Phytopathologia Mediterranea (2015) 54, 3, 524–530
DOI: 10.14601/Phytopathol_Mediterr-16057

SHORT NOTES

Phenotypic and genotypic characterization of Italian *Phytophthora infestans* isolates

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Summary. *Phytophthora infestans* (Mont.) de Bary causes late blight of potato. After the 1970s, several changes have occurred in the European *P. infestans* population, frequently associated with an increased virulence. While the genotypic and phenotypic diversity of *P. infestans* has been studied in-depth in northern and central Europe, only a few reports are available regarding Italian isolates, mainly based on phenotypic traits. We report data of phenotypic and genotypic characteristics of isolates collected from infected potato and tomato plants in different Italian regions in 2006–2008. A prevalence of the A1 mating type and a majority of metalaxyl-resistant isolates were found. Tomato-derived isolates showed fungicide sensitivity, confirming previous reports. One of the isolates showed the rare IIb mitochondrial DNA haplotype. Genetic analyses of the single-sequence repeats (SSRs) and of the internal transcribed spacers gave similar results, although SSRs gave the best discrimination of genotypes.

Key words: late blight, mating type, metalaxyl, haplotype, SSR.

Introduction

Phytophthora infestans (Mont.) de Bary is a heterothallic oomycete, causal agent of late blight in potato and tomato crops. Late blight is one of the most important potato diseases worldwide, affecting all plant organs (leaves, stems and tubers), and causing severe economic losses. Mexico is considered to be the centre of origin and genetic diversity for this pathogen. It has sexual reproduction with two mating types, designated A1 and A2, which occur at approximately equal frequencies, and four mitochondrial haplotypes have been described (Grünwald and Flier, 2005). Before 1980, the worldwide population appeared to reproduce asexually and to consist of a single clonal lineage (US-1) belonging to the A1 type, characterized by a single genotype (one mitochondrial DNA haplotype, one multi-locus random fragment length polymorphism (RFLP) genotype and one di-locus isozyme genotype) (Cooke *et al*., 2011). Since then, major changes have occurred in the pathogen population outside Mexico, due to migration of new strains with potato trading, which has led to more diverse populations including both mating types, sexual reproduction and oospores (Niederhauser, 1991). This spread was considered responsible for increased fungicide resistance, increased pathogen aggressiveness (Day and Shattock, 1997) and superior fitness due to the presence of overwintering oospores (Turkensteen *et al*., 2000).

Monitoring of the pathogen population is crucial for effective implementation of control strategies, and several studies have characterized *P. infestans* in European countries, mostly in the northern-central regions (Cooke *et al*., 2011; Gisi *et al*., 2011; Runno-Paurson *et al*., 2012). Phenotypic markers, such as fungicide resistance, virulence and mating type, made it possible to find differences among isolates (Cooke and Lees, 2004). More recently genotypic markers

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ISSN (print): 0031-9465
ISSN (online): 1593-2095
www.fupress.com/pm

have been employed. These include sequencing of internal transcribed spacer (ITS) regions of the ribosomal gene DNA (Cooke *et al*., 2000), isozyme and allozyme patterns, random fragment length polymorphism (RFLP), mitochondrial DNA (mtDNA) haplotyping, amplified fragment length polymorphism (AFLP), random amplified polymorphic DNA (RAPD), single nucleotide polymorphisms (SNP) and simple sequence repeats (SSR) (Cooke and Lees, 2004). Recently the genome sequence of this important pathogen has been published (Haas *et al*., 2009; Cooke *et al*., 2012).

Late blight is one of the main biotic constraints to potato production in the Mediterranean area and some characterizations of *P. infestans* isolates from Morocco, Tunisia and Algeria have been reported (Hammi *et al*., 2002; Jmour and Hamada, 2006; Corbière *et al*., 2010). Despite the importance of tomato and potato cultivation in Italy, only a few studies on the biology and population structure of this oomycete are available for this country. The first studies on the Italian *P. infestans* population recognized the existence of different races (Black and Mezzetti, 1948; Ciccarone *et al*., 1961). The first report of the occurrence of the A2 mating type in Italy appeared in 1996 (Cristinzio and Testa, 1997), followed by the identification of 38 different physiological races by virulence tests (Cristinzio *et al*., 1998). Since then, the Italian *P. infestans* population has been scarcely investigated, except for two reports, both confirming the presence of the A2 mating type (Collina *et al*., 2004; D'Ascenzo *et al*., 2006). Collina *et al*. (2004) also characterized some *P. infestans* isolates from northern and central Italy by RAPD, finding a main clustering according to location of origin and to a lesser extent toward mating type.

The aim of the present study was to characterize *P. infestans* isolates, collected from different northern and southern regions of Italy, both for phenotypic traits, such as metalaxyl sensitivity and mating type, and genotypically, using molecular markers including mtDNA haplotype, ITS sequences and SSR.

Materials and methods

Sampling, recovery and conservation of *P. infestans* isolates

Leaves and stems showing the typical symptoms of late blight were randomly collected in tomato and potato fields in different Italian areas, from north to south, during 2006, 2007 and 2008 (Table 1, Figure 1). After incubation in a humid chamber at 4°C, pieces of tissue from the sporulating borders of single lesions were put under sterilized potato slices in Petri dishes and incubated at 18–20°C with a 12 h light/dark photoperiod. When abundant mycelium emerged, sporangia were collected and transferred to Rye B agar (Caten and Jinks, 1968).

Two isolates, one from northern and one from southern Italy, collected in 1987 and stored as sporangia in 10% dimethyl sulfoxide (DMSO) solution in liquid nitrogen, were also analyzed. Sporangia were suspended in water and left at 4°C to promote zoospore release until use for leaf infection, which were then treated similarly to the leaves harvested from the field, as described above.

For short-term use and maintenance, isolates were cultured on Rye B agar at 20°C in the dark.

Phenotypic characterization: mating type and metalaxyl sensitivity

The mating type was determined by dual culture in Petri dishes (Day and Shattock, 1997). The isolates were also checked for self-fertility and cultured with the reference strain of the same mating type, to verify the absence of oogonium formation.

In vitro sensitivity to metalaxyl was determined on Rye B agar supplemented with 10 mg mL^{-1} technical grade metalaxyl, according to Day and Shattock (1997). Two metalaxyl tests were carried out, each time using two replicas for the same isolate.

Molecular characterization: mtDNA haplotype, ITS sequencing and SSR analysis

DNA was extracted (Plant Mini kit, Mackerey Nagel) from about 100 mg of mycelium of each *P. infestans* isolate, following the manufacturer's instructions. The mtDNA haplotype was determined according to Griffith and Shaw (1998), exploring the four polymorphic regions P1, P2, P3 and P4. The results obtained after amplification and digestion of the regions can discriminate among the four mtDNA haplotypes (Ia, IIa, Ib, IIb).

The ITS sequences were generated using the primer set ITS5 and ITS4, amplified according to Ristaino *et al.* (1998). Sequences were edited using BioEdit software (Ibis Biosciences). The alignment was

Table 1. *Phytophthora infestans* isolates, locations, year of harvesting, mating type, metalaxyl sensitivity and mitochondrial haplotype.

Isolate (GenBank nr)[a]	Location	Year	Mating type	Metalaxyl sensitivity[b]	Mitochondrial haplotype
From potato					
37_LE (KT363855)	Lecce	2006	A2	R	Ia
38_BO (KT363856)	Bologna	2006	A1	I	Ia
56_BO (KT363857)	Bologna	2008	A1	R	Ia
58_BL (KT363858)	Belluno	2007	A1	I	IIb
59_CS (KT363859)	Cosenza	1987	Nd	Nd	Ia
60_BO (KT363860)	Bologna	1987	A1	I	Ia
81_PR (KT363861)	Parma	2008	A1	R	Ia
83_PR (KT363862)	Parma	2008	A1	I	Ia
From tomato					
105_CN (KT363863)	Cuneo	2006	A1	R	Ia
108_CN (Ns)	Cuneo	2006	A1	I	Ia
498_TO (KT363864)	Torino	2008	A1	S	Ia
600_TO (KT363865)	Torino	2008	A1	S	Ia
Reference strains					
CBS 430.90_A1_NL	The Netherlands	1989	A1	R	Ia
CBS 429.90_A2_NL	The Netherlands	1989	A2	I	Ia

[a] NCBI GenBank accession numbers of ITS sequences; Ns: not submitted.
[b] R: resistant; I: intermediate; S: sensitive. Data obtained 2 weeks after incubation on Rye B agar with 10 mg mL^{-1} metalaxyl. Nd: not determined.

performed by MEGA6.06 software (www.megasoftware.net) using CLUSTAL W, and the dendrogram was constructed with the Neighbour-joining method (Saitou and Nei, 1987), with bootstrapping test (1000 replications). GenBank accession numbers for the isolates are reported in Table 1.

Several ITS sequences were added, retrieved from the *Phytophthora* database (www.phytophthoradb.org): seven from *P. infestans*, one from *P. nicotianae* and two from *P. cactorum*. The last two species belong to the same Clade I as *P. infestans*.

For the SSR analysis, 11 microsatellite markers, previously described by Knapova and Gisi (2002) and Lees *et al.* (2006), were included in the present study; three multiplex amplifications were performed according to the Eucablight Project draft protocol (www.eucablight.org). After electrophoresis, fragment sizes were determined by GeneScan software (Applied Biosystems). The cluster analysis of SSR matrix values was performed using the unweighted pair-group method with arithmetic mean (UPGMA) with Dice's coefficient, and the dendrogram was produced (Nei, 1973) using the GelCompar II software (Applied Maths).

Results

Twelve isolates were successfully recovered from field or liquid nitrogen (Table 1). One of them (59_CS) did not produce enough mycelium for the mating type and metalaxyl sensitivity tests, but only for DNA analyses. Despite the different geographi-

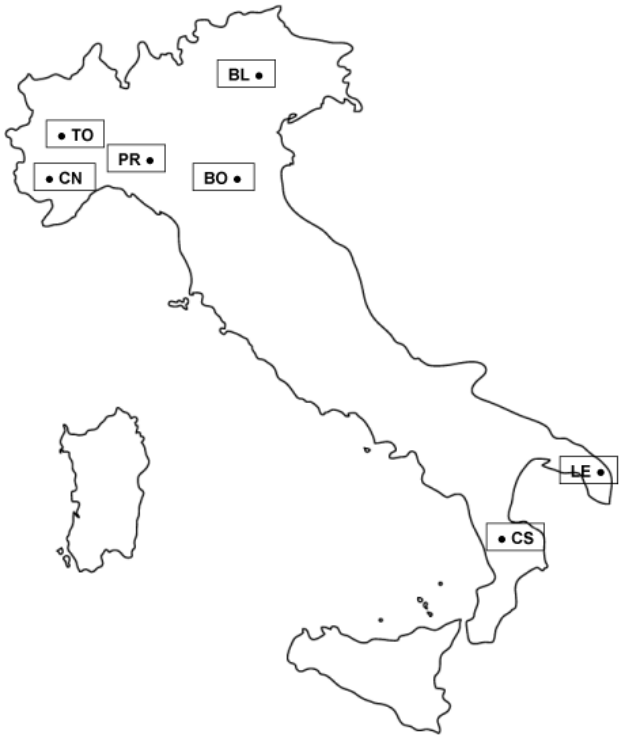

Figure 1. Map of the *Phytophthora infestans* isolate collection sites in Italy. Letters correspond to respective isolate designations (Table 1).

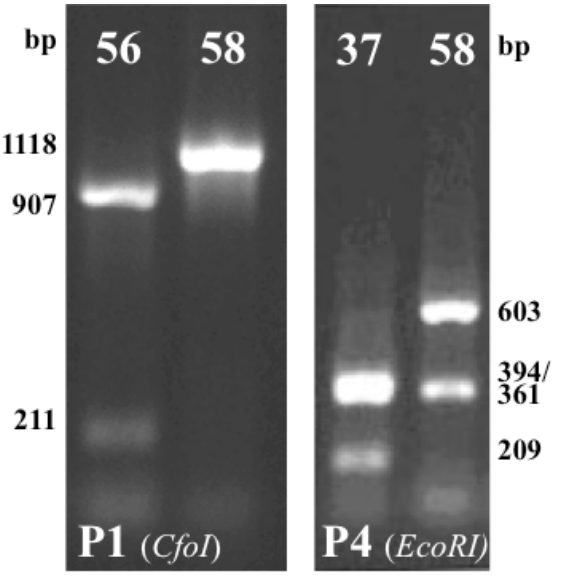

Figure 2. Digestion patterns of *Phytophthora infestans* mtDNA polymorphic regions P1 and P4. Comparison of isolates 37_LE, and 56_BO (Ia haplotype) with isolate 58_BL (IIb haplotype). The digestion product length, in base pair, is indicated on each side.

cal origins of the isolates, from northern to southern Italy, and their collection in different years, they showed very few differences in morphological and molecular assessments.

All isolates belonged to mating type A1, with the exception of strain 37_LE from south-eastern Italy, collected in 2006 (Table 1; Figure 1).

Some diversity was observed in the metalaxyl sensitivity tests: after 2 weeks, the majority of the isolates (82%) were classified as resistant (n=4) or intermediate (n=5), while only 18% are sensitive (Table 1). The same results were obtained after one month of culture.

All the isolates were of the Ia mtDNA haplotype, with the exception of isolate 58_BL, from Alpine region, which gave the IIb pattern type (Table 1 and Figure 2).

The ITS sequencing analysis grouped most of the isolates in one main cluster, together with the published *P. infestans* sequences. Only the isolate 58_BL was slightly distant from the *P. infestans* group, but was clearly separated from *P. nicotianae* and *P. cactorum* (Figure 3A). This isolate was also identified as *P. infestans* by BLAST analysis.

Four SSR markers (D13, Pi33, Pi70, Pi63) did not amplify and one SSR (Pi02) did not give satisfactory amplifications. Using the remaining six SSRs, a considerable number of clusters were detected (Figure 3B): isolate 37_LE was very distant from all the other strains. Isolates 105_CN and 108_CN were grouped together, as well as 38_BO and 56_BO. Some similarity was found in the subgroup of isolates 81_PR, 83_PR, 498_TO and 600_TO. Isolates 58_BL, 59_CS and 60_BO were grouped together (Figure 3B).

Discussion

Although there have been several published reports of *P. infestans* population studies for northern and central Europe, few reports for southern Europe and the Mediterranean areas are available. Studies carried out in Morocco, Tunisia, and Algeria with isolates collected from 1996 to 2009, have characterized the *P. infestans* populations using mating type and metalaxyl sensitivity (Sedegui *et al*., 2000; Ham-

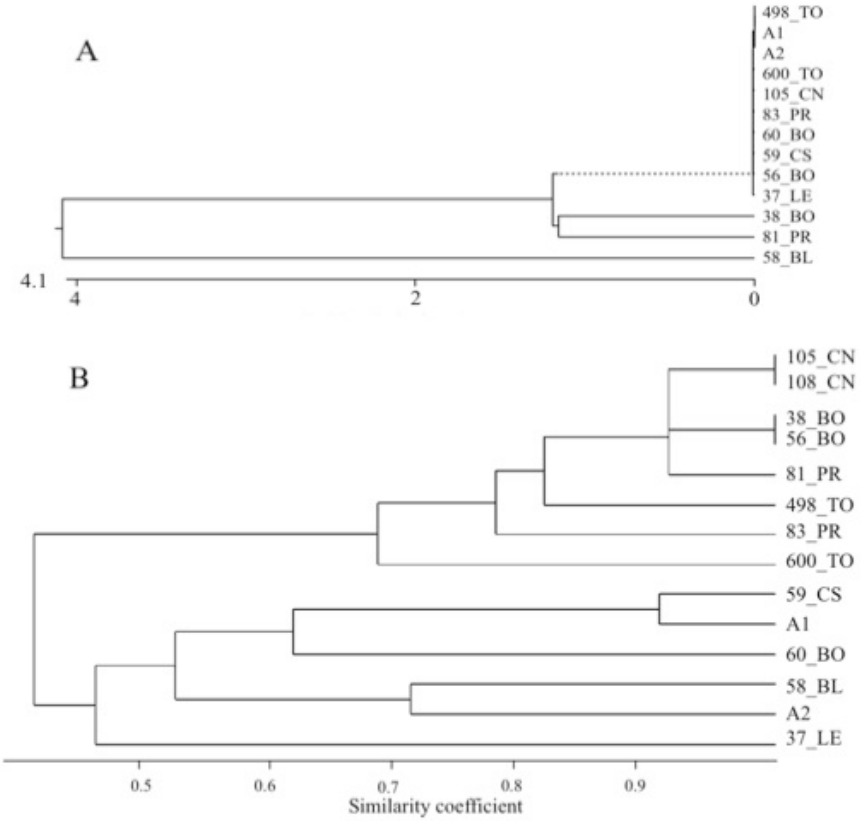

Figure 3. Dendrograms of *Phytophthora infestans* isolates obtained from A) ITS nucleotide sequences aligned by MegAlign, with bootstrap test results on each node, and B) SSR matrix analysis by unweighted pair-group method with average linkage (UPGMA) using Dice's coefficient.

mi *et al*., 2002; Jmour and Hamada, 2006; Corbière *et al*., 2010) and SSR markers (Corbière *et al*., 2010). In these areas the *P. infestans* populations showed an increasing proportion of the A2 type, from 12 % in Tunisia (Jmour and Hamada, 2006) up to 36% or 59% in Morocco (Sedegui *et al*., 2000; Hammi *et al*., 2002) and up to 88% in Algeria (Corbière *et al*., 2010). Conversely, in our study we found a low frequency of the A2 mating type. Thus, the prevalence of A1 type in Italy, reported also by other authors (Collina *et al*., 2004; D'Ascenzo *et al*., 2006), suggests a different situation from that of other Mediterranean areas (except Tunisia), and from that of the central and northern European regions, in the same period (Cooke *et al*., 2011).

The low frequency of metalaxyl sensitive strains (18%) found in this study is consistent with what was reported in other surveys in the Mediterranean area (Corbière *et al*., 2010, Jmour and Hamada, 2006). Moreover, the two sensitive strains (498_TO and 600_TO) were collected from tomato crops, suggesting behavioural differences between tomato and potato-derived isolates toward metalaxyl. Other authors have reported metalaxyl sensitivity for isolates collected from tomato plants (Knapova and Gisi, 2002; Collina *et al*., 2004; D'Ascenzo *et al*., 2006; Corbière *et al*., 2010).

Cooke *et al*. (2006) found a strong relationship between the mtDNA haplotype Ia and fungicide resistance, while the majority of the IIa haplotype isolates was sensitive. According to our data, the first regarding mtDNA haplotypes in Italy and in the Mediterranean area, almost all the strains belong to haplotype Ia, while IIa haplotype was absent. This is consistent with the high resistance frequency observed. The frequencies of mtDNA haplotypes have changed in European populations during the last few years, from a

majority of Ib and IIa types towards Ia type (Cooke *et al*., 2006). Our study confirms this trend and reports frequencies similar to those found in France and Switzerland (Knapova and Gisi, 2002). Only isolate 58_BL, collected in the Alpine region, was of the relatively rare haplotype IIb.

While mtDNA haplotyping can discriminate a maximum of four groups, genetic diversity could be better discerned by analyzing the nuclear genome, through comparison of ITS sequences or SSR markers.

ITS regions were successfully utilized for distinguishing different *Phytophthora* species for evolutionary and taxonomic purposes (Ristaino *et al*., 1998; Cooke *et al*., 2000), and for their traceability (Scibetta *et al*., 2012). According to our results, the ITS regions do not contain enough variability to discriminate among all the tested *P. infestans* isolates. Most of the isolates were grouped together in one main cluster, with only isolate 58_BL was somewhat separated (Figure 3A). This strain was the only one with the mtDNA haplotype IIb. Since the isolate came from a remote mountain environment (the Alpine region), this could explain its different ITS clustering and mtDNA haplotype.

SSR analysis (Figure 3B) made it possible to highlight greater polymorphism in our group of isolates than the ITS analysis. In particular, SSR analysis discriminated the isolates according to location of origin and collection year (old and recent): the two older isolates, 59_CS and 60_BO, collected in 1987, were in the same clusters as the A1_NL and A2_NL reference strains, both collected in 1989 (Figure 3B). In addition, the analysis allowed separation according to mating type, with the A2 isolate 37_LE falling outside the main group in the SSR tree. This analysis also allowed separation of isolate 58_BL, with IIb haplotype, as found in the ITS analysis.

In conclusion, the results presented here, although assessing a small number of isolates, provide information about the characteristics of the Italian *P. infestans* population, mainly collected in 2006–2008, and is a reference point for future studies of larger populations of this pathogen in Italy and in the Mediterranean area.

Acknowledgements

This work was funded by the Italian Ministry for Agriculture, Food and Forestry Policies, VAPABIO project D. M. 91457, September 6, 2005. The contribution of Pier Luigi Burzi to laboratory activities is gratefully acknowledged.

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Accepted for publication: August 14, 2015
Published online: December 31, 2015