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AFLP analyses of California and Mediterranean populations of *Seiridium cardinale* provide insights on its origin, biology and spread pathways

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Summary

Seiridium cardinale is regarded as the most important agent responsible for the disease of cupressaceous hosts referred to as Cypress canker. The fungus was first described in California and is currently reported in all continents. A recent study based on seven SSR loci has suggested that California populations may represent the source of the epidemic in the Mediterranean. In this study, 185 AFLP markers were used on an expanded sample size of 125 isolates to determine whether the Mediterranean population may indeed be derived from the California one and to compare the reproductive modes of populations in the two regions of the world. Additionally, AMOVA, NJ, STRUCTURE, principal component analysis and β-tubulin sequence analyses were employed to infer the presence of genetic structure within and between populations. The distribution of pairwise AFLP similarity coefficients suggests Mediterranean populations are reproducing only clonally, while California populations are reproducing both clonally and sexually. AMOVA indicates Mediterranean and California populations are currently genetically isolated, but NJ and STRUCTURE analyses both suggest ancestral Mediterranean genotypes belong to the California population. No alleles were private to either population, and the presence of identical or quasi-identical genotypes at large distances supports the notion that movement of infected cypress plants is responsible for the global spread of the disease. Surprisingly, STRUCTURE identified a second cluster of Mediterranean genotypes distinct from the basal mixed California–Mediterranean cluster. This second cluster either may have originated from the first one under the selection pressure imposed on the pathogen in the new Mediterranean environments or may be the result of a further introduction from California or elsewhere. Cumulatively, the evidence presented here suggests that *S. cardinale* may be native or long naturalized in California and that two genetically distinct groups are present in the Mediterranean, with obvious implications for further studies on this disease.

1 Introduction

Cupressus species are present worldwide in the temperate zone, playing an important ecological role in natural ecosystems. Many *Cupressus* species are widely cultivated and used in reforestation efforts to provide protection from soil erosion, to produce timber and for ornamental purposes (Della Rocca and Danti 2007). In the Mediterranean area, Italian cypress (*Cupressus sempervirens* L.) is a keystone species for all of the above reasons and holds a special cultural and symbolic meaning (Xenopoulos et al. 1990). An intense trade of cypresses has occurred across continents during the last few decades, with more than 5 million plants produced every year in European nurseries alone (Pichot et al. 1999). Some cypress species are endangered or threatened (IUCN 2011) and are only represented by fragmented relic populations often found in inaccessible areas. The protection of such endangered species is an obvious priority from a conservation perspective with significant consequences for the maintenance of regional biodiversity. Other *Cupressus* species, including *Cupressus macrocarpa* Hartw. ex Gordon and *C. sempervirens*, are valued ornamental trees in the landscape industry and are often considered key elements that define the character and the beauty of the scenery (Xenopoulos et al. 1990; Pozzana 1991).

Cypress canker is regarded as one of the most serious threats to the survival of cypress trees worldwide (Graniti 1998). Three different species of *Seiridium* are known as causal agents of Cypress canker: *Seiridium cardinale* (Wagner) Sutton and Gibson, *Seiridium unicorne* (Cooke and Ellis) Sutton and *Seiridium cupressi* (Guba) Boesewinkel (Graniti 1986). *Seiridium cardinale* is the most aggressive and widespread among the three, and in a recent survey in California, *S. cardinale* was isolated from all but one of surveyed cankers (Della Rocca et al. 2011a). This mitosporic coelomycete was described for the first time in California on *C. macrocarpa* (Wagener 1928, 1939) and was subsequently reported in Oceania (Birch 1933), Europe (Barthelet and Vinot 1944), South America (1947 in Mujica et al. 1980) and Africa (Faddoul 1973). Despite the fact that the first official description of *S. cardinale* was made in 1928, the field notebook of W.L. Jepson describes a disease with symptoms perfectly matching those of cypress canker on Monterey cypresses planted outside their natural range in the California delta in 1900, and cites reports of diebacks occurring as early as 1885 in areas where *C. macrocarpa* was planted and not native (field notebook of W. L. Jepson, 16 May 1900, available at http://ucjeps.berkeley.edu/cgi-bin/read_fb.pl?page_no=3_101).

Seiridium cardinale reproduces asexually wherever it is found. Additionally, Hansen (1956) partially described a perfect stage of the fungus on an old *C. macrocarpa* branch in California, and Della Rocca et al. (2011b) have recently shown California populations lack linkage disequilibrium (LD) at seven SSR markers, suggesting that – aside the well-known mitotic reproductive mode – a sexual reproductive mode may be probable in this US state. In the same study, Della Rocca et al. (2011b) showed that Mediterranean populations of the pathogen could be traced to two distinct introduction events, one in Central

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Italy responsible for the widespread epidemic in the Mediterranean and one limited to Morocco. Both introductions appeared to have been founded by genotypes very closely related to California genotypes. Identical genotypes could not be found between the Mediterranean and California, but many identical genotypes were found in locations over 1000 km apart both in California and in the Mediterranean, indicating a likely human involvement in the spread of the disease. Trade of *C. macrocarpa* in the 1920s between North America and Europe and trade of *C. sempervirens* between Central Italy and many Mediterranean locations starting in the mid-1900s represent likely pathways for the long-distance spread of the pathogen. The fungus can colonize both branches (Panconesi 1990) and cones (Roques and Battisti 1999), thus facilitating its spread independently of the propagation method used. The presence of highly susceptible hosts and climatic conditions conducive to the reproduction of the pathogen have lead to an epidemic in the Mediterranean region, affecting trees both in plantations and in the wild (Solel et al. 1983; Graniti 1986; Raddi et al. 1987; Luisi 1990).

Although the work by Della Rocca et al. (2011b) provided a convincing scenario with regards to a California source and spread pathways of *S. cardinale*, alternative explanations of the data are possible, including a convergent evolution of genotypes introduced from a third location to both California and the Mediterranean. If that were the case, the origin of the pathogen may be in a region different than the US West Coast. The main aim of this study was to use a larger number of AFLP markers on an expanded number of isolates to confirm or refute the introduction scenario favoured by Della Rocca et al. (2011b). A second aim was to determine whether a larger number of variable genetic markers would allow the identification of any meaningful structure within California and/or within the Mediterranean region. The only hint of within-region structure presented by Della Rocca et al. (2011b) was with regards to the presence of two β-tubulin alleles in California as opposed to a single allele anywhere else in the world, but the meaning of this was not further investigated. AFLPs were selected because they generate large numbers of reproducible nuclear markers without the need for any prior knowledge regarding the genome of the organism (Vos et al. 1995), and they are particularly well suited for the study of haploid fungi because there is no loss of genetic information caused by their dominant nature (Moyano et al. 2003). AFLPs have been previously employed for the study of many fungal pathogens (Ivors et al. 2004; Pei et al. 2007; Linzer et al. 2009;), but this is the first time this technique has been used to elucidate the population structure of *S. cardinale*. All analyses performed in this study were different than those used in Della Rocca et al. (2011b) to provide an alternative and independent testing of the hypotheses presented in that study.

2 Materials and methods

2.1 Fungal isolates and culture conditions

One hundred and twenty-five isolates of *Seiridium cardinale*, representing a broad range of geographic locations and hosts from California and the Mediterranean basin, were used for this study (Table 1). The isolates were collected from eight different hosts (*C. sempervirens, Cupressus macnabiana* Murray*, Cupressus dupreziana* Camus*, C. macrocarpa,* ×*Cupressocyparis leylandii* Dallim.*, Juniperus chinensis* L.*, Juniperus phoenicea* L. and *Juniperus oxycedrus* L.) in eight geographical areas (Italy, France, Greece, Algeria, Morocco, Portugal, Spain and California). Two isolates of *S. cupressi* were included as outgroups (Table 1). Isolates were grown on potato dextrose agar (PDA) (Difco, BD, Detroit, MI, USA) and incubated at 25°C for 10 days. A plug from the edge of the colony produced by each isolate was transferred to PDA covered with a cellophane membrane. Subcultures were incubated at 25°C for 15 days before scraping the mycelium off the cellophane with a sterile spatula. Harvested mycelia were lyophilized as previously described (Della Rocca et al. 2011b).

2.2 DNA extraction

A total of 20 mg of lyophilized mycelium of each isolate was ground to a fine powder with a Tissue Lyser (Qiagen, Hilden, Germany). Total genomic DNA was extracted using the mi-Plant Genomic DNA Isolation Kit (Metabion, Martinsried, Germany), following the manufacturer's instructions. DNA was quantified by a Biophotometer (Eppendorf AG, Hamburg, Germany) before being stored at −20°C.

2.3 Sequence analysis

The β-tubulin locus was amplified for 19 isolates (18 from the Mediterranean and 1 from California) (Table 1) using primers Bt1a and Bt1b as previously described (Della Rocca et al. 2011b). Amplicons were sequenced using an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were edited and aligned with MEGA version 4 (Tamura et al. 2007) and compared with sequence accessions in GenBank using NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/) to determine species identity and cluster (A or B) of the isolates as described in Della Rocca et al. (2011b).

2.4 AFLP analysis

AFLP analysis was carried out as described by Vos et al. (1995) with the slight modifications described by Fanci et al. (2009) and Donnarumma et al. (2010). Briefly, purified DNA (80 ng) was digested using endonucleases *Eco*RI and *Mse*I (Fermentas, Glen Burnie, MD, Canada) simultaneously, followed by ligating *Eco*RI and *Mse*I adapters to the restriction fragments using T4 DNA ligase (Fermentas). Following ligation, DNA was amplified by PCR using the labelled *Eco*RI primer (6-carboxyfluorescein-5′-GACTGCGTACCAATTCA) and the *Mse*I primer (5′-GATGAGTCCTGAGTAAG), with a selective A

Table 1. Summary of *Seiridium cardinale* isolates.

Isolate	Host	Provenance	Region	Country	B-tubulin haplotype[1]	GenBank accession
Mediterranean population						
SC37	*Cupress\us macrocarpa*	Rosignano	Tuscany	Italy		
SC53	*Cupressus sempervirens*	Troina	Sicily	Italy	A^1	JQ669787
SC57	*C. sempervirens*	Bastia	Corse	France		
SC58	*C. sempervirens*	Vagliagli	Tuscany	Italy		
SC61	*C. sempervirens*	Fonterutoli	Tuscany	Italy		
SC64	*C. sempervirens*	Guarniente	Tuscany	Italy		
SC67	*C. sempervirens*	Salutio	Tuscany	Italy		
SC69	*C. sempervirens*	Lucignano	Tuscany	Italy		
SC73	*C. sempervirens*	Trasimeno	Umbria	Italy		
SC76	*xCupressocyparis leylandi*	Calenzano	Tuscany	Italy		
SC80	*C. sempervirens*	Calenzano	Tuscany	Italy		
SC84	*C. sempervirens*	Sesto Fiorentino	Tuscany	Italy		
SC88	×*C. leylandi*	Lastra a Signa	Tuscany	Italy		
SC92	*C. sempervirens*	Corte	Corse	France		
SC94	*C. sempervirens*	Galliano	Tuscany	Italy		
SC103	*C. sempervirens*	Roselle	Tuscany	Italy		
SC104	*C. sempervirens*	Serpiolle	Tuscany	Italy		
SC105	*C. sempervirens*	Comiso	Sicily	Italy		
SC106	*C. sempervirens*	Poggio alle mura	Tuscany	Italy	A^1	JQ669782
SC109	*C. macrocarpa*	Santa Rita	Tuscany	Italy		
SC114	*C. sempervirens*	Poggio d'Acona	Tuscany	Italy		
SC115	*C. sempervirens*	Gargiano	Tuscany	Italy		
SC124	*C. sempervirens*	Sassari	Sardinia	Italy		
SC134	*C. sempervirens*	Monte Cavolo	Emilia Romagna	Italy		
SC147	*C. sempervirens*	Var		France		
SC148	*C. sempervirens*	Rouet A		France		
SC149	*C. sempervirens*	Tripolis 2		Greece		
SC150	*C. sempervirens*	16 S Omalos	Crete	Greece		
SC154	*C. sempervirens*	Fès – Ifrane	Fès-Boulmane	Morocco	A	HQ678145
SC155	×*C. leylandii*	Osmannoro	Tuscany	Italy		
SC156	*C. sempervirens*	Ala	Trentino	Italy		
SC157	*C. sempervirens*	Mori	Trentino	Italy	A	HQ678146
SC158	*C. sempervirens*	Mezzocorona	Trentino	Italy	A^1	JQ669789
SC159	*C. sempervirens*	Cannara	Umbria	Italy		
SC160	*C. sempervirens*	S. Marino		S. Marino		
SC162	*C. sempervirens*	Forci 1	Tuscany	Italy		
SC164	*C. sempervirens*	Carrara 2	Tuscany	Italy	A	HQ678147
SC167	*C. sempervirens*	Bolgheri	Tuscany	Italy	A^1	JQ669790
SC168	*C. sempervirens*	S. Miniato	Tuscany	Italy		
SC169	*C. sempervirens*	Montegiovi	Tuscany	Italy		
SC170	*C. sempervirens*	Saturnia	Tuscany	Italy	A^1	JQ669791
SC172	*C. sempervirens*	Arcidosso	Tuscany	Italy		
SC175	*C. macrocarpa*	La Sterza	Tuscany	Italy		
SC176	*C. sempervirens*	Frejus	PACA	France		
SC177	*C. sempervirens*	Volterrana	Tuscany	Italy		
SC178	*C. sempervirens*	S. Gimignano	Tuscany	Italy		
SC179	*C. sempervirens*	Larderello	Tuscany	Italy		
SC181	*C. sempervirens*	Frejus	PACA	France		
SC191	*C. sempervirens*	Kaliani	Peloponnese	Greece		
SC192	*Juniperus phoenicea*	Lefkada	W-Greece	Greece		
SC193	*C. sempervirens*	M. Alexandros	W-Greece	Greece	A^1	JQ669792
SC194	*C. sempervirens*	Spanochori	W-Greece	Greece		
SC195	*C. sempervirens*	Syronta	W-Greece	Greece		
SC196	*C. sempervirens*	Hortata	W-Greece	Greece		
SC197	*C. sempervirens*	Nydri	W-Greece	Greece		
SC249	*C. sempervirens*	Az. Platamona	Sardinia	Italy		
SC250	*C. sempervirens*	Porta Coeli, Naquera	Com. Valenciana	Spain	A^1	JQ669793
SC253	×*C. leylandii*	Imperia Ovest	Liguria	Italy		
SC281	*C.sempervirens*	Imperia Ovest	Liguria	Italy	A^1	JQ669794
SC291	*C. sempervirens*	Bellaria	Emilia Romagna	Italy		

Table 1. Continued

Isolate	Host	Provenance	Region	Country	B-tubulin haplotype[1]	GenBank accession
SC314	*C. sempervirens*	Megali Panagia	Peloponnese	Greece	A[1]	JQ669795
SC316	*C. sempervirens*	Megali Panagia	Peloponnese	Greece		
SC319	*C. sempervirens*	Kalimani	Peloponnese	Greece	A	HQ678149
SC322	*C. sempervirens*	Kalimani	Peloponnese	Greece		
SC323	*C. sempervirens*	Kritharion	Peloponnese	Greece		
SC325	*C. sempervirens*	Kritharion	Peloponnese	Greece		
SC327	*C. sempervirens*	Nea Makri	Attica	Greece		
SC333	*C. sempervirens*	Alepochori	Peloponnese	Greece		
SC334	*C. sempervirens*	Alepochori	Peloponnese	Greece		
SC335	*C. sempervirens*	Nipos	Crete	Greece		
SC336	*C. sempervirens*	Nipos	Crete	Greece	A[1]	JQ669796
SC344	*C. sempervirens*	Agrokepio	Crete	Greece		
SC345	*C. sempervirens*	Agrokepio	Crete	Greece		
SC346	*J. oxycedrus*	Korinthia	Peloponnese	Greece		
SC347	*C. sempervirens*	Asea		Greece		
SC348	*C. sempervirens*	Asea		Greece		
SC354	*C. sempervirens*	Askyfou	Crete	Greece		
SC355	*C. sempervirens*	Askyfou	Crete	Greece		
SC356	*C. sempervirens*	Zourva	Crete	Greece		
SC357	*C. sempervirens*	Zourva	Crete	Greece	A[1]	JQ669797
SC359	*C. sempervirens*	Kaliani	Peloponnese	Greece		
SC363	*C. sempervirens*	Marmari	Central Greece	Greece		
SC364	*C. sempervirens*	Marmari	Central Greece	Greece		
SC366	*C. sempervirens*	Karystos	Central Greece	Greece		
SC367	*C. sempervirens*	Karystos Livadaki	Central Greece	Greece		
SC368	*C. sempervirens*	Karystos	Central Greece	Greece		
SC369	*C. sempervirens*	Karystos Livadaki	Central Greece	Greece	A[1]	JQ669798
SC370	*C. sempervirens*	Kopanaki	Peloponnese	Greece		
SC375	*C. sempervirens*	Fres	Crete	Greece		
SC376	*C. sempervirens*	Fres	Crete	Greece		
SC402	*C. sempervirens*	ATCC–Firenze	Tuscany	Greece		
SC403	*C. sempervirens*	Nea Makri	Attica	Greece		
SC404	*C. sempervirens*	M. Athos	Tracia	Greece		
SC430	*C. sempervirens*	Algieris	Alger	Algeria	A[1]	JQ669799
SC431	*C. sempervirens*	Algieris	Alger	Algeria		
SC502	×*C. leylandii*	Ponte a Ema	Tuscany	Italy	A[1]	JQ669784
SC509	*C. sempervirens*	Bainem		Algeria		
SC510	*C. macrocarpa*	Ain Abbassa setif		Algeria	A[1]	JQ669785
SC511	*C. dupreziana*	Beraki		Algeria		
SC512	*C. dupreziana*	Baunem		Algeria	A[1]	JQ669786
SC513	*C. sempervirens*	Lisbona		Portugal	A[1]	JQ669783
SC514	*C. sempervirens*	Lisbona		Portugal		
Californian population						
SC475	*C. macnabiana*	Berkeley	Alameda	California (Cal) USA	A	HQ678150
SC476	*C. macrocarpa*	Berkeley Marina	Alameda	Cal USA	B	HQ678151
SC477	*C. macrocarpa*	Berkeley Marina	Alameda	Cal USA	A[1]	JQ669788
SC478	×*C. leylandii*	Lodi	San Joaquin	Cal USA		
SC479	×*C. leylandii*	Lodi	San Joaquin	Cal USA	A	HQ678152
SC480	*Juniperus chinensis*	Lodi	San Joaquin	Cal USA	A	HQ678153
SC481	×*C. leylandii*	Lodi	San Joaquin	Cal USA	A	HQ678154
SC482	×*C. leylandii*	Lathrop	San Joaquin	Cal USA	B	HQ678155
SC483	*C. sempervirens*	Lafayette	Contra Costa	Cal USA	B	HQ678156
SC484	×*C. leylandii*	Lafayette	Contra Costa	Cal USA	A	HQ678157
SC485	*C. macrocarpa*	Berkeley	Alameda	Cal USA	B	HQ678158
SC486	×*C. leylandii*	San Diego	San Diego	Cal USA	B	HQ678159
SC487	*C. macrocarpa*	San Diego	San Diego	Cal USA	B	HQ678160
SC488	*C. macrocarpa*	Millbrae	S. Matteo	Cal USA	B	HQ678161
SC489	×*C. leylandii*	Millbrae	S. Matteo	Cal USA	B	HQ678162
SC490	×*C. leylandii*	Hillsborogh	S. Matteo	Cal USA	B	HQ678163
SC491	*C. macrocarpa*	Millbrae	S. Matteo	Cal USA	B	HQ678164
SC492	×*C. leylandii*	Ukiah	Mendocino	Cal USA	A	HQ678165
SC494	×*C. leylandii*	Novato	Marin	Cal USA	B	HQ678166
SC495	×*C. leylandii*	S. Marine	Marin	Cal USA	A	HQ678167
SC497	×*C. leylandii*	Knoll Forest	Marin	Cal USA	A	HQ678168
SC498	×*C. leylandii*	Calistoga	Napa	Cal USA	B	HQ678169

Table 1. Continued

Isolate	Host	Provenance	Region	Country	B-tubulin haplotype[1]	GenBank accession
SC499	*C. sempervirens*	Tiburon	Marin	Cal USA	B	HQ678170
Outgroups						
SCUP1 1567		Bari		Italy		
SCUP2 CBS 122616				Greece		

[1]Isolates used for β-tubulin analysis sequenced in this study, chosen to represent the various subclades identified by the NJ tree.

and G nucleotides at the 3′ end, respectively. Each reaction mixture contained 4 ng DNA, DreamTaq buffer 1× (with 20 mM $MgCl_2$), 0.2 mM dNTP, 0.2 pmols of primer *Eco*RI, 0.6 pmols of primer *Mse*I and 1.5 U of DreamTaq DNA polymerase (Fermentas). PCR was performed in a Mastercycler EP Gradient Thermocycler (Eppendorf AG), with the following cycling profile: 94°C for 30 s, 65°C for 30 s and 72°C for 1 min for 12 cycles, followed by 23 cycles of 94°C for 30 s, 56°C for 30 s and 72°C for 1 min.

Amplified fragments were separated by capillary electrophoresis on an ABI 310 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA), and fragment size was determined using a 50–400-bp internal standard (Rox 400HD). Only fragments between 75 and 300 bp in size were considered, and only unambiguous fragments reaching a threshold intensity of 100 were scored. Bands that were absent in some of the replicates of the same isolate were not considered. Fragment profiles were analysed using GeneMapper 4.0 (Applied Biosystems). All bands were confirmed by a visual check, and a binary data matrix of presence (1) or absence (0) of bands of each size by isolate was generated. AFLPs of isolate ATCC 38654 were produced in five replicates to differentiate between experimental variation due to inconsistent amplification of fragments and true polymorphisms.

2.5 Data analysis

Genetic similarity calculations, NJ tree building and bootstrapping were conducted using TreeCon (Van de Peer and De Wachter 1993, 1994). Genetic similarity measures were calculated using the formula of Link et al. (1995), which calculates distances as the complement of Jaccard's similarity coefficient (S_J). Because the AFLP profiles of five replicates of isolate ATCC 38654 were slightly different, pairwise S_J values obtained from this isolate generated a normal distribution. A prediction interval (PI) was then calculated using a parametric formula in JMP (SAS Institute, Cary, NC, USA), with alpha set at 0.05. The neighbour-joining (NJ) tree was rooted using the two *S. cupressi* isolates, and 1000 bootstrap replicates were performed to assess cluster support. Genotypic diversity (G) and clonal genotype diversity (R) for Mediterranean (excluding Morocco), Californian and whole *S. cardinale* populations were calculated following Stoddart and Taylor (1988) and Mascheretti et al. (2009), respectively. The Shannon's index of genotypic diversity ($I = -1^* (p^*\text{Ln}(p) + q^*\text{Ln}(q))$, the haplotype diversity index ($h = 1 - (p^2 + q^2)$) and the unbiased diversity index ($hu = (N/(N-1))^*h$) were all calculated using the formulas for dominant markers and haploid organisms in GenAlex 6.4. Additionally, rarefaction was used in ADZE1.0 (http://www.stanford.edu/group/rosenberglab/adze.html) to calculate band richness of Californian and Mediterranean *S. cardinale* populations, in a standardized sample size equal to that of the least sampled population (Petit et al. 1998). Distributions of pairwise S_J measures were visualized independently for California and Mediterranean populations to identify the presence of uni- or bimodal distribution patterns, considered to be indicative of single or dual reproductive modes, respectively.

The data set was clone-corrected by removing duplicate samples with multilocus genotypes regarded as identical based on S_J values: the final data set consisted of 105 distinct haploid samples. Bayesian population assignment tests were conducted using STRUCTURE 2.3.3 (Pritchard et al. 2000). Because sexual recombination has been inferred for the California population but not for the European population (Della Rocca et al. 2011b; this article), separate analyses were conducted on the entire sample using both the admixture and non-admixture models in STRUCTURE. Estimation of the number of populations (K) was conducted using three replicate exploratory runs at each level of K. Each run included 10 000 iterations with the first 1000 discarded as burn-in. Both the admixture and non-admixture analyses used the independent allele frequencies model, with lambda set at the program default of 1.0 for exploratory analyses. The optimal level of K was determined by calculating Ln $P(D)$ values for each exploratory run and calculating the delta K statistic of Evanno et al. (2005) using CorrSieve 1.6-5 (Campana et al. 2011). Once the optimal level of K was determined, three replicate exploratory runs were conducted as described above, except that lambda was inferred within STRUCTURE for each run (using a starting value of 1.0). The value of lambda inferred from these runs was used to select a value of lambda for the final program run consisting of 100 000 iterations, the first 10 000 discarded as burn-in. Chain convergence was assessed by examining the output graphs of alpha *vs.* program run number provided by STRUCTURE.

A principal component analysis (PCA) was performed on the AFLP profile data using STATISTICA 7.0® software; centroids and standard deviations (SD) of each β-tubulin group were calculated on factor 1 and 2 coordinates. Analysis of molecular variance (AMOVA) was performed using ARLEQUIN version 3.1 (Excoffier et al. 1992; Excoffier et al. 2005) with populations from the Mediterranean and California regarded as distinct.

3 Results

3.1 AFLP analysis of *Seiridium cardinale* populations

A total of 185 AFLP unambiguous bands were scored, 144 of which (77.84%) were polymorphic. No AFLP bands were private to either the Mediterranean or Californian population. Pairwise S_J values for the entire sample ranged from 0.4586 to 0.9798 (mean 0.6887 ± 0.0953 SD). Pairwise S_J values for five replicates of isolate ATCC 38654 ranged between 0.9029 and 0.9899, resulting in a mean 95% PI ranging between 0.94 and 0.96 for identical isolates within the entire sample. Thus, all isolates with a S_J equal to or >0.94 were considered to be identical, and differences were attributed to procedural error. Pairs of isolates with S_J values >0.94 were found mostly either within Greece, Italy, in Italy and Greece, or in Italy and Spain (Table 2). Although AFLPs identified 111 different genotypes using the lower limit of the 95% PI, if the upper limit is used, the number of distinct genotypes is 119. The Stoddart and Taylor's index (G) and the clonal genotype diversity (R) were calculated using both PI limits of S_J values from replicates of ATCC 38654 to define identical genotypes, but results were not significantly different using either PI value (Table 3). G indicated that genotypic diversity was high within the Mediterranean population, but when R was calculated, both indices indicated that only the California population was made up of all different genotypes. Shannon's and the two haplotype diversity indices were almost identical for California and the Mediterranean. Band richness values (Br) for Californian and Mediterranean populations were also almost identical (Table 3).

S_J values for Mediterranean populations ranged from 0.4586 to 0.9798 (range 0.5212; mean 0.7230 ± 0.0911 SD) with the largest pairwise distance being between isolates SC181 (Southern France) and SC154 (Morocco). The distribution of S_J for the Mediterranean population is unimodal with the maximum falling between 0.676 and 0.725 (Fig. 1). S_J values for California samples ranged between 0.5748 and 0.9223 (range 0.3475; mean 0.7136 ± 0.0815 SD). The largest S_J pairwise distance was between isolates SC479 (Lodi, San Joaquin County) and SC492 (Ukiah, Mendocino County). The distribution of S_J pairwise values in the Californian population was bimodal with two maxima, the first between 0.626 and 0.675 and the second between 0.776 and 0.825 (Fig. 1). The mean pairwise S_J value calculated within California (S_{cal}) was not different from the mean pairwise S_J value calculated for the Mediterranean (S_{med}); however, S_{tot} (mean 0.689 ± 0.095 SD) was significantly smaller than both S_{cal} and $S_{med.}$ ($F = 210.5$, $p < 0.01$).

AMOVA results showed that 70.23% of the genetic variation was partitioned within *S. cardinale* populations, while 29.77% was partitioned between Mediterranean and Californian populations. Both values were significant at p < 0.01. The NJ tree shows all *S. cardinale* isolates as a monophyletic group with a 100% bootstrap value (Fig. 2). Within the large and complex *S. cardinale* clade, California isolates are in four of five adjacent basal subclades, with at least one subclade containing both Mediterranean and California isolates (Fig. 2). All other distal subclades include only Mediterranean isolates. Bootstrap support was low for the nodes of all major clades within *S. cardinale*, except for two (one in California and one in the Mediterranean). A few isolates were grouped in small distal clades within the larger ones, with good bootstrap support. Moroccan isolates were in an intermediate position between the major California clades and the outgroup isolates (Fig. 2).

Table 2. Pairs of *Seiridium cardinale* isolates having a S_J > 0.94, and thus considered as representatives of the same genotype.

Couples of isolates	Provenance
SC192–SC336	Islands Greece–Islands Greece
SC367–SC178	Islands Greece– Central Italy
SC124–SC359	Central Italy (Sardinia)–Peloponnese Greece
SC314–SC191	Peloponnese Greece–Peloponnese Greece
SC314–SC359	Peloponnese Greece–Peloponnese Greece
SC73–SC250	Central Italy–Spain
SC164–SC291	Central Italy–Northern Italy
SC336–SC333	Islands Greece–Peloponnese Greece
SC359–SC336	Peloponnese Greece–Islands Greece
SC323–SC322	Peloponnese Greece–Peloponnese Greece
SC323–SC191	Peloponnese Greece–Peloponnese Greece
SC323–SC359	Peloponnese Greece–Peloponnese Greece
SC175–SC162	Central Italy–Central Italy
SC124–SC325	Central Italy (Sardinia)–Peloponnese Greece

Table 3. Indices of genetic diversity calculated using the lower and upper limit of the 95% prediction interval of S_J obtained for five replicates of the same isolate to identify identical genotypes: R = clonal genotype diversity, G = Stoddart and Taylor's index. I = Shannon's index, h = haplotype diversity index, hu = unbiased diversity, Br = band richness in a standardized sample size equal to the smallest population. *I, h,* hu and Br were all calculated using the binary AFLP data; Br was calculated using rarefaction.

Population	R_{lower}	R_{upper}	G_{lower}	G_{upper}	I	h	hu	Br
All	0.887	0.952	93.563	109.266	–	–	–	–
California	1	1	23	23	0.262	0.171	0.179	1.557
Mediterranean	0.861	0.941	72.250	86.700	0.274	0.172	0.173	1.602

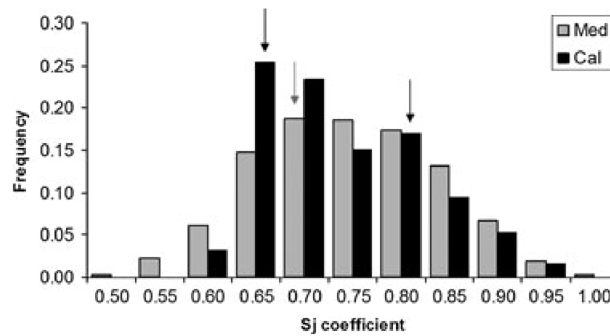

Fig. 1. Distributions of pairwise Jaccard coefficient of similarity (S_J) calculated using AFLP data within Mediterranean and Californian *Seiridium cardinale* populations. Frequency histograms show the distributions of pairwise S_J coefficients for 102 Mediterranean (n = 5151) and 23 California (n = 253) isolates. Arrows point to the maxima for the bimodal California (Cal) and for the unimodal Mediterranean (Med) distributions.

Comparison of delta K values using both genome admixing and non-admixing models in STRUCTURE strongly supported selection of $K = 2$ as the optimal number of populations. For the admixture model, $K = 2$ corresponded to a delta K value of 364.273, over an order of magnitude higher than next highest value (28.920 for $K = 7$). For the non-admixture model, $K = 2$ corresponded to a delta K value of 1.169, compared to a next highest value of 15.011 (for $K = 3$). The estimated lambda value for the admixture model was 0.3, and for the non-admixture model, 0.5. Results of both models were highly congruent (Fig. 3). Using the admixing genome model, Californian isolates and a number of Mediterranean isolates appeared as being admixtures with a prevalence of one genome over the other. These same isolates appeared as representative of a single cluster (Cluster 1) in the non-admixing model, while all remaining Mediterranean isolates clearly belonged to the genome under-represented in admixtures (using the genome admixing model) and to a second cluster (Cluster 2) using the non-admixing model (Fig. 3). Cluster 1 isolates (or admixed genotypes in the genome mixing model) were all basal to Cluster 2 isolates in the NJ tree (Fig. 2).

Irrespective of STRUCTURE cluster, all 18 Mediterranean isolates sequenced in this study had a β-tubulin sequence (haplotype A) identical to the one found in all Mediterranean isolates sequenced in a previous study (Della Rocca et al. 2011b). Isolate SC477 from California was found in the same clade as several Mediterranean isolates and also belonged to β-tubulin haplotype A. The PCA of the California sample showed that haplotype A isolates from California clustered separately from haplotype B isolates, and two isolates from Southern California clustered very tightly with Northern California isolates (Fig. 4). No significant clustering was evident among Mediterranean isolates, all belonging to β-tubulin haplotype A.

4 Discussion

The genetic analyses presented in this study are based on a much larger number of markers than those previously utilized (Della Rocca et al. 2011b), and their results not only support previous findings, but also highlight novel interesting details on the recent history and biology of the Cypress canker fungus. The large number of AFLPs used in this study revealed a greater amount of genetic variability than that detected with seven SSRs in a previous study (Della Rocca et al. 2011b), thus increasing our ability to infer pathways of spread of the pathogen and relationships among groups of isolates. The genotypic diversity detected in the Mediterranean population is higher than that previously reported using seven SSR markers (Della Rocca et al. 2011b); this result is not surprising given the number and the variability of the AFLP markers employed in this study. In the Mediterranean, the fungus appears to be reproducing only clonally (Graniti 1998; Della Rocca et al. 2011b; this article): mutations or mitotic recombination events are likely to occur frequently and to accumulate in the absence of sexual reproduction. Additionally, the ongoing epidemic in the Mediterranean is in contrast to the limited outbreaks currently present in California, and polymorphisms are likely to accumulate at a relatively fast rate in the very large Mediterranean population. Because sampling was extensive and isolates came from distant sites, it is not surprising that most Mediterranean genotypes presented some differences in AFLP profiles. However, despite the extensive sampling scheme, identical genotypes were only found in the Mediterranean and never in California. This difference in genotypic richness is exemplified by values of the R index indicating a relative greater genetic diversity in California. The Shannon index and the haplotype diversity indices show the California and the Mediterranean populations to be comparably diversified. Rarefaction determined that band richness was identical in the two world regions; hence, the observed difference in genotypic diversity cannot be attributed to biases in amplification of the AFLP markers between the two populations.

While the average and range of pairwise genetic similarity values (S_J) were comparable between the Mediterranean and California, fungal populations from the two different regions had two clearly distinct patterns of distribution of S_J values. The unimodal distribution in Mediterranean populations indicates a single-clonal reproductive mode, while the bimodal distribution in California is better explained by the presence of both a clonal (peak with higher S_J value) and a sexual (peak with lower S_J value) reproductive modes. This finding is perfectly in agreement with field observations and genetic analyses presented by Della Rocca et al. (2011b), who suggested sexual and clonal reproduction may be occurring in California, while Mediterranean populations appeared to be reproducing only clonally.

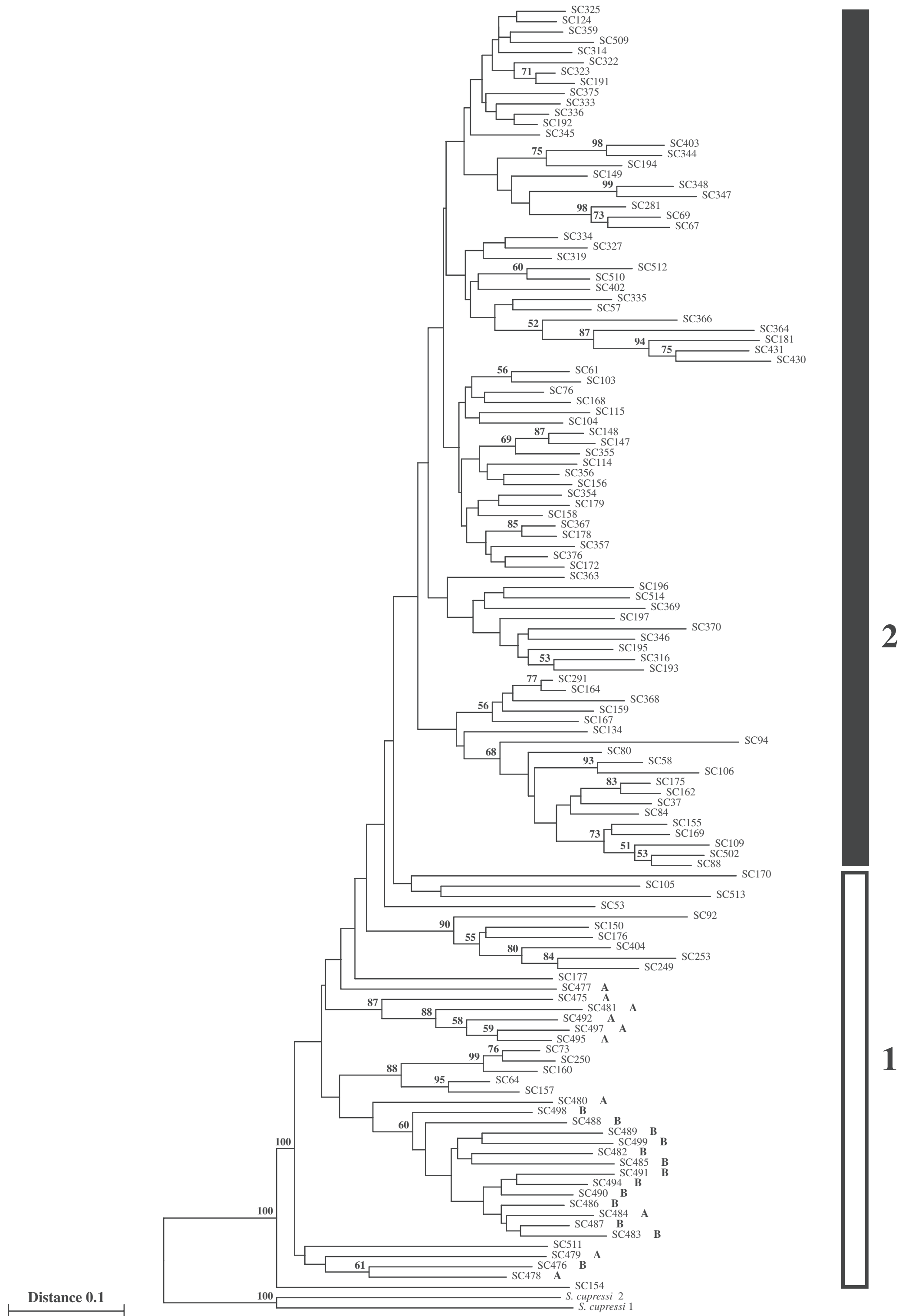

Fig. 2. NJ trees calculated using pairwise Jaccard genetic similarity coefficients (S_J). Bootstrap values >50% appear above branches. Lateral bars (1 and 2) indicate clusters inferred in the structure analysis. Letters A and B indicate the β-tubulin haplotype of each Californian isolate. All Mediterranean isolates belong to the haplotype A.

The fact that California and Mediterranean isolates do not represent a single panmictic population with significant current intercontinental gene flow is exemplified by the large and significant component of genetic variability identified by AMOVA between the two populations and by the fact that the average pairwise S_J calculated for all samples was significantly lower than that calculated for the Mediterranean and California populations independently. It should be noted, however,

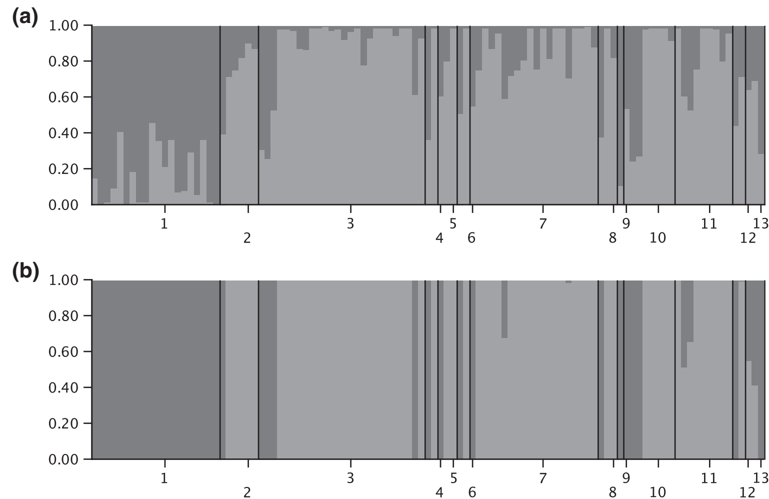

Fig. 3. STRUCTURE bar plots for $K = 2$ using the genome admixing (a) and non-admixing (b) models. Populations are as follows: 1 = California, 2 = Algeria, 3 = Central Italy, 4 = Sardinia (Central Italy), 5 = France, 6 = Corse (France), 7 = Islands Greece, 8 = Mainland Greece, 9 = Morocco, 10 = Northern Italy, 11 = Peloponnese Greece, 12 = Portugal, 13 = Sicily, 14 = Spain.

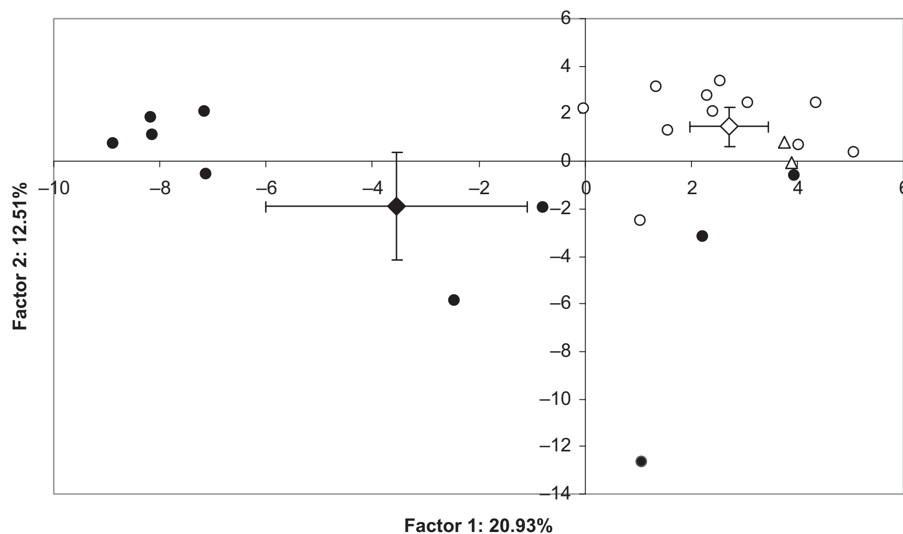

Fig. 4. Principal component analysis based on AFLP profiles of Californian isolates. Filled circles = isolates belonging to β-tubulin haplotype A; open circles = isolates belonging to β-tubulin haplotype B. Rhombs are the centroids of each group of isolates belonging to the two different haplotypes, and bars indicate the standard deviations. Δ = isolates from southern California counties (San Diego).

that the difference in S_J between regions is only slightly higher than that detected within regions. This is in contrast with the much greater diversity reported between rather than within long-isolated lineages in other systems (Ivors et al. 2004), indicating that it is possible that Mediterranean and California populations may be currently isolated, but may have derived from one another in relatively recent times. The fact that no AFLP markers were private to either California or the Mediterranean further confirms the likelihood that one population is derived from the other.

The human-mediated long-distance transport of fungal individuals has been previously suggested based on genotypic identity of isolates coming from locations hundreds of kilometres apart (Della Rocca et al. 2011b). The current study confirms this is an extremely likely pathway of spread in three ways: (i) identical AFLP genotypes were found in Italy and Greece as well as in Italy and Spain (ii) PCA reveals no clustering of Mediterranean isolates based on geography, suggesting fungal individuals are moved frequently within this region; and (iii) PCA reveals a clustering of isolates from Northern and Southern California that had been identified as being identical with the less discriminating SSR markers used in a previous study (Della Rocca et al. 2011b). No identical genotypes or PCA clustering of isolates from the two different world regions were detected, suggesting that intercontinental movement is an infrequent event. The human role in the spread of *S. cardinale* between Italy and Greece through the trade of infected plants is potentially explained by the high volume of Cypress plants sold for decades to Greece by nurseries in Tuscany.

The NJ tree indicated that all isolates morphologically identified as *S. cardinale*, whether from Europe, North Africa or North America, belonged to a well-supported (100% bootstrap) monophyletic clade, confirming the validity of *S. cardinale* as a species. Several *S. cardinale* subclades are identified by the NJ analysis, but these clades are poorly supported statistically and without any clear association with host or provenance. However, three of five basal clades include exclusively California isolates, one includes only Mediterranean isolates, and one includes both California and Mediterranean isolates. This clade of mixed provenance included the two isolates (SC477 and SC170) that had been identified as the most closely related genotypes between California and the Mediterranean based on 7 SSR loci (Della Rocca et al. 2011b). Additionally, the basal positioning of California isolates is suggestive that California populations represent a source for the Mediterranean infestation. The likelihood that this connection may have occurred simply by chance is very low, considering the large number of variable markers employed. Only one of the two known β-tubulin haplotypes was found in all 18 Mediterranean isolates sequenced in this study, irrespective of their position on the NJ tree and of the STRUCTURE cluster in which they fall. The same β-tubulin haplotype was also found in the California isolate SC477, the likely California progenitor of the Mediterranean population found in the Mediterranean–California mixed clade. Interestingly, PCA of AFLP data clusters California isolates in two groups that correspond to their β-tubulin haplotype. This result is based on a large number of nuclear AFLP markers and suggests that such two haplotypes are not merely two alleles at one locus, but may be associated with two distinct groups of isolates, potentially characterized by genotypic and phenotypic differences. This finding strengthens the use of β-tubulin as a valid marker in further studies on the diversity of *S. cardinale* populations worldwide.

Both STRUCTURE analyses revealed that all five basal clades in the NJ tree, independent of continental origin, belonged to the same cluster (Cluster 1), providing convincing evidence of the genetic relatedness between California and Mediterranean isolates. Because the presence of sexual reproduction is probable in California based on the description of the sexual stage (Hansen 1956) and the absence of LD (Della Rocca et al. 2011b), the genome admixing model may appropriately represent the STRUCTURE of its fungal population. Using this model, STRUCTURE revealed that California isolates could be mostly regarded as admixtures of the two clusters (i.e. genomes) identified by the overall analysis. The non-admixing model instead may be more appropriate to describe clonally reproducing Mediterranean populations (Della Rocca et al. 2011b). Using this model, Mediterranean populations contain two clusters, of which only one (i.e. Cluster 1) is present in California. Cluster 2 may be explained in one of two ways: (i) it may be the result of a second introduction from a population not sampled in California or elsewhere or (ii) it may be the result of a genetic process that occurred in the Mediterranean in some individuals belonging to Cluster 1, founding a distinct population that then expanded in the Mediterranean. The fact that most California individuals clearly contain both genomes when analysed by STRUCTURE using the genome-admixing model makes this second hypothesis as likely as the first one, but we cannot make a definitive choice between the two hypotheses.

Finally, the bimodal distribution of Jaccard's pairwise coefficients of similarity in California is congruent with the presence of both sexual and asexual reproduction in this US state. As stated by Della Rocca et al. (2011b), this observation, combined with the fact that all California cypresses are resistant to the disease when in their natural geographic and climatic range, is indicative that *S. cardinale* may be endemic to California. Further work on Cluster 2 isolates in the Mediterranean in a study including isolates from other regions of the world may help to determine whether the pathogen may also be endemic to other parts of the world and/or may be evolving rapidly in the Mediterranean, possibly under strong novel selection pressures encountered in habitats only recently colonized by this fungus. Whether the result of two introductions or of a rapid evolutionary process, the ability to differentiate two groups of genotypes within Mediterranean populations provides an important opportunity to study the evolution and adaptation of an exotic fungus in a new environment.

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