THE ASSESSMENT OF THE GENETIC VARIABILITY IN NORTHERN ITALY OF THE INTRODUCED CYPRESS CANKER AGENT (*SEIRIDIUM CARDINALE*): A MOLECULAR APPROACH

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Abstract

Over the past half century a destructive blight of *Cupressus* spp., caused by *Seiridium cardinale*, has spread worldwide from North America, devastating forests, plantations, and ornamental cypresses. The epidemic has been particularly severe in the Mediterranean region, on *C. sempervirens*. Three species of *Seiridium*, *S. cardinale*, *S. cupressi*, and *S. unicorne*, are associated with cypress canker. To test the genetic variability of the Italian population of *S. cardinale*, a large sample of different isolates was analysed by RAPD (Random amplified polymorphic DNA) markers and compared with a number of other *Seiridium* spp. isolates from various hosts growing in different parts of the world. The results indicated a high level of homogeneity in the North-Italian population of the fungus, whereas certain variability was recognized in isolates from other hosts and other species. The isolates belonged to the North-Italian population appear to be very similar from the molecular comparison and in the same group was included also the isolate from Greece. The results are discussed in relation to the introduction and spread of the fungus in Europe.

Keywords: molecular markers, RAPD, *Cupressus sempervirens*, population genetics, genetic variability, introduced pathogens, fungi.

1. Introduction

Cypress canker, caused by *Seiridium cardinale* (Wagener) Sutton & Gibson, is one of the most significant canker diseases of woody plants, affecting trees in natural forests, plantations and the urban environment worldwide (Graniti, 1998; Panconesi, 1990).
This mitosporic coelomycete, probably originating from California, USA on native trees of the family *Cupressaceae* (Wagener, 1939), was accidentally introduced and reported in Europe around the middle of the 20th century (Barthelet & Vinot, 1944; Grasso, 1951). The presence of highly susceptible hosts and climatic conditions favorable for asexual reproduction of the pathogen facilitated its establishment and spread in the Mediterranean area, where it has caused destructive and recurrent epidemics of canker that have decimated the host species in ornamental contexts, in windbreaks and in forest (Graniti, 1986; Raddi *et al*., 1987).
However, several authors have disputed the separation of *Seiridium* into three species: *S. cupressi* (Guba) Boes. *S. unicorne* (Cke. & Ell.) Sutt. and *S. cardinale* based on morphology (Swart, 1973; Chou, 1989), and recently molecular evidence have supported this view (Viljoen *et al*., 1993; Barnes *et al.*, 2001; Krokene *et al.*, 2004). However, the host ranges described for these three species appears quite separated (Graniti, 1998).

Great variation exists in the susceptibility of different species within the *Cupressaceae* to *S. cardinale* infection (Andreoli, 1979; Grasso and Ponchet, 1979; Raddi, 1979; Graniti, 1998). Intraspecific variation in resistance has also been reported in *C. sempervirens* (Ponchet and Andreoli, 1979; Xenopoulos, 1990, 1991; Teissier Du Cros *et al*., 1991), with evident variations within provenances and families from controlled crosses (Santini & Lonardo, 2000). Effective exploitation of these genetic resistance sources may enable the replacement of stands damaged by canker with more resistant selected clones. As a matter of fact, several clones have patented so far by the breeding programs carried out in Italy and France in the last 25-30 years (Teissier Du Cros *et al*., 1991) and a new breeding program started in North Italy (La Porta *et al*., 2004) intend to select clones resistant to canker and to cold stress.
The aim of this study was to compare the genetic variability among different provenance of North Italian *S. cardinale* isolates with the use of some close-related *Seiridium* spp outgroups. The final aim is to confirm and clarify also for Northern Italy the supposed low variability of *S. cardinale* (Moricca *et al*., 2000), due to the missing of sexual reproduction and to the supposed introduction of few virulent genotypes of the fungus. These information are crucial for the maintenance of resistance stability in any breeding program before to obtain and to release the resistant patented cypress clones.

2. Materials and methods

2.1 Fungal cultures and DNA extraction

A total of 82 isolates found on various hosts of the family *Cupressaceae*, were used (Tab. 1). Seventy-seven isolates of *Seiridium cardinale* were used for the genetic characterization of the North-Italian population (Fig. 1), and five *Seiridium* spp. isolates from different countries and different hosts were used for comparison. Among the latter five isolates included in this study, three of them served as outgroups in the phylogenetic analysis.

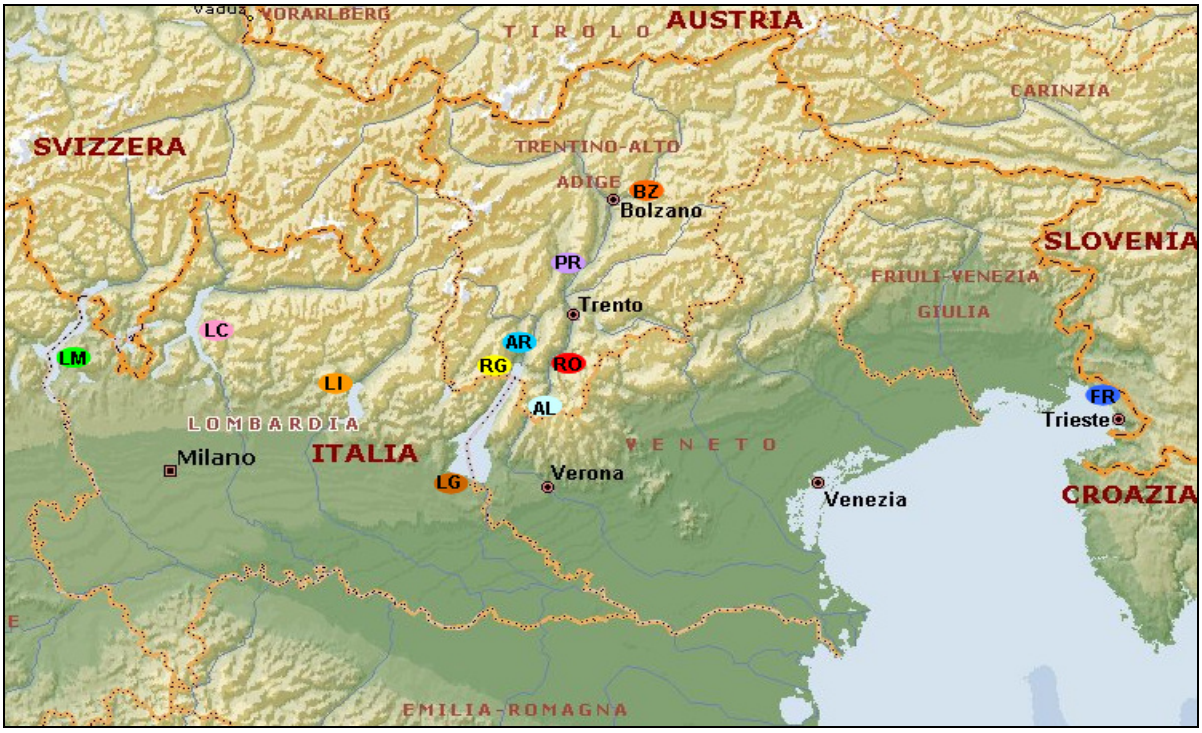

Figure 1 - Map of distribution of the 11 Italian populations of S. cardinale *analyzed in this study.*

Table 1 – Eighty-two isolates of *Seiridium cardinale* (79) and *Seiridium* spp. (3) analyzed in this study.

Fungus	Code	Locality	Province or state	Latitude	Longitude	Collector	Host
S. cardinale	AL1	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL2	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL3	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL4	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL5	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL6	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL7	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL8	Ala	Trentino Alto Adige	45°46'06"	11°00'05"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL9	Avio	Trentino Alto Adige	45°44'15"	10°55'56"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AL10	Avio	Trentino Alto Adige	45°44'15"	10°55'56"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AR1	Arco	Trentino Alto Adige	44°55'03"	10°52'41"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AR2	Arco	Trentino Alto Adige	44°55'03"	10°52'41"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AR3	Arco	Trentino Alto Adige	44°55'03"	10°52'41"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AR4	Arco	Trentino Alto Adige	44°55'03"	10°52'41"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	AR5	Arco	Trentino Alto Adige	44°55'03"	10°52'41"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	RO1	Mori	Trentino Alto Adige	45°51'09"	10°58'21"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	RO2	Mori	Trentino Alto Adige	45°51'09"	10°58'21"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	RO3	Rovereto	Trentino Alto Adige	45°53'40"	11°01'58"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	RO4	Rovereto	Trentino Alto Adige	45°53'40"	11°01'58"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	BZ1	Bolzano	Trentino Alto Adige	46°29'22"	11°20'39"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	BZ2	Bolzano	Trentino Alto Adige	46°29'22"	11°20'39"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	PR1	Piana Rotaliana	Trentino Alto Adige	46°13'17"	11°06'07"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	PR2	Piana Rotaliana	Trentino Alto Adige	46°13'17"	11°06'07"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	PR3	Piana Rotaliana	Trentino Alto Adige	46°13'17"	11°06'07"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	PR4	Piana Rotaliana	Trentino Alto Adige	46°13'17"	11°06'07"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	PR5	Piana Rotaliana	Trentino Alto Adige	46°13'17"	11°06'07"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	PR6	Piana Rotaliana	Trentino Alto Adige	46°13'17"	11°06'07"	G. Frigimelica, A. Zocca	*C. sempervirens*
S. cardinale	RG1	Nago	Garda Lake	45°53'03"	10°52'55"	G. Piva	*C. sempervirens*
S. cardinale	RG2	Nago	Garda Lake	45°53'03"	10°52'55"	G. Piva	*C. sempervirens*
S. cardinale	RG3	Riva del Garda	Garda Lake	45°53'32"	10°50'37"	G. Piva	*C. sempervirens*
S. cardinale	RG4	Riva del Garda	Garda Lake	45°53'32"	10°50'37"	G. Piva	*C. sempervirens*
S. cardinale	LG1	Gargnano	Garda Lake	45°41'48"	10°39'36"	G. Piva	*C. sempervirens*
S. cardinale	LG2	Gargnano	Garda Lake	45°41'48"	10°39'36"	G. Piva	*C. sempervirens*
S. cardinale	LG3	Limone	Garda Lake	45°49'16"	10°47'05"	G. Piva	*C. sempervirens*
S. cardinale	LG4	Limone	Garda Lake	45°49'16"	10°47'05"	G. Piva	*C. sempervirens*
S. cardinale	LG5	Pieve	Garda Lake	45°46'07"	10°44'21"	G. Piva	*C. sempervirens*
S. cardinale	LG6	Pieve	Garda Lake	45°46'07"	10°44'21"	G. Piva	*C. sempervirens*
S. cardinale	LG7	Pieve	Garda Lake	45°46'07"	10°44'21"	G. Piva	*C. sempervirens*
S. cardinale	LG8	Pieve	Garda Lake	45°46'07"	10°44'21"	G. Piva	*C. sempervirens*
S. cardinale	LG9	Sirmione	Garda Lake	45°29'56"	10°36'20"	G. Piva	*C. sempervirens*
S. cardinale	LG10	Sirmione	Garda Lake	45°29'56"	10°36'20"	G. Piva	*C. sempervirens*
S. cardinale	LG11	Sirmione	Garda Lake	45°29'56"	10°36'20"	G. Piva	*C. sempervirens*
S. cardinale	LG12	Sirmione	Garda Lake	45°29'56"	10°36'20"	G. Piva	*C. sempervirens*
S. cardinale	LG13	Sirmione	Garda Lake	45°29'56"	10°36'20"	G. Piva	*C. sempervirens*
S. cardinale	LG14	Sirmione	Garda Lake	45°29'56"	10°36'20"	G. Piva	*C. sempervirens*
S. cardinale	LG15	Valeggio sul Mincio	Garda Lake	45°21'04"	10°43'54"	G. Piva	*C. sempervirens*
S. cardinale	LG16	Valeggio sul Mincio	Garda Lake	45°21'04"	10°43'54"	G. Piva	*C. sempervirens*
S. cardinale	LG17	Valeggio sul Mincio	Garda Lake	45°21'04"	10°43'54"	G. Piva	*C. sempervirens*
S. cardinale	LG18	Valeggio sul Mincio	Garda Lake	45°21'04"	10°43'54"	G. Piva	*C. sempervirens*
S. cardinale	LG19	Garda	Garda Lake	45°34'53"	10°42'14"	G. Piva	*C. sempervirens*
S. cardinale	LG20	Malcesine	Garda Lake	45°46'23"	10°48'30"	G. Piva	*C. sempervirens*
S. cardinale	LG21	Sirmione	Garda Lake	45°21'04"	10°43'54"	G. Piva	*C. sempervirens*
S. cardinale	LM1	Verbania	Lake Maggiore	45°56'10"	8°33'10"	G. Piva	*C. sempervirens*
S. cardinale	LM2	Verbania	Lake Maggiore	45°56'10"	8°33'10"	G. Piva	*C. sempervirens*
S. cardinale	LM3	Verbania	Lake Maggiore	45°56'10"	8°33'10"	G. Piva	*C. sempervirens*
S. cardinale	LM4	Verbania	Lake Maggiore	45°56'10"	8°33'10"	G. Piva	*C. sempervirens*
S. cardinale	LM5	Verbania	Lake Maggiore	45°56'10"	8°33'10"	G. Piva	*C. sempervirens*
S. cardinale	LM6	Laveno	Lake Maggiore	45°55'07"	8°36'59"	G. Piva	*C. sempervirens*
S. cardinale	LM7	Laveno	Lake Maggiore	45°55'07"	8°36'59"	G. Piva	*C. sempervirens*
S. cardinale	LM8	Laveno	Lake Maggiore	45°55'07"	8°36'59"	G. Piva	*C. sempervirens*
S. cardinale	LM9	Laveno	Lake Maggiore	45°55'07"	8°36'59"	G. Piva	*C. sempervirens*
S. cardinale	LM10	Azzate	Varese Lake	45°47'02"	8°47'28"	G. Piva	*C. sempervirens*
S. cardinale	LC1	Bellagio	Como Lake	45°59'08"	9°15'22"	G. Piva	*C. sempervirens*
S. cardinale	LC2	Bellagio	Como Lake	45°59'08"	9°15'22"	G. Piva	*C. sempervirens*
S. cardinale	LC3	Bellagio	Como Lake	45°59'08"	9°15'22"	G. Piva	*C. sempervirens*
S. cardinale	LC4	Dervio	Como Lake	46°04'36"	9°18'08"	G. Piva	*C. sempervirens*
S. cardinale	LC5	Bellano	Como Lake	46°02'31"	9°17'46"	G. Piva	*C. sempervirens*
S. cardinale	LC6	Moltrasio	Como Lake	45°51'59"	9°06'03"	G. Piva	*C. sempervirens*
S. cardinale	LC7	Moltrasio	Como Lake	45°51'59"	9°06'03"	G. Piva	*C. sempervirens*
S. cardinale	LI1	Sarnico	Iseo Lake	45°39'53"	9°57'11"	G. Piva	*C. sempervirens*
S. cardinale	LI2	Lovere	Iseo Lake	45°48'51"	10°04'15"	G. Piva	*C. sempervirens*
S. cardinale	LI3	Clusane	Iseo Lake	45°04'37"	9°59'58"	G. Piva	*C. sempervirens*
S. cardinale	LI4	Marone	Iseo Lake	45°44'03"	10°05'22"	G. Piva	*C. sempervirens*
S. cardinale	LI5	Iseo	Iseo Lake	45°39'14"	10°02'46"	G. Piva	*C. sempervirens*
S. cardinale	FR1	Bassovizza	Friuli Venezia Giulia	45°38'31"	13°51'04"	G. Frigimelica	*C. sempervirens*
S. cardinale	FR2	Bassovizza	Friuli Venezia Giulia	45°38'31"	13°51'04"	G. Frigimelica	*C. sempervirens*
S. cardinale	FR3	San Dorligo della Valle	Friuli Venezia Giulia	45°36'37"	13°51'04"	G. Frigimelica	*C. sempervirens*
S. cardinale	GR	Kos	Greece			P. Tsopelas	*C. sempervirens*
S. cardinale	CI		Chile			A. Wingfield	*Cupressus* sp.
S. cupressi	NZ		New Zealand			S. Chou	*Cupressus macrocarpa*
S. unicorne	P		Portugal			A. Graniti	*Cupressus* sp.
S. eucalypti	A		Australia			Z. Q. Yuan	*Eucalyptus delegatensis*

The North-Italian isolates were obtained from cones or active infections from branches and twigs of diseased and declining trees. The cones have been placed in a most chamber with the aim to favours and to speed up the appearance of the conidia.
Single-spore isolates were isolated and cultured in Petri dishes on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) at 25°C in darkness and than maintained in PDA tubes at

4°C (Fig. 2). For DNA extraction the cultures were grown on cellophane disc placed on agarified PDA (20 g/l PDA, 20 g/l agar; Difco Laboratories, Detroit, USA) in Petri dishes for 15-20 days at 25°C. Once the mycelia had covered the discs, they were lifted from the cellophane membrane and grounded in a mortar with liquid N_2.
Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in agreement with the indications of the producer.

Figure 2 – Example of isolates using in this study growing in Petri dishes.

2.2 DNA amplification

Amplification of fungal DNA was performed in volumes of 25 µl according to the following reaction mixture: 1X Taq DNA polymerase buffer (10 mM Tris-HCl pH 8.3, 1,5 mM $MgCl_2$, 50 mM KCl, 0,1 mg gelatin); 0,2 mM each of dATP, dCTP, dGTP and dTTP; 0,3 µM of each primer RAPD; 0,75 units of Taq DNA polymerase (Bioline, Randolph, USA) and 20 ng of genomic DNA. They have been tested 60 primers (Kits B, E and P, Operon Technologies, Alameda, USA) of which 8 primers they have been selects in order to execute the genetic analysis in how much show clear polymorphism and pattern of banding repeatability. Therefore, they have been characterized the optimal temperatures of annealing for every single primer selected.
The eight primers used were the following: OPP8 T°an (annealing temperature) 50°C, OPP6 T°an 50°C, OPP12 T°an 49°C, OPP14 T°an 50°C, OPB11 T°an 42°C, OPB18 T°an 42°C, OPE14 T°an 42°C and OPB10 T°an 50°C.
Incubation was performed in a Mastercycler ep gradient S thermal cycler (Eppendorf AG, Hamburg, Germany) using the following cycle parameters: 94°C for 60 s, from 42°C to 50°C in relation of the primer used for 60 s and 72°C for 60 s, using the maximum ramp time between each temperature. The total number of cycles was 35, with an initial denaturation step of 5 min at 94°C and a final extension step of 10 min at 72°C. A negative control with all reagents except DNA was included in all reactions.
The amplification products were separated in 1,5% agarose gels (AquaPor HR GTAC, Atlanta, USA) in 0,5X TBE buffer (Tris-acetate 40 mM, EDTA 1 mM, pH 8), at a constant voltage of 100 V for 5 h at room temperature, and stained with 0,1% ethidium bromide. The results were observed under UV light, and photographed with a digital camera.
The molecular size of the amplification products was estimated using HyperLadder II (Bioline, Randolph, USA).

2.3 Data analysis

A comparison of each profile was carried out on the basis of presence/absence (1/0) of amplification products of the same length. A binary matrix combined the complete data records for all the isolates from the eight RAPD primers. Genetic distance was calculated with the GenAlEx 6 software using the formula of Nei's standard genetic distance (Nei, 1972; Nei 1978) between pairs of populations. Dendrograms were constructed by means of the UPGMA (unweighted pair grouping by mathematical averaging) methods using the MEGA3 software (version BETA).
In addition, with all isolates was estimated the RAPD groups (clusters) to avoid defining every clonally related pair of haplotypes as a separate group.
Each such UPGMA-defined group of isolates was considered as a separate genetic lineage.

3. Results

The total number of 82 isolates was used in the work. The sampling along Trentino-South Tyrol and the biggest lakes in North Italy provided a total of 77 isolates (Fig. 1). Some extra 5 isolates were provided by several other colleagues (P. Capretti, P. Tsopelas and A. Wingfield; Tab. 1) including 3 isolates from *Seiridium* outgroup species: *S. cupressi*, *S. unicorne* and *S. eucalypti*.
The electrophoretic profiles of the isolates amplified by RAPDs exhibited amplified fragments ranging from 200 to 2300 bp. The total number of the fragments was 137 and only one of them was common to all the isolates, including the outgroups. Out of 137 fragments only 91 were found in the *Seiridium cardinale* and among them, 57 showed to be polymorphic.
The analyses of the RAPD amplifications with 8 primes revealed a substantial low genetic variability among the 11 Italian populations. As a matter of fact the UPGMA cluster analysis groups all the Italian populations very close each other even though they are not totally identical (Fig. 3). Only the *S. cardinale* from Chile was clustered at significant distance from the other *S. cardinale* isolates from Italy and Greece (Fig. 4).

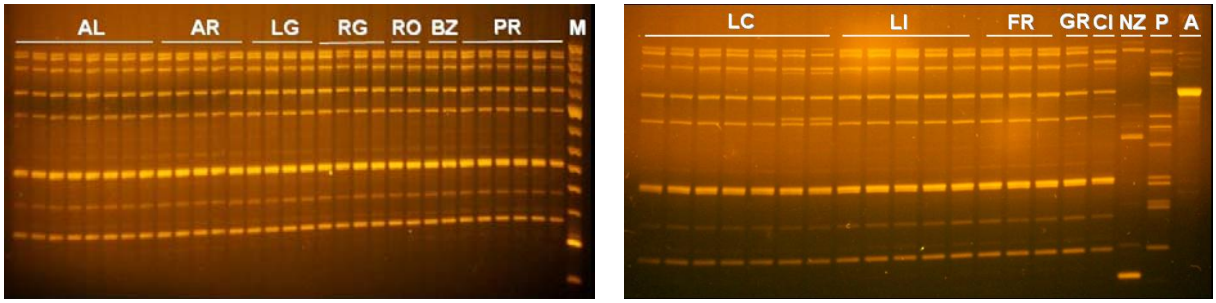

Figure 3 - Example of RAPD profiles generated by primers OPP 8 for selected Seiridium *spp. isolates. The acronyms of the populations are the following: AL (Ala), AR (Arco), LG (Garda Lake), RG (Riva del Garda), RO (Rovereto), BZ (Bolzano), PR (Piana Rotaliana), LC (Como Lake), LI (Iseo Lake), FR (Friuli Venezia Giulia), GR (Greece), CI (Chile), NZ (New Zealand), P (Portugal), A (Australia).*

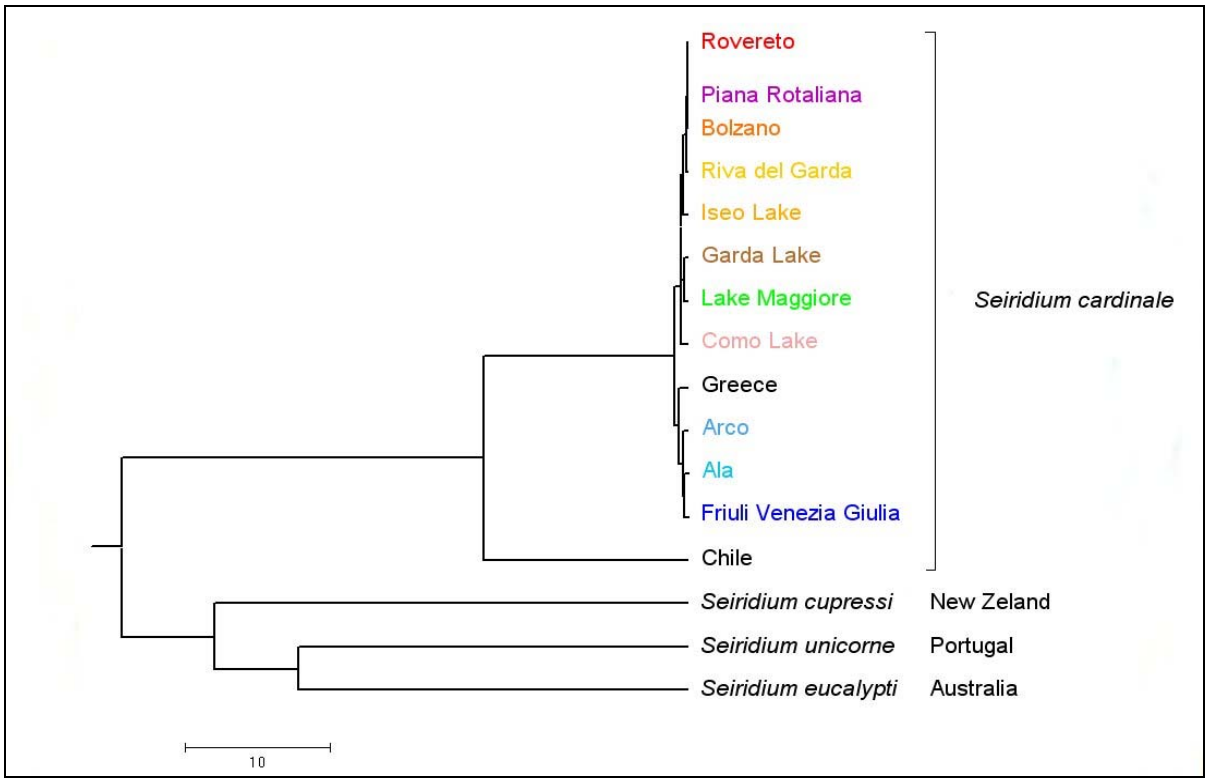

Figure 4 – Dendrogram produced using UPGMA cluster analysis of genetic distances between the Seiridium *populations: 13 of* S. cardinale *from Italy, Greece and Chile and 3 other* Seiridium *spp. used as outgroups.*

Table 2 - Number and composition of RAPD groups and proportion of haplotypes individuals in the populations of *S. cardinale*.

RAPD group	Number of isolates	Ala	Arco	Rovereto	Bolzano	Piana Rotaliana	Riva del Garda	Garda Lake	Lake Maggiore	Como Lake	Iseo Lake	Friuli Venezia Giulia	Greece	Chile
1	**15**			2		3	2	2	3	2	1			
2	**15**	1	2	2	2	3	1	1	1	1	1			
3	**4**							4						
4	**4**	3	1											
5	**3**							3						
6	**3**							2	1					
7	**3**	1	1									1		
8	**3**								3					
9	**2**							2						
10	**2**							1				1		
11	**2**	2												
12	**2**	2												
13	**2**									2				
14	**2**	1									1			
15	**1**									1				
16	**1**									1				
17	**1**								1					
18	**1**							1						
19	**1**							1						
20	**1**							1						
21	**1**							1						
22	**1**										1			
23	**1**											1		
24	**1**						1							
25	**1**										1			
26	**1**												1	
27	**1**		1											
28	**1**								1					
29	**1**							1						
30	**1**							1						
31	**1**													1
Different RAPD groups		6	4	2	1	2	3	13	6	5	5	3	1	1
Number of isolates	**79**	10	5	4	2	6	4	21	10	7	5	3	1	1

4. Discussion and conclusion

The use of RAPD technique is quite convenient especially when there is poor previous knowledge about markers and sequences on the studied organism to apply other techniques (Martinez *et al.*, 2006; Monteleone *et al*., 2006; Valladares *et al.*, 2006; Diaz *et al.*, 2001; Lynch & Milligan, 1994) including fungi (Nagy *et al.*, 2003; Dettman & Kamp, 2001; Santini & Capretti, 2000; Hoegger *et al.*, 1996). To improve the performance and the repeatability of this technique it is advisable to have higher stringency. It is possible to obtain this high performance selecting the RAPD primers that are able to amplify polymorphic fragments at higher annealing temperature (Sitthiphrom *et al.*, 2005; HongYan *et al.*, 2001; Perez-Artes *et al*., 2001; Wolf *et al.*, 1999). In this study the 8 RAPD primers used were selected on a base of 80 different Operon primers and their annealing temperature were 50°C for 4 of them, 49°C for one, 42°C for the remain three of them. This kind of selection allowed a more stable results.
This results are coherent with the common source of introduced isolates in Europe from the American continent about 60 years ago and suggests an occurring of a strong genetic bottleneck event.
The results of the analysis of the Italian population of *S. cardinale* indicated a high level of homogeneity in the population, confirming the findings of Moricca *et al*. (2000) who studied ITS2 region of ribosomal DNA in 18 isolates collected in Europe and Turkey. Considering the number of samples analysed, this study suggests that *S. cardinale* is very homogeneous if not even para-clonal population, having the almost the same genotype in the whole of Italy and probably also throughout Europe. The pathogen, that for the first time was isolate in California in the 1928 (Wagener, 1939), was probably introduced in Europe and in Italy from USA (Graniti, 1998).
The finding that also the isolate from Greece didn't show any significant difference with the Italian populations is emblematic of the high level of genetical homogeneity of this fungus at least in Europe. This situation is even more evident in *S. cardinale*, because the sexual cycle is not known or in any case extremely rare (Graniti, 1998).
The other species of *Seiridium* analysed in this work showed high significant differences each other and they where totally separated from the *S. cardinale* isolates as it was also found by Krokene *et al*. (2004). Similar results were found for several other virulent pathogens when they were introduced in a new environment or/and on the new host (Engelbrecht *et al*., 2004; Ristaino *et al*., 2001; Santini & Capretti, 2000).
In conclusion, despite the fact that would be useful to confirm such results with other molecular markers, it seem quite clear from these preliminary data that *S. cardinale* has a narrow genetic homogeneity at least in the European continent. This fact, in absence of further introductions of higher virulent isolates of *S. cardinale* or other *Seiridium* spp., consents us to be relatively trustful regard the stability of the canker resistance and the sustainability of the future issue on the market of resistant cypress clones.

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