The Genetic Structure of Cypress Canker Fungus in Italy using RAPD and Minisatellite Markers

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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*. Seventy-seven isolates of *Seiridium cardinale* have been collected for the genetic characterization of the North-Italian populations of the fungus. Five *Seiridium* spp. isolates from different countries and different hosts growing in different parts of the world were used for comparison. The structure of the population has been analysed by means of Random Amplified Polymorphic DNAs (RAPDs) and Direct Amplification of Minisatellite-Region DNA (DAMD) PCR marker techniques by using the M13 core sequence. The results indicated a very high level of homogeneity in the North-Italian population of the fungus, whereas a 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 with both type of markers. The isolate from Greece was included in the same group of the Italian isolates. Only the *S. cardinale* from Chile was clustered at significant distance from the other *S. cardinale* isolates from Italy and Greece. The genetic homogeneity of the fungus in Italy suggests that this population has gone through a recent genetic bottleneck, perhaps from the introduction in Europe of few genotypes of the fungus. This supports the hypothesis that the pathogen was introduced to Europe during World War II on infected wood material from the United States. The results are discussed in relation to the introduction and spread of the fungus in Europe.

molecular markers / *Cupressus sempervirens* / *Seiridium cardinale* / population genetics / genetic variability / tree pathogen

Kivonat – A ciprusok kéregrákját okozó gomba genetikai struktúrája RAPD és miniszatellit markerekkel. A *Cupressus* fajokat pusztító *Seiridium cardinale* a múlt század második felében terjedt el Észak Amerikából világszerte, erdőkben, ültetvényekben és díszítő fajtákon. A járvány a mediterrán vidékeken különösen a *C. sempervirens* fajt pusztítja. A gomba észak-olaszországi populációjának jellemzésére hetvenhét *Seiridium cardinale* izolátumot gyűjtöttünk. Összehasonlításra öt, különböző országokból, különböző gazdanövényekről és világrészekről származó *Seiridium* spp. izolátumot használtunk. A populáció szerkezetét RAPD és DAMD PCR marker technikákkal elemeztük az M13 magszekvencia alkalmazásával. Az eredmények a gomba észak-olaszországi populációjában magas fokú homogenitást mutattak, míg más gazdanövények és más fajok esetében bizonyos változékonyság fordult elő. Az észak-olaszországi populációhoz tartozó izolátumok molekuláris összehasonlításban mindkét markertípus esetében nagyon hasonlítottak. A görögországi izolátum is az olasz izolátumok csoportjába tartozott. Egyedül a chilei *S. cardinale* izolátum különbözött szignifikánsan a többi,

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olaszországi és görögországi *S. cardinale* izolátumtól. Az olasz izolátumok genetikai homogenitása arra utal, hogy a gomba nemrég palacknyak-hatáson ment át (egyedlétszáma erősen lecsökkent), valószínűleg néhány genotípusának európai behurcolása óta. Ez alátámasztja azt a hipotézist, hogy a gomba a második világháború során, fertőzött faanyaggal került be Európába az USA-ból. Az eredményeket a gomba európai behurcolása és terjedése vonatkozásában vitatjuk meg.

Molekuláris markerek / *Cupressus sempervirens* / *Seiridium cardinale* / populációgenetika / genetikai változatosság / f a kórokozó

1 INTRODUCTION

The common cypress (*Cupressus sempervirens* L., Cupressaceae) has an important role in the characterization of Mediterranean landscape mainly for its aesthetic functions. This tree species grows under various Mediterranean climates (Emberger et al. 1963), from sea level up to 2000 m or more on a variety of soil types and in a variety of plant association (Zohary 1973).

The cypress is an hardy tree which grows in most environmental conditions although it dislikes severe winter temperatures. Two varieties can be found, var. pyramidalis, the most used as ornamental, and var. horizontalis with its broader more irregular crown.

Cypress has demonstrated to be an excellent pioneer species for reforestation of rocky, argillaceous, limestone, barren and superficial lands. It prevents the hydrogeological erosion and constitutes a source of yield for the good quality of its wood. It is also used like windbreak plant. The cypress., is native to Asia Minor and Persia and part of Greece, but nowadays it grows also along all Mediterranean basin: France, Spain, Portugal, former Yugoslavia and Italy as well as in North Africa (Teissier du Cros et al. 1999) where it was introduced presumably during the Roman era or even before, since the Phoenicians and Etruscans (Santini – Di Lonardo 2000, Giannelli – Bezzini 2002).

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 (Swart 1973, Chou 1989), even though *S. cardinale* seems to be the most dangerous in Europe on *C. sempervirens*.

Great variation exists in the susceptibility of different species of *Cupressaceae* to *S. cardinale* infection (Andreoli 1979, Raddi 1979). Intraspecific variation in resistance has also been reported on *C. sempervirens* (Ponchet – Andreoli 1979, Xenopoulos 1990, 1991), with marked variations within provenances and families from controlled crosses (Graniti 1998).

The low temperatures, that the cypress has often to stand in the Northern area of the species distribution, act indirectly to increase the strength of penetration of *S. cardinale* spores by means of microlesions created by frost (Teissier du Cros et al. 1999). Several cypress improvement programs for resistance to canker and cold were set up with the attempt to cultivate resistant clones throughout wide-reaching territories and areas with highly diverse pedoclimatic conditions (Santini – Di Lonardo 2000, La Porta et al. 2004).

The possibility that, despite its cold susceptibility, future climate changes may render the North of Italy more favourable to cypress cultivation could aid the increasing spread of this arboreal plant on the amenities and encourages studies on the acclimatation of this conifer in the Italian northern regions (La Porta et al. 2004).

Effective exploitation of the cypress genetic resistance sources may enable the replacement of stands damaged by canker and by cold with more resistant selections.

As a matter of fact, so far several clones have patented by the breeding programs carried out in Italy and France in the last 15-20 years (Panconesi 1990, Teissier Du Cros et al. 1991, 1999, Danti et al. 2006) and a new breeding program started in North Italy (La Porta et al. 2004) intend to select clones resistant to canker and to cold. However, a strong effect of environment and of environment by genotype interaction on cypress clones has been noted (Santini et al. 1997, Giannini – Raddi 1992).

In this context it is very important to assess the real genetic structure and variability of the pathogen populations because an hypothetical high variability of the fungus may increase the possible outcome of hypervirulent strains that could compromise the stability of the acquired resistance of the patented clones (Santini et al. 1997). At the same time, an hopefully low variability of the pathogen would make easier the selection work of the strains used for the inoculation tests and more trustable and reliable the selection work.

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 clarify the supposed low genetic variability of *S. cardinale* in Northern Italy 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 (*Table 1*). Seventy-seven isolates of *Seiridium cardinale* were used for the genetic characterization of the North-Italian population, and five *Seiridium* spp. isolates from different countries and different hosts were used for comparison.

This five isolates were included in this study to serve as out-group in the phylogenetic analysis. The North-Italian isolates were obtained from cones or infections of diseased and dead trees.

Cones and infected barks have been placed in humid room to the aim to favour the appearance of reproductive structures of the fungus, the conidia.

Single-spore isolates were cultured in Petri dishes on potato dextrose agar (PDA; Difco Laboratories, Detroit, USA) at 25°C in the dark and maintained in tubes on PDA at 4°C. For DNA extraction, cultures were grown on cellophane disc placed on potato dextrose agar (20 g/liter PDA, 20 g/liter agar; Difco Laboratories, Detroit, USA) in Petri dishes for 15-20 days at 25°C.

Once the mycelia had covered the disc, they were lifted from the cellophane membrane, frozen using liquid N2 and ground in a mortar with liquid N2.

Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in agreement with the indications of the producer.

2.2 DNA amplification

Amplification of fungal DNA was obtained with eight Operon RAPD markers (Operon Kits B, E and P, Operon Technologies, Alameda, USA) with the optimized annealing temperatures: OPP8 at 50°C, OPP6 at 50°C, OPP12 at 49°C, OPP14 at 50°C, OPB11 at 42°C, OPB18 at 42°C, OPE14 at 42°C and OPB10 at 50°C.

Table 1. Seiridium cardinale (79) and other Seiridium spp. (3) isolates analysed in this study.

PROVENANCE	LOCALITY	CODE	LATITUDE	LONGITUDE	FUNGUS	HOST	COLLECTOR
TRENTINO - SOUTH TYROL	ALA	AL1	45°46'06"	11°00'05"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. FRIGIMELICA
	ALA	AL2	45°46'06"	11°00'05"			
	ALA	AL3	45°46'06"	11°00'05"			
	ALA	AL4	45°46'06"	11°00'05"			
	ALA	AL5	45°46'06"	11°00'05"			
	ALA	AL6	45°46'06"	11°00'05"			
	ALA	AL7	45°46'06"	11°00'05"			
	ALA	AL8	45°46'06"	11°00'05"			
	AVIO	AL9	45°44'15"	10°55'56"			
	AVIO	AL10	45°44'15"	10°55'56"			
	ARCO	AR1	44°55'03"	10°52'41"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. FRIGIMELICA
	ARCO	AR2	44°55'03"	10°52'41"			
	ARCO	AR3	44°55'03"	10°52'41"			
	ARCO	AR4	44°55'03"	10°52'41"			
	ARCO	AR5	44°55'03"	10°52'41"			
	MORI	RO1	45°51'09"	10°58'21"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. FRIGIMELICA
	MORI	RO2	45°51'09"	10°58'21"			
	ROVERETO	RO3	45°53'40"	11°01'58"			
	ROVERETO	RO4	45°53'40"	11°01'58"			
	BOLZANO	BZ1	46°29'22"	11°20'39"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. FRIGIMELICA
	BOLZANO	BZ2	46°29'22"	11°20'39"			
	PIANA ROTALIANA	PR1	46°13'17"	11°06'07"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. FRIGIMELICA
	PIANA ROTALIANA	PR2	46°13'17"	11°06'07"			
	PIANA ROTALIANA	PR3	46°13'17"	11°06'07"			
	PIANA ROTALIANA	PR4	46°13'17"	11°06'07"			
	PIANA ROTALIANA	PR5	46°13'17"	11°06'07"			
	PIANA ROTALIANA	PR6	46°13'17"	11°06'07"			
GARDA LAKE	NAGO	RG1	45°53'03"	10°52'55"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. PIVA
	NAGO	RG2	45°53'03"	10°52'55"			
	RIVA DEL GARDA	RG3	45°53'32"	10°50'37"			
	RIVA DEL GARDA	RG4	45°53'32"	10°50'37"			
	GARGNANO	LG1	45°41'48"	10°39'36"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. PIVA
	GARGNANO	LG2	45°41'48"	10°39'36"			
	LIMONE	LG3	45°49'16"	10°47'05"			
	LIMONE	LG4	45°49'16"	10°47'05"			
	PIEVE	LG5	45°46'07"	10°44'21"			
	PIEVE	LG6	45°46'07"	10°44'21"			
	PIEVE	LG7	45°46'07"	10°44'21"			
	PIEVE	LG8	45°46'07"	10°44'21"			
	SIRMIONE	LG9	45°29'56"	10°36'20"			
	SIRMIONE	LG10	45°29'56"	10°36'20"			
	SIRMIONE	LG11	45°29'56"	10°36'20"			
	SIRMIONE	LG12	45°29'56"	10°36'20"			
	SIRMIONE	LG13	45°29'56"	10°36'20"			
	SIRMIONE	LG14	45°29'56"	10°36'20"			
	VALEGGIO SUL MINCIO	LG15	45°21'04"	10°43'54"			
	VALEGGIO SUL MINCIO	LG16	45°21'04"	10°43'54"			
	VALEGGIO SUL MINCIO	LG17	45°21'04"	10°43'54"			
	VALEGGIO SUL MINCIO	LG18	45°21'04"	10°43'54"			
	GARDA	LG19	45°34'53"	10°42'14"			
	MALCESINE	LG20	45°46'23"	10°48'30"			
	SIRMIONE	LG21	45°21'04"	10°43'54"			
LAKE MAGGIORE	VERBANIA	LM1	45°56'10"	8°33'10"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. PIVA
	VERBANIA	LM2	45°56'10"	8°33'10"			
	VERBANIA	LM3	45°56'10"	8°33'10"			
	VERBANIA	LM4	45°56'10"	8°33'10"			
	VERBANIA	LM5	45°56'10"	8°33'10"			
	LAVENO	LM6	45°55'07"	8°36'59"			
	LAVENO	LM7	45°55'07"	8°36'59"			
	LAVENO	LM8	45°55'07"	8°36'59"			
	LAVENO	LM9	45°55'07"	8°36'59"			
	AZZATE	LM10	45°47'02"	8°47'28"			
COMO LAKE	BELLAGIO	LC1	45°59'08"	9°15'22"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. PIVA
	BELLAGIO	LC2	45°59'08"	9°15'22"			
	BELLAGIO	LC3	45°59'08"	9°15'22"			
	DERVIO	LC4	46°04'36"	9°18'08"			
	BELLANO	LC5	46°02'31"	9°17'46"			
	MOLTRASIO	LC6	45°51'59"	9°06'03"			
	MOLTRASIO	LC7	45°51'59"	9°06'03"			
ISEO LAKE	SARNICO	LI1	45°39'53"	9°57'11"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. PIVA
	LOVERE	LI2	45°48'51"	10°04'15"			
	CLUSANE	LI3	45°04'37"	9°59'58"			
	MARONE	LI4	45°44'03"	10°05'22"			
	ISEO	LI5	45°39'14"	10°02'46"			
FRIULI VENEZIA GIULIA	BASSOVIZZA	FR1	45°38'31"	13°51'04"	*S. CARDINALE*	*C. SEMPERVIRENS*	G. FRIGIMELICA
	BASSOVIZZA	FR2	45°38'31"	13°51'04"			
	SAN DORLIGO DELLA VALLE	FR3	45°36'37"	13°51'04"			
GREECE	KOS	GR			*S. CARDINALE*	*C. SEMPERVIRENS*	P. TSOPELAS
CHILE		CI			*S. CARDINALE*	*CUPRESSUS* SP.	A. WINGFIELD
NEW ZEALAND		NZ			*S. CUPRESSI*	*C. MACROCARPA*	S. CHOU
PORTUGAL		P			*S. UNICORNE*	*CUPRESSUS* SP.	A. GRANITI
AUSTRALIA		A			*S. EUCALYPTI*	*EUCALYPTUS DELEGATENSIS*	Z. Q. YUAN

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.

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 structure of the population was analyzed also by use of the core sequence of M13 minisatellite DNA (Innovagen, Lund, Sweden) (Stenlid et al. 1994). Amplification was carried in the above described Mastercycler. The cycling parameters with this primer were: 7 min pre-denaturation at 93°C; 45 cycles of denaturing: at 95°C for 30 s, annealing at 48°C for 30 s; extension 72°C for 30 s; and final extension 72°C for 10 min.

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.

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 and another from minisatellite M13.

Genetic distance was calculated with the GenAlEx 6 software using the formula of Nei's standard genetic distance (Nei 1972, 1978) between pairs of populations. A dendrogram for the RAPD primers was constructed by means of the UPGMA (Unweighted Pair Grouping by Mathematical Averaging) methods using the MEGA3 software (BETA version).

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. Some extra 5 isolates were provided by several other colleagues (P. Capretti, P. Tsopelas, A. Wingfield; *Table 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. An example of the amplificated fragments are shown for RAPDs (*Figure 1*) and DAMDs (*Figure 2*).

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 (*Figure 3*). Only the *S. cardinale* from Chile was clustered at significant distance from the other *S. cardinale* isolates from Italy and Greece. About the other three species of *Seiridium* used as outgroups, all of three species were separated with RAPD primers from the *S. cardinale* strains. However, *S. cupressi* and *S. eucalypti* were closer each other than *S unicorne* with both molecular markers. The results obtained by DAMDs markers substantially confirm the same results observed by RAPDs.

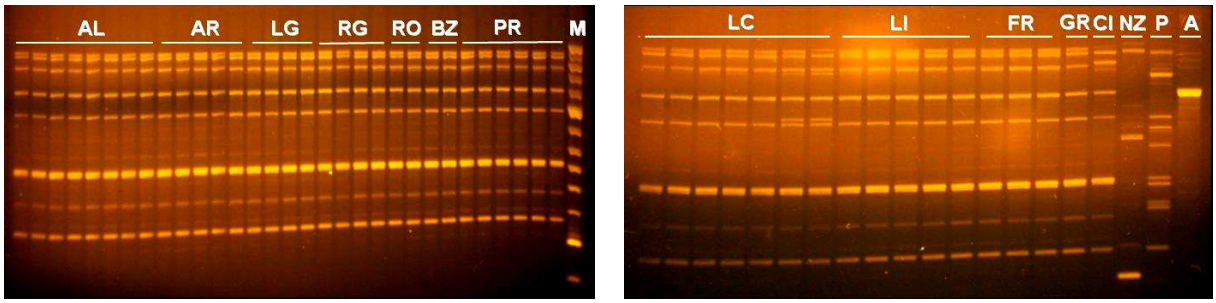

Figure 1. Example of RAPD profiles generated by primers OPP 8 for selected Seiridium spp. isolates. The acronyms of the populations are the following: AL (Ala), AR (Arco), LG (Garda Lake), RG (Riva del Garda), RO (Rovereto), BZ (Bolzano), PR (Piana Rotaliana), LC (Como Lake), LI (Iseo Lake), FR (Friuli Venezia Giulia), GR (Greece), CI (Chile), NZ (New Zealand), P (Portugal), A (Australia).

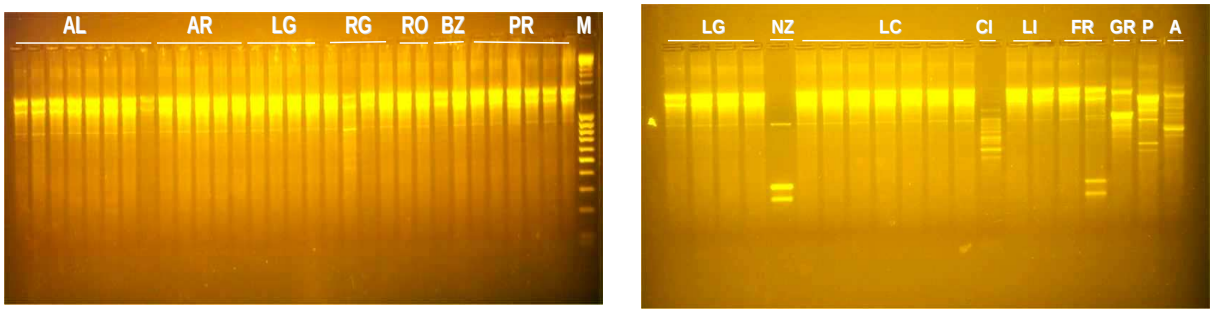

Figure 2. Example of minisatellite profiles generated by primers OPP 8 for selected Seiridium spp. isolates. The acronyms of the populations are the following: AL (Ala), AR (Arco), LG (Garda Lake), RG (Riva del Garda), RO (Rovereto), BZ (Bolzano), PR (Piana Rotaliana), LC (Como Lake), LI (Iseo Lake), FR (Friuli Venezia Giulia), GR (Greece), CI (Chile), NZ (New Zealand), P (Portugal), A (Australia).

4 DISCUSSION AND CONCLUSION

The results of the analysis of the Northen Italian populations of *S. cardinale* indicate a high level of homogeneity among them.

Viljoen et al. (1993) using sequences of ITS genes did not find any difference among and between 12 strains of *Seiridium*: *S. cardinale*, *S. cupressi* and *S. unicorne*, concluding that the three species are synonyms. Also Moricca et al. (2000), who compared by ITS2 of rDNA, didn't find any difference among five strains of Central and Southern Italian *S. cardinale* and with other European ones. Using ß-tubulin and histone sequences, Barnes et al. (2001) found very limited genetic variability among four strains of *S. cardinale*.

Later Krokene et al. (2004) was able to distinguish among the three *Seiridium* species, but no difference was detected among the three *S. cardinale* strains analysed. Regard *S. cardinale*, generally in all these previous works few strains were used in the analysis and even fewer of them were isolated from Italy. Sometimes, same strains were used in different works. A wide collection of strain was never analysed before to figure out definitely the variability among *S. cardinale*.

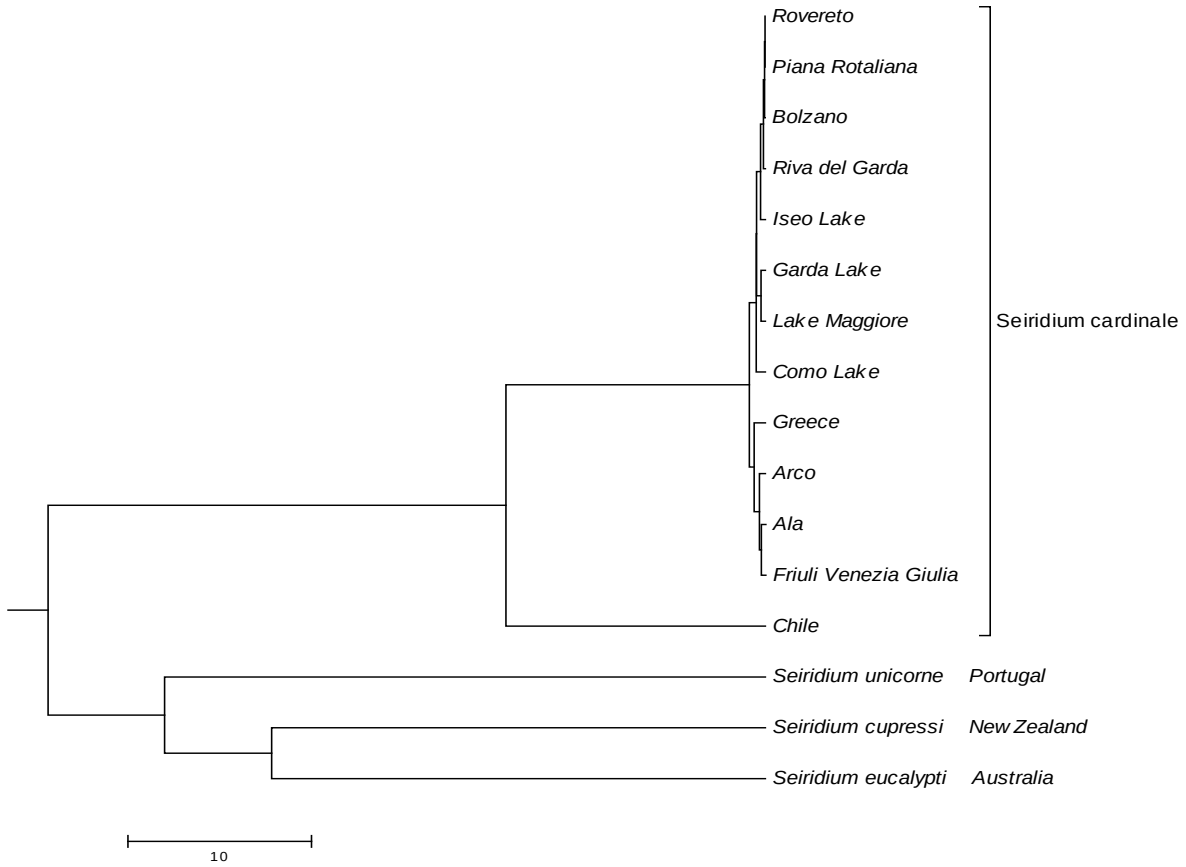

Figure 3. Dendrogram based on the similarity index matrix calculated from analysis of RAPD fragments obtained through the amplification of eight RAPD primers

In view of the extreme susceptibility of *C. sempervirens* to bark canker disease, however, it is also possible that only one or very few close relative strains of the fungus were introduced to Europe. Here they found extremely favorable conditions for their spread without facing an effective selection pressure from the host tree.

Based on our data, different populations of the fungus are morphologically indistinguishable from each other and are generally not separable on the basis of the host provenances they infect.

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 (Lynch – Milligan 1994, Diaz et al. 2001, Martinez et al. 2006, Monteleone et al. 2006, Valladares et al. 2006) including fungi (Hoegger et al. 1996, Santini – Capretti 2000, Dettman – Kamp 2001, Nagy et al. 2003). 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 (Wolf et al. 1999, Perez-Artes et al. 2000, HongYan et al. 2001, Sitthiphrom et al. 2005). In this study the 8 RAPD primers used were selected on a base of 80 different Operon primers and their annealing temperature were 49-50°C for 5 of them, while only for the remain three was 42°C. This kind of selection allowed a more stable results. The small differences osserved among the Italian *S. cardinale* strains were likely barely significant. Even though the preliminary data have to be confirmed by further analyses, the DAMD marker seem to show in this study a perfect similar pattern of amplified fragments

among the Italian strains even without the small minor differences showed by RAPDs. These results between the two markers types are similar with those obtained by other authors (Santini – Capretti 2000, Bhattacharya – Ranade 2001, Sabir 2006).

However, the high degree of similarity in *S. cardinale* is coherent with the common source of introduced isolates in Europe from the American continent about 60 years ago and suggest an occurring of a strong genetic bottleneck event.

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, at present, the sexual cycle is not known and 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 (Santini – Capretti 2000, Ristaino et al. 2001, Engelbrecht et al. 2004).

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 acquired in the breeding programmes and the sustainability of the future issue on the market of resistant cypress clones.

Acknowledgements: The authors wish to thank several colleagues: Prof. Paolo Capretti, Prof. Panaghiotis Tsopelas and Prof. Michael Wingfield to provide foreign isolates. Dr. Corrado Zanini and Dr. Maria Chiara Valgimigli are also acknowledged for the help in the collection of some isolates. The authors are gratefully acknowledging Mr. Emanuel Endrizzi from IASMA for the expert technical assistance and Dr. Gabriella Frigimelica from University of Padua. This work is a part of the project ECOCYPRE (Ecological assessment and sustainable management of cypress in the landscape of Trentino) financially supported by the Provincia Autonoma of Trento with deliberation no. 437 to Dr. Nicola la Porta.

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