.1.2 Gummosis, Foot Rot, and Fibrous Root Rot

Citrus gummosis was first described in the Azores in 1834 (Fawcett, 1925). Foot rot is similar, except the lesion is above the bud union (Timmer et al., 1993). Fibrous root rot involves the small feeder roots that are produced in the upper soil layer. These diseases occur in every citrus-growing area worldwide and were most likely spread on infected rootstocks. *Phytophthora citrophthora* and *P. parasitica* (*P. nicotianae*) are both causal agents of gummosis and root rot, but in Mediterranenan type climates where rain falls in winter and summers are dry, *P. citrophthora* is the most important cause of gummosis and root rot because it is mainly active during the mild winter months (Fig. I.7). The types of lesions on the trunks of citrus trees caused by *P. citrophthora* and *P. parasitica* vary in appearance among cultivars (Klotz 1973). Fibrous root rot symptoms caused by *P. citrophthora* or *P. parasitica* include a brown discoloration and dieback of feeder roots. Distinct 'frog-eye' lesions are obvious on highly susceptible rootstocks (Klotz and Calavan, 1978). Fibrous root rot leads to a slow decline of trees; in some cases trees survive and bear fruit despite of the infection. Root rot is distinguished from gummosis by the lack of gum exsudation and the dusky to black discoloration of the wood below the bark (Klotz et al. 1967). Fibrous root rot can also be caused by anaerobic conditions in soil resulting from excess soil water or salinity (Lutz and Menge, 1986; Timmer et al., 1993).

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 used, hypoxia resulting from water saturation of the soil increases the susceptibility of roots 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 status 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 which is when the first spring leaf flush has reached about three-quarters of its maximum development (Cacciola and Magnano di San Lio, 2008). Treatment with systemic products can only be justified if soil inoculum density reaches 10-15 propagules/cm^3 because of 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 particularly susceptible to this disease.

Fig. I.7 - Gummosis on stem *Citrus sinensis.*

Chapter II: Material and Methods

II.1 Isolation of *Phytopthora citrophthora* from soil samples

Soil was sampled from diseased plants that were declining but not dying. First, the organic litter layer was removed. Two or three soil monoliths were taken from the rhizosphere of each tree. Rhizosphere soil containing fine roots was taken from all monoliths and bulked to a mixed sample of ca 1-3 L. The soil was mixed thoroughly before baiting. In case that the soil sample was dry it was moistened and incubated for 2-3 days at 18-20 °C before baiting. Then, the soil was flooded with distilled water keeping the distance between the surface of the soil and the waterline at around 3-4 cm. All the litter and debris floating on the surface of the water was collected and concentrated to the margin. The remaining debris was removed with a sieve and then with paper tissue. After 2 hours of sedimentation of the soil, the baiting leaves were floated on the water and incubated at 18-20 °C. The leaves used in this work were *Ceratonia siliqua, Robinia pseudoacacia, Citrus limon* (Fig. II.1).

After 24-48 hours the leaves becoming blackish or showing dark spots were examined under the light microscope for the presence of sporangia. Then leaf lesions were cut into small (2-3 mm) segments, dipped on filter paper to remove excess water, and plated onto selective PARPNH - agar and incubated at 20 °C in the dark. The plated leaf segments were examined daily under the stereomicroscope for outgrowing *Phytophthora* and *Pythium* hyphae. Usually, *Phytophthora* grew from the plated leaf stripes after 24 to max 48 hours. The subcultured colonies were examined daily for infestation by bacteria or *Pythium* spp. All new cultures were regularly purified by using the pancake method. An agar piece from the growing edge of a new culture was put below the agar of a fresh V8A-plate (which is lifted using a sterilized spoon etc.). After 24-48 hours first hyphae appeared at the surface. Then a very shallow pieces of agar from the surface was transfered to V8A or carrot agar.

Fig. II.1 - Necrotic spots caused by *Phytophthora citrophthora on Ceratonia silique (A), Citrus limon(B) and Robinia pseudoacacia*

II. 2 Identification of isolates proviosonally referred to *P. citrophthora*

Phytophthora isolates were identified using descriptions in literature (Erwin and Ribeiro, 1996). The isolates were grown in a 9 cm petri- dish add sterile pea broth, (Bird's Eye Frozen Peas 125g/Ltr, boil for 1 hour, pour through muslin, adjust volume back to starting volume, adjust pH to 6.25, autoclave) after in a pea broth were added a plugs of actively growing isolate and incubated at room temperature. Using a sterile spatula (flamed) the mycelial mat was removed, excise the agar plug, blot mat on tissue. The mycelial was washed in sterile distilled water, twice- discarding washing solution each time and was transferred to labelled 1.5 ml micro tube. Tubes were saled with parafilm (perforated) and placed in freeze drier preferably over night till completely dry, then were stored at room temperature.

All isolates provisionally referred to *P. citrophthora*, on the basis of colony morphology petaloid in PDA, dense cottony like, or rosette, or stellate, or patternless on V8C agar at 24°, sporangium morphology (ovoid, papillate and persistent) as well as ITS sequences.

II. 3. *Phytophthora citrophthora* isolates

One hundred twenty-three isolates from different geographic regions and International culture collections mainly sourced from citrus were used in this study (Table II.1). Isolates were maintained on V8 agar at 16 °C for routine stock cultures.

Seventhy-five Italian isolates of *P. citrophthora* were compared with isolates collected in Algeria, Australia, China, Jordan, Morocco, New Zealand, Poland, Portugal, Spain, Taiwan and Vietnam (Table II. 1 and Fig. II. 2).

Table II.1 - Isolates of *Phytophthora* included in the study, their host, source, geographic origin, isolation date and collector. Italian isolates were sourced from the following Sicilian orchards Azienda Xirumi (Scordia, Catania), Azienda Serravalle (Mineo, Catania), Azienda Crisciunà (Regalbuto, Enna).

Isolate code	Host	Source	Location	Date	Collector
AX 1aC	*Citrus aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 1b R	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX1bC	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 1c L	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 1c C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 1aR	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 2a L	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 2bC	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 2dL	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX2dC	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX2eL	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 3a L	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 3b C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 3bL	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 3d L	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli

AX 3e L	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 3f L	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 4a C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 5aC	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 6aL	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 6a R	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 6a C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 6b R	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 7a C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 7b C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 7c C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX7d C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 8aC	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 8b C	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 9a R	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
AX 9b R	*C. aurantium*	Rhizosphere soil	Italy (Az. Xirumi)	April 2014	M. Evoli
CC 1a L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 1b L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 1cL	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 2aL	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 2bL	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 3a C	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 3a R	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 3b C	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 3b L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 3b R	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC4a L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC4c L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 4b L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli

CC 5b C	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 5b L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 6a C	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 6b L	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 7b C	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC 12a R	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC13A R	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC13b R	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
CC15A C	*C. aurantium*	Rhizosphere soil	Italy (Az.Crisciunà)	April 2014	M. Evoli
TS 1a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 2a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 2e	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 3a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 3b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 3c	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 4a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 4b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 4e	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 5a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 5b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 7a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 7b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 8a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 8b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS F9	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 10a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 10b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 12a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS 12b	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli

TS 13 a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
TS15a	*C. sinensis*	Rhizosphere soil	Italy (Az.Serravalle)	April 2014	M. Evoli
SCRP180	*C. grandis*	n.k.	New Zealand	n.k.	n.k.
SCRP 181	*C. aurantium*	n.k.	Australia	n.k.	n.k.
SCRP182	*Citrus* orchard	n.k.	Jordan	n.k.	n.k.
SCRP 183	*Hevea brasiliensis*	n.k.	China	n.k.	n.k.
SCRP 188	n.k.	n.k.	China	n.k.	n.k.
CH 201	*Citrus* spp.	Soil sample	Vietnam	2006	C. Olsson
CH 273	*C. sinensis*	Fruit	Vietnam	2006	C. Olsson
CH274	*C. sinensis*	Fruit	Vietnam	2006	C. Olsson
CH276	*C. paradise*	Fruit	Vietnam	2006	C. Olsson
CH277	*C. paradise*	Fruit	Vietnam	2006	C. Olsson
CH278	*C. paradise*	Fruit	Vietnam	2006	C. Olsson
CH282	*C. paradise*	Fruit	Vietnam	2006	C. Olsson
NED1	*C. sinensis*	Fruit	Mostaganem, (Algeria)	April 2015	Fatma Zohra
E1-4-14	*C. sinensis*	Fruit	Mostaganem (Algeria)	June2014	Fatma Zohra
E4-1-14	*C. clementina*	Fruit	Mostaganem (Algeria)	May2014	Fatma Zohra
TW 62		River water	Taiwan, Taipei City	March 2013	T. Jung
TW 132		River water	Taiwan, Hualien County	August 2013	T. Jung
TW 132 a		River water	Taiwan, Hualien County	August 2013	T. Jung
TW 132 d		River water	Taiwan, Hualien County	August 2013	T. Jung
TW 133		River water	Taiwan, Hualien County	August 2013	T. Jung
TW 161	*Styrax suberifolia*	Rhizosphere soil	Taiwan, Hualien County	August 2013	T. Jung
TW 165	*Cinnamomum camphora*	Rhizosphere soil	Taiwan, Hualien County	August 2013	T. Jung
TW 167	*C. camphora*	Rhizosphere soil	Taiwan, Hualien County	August 2013	T. Jung
TW 168	*C. camphora*	Rhizosphere soil	Taiwan, Hualien County	August 2013	T. Jung
TW 172	*C. camphora*	Rhizosphere soil	Taiwan, Hualien County	August 2013	T. Jung
TW 242	*Trema orientalis*	Rhizosphere soil	Taiwan, Taitung County	August 2013	T. Jung
TW 243	*T. orientalis*	Rhizosphere soil	Taiwan, Taitung County	August 2013	T. Jung
TW 244	*T. orientalis*	Rhizosphere soil	Taiwan, Taitung County	August 2013	T. Jung

TW 249	mixed broadleaved forest	Rhizosphere soil	Taiwan, Hualien County	August 2013	T. Jung
TW 343		River water	Taiwan, Miaoli County	September 2013	T. Jung
TW 369		River water	Taiwan, Taichung County	September 2013	T. Jung
TW 386	*Quercus tarokoensis*	Rhizosphere soil	Taiwan, Yilan County	September 2013	T. Jung
TW 387	*Q. tarokoensis*	Rhizosphere soil	Taiwan, Yilan County	September 2013	T. Jung
Rio 2011/12/1		River water	Portugal, Faro district	July 2011	T. Jung
Rio 2011/12/2		River water	Portugal, Faro district	July 2011	T. Jung
Rio 2011/12		River water	Portugal, Faro district	July 2011	T. Jung
Rio 2011/8		River water	Portugal, Faro district	July 2011	T. Jung
Rio 2011/8/1		River water	Portugal, Faro district	July 2011	T. Jung
Rio 2011/8/2		River water	Portugal, Faro district	July 2011	T. Jung
Rio 2011/3/2		River water	Portugal, Faro district	July 2011	T. Jung
927b suelo 1	n.k.	n.k.	Spain	n.k.	A. Perez-Sierra
927b suelo 2	n.k.	n.k.	Spain	n.k.	A. Perez-Sierra
Marrakech citrus 3	*Citrus limon*	Rhizosphere soil	Marrakech, Morocco	October 2012	T. Jung
Marrakech myrthus5/2	*Myrthus* sp.	Rhizosphere soil	Marrakech, Morocco	October 2012	T. Jung
P. citrophthora Pinus s.	*Pinus silvestris*	n.k.	Poland	n.k.	L. Orlikowski
P. citrophthora Abies alba	*Alba abies*	n.k.	Poland	n.k.	L. Orlikowski
P. citrophthora silver	n.k.	n.k.	Poland	n.k.	L. Orlikowski
P. citrophthora nursery	n.k.	n.k.	Poland	n.k.	L. Orlikowski

Fig. II.2 - Geographic origin of the isolates of *Phytophthora citrophthora* used in this study

II.4 DNA extraction

Genomic DNA was extracted using PowerPlant® Pro DNA isolation Kit. Mycelium was scraped from PDA plates and 50 mg were added to a bead tube along with a buffer PD1 Phenolic Separation Solution used for dissociates phenolic from the nucleic acids, supplied from a kit for rapid homogenization. Cell lysis and DNA release occured by both mechanical and chemical methods by a Retsch Mixer Mill MM301. Released genomic DNA was cleared by PCR inhibitors using IRT and then DNA was captured on a silica membrane in a spin column format. Finally, DNA was washed and eluted from the silica membrane using 50 µl of elution of PD7 composed of 10mM Tris, pH 8.0, with this low salt buffer and the neutral pH protects DNA during storage. DNA was stored at -20 °C until further analysis.

II.5 Analysis of the ITS region

The ITS1-5.8S-ITS2 Internal transcribed spacer (ITS) regions of ribosomal DNA (rDNA) were amplified using the universal primer pair ITS-6 (5'-GAAGGTGAAGTCGTAACAAGG-3')

and ITS-4 (5’- TCCTCCGCTTATTGATATGC-3’) (Cooke et al., 2000). PCR amplification was performed using 200 ng of genomic DNA as template. PCR consisted of 95°C for 5 min, 35cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 30 s and a final cycle of 72°C for 10 min. Amplicons were analyzed by electrophoresis, and sequenced in both directions by an external service (Macrogen). The Basic Local Alignment Search Tool (BLASTN) was used to compare sequences from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov).

II.6 Analysis of mitochondrial regions

To amplify different regions of mitochondrial DNA different primers, described in Schena and Cooke (2006) and Schena et al. (2008), were selected. The mitochondrial pairs of primers used were Mt15F-5R (Mt15F 5’-TTGCCAAGGTTAATGTTGAGG-3’ and Mt5R 5’-TTGCATGTGTTAAGCATACCG-3’) covering the whole region between TrnG and Rns genes (Table II.2 and Fig. II.3). Fragment length was approximately 500bp. The PCR reactions were performed as follows: in a total volume of 25µl containing 200 ng of genomic DNA, dNTP10mM 0.5µl, 2mM MgCl2, 5Xgo taq colourless Buffer 5µl, Go Taq® DNA polymerase 5U/µl 0.2µl (Go Taq® Flexi DNA Polymerase Promega Corporation) and 1 µM of primers. PCR amplification conditions consisted of: 1cycle of 95 °C for 2 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s; and a final cycle of 72 °C for 10 min. A successful amplification was confirmed by gel electrophoresis on 1% agarose gel and SYBR safe staining.

Table II.2 - Mitochondrial primers used in this study

Target DNA and amplified region	Primers	Sequences	Amplifications conditions
Mitochondrial DNA			
1 TrnG-Rns	Mt 15 F Mt 5R	TTGCCAAGGTTAATGTTGAGG TTGCATGTGTTAAGCATACCG	Annealing 58 °C 2mM MgCl
2 TrnG-TrnY	Mt 15 F Mt 3R	TTGCCAAGGTTAATGTTGAGG GGAGAAAGTAGGATTCGAACCT	Annealing 58 °C 2mM MgCl
3 TrnY-rns	Mt 2F Mt 5R	TGGCAGACTGTAAATTTGTTGAA TTGCATGTGTTAAGCATACCG	Annealing 58 °C 2mM MgCl

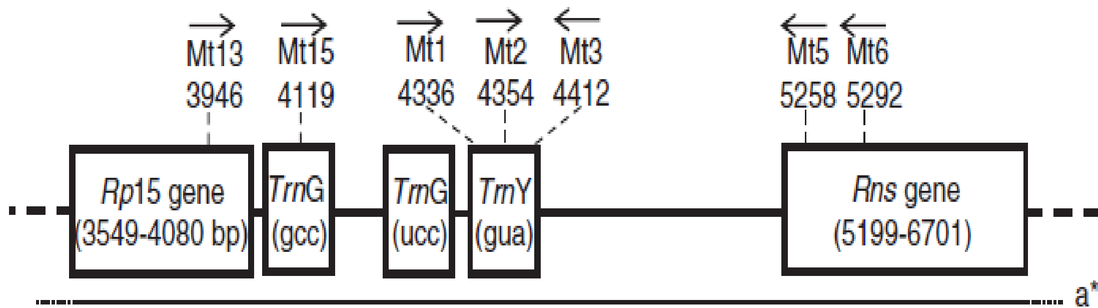

Fig. II.3 - Schematic representation of mitochondrial DNA regions examined in Schena and Cooke (2006).

II.7 Nuclear markers

To amplify different regions of nuclear DNA, SSRs were selected as markers from the study of Schena et al (2008) which were developed to study *Phytophtora* populations but were never tested for *Phytophthora citrophtora*.

The pair of SSRs markers tested in this study are listed in Table 4. Only I13F-I14R (I13F-5' GCCTGTGGAYGAGAATGGYS-3') and I14R (5-CAGATCCACGACACCRGGY-3'), produced a significant amplification with a sequence length of about 400 bp (Table 4).

PCR reactions were performed as follows: in a total volume of 15 µl containing 200 ng of genomic DNA, 5Xgo taq colourless Buffer 3µl, dNTP10mM 0.5µl, 1,7 mM MgCl2, Go Taq® DNA polymerase 5U/µl 0.2µl (Go Taq® Flexi DNA Polymerase Promega Corporation) and 1µM of primers. PCR amplification conditions consisted of: 1 cycle of 94 °C for 3 min; 40 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s; and a final cycle of 72 °C for 10 min. A successful amplification was confirmed by gel electrophoresis on 1% agarose gel and SYBR safe staining.

Table II.3 - SSR markers used in this study

Primers	Sequences	Amplifications conditions
S29F S30R	MGCAAGAAGGCGTCGTA CCTTCATCATGAGCTTCTGG	Annealing 58 °C 1.7mM MgCl
I13 F I14 R	GTCTGCGCTGTCGGAACT TRATGATGCGGTTCATCTCG	Annealing 58 °C 1.7mM MgCl
I5F I6R	CATCAACAAGTGCTCGTWCS TAGTCRAYGTTCTTGTTGTTCA	Annealing 58 °C 1 mM MgCl

II.8 Sequence analysis

All ITS, mitochondrial and nuclear DNA SSRs sequence chromatograms were analyzed. Sequences were aligned and checked for single nucleotide polymorphisms (SNPs) or indels using MEGA 6 tool, MUSCLE (http://www.ebi.ac.uk/Tools/msa/muscle/) and Multalin (http://multalin.toulouse.inra.fr/multalin/).

All polymorphic sites observed in the alignment were checked back in the chromatograms. Heterozygous SNPs identified in the nuclear coding regions by the presence of double peaks were marked with standard degeneracy codes (W = T/A; S= C/T; Y = C/T; M = A/C; R = A/G; K = G/T; N = any base).

Haplotypes were identified in mitochondrial sequences by aligning all hundred twenty-five isolates of sequences with ElimDupes (https://hcv.lanl.gov/content/sequence/ELIMDUPES/elimdupes.html).

II.9 Phylogenetic analyses based on ITS, mitochondrial and nuclear DNA SSRs sequences

To analyze the population structure with regard to geographic origin, host and matrix source of the isolates, phylogenetic analyses of mitochondrial regions were perfomed. Phylogenetic analysis was conducted using maximum likelihood and Tamura-Nei model. To achieve this, sequences were aligned and compared using the software MAFFT and MEGA6 (Hall, 2013). Phylogeny reconstruction was performed with MEGA6 (Hall, 2013) using Maximum Likelihood statistical method. Bootstrap values were obtained from 1000 repetitions.

Chapter III: Results and Discussion

III.1 Primers and PCR conditions optimization

Preliminary to the population genetic study of *P. citrophthora* suitable primer pairs were selected and PCR conditions optimized. This step was performed during the stay at The James Hutton Institute (UK) under the supervision of David Cooke and Leonardo Schena (Mediterranea University of Reggio Calabria, Italy). The mitochondrial region between TrnG and TrnY (TrnG–TrnY; (Fig.II.3) genes had been previously amplified and sequenced from 25 different *Phytophthora* species, including *P. citrophthora* (Schena and Cooke, 2006). In this study, Mt15F–Mt3R and Mt2F-Mt5R primer pairs were successfully used to amplify the regions between TrnG and TrnY (263 bp), and TrnY and Rns genes (~300 bp), respectively, comprising two mitochondrial regions. Therefore, to amplify the entire region from TrnG and Rns genes (~600 bp), Mt15F and Mt5R primer pair was chosen to study *P. citrophthora* population. PCR conditions were optimized as described in Materials and Methods section. The PCR reactions were performed assaying the $MgCl_2$ concentration (1.25 and 2 mM $MgCl_2$). As shown in figure III.1 A and B the best results in agarose gel was obtained using 2 mM $MgCl_2$ concentration.

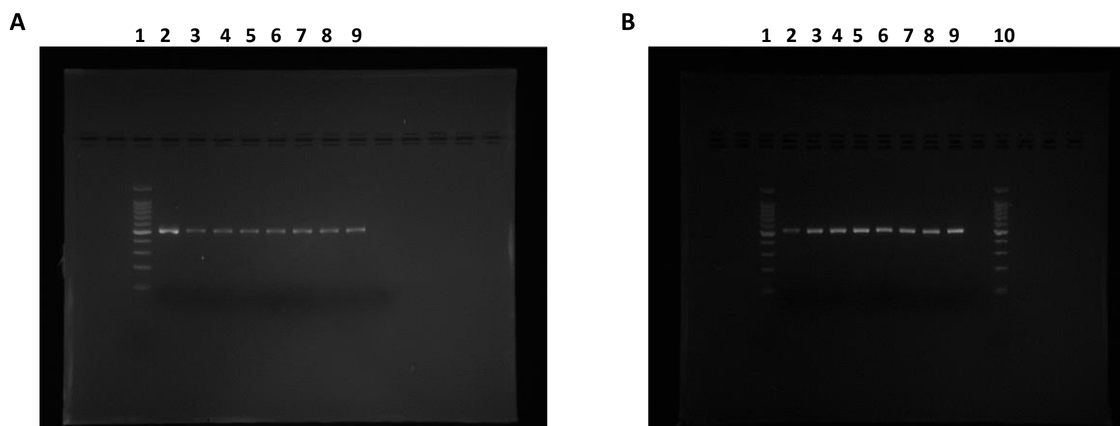

Fig. III.1 A-B - Electrophoresis in 1% agarose gel in TAE buffer stained with red gel. A- The amplification was performed with the primers Mt15F-Mt5R using 1.25 mM $MgCl_2$. Line 1: ladder 1kb; Line 2: AX 1aC, AX9bR, CH274,CH273, TS3a, CC2aL, TS15a, TS13a B- The amplification was performed with the primers Mt15F-Mt5R using 2 mM $MgCl_2$ Line 1: ladder 1kb; Line 2: AX 1aC, AX9bR, CH274,CH273, TS3a, CC2aL, TS15a, TS13a and ladder 1kb.

For the nuclear regions three pairs of SSRs marker were tested, S29F-S30R, I5F-I6R, I13F-I14R but only the last couple I13F-I14R produced a significant amplification with a sequence length of around 400 bp, whereas the other primers combinations produced complex profiles (Fig. III. 2). These two primers were selected among a total number of 62 different degenerate primers (12 for *P. infestans*, 18 for *P. ramorum*, and 32 for *P. sojae*) described in Schena et al. (2008). They were successfully used in Schena et al. (2008) to amplify fragments from isolates of *P. alni subsp. alni, P. cambivora, P. cinnamomi, P. citricola, P. europaea, P. fragariae* var. *rubi, P. ilicis, P. infestans, P. inundata, P. lateralis, P. nemorosa, P. pseudosyringae, P. psychrophila, P. quercina, P. ramorum* and *P. sojae*. In this study, the primer pair I13F-I14R were used to study the genetic variability of *P. citrophthora* for the first time. Moreover, amplification conditions for these primers were optimized. The PCR reactions were performed assaying the $MgCl_2$ concentration (0.7, 1 and 1,7 mM $MgCl_2$). As shown in figure III. 2 the best results in agarose gel was obtained using 1.7 mM $MgCl_2$ concentration.

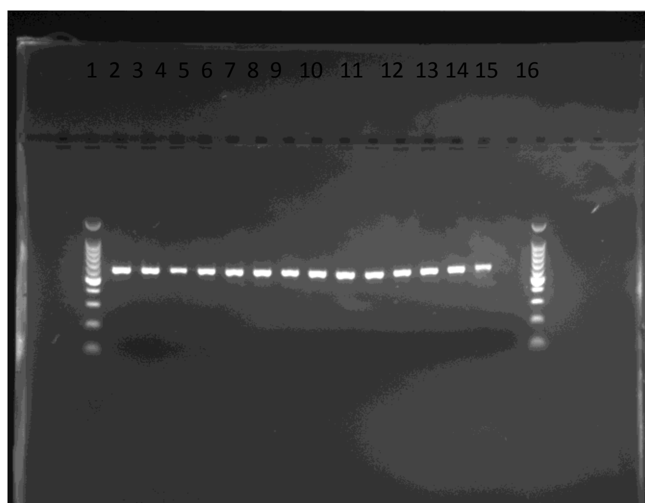

Fig. III.2 - Electrophoresis in 1% agarose gel in TAE buffer stained with red gel. The amplification was performed with the primers I13F-I14R using 1.7 mM $MgCl_2$. Line 1: ladder 1kb, AX 1aC, AX 6bL, AX9bR, CH274, CH273, TS3a, TS15a, TS13a, TS3c, CC2aL, CC3aC, CC6aC, CC13aR, CC13bR, ladder 1kb.

III. 2 Analysis of ITS sequences

One hundred twenty-three isolates of *Phytophthora* belonging to clade 2 from different hosts and geographic origins were identified by colony morphology and ITS sequences analysis. Alignment was performed using 700 bp fragments of ITS sequences. Seventy-five Italian isolates of *P. citrophthora* were compared with worldwide isolates sourced in Algeria, Australia, China, Jordan, Morocco, New Zealand, Poland, Portugal, Spain, Taiwan and Vietnam. All ITS sequences, except TW132a, TW132d, TW343 and TW369, ranged between 99 and 100% homology with other deposited reliable sequences of *P. citrophthora* reported in GenBank. Therefore, on the basis of ITS sequences all these isolates were preliminary identified as *P. citrophthora*. Whereras the remaining sequences of isolates TW132a, TW132d, TW343 and TW369 showed 100% homology with both *P.*

capsici and *P. tropicalis* and these isolates were considered as out-group. The analysis of ITS sequences with intraspecific diversity is summarized in Table III.5. Heterozygous SNPs were identified in the ITS regions by the presence of double peaks in both forward and reverse sequences and were marked with standard degeneracy codes. As also reported by Uddin et al. (2007), rDNA ITS sequences showed no consistent association with the geographic origin of isolates or host from which isolates were sourced. Alignment was performed using 700 bp fragments of ITS sequences. Accordingly, all 75 Italian isolates sourced from citrus soil, isolates CH273 and CH274 sourced from *C. sinensis* in Vietnam, isolates Rio2011-8-2 and Rio2011-8 sourced from river water in Portugal, isolates 927bsuelo1 and 927bsuelo2 from Spain, isolates Marrakech citrus 3 and Marrakech myrthus5/2 sourced from *Citrus limon* and *Myrthus* sp. in Morocco, isolates *P. citrophthora Pinus silvestris*, *P.citrophthora* silver, *P. citrophthora* nursery, and *P. citrophthora Abies alba* from Poland as well as isolates E-1-4-14, E-4-1-14 and NED1 sourced from citrus soil in Algeria showed identical ITS sequences (ITS Group 1). Interestingly, all these isolates were obtained in different years and from different sources, including river water (Table II.1). In contrast, the majority of the isolates from Vietnam (CH201, CH276, CH277, CH278 and CH282) that were all isolated from *C. paradisi* showed 6 variable nucleotides (Table III.1) in the same position and were included in ITS Group 2; this is the only case of a strict correlation between ITS grouping and host species. ITS Group 3 included isolates Rio2011-8-14, Rio2011-12-14, Rio2011-12-24 and Rio 2011-3-24 all recovered from river water in Portugal differing in 4 nucleotides with respect to ITS Group 1. Isolates from the collection of the James Hutton Institute (UK) formed four distinct ITS groups represented by isolate SCRP180 sourced from citrus in New Zealand (ITS Group 4), SCRP181 collected from citrus in Australia (ITS Group 5), isolate SCRP182 from *Citrus* sp. in Jordan and isolate SCRP183 from *Hevea* sp. in China (ITS Group 6), and isolate SCRP188 from China (ITS Group 7) differing by 2, 4, 2 and 6 nucleotides from ITS Group 1, respectively. ITS Group 8 was represented by isolates TW165 and TW242 from *Cinnamomum camphora* and *Trema orientalis*, respectively, sourced in Taiwan differing by 4 nucleotides from ITS Group 1. ITS Group

9 was constituted by TW243 and TW244 isolates from *Trema orientalis* differing by 5 nucleotides from ITS Group 1. The analysis of sequences of isolates of ITS Groups 8 and 9 are very close to *P. botryosa* and *P. meadii*. The last group (ITS Group 10) included only isolates from Taiwan, TW167, TW168 sourced from *Cinnamomum camphora,* and isolates TW133 from river water and TW249 from a mixed broad leaved forest. Interestingly, in ITS Groups 4, 6, 7, 9 and 10 different heterozygous SNPs were identified.

Furthermore, all ITS sequences of isolates analysed in this study were compared with the newly described species of Clade 2a, *P. occultans* and *P. terminalis* (Man in't Veld et al.; 2015), which are closely related to *P. citrophthora*. Effectively, alignment of sequences of *P. occultans* JX978155, *P. terminalis* JX978167, *P. citrophthora* JN618715 and AX1aC (Italian isolate from *C. aurantium* see Table II.1) showed as lower value of Percent Identity (http://www.ebi.ac.uk/Tools/services/rest/muscle) of 99.6 (data not shown). Therefore, on the basis of ITS sequences analysis (Table III.1). These results are in agreement with those reported in Eastern Corsica where a variability of ITS sequences of *P. citrophthora* population from citrus was also observed (Cohen et al., 2003).

Table III.1 - *Phytophthora citrophthora* ITS genotypes identified in the present study.

ITS Group	N° of duplicates	Duplicate sequenze	1	9	43	54	65	194	196	309	310	325	605
1	92	AX1aC[1]AX1bR[1], AX1bC[1], AX1cL[1], AX1cC[1], AX1aR[1], AX2aL[1], AX2bC[1], AX2dL[1],AX2dC[1], AX2eL[1],AX3aL[1], AX3bC[1], AX3bL[1], AX3dL[1], AX3eL[1], AX3fL[1] , AX4aC[1], AX5aC[1], AX6aL[1], AX6aR[1],AX6bR[1], AX6aC[1], AX7aC[1], AX7bC[1], AX7dC[1], AX8aC[1], AX8bC[1], AX9aR[1], AX9bR[1],AX1aL[1],CC2aL[1],CC3aC[1],CC3aR[1],CC2bL[1] CC3bC[1], CC3bL[1], CC3bR[1], CC4aL[1], CC4bL[1], CC4cL[1], CC5bC[1],CC5bL[1], CC6aC[1], CC1cL, CC6bL[1], CC7bC[1], CC12aR[1], CC13aR[1], CC13bR[1],CC15aC[1],TS1a[1], TS2a[1], TS2e[1], TS3a[1], TS3b[1], TS3C[1],TS4a[1], TS4b[1], TS4e[1],TS5b[1], TS7a[1], TS7b[1], TS8a[1], TS8b[1], TSf9[1], TS10a[1], TS5a[1], TS10b[1],TS12a[1], TS12b[1], TS13a[1], TS15a[1], TS15c[1], CH273[2],CH274[2],TW387[3], TW172[3], TW386[3], Rio2011-8-2[4], Rio2011-8[4], E1-4-14[5], E4-1-14[5], NED1[5], 927bsuelo1[6], 927bsuelo2[6],Marrakech myrthus5/2[11], Marrachech citrus3[11], P. citrophthora Pinus silvestris [12], P. citrophthora silver[12], P. citrophthora nursery[12], P. citrophthora Abies alba	G	C	C	C	A	C	C	A	T	A	G
2	5	CH201[2], CH276[2], CH277[2], CH278[2], CH282[2]	.	.	.	.	G	.	.	T	C	C	.
3	5	Rio2011-12[4], Rio2011-8-1[4], Rio2011-12-1[4], Rio2011-12-2[4], Rio 2011-3-2[4]	.	.	.	.	.	.	.	T	C	C	.
4	1	SCRP180[7]	.	A	.	.	.	Y	.	.	.	.	.
5	1	SCRP181[8]	.	A	.	.	.	.	.	T	C	C	.
6	2	SCRP182[9], SCRP 183[10]	.	.	Y	.	.	T	.	.	.	.	.
7	1	SCRP188[10]	.	A	.	.	R	Y	.	W	Y	M	.
8	2	TW165[3], TW242[3]	T	.	.	T	.	.	.	T	C	.	.
9	2	TW243[3], TW244[3]	.	.	.	Y	.	.	.	W	C	M	S
10	4	TW167[3], TW168[3], TW133[3], TW249[3]	K	.	.	T	.	.	Y	W	C	.	W

[1]isolates from Italy; [2]isolates from Vietnam; [3]isolates from Taiwan;[4]isolates from Portugal;[5]isolates from Algeria; [6]isolates from in Spain; [7]isolates from New Zealand; [8]isolates from Australia; [9]isolates from Jordan; [10]isolates from China; [11] Isolates from Morocco; [12]Isolates from Poland.

III. 3 Analysis of mitochondrial regions

Isolates of *P. citrophthora* were further characterized by sequencing the variable intergenic region of the mitochondrial DNA between TrnG and Rns genes, comprising two mitochondrial regions. Chromatograms of all sequences were analyzed and aligned showing a different level of variation within the whole amplicon. Alignment was performed using 500 bp fragments of mitocondrial sequences (Annex 2). Results of the alignment performed with ElimDupes to compare and align sequences as well as to identify or eliminate duplicates are reported in Table III.2. The analysis of the mitochondrial sequences allowed the identification of 6 different haplotypes (H); however, these haplotypes only partly overlapped with those identified by ITS sequencing. Interestingly, by comparing mitochondrial sequences it was possible to identify an intraspecific diversity in the group of Italian isolates that was not possible to highlight by ITS sequences analysis. The majority of Italian isolates were included in the same haplotype (H1) that also included isolates CH273 and CH274 sourced from *C. sinensis* in Vietnam. The other isolates from ITS Group 1 formed two distinct haplotypes, H2 and H3, respectively. In particular, an insertion indel of 8 nucleotides (ATTATTAA) was detected in the sequences of the following isolates of group H2: isolate CC1cL from Italy, all isolates from Algeria (E-1-4-14, E-4-1-14 and NED1 sourced from citrus soil), isolates Rio2011-12, Rio2011-12-1, Rio2011-12-2, Rio 2011-8-2¸ Rio2011-3-2, from river water in Portugal, isolate SCRP180 from New Zealand, isolate SCRP181 from Australia, isolate SCRP182 from Jordan and isolate SCRP183 from China. Interestingly, these isolates differed from haplotype 1 only for the insertion of this indel. In H3, in addition to the above-mentioned indel, other 5 variable nucleotides were detected in the whole sequences and included isolates CH276, CH277, CH278, CH282, CH201 from *C. paradisi* in Vietnam and SCRP188 from China (Tab. III.1). In H4, constituted by Rio2011-8 from river water in Portugal, 927bsuelo1, 927bsuelo2 from Spain, the two isolates Marrakech citrus3 and Marrakech myrthus5/2 sourced from *Citrus limon* and *Myrthus* sp., respectively, and the four isolates *P. citrophthora Pinus*

silvestris, *P.citrophthora* silver *P.citrophthora* nursery, *P. citrophthora Alba abies* from Poland, in addition to the same insertion indel of 8 nucleotides (ATTATTAA), 2 variable nucleotides were observed. The H5 corresponds to the ITS Group 8. The H6 is constituted by the rest of isolates from Taiwan. TW133 from river water, TW161 from *Styrax suberifolia*, TW167 and TW168 from *Cinnamomun camphora*, TW243, TW244 from Trema orientalis, TW249 from mixed broad-leaved forest and TW386 from *Quercus tarkoensis*.

Several haplotypes characterized by different indels and variable nucleotides were also reported in trnY/rns region in *P. nicotianae* by Mammella et al. (2011; 2013).

Table III.2 - *Phytophthora citrophthora* mitochondrial haplotypes identified in the present study

Haplotype	N° of duplicates	Duplicate sequenze	58	60	269	275	292	295	299	375	376	377	378	379	380	381	382
1	76	AX1aC[1]AX1bR[1], AX1bC[1], AX1cL[1], AX1cC[1], AX1aR[1], AX2aL[1], AX2bC[1], AX2dL[1],AX2dC[1], AX2eL[1],AX3aL[1], AX3bC[1], AX3bL[1], AX3dL[1], AX3eL[1], AX3fL[1] , AX4aC[1], AX5aC[1], AX6aL[1], AX6aR[1],AX6bR[1], AX6aC[1], AX7aC[1], AX7bC[1], AX7dC[1], AX8aC[1], AX8bC[1], AX9aR[1], AX9bR[1],AX1aL[1],CC2aL[1],CC3aC[1],CC3aR[1],CC2bL[1] CC3bC[1], CC3bL[1], CC3bR[1], CC4aL[1], CC4bL[1], CC4cL[1], CC5bC[1],CC5bL[1], CC6aC[1], , CC6bL[1], CC7bC[1], CC12aR[1], CC13aR[1], CC13bR[1],CC15aC[1],TS1a[1], TS2a[1], TS2e[1], TS3a[1], TS3b[1], TS3C[1],TS4a[1], TS4b[1], TS4e[1], TS5b[1], TS7a[1], TS7b[1], TS8a[1], TS8b[1], TSf9[1], TS10a[1], TS5a[1], TS10b[1],TS12a[1], TS12b[1], TS13a[1], TS15a[1], TS15c[1], CH273[2], CH274[2]	G	A	-	C	T	G	A	-	-	-	-	-	-	-	-
2	15	CC1cL[1], TW172[3], TW387[3], Rio2011-12[4], Rio2011-8-2[4], Rio2011-12-1[4], Rio2011-12-2[4], Rio2011-3-2[4], E1-4-14[5], E4-1-14[5], NED1[5], SCRP180[7], SCRP181[8], SCRP182[9], SCRP183[10]	.	.	.	.	.	.	.	A	T	T	A	T	T	A	A
3	6	CH201[2], CH276[2], CH277[2], CH278[2], CH282[2], SCRP188[10]	T	T	.	T	.	A	T	A	T	T	A	T	T	A	A
4	9	Rio2011-8[4], 927bsuelo1[6], 927bsuelo2[6], Marrakech myrthus5/2[11], Marrachech citrus 3[11], *P. citrophthora* Pinus silvestris [12], *P. citrophthora* silver[12], *P.citrophtora* nursery[12], *P. citrophthora* Abies alba[12]	.	.	T	.	.	A	.	A	T	T	A	T	T	A	A
5	2	TW165[3],TW242[3]	T	.	.	.	G	A	T	A	T	T	A	T	T	A	A
6	8	TW133[3],TW161[3] ,TW167[3],TW168[3],TW243[3],TW244[3] ,TW249[3] TW386[3]	.	.	.	.	G	A	T	A	T	T	A	T	T	A	A

[1]isolates from Italy; [2]isolates from Vietnam; [3]isolates from Taiwan;[4]isolates from Portugal;[5]isolates from Algeria; [6]isolates from in Spain; [7]isolates from New Zealand; [8]isolates from Australia; [9]isolates from Jordan; [10]isolates from China; ; [11] Isolates from Morocco; [12]Isolates from Poland

III.4 Analysis of SSR nuclear regions

Finally, the genotypes identified in the nuclear region according to variable SSR regions with primers I13F-I14R are summarized in Table III.3. Alignment was performed using 400 bp fragments of nuclear sequences (Annex 3). Nine SNPs were detected in the *P. citrophthora* population analyzed and 7 nuclear genotypes (NG) were identified. The most numerous NG (g1) is constituted of the same isolates grouped in H1 (Table III.2), except for isolate SCRP183 sourced from *Hevea* sp. in China that in the mitochondrial sequences was included in H2 and for TW 172 from *Cinnamomum camphora* and TW387 from *Quercus tarokoensis*. In fact, isolates of H2 were distributed in 2, 5 and 6 NG (g2, g5 and g6) (Table III.3). In particular, in g2 the following isolates were included: CC1cL sourced from rhizosphere soil in Italy, Rio2011-12, Rio2011-12-2, Rio2011-8, all sourced from river water in Portugal and isolates from Algeria, E1-4-14 and NED1, from *Citrus sinensis* and E4-1-14 sourced from *C. clementina*. The g5 was constituted by isolate SCRP180 from *Citrus* sp. in New Zealand, whereas two isolates, SCRP181 from *Citrus* sp. in Australia and SCRP182 from *Citrus* sp. in Jordan were included in g6. Similarly, isolates of H3 were distributed in g3, g4 and g7. In fact, g3 was constituted of isolates CH201 from *Citrus* sp. and CH276 from *C. paradisi* in Vietnam whereas g4 is constituted of isolates CH277, CH278 and CH282 sourced from *C. paradisi* in Vietnam. The g7 is represented by only one isolate, SCRP188 from China. All groups, except isolates of g1, showed different heterozygous sites.

Results from both mitochondrial and nuclear SSRs may be a better approach to the study the variability in *P. citrophthora* population.

Table III.3 - *Phytophthora citrophthora* genotypes identified according to variable SSRs region amplified with primers I13F-I14R

NG[a]	N° of duplicates	Duplicate sequences	177	195	299	306	328	330	340	348	350
1	**79**	**AX1aC[1]AX1bR[1], AX1bC[1], AX1cL[1], AX1cC[1], AX1aR[1], AX2aL[1], AX2bC[1], AX2dL[1],AX2dC[1], AX2eL[1],AX3aL[1], AX3bC[1], AX3bL[1], AX3dL[1], AX3eL[1], AX3fL[1] , AX4aC[1], AX5aC[1], AX6aL[1], AX6aR[1],AX6bR[1], AX6aC[1], AX7aC[1], AX7bC[1], AX7dC[1], AX8aC[1], AX8bC[1], AX9aR[1], AX9bR[1],AX1aL[1],CC2aL[1],CC3aC[1],CC3aR[1],CC2bL[1] CC3bC[1], CC3bL[1], CC3bR[1], CC4aL[1], CC4bL[1], CC4cL[1], CC5bC[1],CC5bL[1], CC6aC[1] , CC6bL[1], CC7bC[1], CC12aR[1], CC13aR[1], CC13bR[1],CC15aC[1],TS1a[1], TS2a[1], TS2e[1], TS3a[1], TS3b[1], TS3C[1],TS4a[1], TS4b[1], TS4e[1],TS5b[1], TS7a[1], TS7b[1], TS8a[1], TS8b[1], TSf9[1], TS10a[1], TS5a[1], TS10b[1],TS12a[1], TS12b[1], TS13a[1], TS15a[1], TS15c[1], CH273[2], CH274[2]**,SCRP 183[10] **TW172[3], TW387[3],**	G	G	T	C	G	A	C	C	T
2	11	**CC1cL[1],E1-4-14[5], E4-1-14[5], NED1[5], Rio2011-12[4], Rio2011-12-1[4] ,Rio2011-12-2[4], Rio2011-8[4] , Rio2011-3-2[4] ,Rio 2011-8-1[4] , Rio 2011-8-2[4] ,**	.	.	.	Y	R	W	Y	S	W
3	2	**CH201[2],CH276[2]**	A	C	C	.	S	.	Y	.	A
4	3	**CH277[2],CH278[2], CH282[2]**	A	C	Y	.	.	.	Y	Y	W
5	1	**SCRP180[7]**	.	.	.	T	-	.	.	.	K
6	2	**SCRP181[8],SCRP182[9]**	.	.	.	Y	-	.	.	.	.
7	1	SCRP188[10]	R	.	.	.	.	.	Y	.	A

NG[a]: Nuclear genotype

[1]isolates from Italy; **[2]isolates from Vietnam**; **[3]isolates from Taiwan**;**[4]isolates from Portugal**;**[5]isolates from Algeria**; **[7]isolates from New Zealand**; **[8]isolates from Australia; [9]isolates from Jordan; [10]isolates from China**

III.5 Phylogenetic analysis

Phylogenetic analysis was not possible for the nuclear data (ITS and nuclear region SSRs) due to the high level of heterozygosity; therefore, phylogenetic analyses was conducted using mitochondrial sequences. Phylogenetic tree (Fig. III.3) confirmed the intraspecific variability observed in the mitochondrial DNA region comprised from TrnG and Rns genes of all isolates of *P. citrophthora* analyzed. According to table III.2, in phylogenetic tree is evident the presence of the 7 haplotypes well separated between them. Finally, as espected the out-grouping represented by the sequences of isolates TW132a, TW132d, TW343 and TW369 clustered alone in a different group. These results suggest that the analysis of the mitochondrial region between TrnG and Rns genes, using the pair primers Mt15F–Mt5R, may be very useful to study the intraspecific variability in *P. citrophthora* as already reported for *P. nicotianae* (Mammella et al., 2011, 2013).

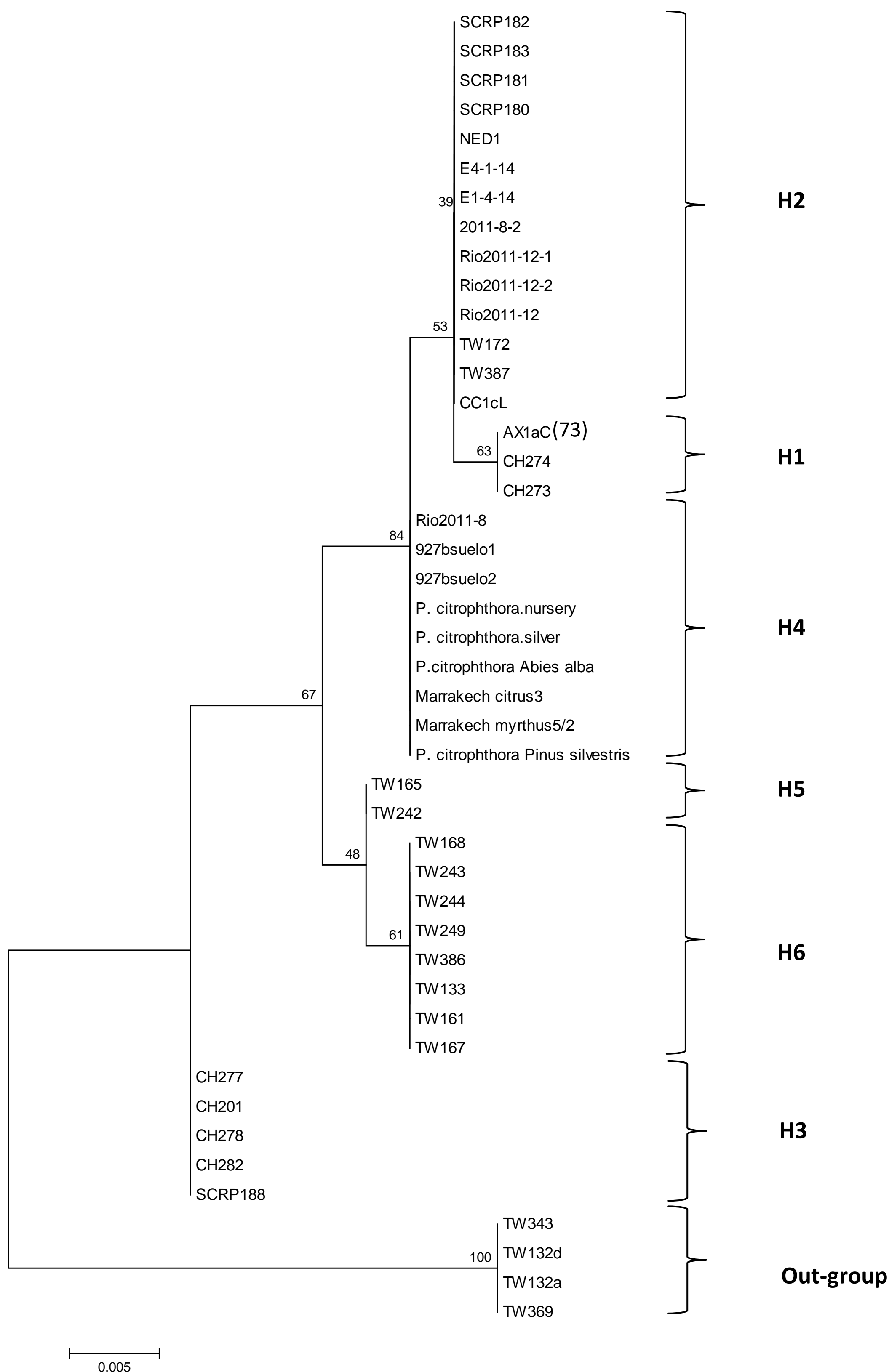

Fig. III.3 - Phylogenetic relationships of mitochondrial sequences of all isolates analysed in this study. In the phylogenetic tree, only two representative *P. citrophthora* Italian isolates (AX1aC and CC1cL) were reported. In brackets, the number of Italian isolates sharing the same mitochondrial sequence of isolate AX1aC.

Conclusion

In this study, both mitochondrial and nuclear markers were successfully used to study the distribution and the genetic variability of isolates of *P. citrophthora* from different hosts and various geographic origins. In fact, results show that mitochondrial markers flanking the regions between the genes TrnG-Rns, which are maternally inherited, are useful for studying the populations variability of *P. citrophthora,* also within Italian isolates. All these results taken toghether showed that an evident intraspecific genetic variability in *P. citrophthora* is detected also in isolates with the same geographical origin (e.g. Vietnam and Taiwan), as already shown in *P. nicotianae*, *P. infestans, P. sojae* and *P. ramorum* (Mammella et al., 2013). Most likely, no consistent association of haplotypes or genotypes with the geographic location or host or year of isolation was observed. Supporting this hypothesis, Cohen et al. (2003), in a study of intraspecific variation of ITS sequences in *P. citrophthora* from citrus in Eastern Corsica, showed that no obvious association could be observed between a given *P. citrophthora* genotype and rootstock and/or cultivar (scion). Altogether these results are a circumstantial evidence that the genetic diversity originates from recombination events which is of particular relevance in a species whose sexual reproductive structures have not been observed so far. Therefore, this hypothesis deserves further investigation.

In conclusion, this molecular approach already applied for *P. nicotianae* was successfully extended to *P. citrophthora* and could be easily exploited to study the intraspecific variability of other *Phytophthora* species as well as to other plant pathogens. Interestingly, this study lays the basis for the use of these markers as an effective DNA barcoding method to revise and reorganize the taxonomy of *Phytophthora* clade 2, clearly separating already known species whose borders presently overlap and very probably leading to the description of new species or hybrids that in the present classification are included in complex taxonomic entities. Interestingly, the genetic homogeneity of the Italian isolates of *P. citrophthora* recovered in citrus growing areas could

confirm that they are a clonal population, probably originating from the epidemic gummosis caused by *Phytophthora* during the 19th century in the Mediterranean Basin.

In addition, the occurrence of the same genotype in isolates sourced from *Citrus sinensis* in Vietnam, from Tarocco 'Scirè' in Azienda Serravalle in Sicily (Italy) and from *Citrus aurantium* in Italy may indicate a common origin of these two geographically distant populations. Altogether these results are indirect evidence that the low genetic diversity may come from recombination events despite the sexual form of this species has not been so far observed.

Concluding remarks and future perspectives

In the present study an approach based on the analysis of polymorphic mitochondrial and nuclear DNA regions has been developed and utilised to study intraspecific variability in *P. citrophtora.* 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. citrophthora* 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. citrophthora*. The application of the present method to characterize isolates of *P. citrophthora* from different countries in different continents 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 spread and the host specialization of *P. citrophthora*. 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 characterize *P. citrophthora* in an individual population and between populations. In fact, polymorphisms found in *P. citrophthora* 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 an additional panel of 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.

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