Sequence and Simple-Sequence Repeat Analyses of the Fungal Pathogen *Seiridium cardinale* Indicate California Is the Most Likely Source of the Cypress Canker Epidemic for the Mediterranean Region

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ABSTRACT

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Seiridium cardinale is the pathogenic fungus of unknown origin responsible for a world pandemic known as cypress canker affecting several species of *Cupressaceae* in both the Northern and Southern Hemisphere. In this study, a comparative genetic analysis of worldwide populations was performed using sequence analysis of a portion of the β-tubulin locus and seven polymorphic simple-sequence repeat (SSR) loci on 96 isolates. Sequence analysis identified two distinct β-tubulin alleles, both present in California. Only one of the two alleles was detected in the Mediterranean basin, while two isolates from the Southern Hemisphere were characterized by the presence of the allele absent from the Mediterranean. SSRs identified a total of 46 multilocus genotypes (MGs): genotypic diversity was always higher in the California population, and calculations of the index of association (I_A) determined the presence of linkage disequilibrium associated with the absence of sexual reproduction only in the Mediterranean population but not in California. In 50 instances, the same MG was found at great geographic distances, implying a role played by humans in spreading the disease. Network analysis performed on SSR data identified three clusters of MGs: California, Morocco, and the rest of the Mediterranean. Both the Morocco and the Mediterranean clusters were linked to the California cluster. Coalescent analysis identified insignificant migration between California and Italy, as expected in the presence of a single introduction event, and very high migration from Italy into Greece, as expected of an outbreak still in exponential growth phase and starting from an Italian source.

Additional keywords: coalescent analysis, *Cupressus*, genetic diversity, microsatellite, network analysis.

Seiridium cardinale (W. W. Wagener) B. Sutton & I. A. S. Gibson is the pathogenic fungus responsible for a world pandemic known as cypress canker. Although two other *Seiridium* spp. (namely, *S. unicorne* and *S. cupressi*) can also cause a similar disease, their overall abundance and virulence are clearly lower when compared with those of *S. cardinale* (30). Disease symptoms include lethal bark canker on many members of the family *Cupressaceae*, including *Cupressus*, *Thuja*, *Juniperus*, *Calocedrus*, and *Chamaecyparis* spp. and the intergeneric hybrid ×*Cupressocyparis leylandii* (*Chamaecyparis nootkatensis* ♀ × *Cupressus macrocarpa* ♂) (26,34,61,71,72). Cankers may expand relatively slowly on the main stem but can be numerous and relatively rapid-growing on branches, resulting in visible dieback of different portions of the tree canopy that eventually lead to tree mortality. Where high density of susceptible species exist and in the presence of warm and humid conditions known to favor spread of the disease, infection and mortality levels can be ≤95%, with obvious ecological and economic consequences (50,74). Cypress canker has had a major economic impact during its worldwide spread of the last 50 years; in fact, cypress trees are not only important components of natural ecosystems but are also highly valued ornamental species and represent some of the most common trees employed to create windbreaks for agricultural purposes. In the Mediterranean region, cypress trees also have a great cultural relevance, making the impact of this disease particularly worrisome (30,49).

The first epidemic of cypress canker caused by *S. cardinale* was reported in 1928 in California and resulted in the quick demise of many plantations of *C. macrocarpa* in the interior areas of the state, where this tree species does not occur naturally (74,75). Since this initial report, the disease has been described in New Zealand (12), Europe (2,6,15,33,66,69), Australia (68), South America (4,46,61), South Africa (76), and North Africa (18,25) as a true pandemic (30). The sudden recent appearances of the disease and the extremely high levels of mortality experienced in many areas around the globe suggest that the disease and its causal agent are not cosmopolitan by ancient history but, rather, have been introduced into most locations, presumably through human activities. Because cypress trees are traded widely as ornamental plants, this remains the most plausible means of transport across continents. Alternatively, the movement of infected cypress wood may also play a role in the long-distance movement of the pathogen. However, even though the fungus can survive for a long period in the wood of cypress without loss of pathogenicity, sporulation on dead wood or timber in use is reported to be unlikely (47,51); hence, the pathogen introduced in wood may not be able to spread from the initial introduction point.

Various hypotheses have been formulated on the possible origin of the disease but none of them have been substantiated by direct or even corroborating evidence. The first hypothesis is that *S. cardinale* might exist as a weak pathogen on some as-yet-un-

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identified hosts in the western United States and that plantings of *C. macrocarpa* away from its natural coastal range may have facilitated a host jump. The second hypothesis suggests that the pathogen may have arisen as a variant (mutation or hybridization) from a normally saprophytic indigenous fungus. A third possibility implies a source other than California and a dispersal pattern to other regions of the world that may or may not go through California (75). For instance, the fungus could be native to East or South Asia where all indigenous *Cupressus* spp. are characterized by a high degree of resistance to *S. cardinale* (53, 55). The plausibility of this hypothesis is supported, in part, by the early distribution of the disease in Pacific-rim states such as California (75), New Zealand (12), and Chile (in 46).

Identifying the area of origin of the pathogen and its routes of spread is of paramount importance. Studying native populations of the pathogen in the ecosystem in which they have coevolved with their hosts may help better understand the biology and epidemiology of the pathogen, and may further allow the identification of the various evolutionary strategies that have led to resistance in plants. More urgently, understanding the source of the pathogen and its mode of spread may help prevent both the introduction of the pathogen in new areas and the introduction of substantially different pathogen genotypes into areas where the pathogen has already arrived. This last aspect is particularly relevant because the most valuable form of control lies in the resistant cypress lines that have already been developed (3,19, 50,58,59), a strategy and a 30-year-long effort that could be nullified by the introduction of substantially different genotypes of the pathogen.

A first necessary approach to infer the source of a pathogen is to perform a comparative analysis of the genetic diversity of populations from different areas and determine the relationships among these populations. The hypothesis is that source populations should be more diverse while derived populations should be less diverse and nested within source populations. Similar analyses have been successfully done for many exotic forest diseases, including chestnut blight, sudden oak death, white pine blister rust, canker stain of plane, and North American Heterobasidion root rot in Italy (22,23,28,32,35,39,42,48). Additionally, genetic analyses allow us to infer whether sexual reproduction may be effectively ongoing in a population. This aspect is particularly relevant not only for its epidemiological and co-evolutionary implications but also because sexual reproduction in most fungal pathogens requires the presence of at least two mating types. Historically, many introduced forest diseases are locally characterized by the presence of a single mating type, whereas both mating types are normally present in the area of origin (unless, of course, the organism in question has lost its ability to reproduce sexually) or when both mating types of a pathogen are introduced. *S. cardinale* is considered to be an anamorphic fungus reproducing agamically through conidia made of haploid cells. The genus *Leptosphaeria*, the perfect stage of *S. cardinale*, was reported only once on dying and dead cypress trees over a large area of north central California but it was only partially described (33), and it has been deemed too incomplete to be fully recognized.

Very limited information is available on variability of *S. cardinale* within and between regions. Published reports either focus on *S. cardinale* genotypes from a single region or focus on the evolutionary relationships between *S. cardinale* and its sister species. If we exclude the very preliminary tests described in the first report on this pathogen (75), most analyses of variation within *S. cardinale* have been performed with regard to virulence and exclusively on a few European isolates. Results have indicated a substantial uniformity among isolates of the fungus, in agreement with the expectations for an introduced exotic species (55,57,77). Only a few studies have used molecular techniques to look at genetic diversity within *Seiridium* isolates. Most studies have focused on comparisons among rather than within species (5,36,45,60,71,73). With the exception of Viljoen et al. (73), who suggested the existence of a single, morphologically, variable species, these studies all supported the distinction of *S. cardinale*, *S. cupressi*, and *S. unicorne* as separate species; however, all failed to detect variability among *S. cardinale* isolates. Randomly amplified polymorphic DNAs indicated a high level of homogeneity among 77 *S. cardinale* isolates from northern Italy and 1 isolate from Greece (53) but the lack of representation of a region outside of Europe in the sample precluded any conclusions on the origin of the pathogen or on the source of the European population.

In this study, we analyze β-tubulin sequences (36) and seven polymorphic simple-sequence repeats (SSRs) (20) on a total of 96 isolates of *S. cardinale* from California, several Mediterranean countries, Chile, and New Zealand. We use sequence analysis to confirm identity of the isolates employed in the study and to search for a worldwide pattern in distribution of haplotypes. SSR data instead are used to (i) determine presence of linkage disequilibrium (LD) in the data set to verify the potential for presence of cryptic sex, (ii) calculate indices of genotypic and genetic diversity inside and outside California, and, (iii) perform a coalescent analysis and a minimum spanning network (MSN) to highlight the relationship between California and Mediterranean isolates, as well as intraregional relationships. Results clearly indicate that California is the most likely source of two distinct infestations in the Mediterranean and a possible candidate for the area of origin of this pathogen. Additionally, distribution of genotypes is consistent with long-distance human-mediated movement of the pathogen. Implications for the future of the susceptible hosts and for trade regulations will be discussed.

MATERIALS AND METHODS

Sampling. A total of 96 *Seiridium cardinale* isolates was obtained in 2007 to 2008 through a sampling effort of symptomatic *Cupressaceae* spp. from seven Mediterranean countries and eight Californian counties (Table 1). Additionally, 18 β-tubulin sequences (8 of *S. cardinale*, 3 of *S. unicorne*, and 7 of *S. cupressi*) from GenBank (10) were also included in the neighbor-joining (NJ) analysis. GenBank accession numbers of sequences not generated in this study are provided in the taxon labels of Figure 1.

Fungal isolation. Small bark fragments (≈3 by 3 mm) cut from the margin of cankered tissues were placed on potato dextrose agar (PDA) (potato dextrose broth [PDB] at 20 g/liter + agar at 20 g/liter) in petri dishes and maintained at 25°C in the dark. White to gray or olive, cottony colonies with a salmon-orange reverse side, obtained from plated fragments, were separately transferred on 1% malt extract agar (malt extract at 10 g/liter + agar at 20 g/liter) supplemented with autoclaved cypress seed. The plates were then incubated at 18°C under mixed white and near-ultraviolet light (400 to 200 nm), set to provide 12-h light-and-dark cycles to induce sporulation of the fungus. After 3 to 4 weeks, monoconidial cultures were obtained from acervuli (i.e., asexual sporulating structures producing mitospores known as conidia) of the fungus developed on seed (16,44,68,75). Seed bearing acervuli of each isolate were collected and stored in hermetic plastic tubes at –20°C.

DNA extraction and polymerase chain reaction amplification. For DNA extraction, the cultures were grown on cellophane discs placed on PDA (PDB at 20 g/liter + agar at 20 g/liter) in petri dishes for 15 to 20 days at 25°C. The colonies were then lifted from cellophane and freeze dried for 24 h. Genomic DNA was extracted using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) in agreement with the indications of the producer.

A portion of the β-tubulin locus was amplified for a subset of 21 California and 5 Mediterranean isolates using primers Bt1a

and Bt1b and conditions described by Barnes et al. (5). The amplicons were sequenced on an Applied Biosystems 3130xl Genetic Analyzer (Life Technologies, Carlsbad, CA). Seven polymorphic nuclear microsatellites developed and characterized for *S. cardinale* (20) were used in this study. Polymerase chain reaction (PCR) mixtures and cycling conditions were the same as those described by Della Rocca et al. (20).

Analyses. Sequences were manually edited and aligned using Sequencher 4.2 (Gene Codes Corporation, Ann Arbor, MI) before being analyzed using Geneious Pro 5.1.6. (21) (Biomatters Ltd., Auckland, New Zealand). An NJ tree was generated for the selected isolates, including sequences of isolates of *S. cardinale* from Chile and New Zealand deposited in GenBank (AF320504.1 and AF320500.1). Isolates of *S. cupressi* and *S. unicorne* were used as outgroups. The Tamura-Nei genetic distance model was used and bootstrap support was calculated with 1,000 replicates. Additionally, sequences were exported as FastA-formatted files, which were converted to a "relaxed" Phylip format (i.e., allowing taxon labels to occupy ≤256 characters) using a Perl script available on the webpage of A. Stamatakis (http://www.kramer.in.tum.de/exelixis/software.html). Maximum likelihood (ML) phylogenetic analyses were performed using RAxML-HPC2 v7.2.8 (64) implemented on the CIPRES Portal v2.2 at the San Diego Supercomputer Center (43). Analyses included 200 inferences on the original alignment and 1,000 bootstrap replicates, using the GTR+GAMMA model for both bootstrapping and final ML optimization and using default parameter settings. Trees were visualized with FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree). All sequences generated in the course of this study were deposited in GenBank (Table 1).

Size of amplicons obtained with primers flanking SSRs was transformed to number of repeats and used for all further analyses. The index of association I_A was calculated using the program MULTILOCUS v1.3 (1) independently for isolates from California and from the Mediterranean, excluding Moroccan genotypes which were characterized by significantly different allelic sizes than all other genotypes from the same region (see Results). I_A is a measure of LD – the number of loci at which pairs of individuals differ; this pairwise "distance" is calculated and its variance compared with that if there was no LD.

TABLE 1. Summary of *Seiridium cardinale* sampling locations

Isolate	Host	Provenance	Country	Latitude N	Longitude	Multilocus genotype	GenBank β-tubulin accession
430	*Cupressus sempervirens*	Algiers	Algeria	36° 45′ 21″	3° 02′ 45″	MG36	…
431	*C. sempervirens*	Algiers	Algeria	36° 45′ 21″	3° 02′ 45″	MG35	…
92	*C. sempervirens*	Corte	France	42° 18′ 20″	9° 09′ 03″	MG33	…
57	*C. sempervirens*	Bastia	France	42° 42′ 03″	9° 26′ 56″	MG30	…
176	*C. sempervirens*	Frejus	France	43° 25′ 27″	6° 43′ 26″	MG30	…
181	*C. sempervirens*	Frejus	France	43° 25′ 27″	6° 43′ 26″	MG27	…
251	*C. sempervirens*	Amamine	France	43° 57′ 57″	4° 12′ 49″	MG30	HQ678148
150	*C. sempervirens*	Omalos	Greece	35° 20′ 38″	23° 54′ 17″	MG39	…
150b	*C. sempervirens*	Omalos	Greece	35° 20′ 38″	23° 54′ 17″	MG43	…
335	*C. sempervirens*	Nipos	Greece	35° 22′ 45″	24° 10′ 10″	MG42	…
336	*C. sempervirens*	Nipos	Greece	35° 22′ 45″	24° 10′ 10″	MG42	…
337	*C. sempervirens*	Nipos	Greece	35° 22′ 45″	24° 10′ 10″	MG42	…
344	*C. sempervirens*	Agrokepio	Greece	35° 18′ 55″	24° 12′ 42″	MG30	…
345	*C. sempervirens*	Agrokepio	Greece	35° 18′ 55″	24° 12′ 42″	MG40	…
375	*C. sempervirens*	Fres	Greece	35° 22′ 54″	24° 08′ 42″	MG30	…
368	*C. sempervirens*	Karistos	Greece	38° 00′ 57″	24° 24′ 41″	MG40	…
366	*C. sempervirens*	Karistos	Greece	38° 00′ 57″	24° 24′ 41″	MG42	…
404	*C. sempervirens*	M. Athos	Greece	40° 26′ 00″	23° 50′ 33″	MG31	…
191	*C. sempervirens*	Kaliani	Greece	37° 53′ 18″	22° 29′ 50″	MG44	…
359	*C. sempervirens*	Kaliani	Greece	37° 53′ 18″	22° 29′ 50″	MG30	…
192	*Juniperus phoenicea*	Lefkas	Greece	38° 49′ 49″	20° 42′ 25″	MG34	…
194	*C. sempervirens*	Spanochori	Greece	38° 47′ 00″	20° 40′ 18″	MG34	…
196	*C. sempervirens*	Hortata	Greece	38° 43′ 01″	20° 36′ 09″	MG30	…
314	*C. sempervirens*	Megalopoli	Greece	37° 24′ 03″	22° 08′ 28″	MG37	…
322	*C. sempervirens*	Kalimani	Greece	38° 06′ 55″	22° 57′ 08″	MG31	…
319	*C. sempervirens*	Kalimani	Greece	38° 06′ 55″	22° 57′ 08″	MG30	HQ678149
323	*C. sempervirens*	Kritharion	Greece	38° 02′ 44″	21° 43′ 27″	MG30	…
325	*C. sempervirens*	Kritharion	Greece	38° 02′ 44″	21° 43′ 27″	MG30	…
334	*C. sempervirens*	Alepochori	Greece	37° 59′ 01″	21° 47′ 54″	MG30	…
333	*C. sempervirens*	Alepochori	Greece	37° 59′ 01″	21° 47′ 54″	MG36	…
328	*C. sempervirens*	Nea Makri	Greece	38° 04′ 54″	23° 58′ 57″	MG30	…
331	*C. sempervirens*	Nea Makri	Greece	38° 04′ 54″	23° 58′ 57″	MG29	…
403	*C. sempervirens*	Nea Makri	Greece	38° 04′ 54″	23° 58′ 57″	MG31	…
70	*C. sempervirens*	Montecavolo	Italy	44° 37′ 54″	10° 32′ 11″	MG24	…
118	*C. sempervirens*	Bellaria	Italy	44° 08′ 22″	12° 28′ 24″	MG30	…
253	×*Cupressocyparis leylandii*	Imperia	Italy	43° 53′ 57″	8° 02′ 17″	MG30	…
249	*Cupressus sempervirens*	Platamona	Italy	40° 48′ 07″	8° 28′ 40″	MG30	…
56	*C. sempervirens*	Sassari	Italy	40° 42′ 49″	8° 31′ 41″	MG30	…
53	*C. sempervirens*	Troina	Italy	37° 47′ 42″	14° 36′ 35″	MG23	…
105	*C. sempervirens*	Comiso	Italy	36° 56′ 44″	14° 36′ 14″	MG22	…
105b	*C. sempervirens*	Comiso	Italy	36° 56′ 44″	14° 36′ 14″	MG22	…
64	*C. sempervirens*	Guarniente	Italy	43° 32′ 26″	11° 53′ 20″	MG30	…
115	*C. sempervirens*	Gargiano	Italy	43° 39′ 12″	11° 51′ 02″	MG27	…
168	*C. sempervirens*	S. Luce	Italy	43° 28′ 17″	10° 33′ 49″	MG30	…
165	*C. sempervirens*	Fosdinovo	Italy	44° 07′ 59″	10° 01′ 03″	MG30	…
165b	*C. sempervirens*	Fosdinovo	Italy	44° 07′ 59″	10° 01′ 03″	MG30	…
164	*C. sempervirens*	Carrara	Italy	44° 04′ 54″	10° 06′ 26″	MG40	HQ678147
167	*C. sempervirens*	Bolgheri	Italy	43° 13′ 54″	10° 36′ 01″	MG29	…

(*continued on next page*)

The following indices of diversity were calculated as described by Mascheretti et al. (38): (i) clonal genotype diversity, $R = (G - 1)/(N - 1)$, where G is the number of multilocus genotypes (MGs) present in a sample and N is the sample size; (ii) Stoddart and Taylor's Index, $G = 1/\Sigma p_i^2$, (63) where p_i is the frequency of the ith MG; and (iii) gene diversity (expected heterozygosity), estimated as

$$H_E = 1 - \frac{1}{m}\sum_{l=1}^{m}\sum_{i=1}^{k} p_i^2$$

where p is the frequency of the ith of k alleles, averaged over each l of m loci. Calculations were performed on the same groupings of isolates described above for I_A analysis.

To avoid the limitations of phylogenetic approaches that inevitably result in bifurcated relationships among groups of genotypes, we opted to use the data instead to determine a MSN for the entire dataset based on the reconstruction of all possible linkages among genotypes. MGs were first determined by identifying unique combinations of alleles, and a pairwise matrix of genetic distances among all MGs was then calculated using the robust method of Bruvo et al. (14) in the program GenoDive 2.0b10 (40) using the stepwise mutation model. The program MINSPNET (24) was used to create a MSN that depicts the relationships among all MGs, based on Bruvo's distances, and visualized using Graphviz 2.26.3 (27) (http://www.graphviz.org).

MIGRATE-N v3.1.6 (8) was used to perform coalescent analyses and determine extent and direction of migration among the following regions: California, Italy, and Greece. The choice of these three regions for the coalescent analysis was dictated by (i) a significant number of genotypes available for each of these regions and (ii) the sequence of reports of the disease clearly described first in California, then in Italy, and only later in Greece and other Mediterranean regions. A Bayesian inference method was used (7,9) to estimate direction migration rates between these three populations. Microsatellite evolution was modeled using the Brownian motion model; genealogies were started with the automatic unweighted pair-group method with arithmetic means-based start; and an assumption of a constant mutation rate for all loci, burn in of 10,000, and adaptive heating with four chains were used in the analysis. Migration, M, was estimated as the immigration rate m divided by the mutation rate μ. Prior distribution parameters for θ and M were assumed to be uniform for

TABLE 1. (*continued from preceding page*)

Isolate	Host	Provenance	Country	Latitude N	Longitude	Multilocus genotype	GenBank β-tubulin accession
167b	*C. sempervirens*	Bolgheri	Italy	43° 13′ 54″	10° 36′ 01″	MG29	…
44	*C. sempervirens*	Bolgheri	Italy	43° 13′ 54″	10° 36′ 01″	MG30	…
402	*C. sempervirens*	Firenze	Italy	43° 45′ 13″	11° 19′ 21″	MG38	…
104	*C. sempervirens*	Serpiolle	Italy	43° 49′ 14″	11° 15′ 09″	MG30	…
76	×*Cupressocyparis leylandii*	Calenzano	Italy	43° 51′ 06″	11° 10′ 05″	MG45	…
178	*Cupressus sempervirens*	San Gimignano	Italy	43° 28′ 04″	11° 02′ 25″	MG30	…
179	*C. sempervirens*	Larderello	Italy	43° 14′ 04″	10° 53′ 26″	MG30	…
61	*C. sempervirens*	Fonterutoli	Italy	43° 26′ 12″	11° 18′ 29″	MG30	…
114	*C. sempervirens*	Poggio d'Acona	Italy	43° 38′ 04″	11° 52′ 31″	MG31	…
103	*C. sempervirens*	Roselle	Italy	42° 48′ 52″	11° 07′ 30″	MG30	…
170	*C. sempervirens*	Saturnia	Italy	42° 39′ 48″	11° 30′ 39″	MG26	…
172	*C. sempervirens*	Arcidosso	Italy	42° 52′ 21″	11° 32′ 23″	MG32	…
157	*C. sempervirens*	Mori	Italy	45° 51′ 09″	10° 58′ 34″	MG40	HQ678146
156	*C. sempervirens*	Ala	Italy	45° 45′ 25″	11° 00′ 01″	MG36	…
158	*C. sempervirens*	Mezzocorona	Italy	46° 12′ 50″	11° 07′ 11″	MG30	…
73	*C. sempervirens*	Lago Trasimeno	Italy	43° 11′ 45″	12° 07′ 49″	MG30	…
159	*C. sempervirens*	Cannara	Italy	43° 00′ 49″	12° 37′ 05″	MG28	…
63	*C. sempervirens*	Cannara	Italy	43° 00′ 49″	12° 37′ 05″	MG25	…
154	*C. sempervirens*	Fès-Ifrane	Morocco	33° 42′ 57″	5° 00′ 45″ O	MG46	HQ678145
154b	*C. sempervirens*	Fès-Ifrane	Morocco	33° 42′ 57″	5° 00′ 45″ O	MG46	…
154c	*C. sempervirens*	Fès-Ifrane	Morocco	33° 42′ 57″	5° 00′ 45″ O	MG46	…
513	*C. sempervirens*	Conde dos Arcos	Portugal	38° 46′ 14″	9° 06′ 31″ O	MG7	…
514	*C. sempervirens*	Museu da Cidade	Portugal	38° 42′ 45″	9° 08′ 01″ O	MG41	…
160	*C. sempervirens*	S. Marino	S. Marino	43° 56′ 19″	12° 26′ 37″	MG30	…
250	*C. sempervirens*	Naquera	Spain	39° 39′ 31″	0° 25′ 32″	MG30	…
475	*C. macnabiana*	Berkeley	California	37° 52′ 27″	122° 14′ 18″ O	MG17	HQ678150
476	*C. macrocarpa*	Berkeley Marina	California	37° 51′ 52″	122° 19′ 02″ O	MG4	HQ678151
477	*C. macrocarpa*	Berkeley Marina	California	37° 51′ 52″	122° 19′ 02″ O	MG10	…
485	*C. macrocarpa*	Berkeley	California	37° 51′ 32″	122° 17′ 55″ O	MG11	HQ678158
483	*C. sempervirens*	Lafayette	California	37° 53′ 42″	122° 07′ 18″ O	MG5	HQ678156
484	×*Cupressocyparis leylandii*	Lafayette	California	37° 53′ 45″	122° 07′ 01″ O	MG20	HQ678157
494	×*C. leylandii*	Novato	California	38° 07′ 11″	122° 35′ 08″ O	MG13	HQ678166
495	×*C. leylandii*	S. Marine	California	37° 52′ 33″	122° 26′ 42″ O	MG13	HQ678167
497	×*C. leylandii*	Knoll Forest	California	38° 00′ 56″	122° 41′ 06″ O	MG15	HQ678168
499	*Cupressus sempervirens*	Tiburon	California	37° 52′ 59″	122° 28′ 14″ O	MG16	HQ678170
492	×*Cupressocyparis leylandii*	Ukiah	California	39° 05′ 25″	123° 13′ 15″ O	MG12	HQ678165
498	×*C. leylandii*	Calistoga	California	38° 34′ 24″	122° 34′ 25″ O	MG1	HQ678169
486	×*C. leylandii*	San Diego	California	32° 45′ 54″	117° 13′ 42″ O	MG20	HQ678159
487	*Cupressus macrocarpa*	San Diego	California	32° 45′ 54″	117° 13′ 42″ O	MG21	HQ678160
478	×*Cupressocyparis leylandii*	Lodi	California	38° 03′ 26″	121° 15′ 01″ O	MG9	…
479	×*C. leylandii*	Lodi	California	38° 03′ 26″	121° 15′ 01″ O	MG3	HQ678151
480	*J. chinensis*	Lodi	California	38° 03′ 27″	121° 13′ 24″ O	MG8	HQ678153
481	×*C. leylandii*	Lodi	California	38° 03′ 29″	121° 16′ 39″ O	MG14	HQ678154
482	×*C. leylandii*	Lathrop	California	37° 49′ 33″	121° 17′ 11″ O	MG18	HQ678155
488	*Cupressus macrocarpa*	Millbrae	California	37° 32′ 50″	122° 22′ 24″ O	MG19	HQ678161
489	×*Cupressocyparis leylandii*	Millbrae	California	37° 33′ 55″	122° 23′ 15″ O	MG20	HQ678162
490	×*C. leylandii*	Hillsborough	California	37° 33′ 25″	122° 22′ 47″ O	MG6	HQ678163
491	*Cupressus macrocarpa*	Millbrae	California	37° 33′ 32″	122° 22′ 57″ O	MG2	HQ678164

both: 0 to 0.1 (mean 0.01, Δ 0.01, bins 200) for θ and 0 to 1,000 (mean 100, Δ 100, bins 200) for *M*. The number of recorded steps after burn-in was 5,000.

RESULTS

Based on morphology (data not shown), the isolates employed in all aspects of this study were considered to be *S. cardinale*. Analysis of a portion of the β-tubulin locus for a randomly selected subset of these isolates confirmed that putative *S. cardinale* isolates in this study, independent of provenance, belonged to the same species, and they all closely or perfectly matched GenBank accessions of *S cardinale* isolates. All *S. cardinale* isolates were clearly distinct from *S. unicorne* and *S. cupressi*, and isolates putatively assigned to one of the three species fell into three statistically supported clades in both the NJ and ML analyses. NJ results are shown in Figure 1 (TreeBASE Study 11435). Both NJ and ML analyses revealed the presence of two distinct β-tubulin haplotypes within *S. cardinale*, and both were present in California, whereas a single haplotype was present among isolates from the Mediterranean. Sequences of two isolates, one from Chile and one from New Zealand, perfectly matched the sequence of the second California haplotype, which is absent from the Mediterranean (Fig. 1) (GenBank accession numbers HQ678145 to HQ678171, JF271675 to JF27167).

The I_As calculated for the California and Mediterranean populations (excluding Morocco) using SSR data were strikingly different. Whereas association among alleles appeared to be random in the California population (I_A = –0.041, P = 0.556), a significant (I_A = 0.585, P = 0.009) LD was found for the Mediterranean population. The Stoddart and Taylor's Index (*G*), clonal genotype diversity (*R*), and expected heterozygosity (H_E) indices are shown in Table 2 and were always much higher for the Californian than for the Mediterranean population.

In total, 46 MGs were identified: 20 in California, 25 in the Mediterranean region, and 1 in Morocco. Within the Mediterranean region, 14 MGs were from Italy, 10 were exclusively from outside Italy (Algeria, France, Greece, and Portugal), and 6 were found both in and outside Italy (Algeria, France, Greece, and Spain). In all, 50 isolates belonging to seven MGs were found in locations at great distances from one another both in California (2 isolates from one MG in the San Francisco Bay Area and San

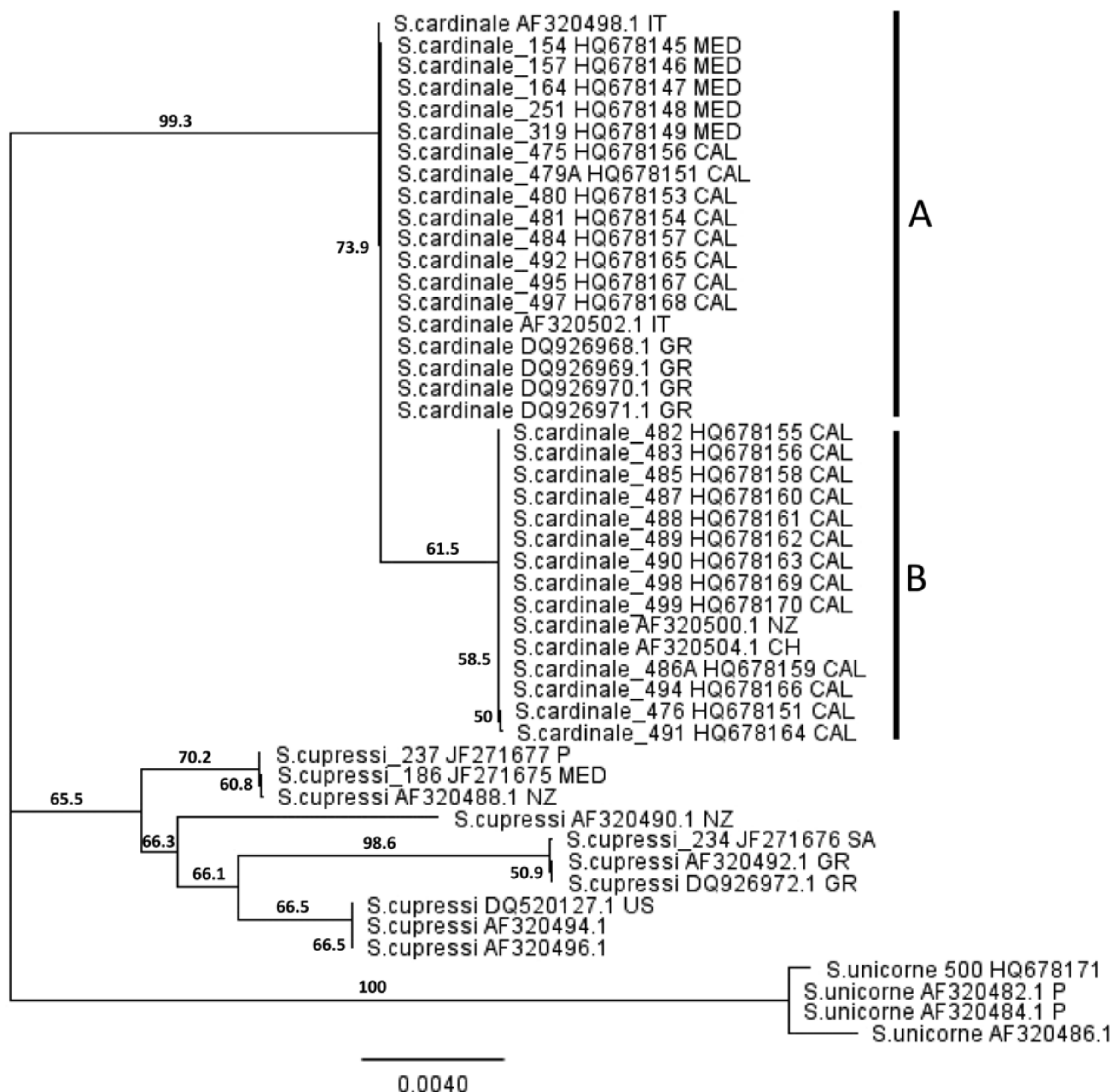

Fig. 1. Neighbor-joining tree based on *Seiridium cardinale* β-tubulin sequences from Californian and Mediterranean samples together with sequences from Chile and New Zealand from GenBank, and using *S. unicorne* and *S. cupressi* as outgroups. CH = Chile, IT = Italy, GR = Greece, MED = Mediterranean, NZ = New Zealand, P = Portugal, SA = South Africa, CAL = California, US = United States. Two different haplotypes (A and B) within *S. cardinale* are indicated.

Diego County) and in the Mediterranean at distances >1,000 km and in regions separated by the Mediterranean Sea (48 isolates representing six MGs found in Spain and Greece or in Algeria, Greece, and Italy). However, no overlap between genotypes was found between California, Morocco, and the remaining countries sampled in the Mediterranean region. The MSN (Fig. 2) shows the presence of three distinct clusters of MGs in California, Morocco, and the Mediterranean region and indicates that the Mediterranean and Moroccan genotypes are each independently linked to Northern California genotypes. The vast Mediterranean infestation appears to have been generated from a single sequence of individual genotypes, all exclusively from central Italy: genetic Bruvo distance (bd) between the MG ancestral to the entire Mediterranean population (MG26) and its closest California relative (MG10) is small (0.286 bd) and within the range of distances among MGs within both California (0.071 to 0.286 bd) and the Mediterranean (with the exclusion of the Moroccan infestation) (0.071 to 0.348 bd). By contrast, the distance of the Moroccan genotype to its closest California relative is somewhat larger (0.393 bd), possibly indicating lack of sampling of intermediate genotypes.

Coalescent analysis detected only minimal migration between California and Greece or Italy, independent of direction, whereas a very significant migration was calculated from Italy into Greece. A lower but significant value of migration back into Italy from Greece was also obtained (Table 3).

TABLE 2. Indices of genetic diversity[a]

Population	R	G	H_E
All	0.474	8.991	0.413
California	0.864	12.903	0.425
Mediterranean (without Morocco)	0.348	0.629	0.239

[a] R = clonal genotype diversity, G = Stoddart and Taylor's index, and H_E = gene diversity (expected heterozygosity).

DISCUSSION

Results of this study confirm the existence of three distinct *Seiridium* spp.: *S. cardinale*, *S unicorne*, and *S cupressi.* Additionally, the study identified previously unknown genetic variability within *S. cardinale* and suggests that California may be the source of the epidemic in the Mediterranean.

Two β-tubulin alleles were detected for the first time within this locus and both were present in California. Only one of these alleles was found in the well-represented Mediterranean population, while the other allele was found in California and in two samples from Chile and New Zealand. Unfortunately, the minimal sampling intensity does not allow us to exclude the presence of both clades in countries of the Southern Hemisphere; however, this finding is the first to highlight significant molecular difference, even if at a single locus, within this species.

In general, higher genetic diversity, is expected in source populations of a pathogen (23,35,37,41,42) compared with introduced populations, which have obviously undergone the genetic bottleneck of the founding effect. However, genotypic diversity may become higher than expected in the case of unchecked expansion of populations of introduced pathogens colonizing naïve ecosystems (11,38). We hypothesized that the epidemic levels observed in the Mediterranean may have led to a large number of closely related genotypes but that indices of diversity should remain higher in California, were that state to be a possible source of the pathogen. The SSRs employed in this study were polymorphic and detected a total of 46 MGs: 25 in the Mediterranean (excluding the Moroccan single genotype) and 20 in California. However, the Mediterranean region was sampled much more

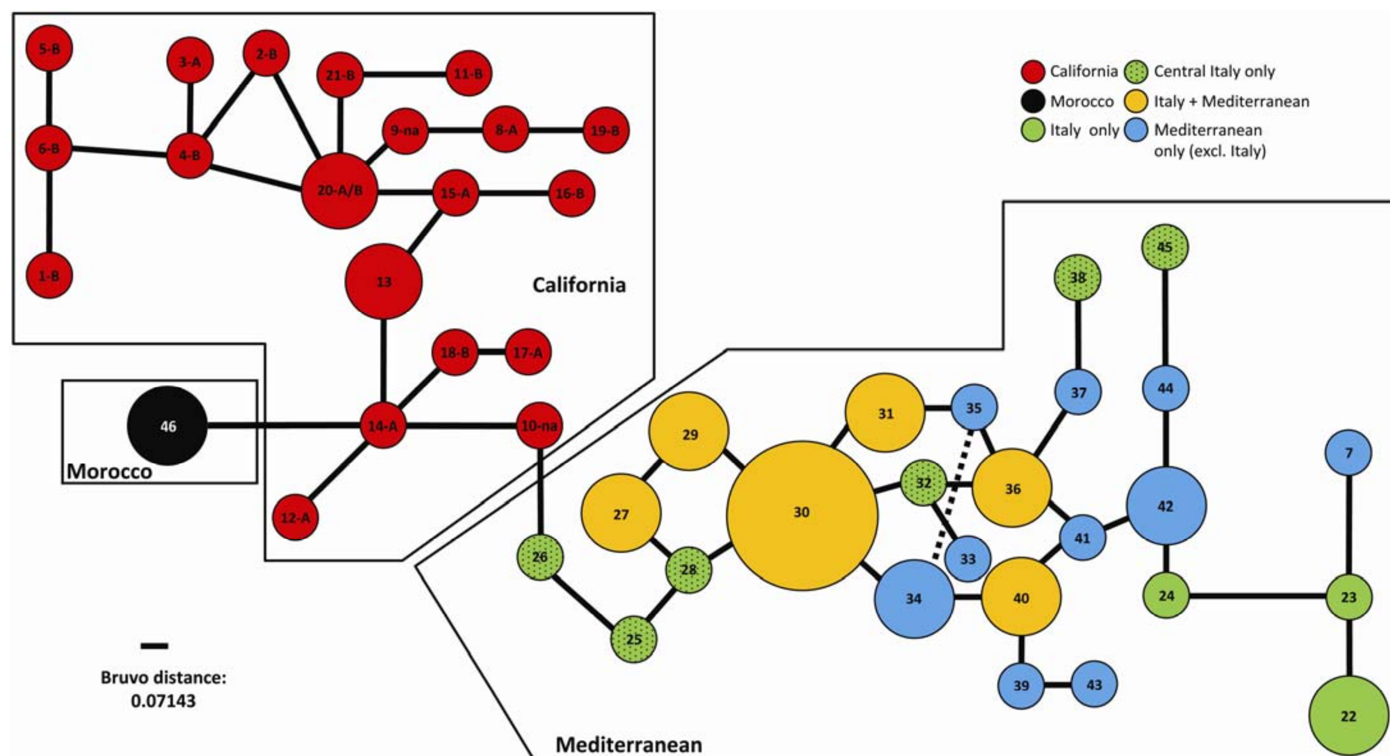

Fig. 2. Minimum spanning network (MSN) showing relationships between multilocus genotypes (MGs) in Californian and Mediterranean populations. Each node represents a different MG and nodes are scaled to reflect the numbers of individuals sharing the same MGs: small = singleton MG, medium = 2 to 5 individuals with same MG, large = 30 individuals with same MG. Labeling with A, B, or na within nodes relates to whether individuals with that MG were found within clade A or B or not included in the analysis, respectively, in the *Seiridium cardinale* neighbor-joining tree (Fig. 1). Dotted line between nodes 34 and 35 indicates where the branch length was unable to be scaled properly without overlap. Distance is the equivalent of the shortest distances in the network; for example, equal length to those between 30 and 32 or 32 and 36.

extensively than California. Because indices of genotypic and genetic diversity take into consideration sampling intensity, all three indices calculated in this study were significantly higher for California than for the Mediterranean region, indicating California as a likely source for the pathogen.

In most countries affected by the canker pandemic, no sexual stage of the fungus has ever been described, and the pathogen is regarded as reproducing exclusively asexually. However, the sexual stage of this pathogen was incompletely described, once, in California (33). We used SSR data to determine whether LD (i.e., the absence of random assortment among unlinked alleles) may result due to the lack of sexual reproduction. Calculations of the I_A, surprisingly, revealed that LD was only present in the Mediterranean population and that the allelic distribution of the California population did not show signs of LD, suggesting that sexual reproduction may be ongoing in this U.S. state. The fact that the same genotype was only found twice at significant distances in California as opposed to 48 times in the Mediterranean is also indicative of a different reproductive strategy in the two regions of the world. An alternative, albeit less conservative, explanation of the Californian I_A may be that multiple introductions of significantly different genotypes and both mating types may have occurred from a third location into California.

Because relationships among individuals within species (or at least within metapopulations belonging to a species) cannot properly be visualized by distance–based approaches resulting in bifurcating representations, we opted to analyze our SSR data through a network analysis. All isolates from California and all isolates from the Mediterranean (excluding the Moroccan genotype) formed two distinct clusters of genotypes. The Moroccan genotype represented a third, distinct "Moroccan" cluster. Both the Mediterranean and the Moroccan genotypes were independently linked to the California cluster. Although no overlap of identical genotypes was found among the three clusters, distance between Californian and genotypes from the other two regions was comparable with distances detected between genotypes within region. A single connection between the Californian and Mediterranean clusters through a series of three genotypes exclusively present in central Italy is best interpreted as depicting a single and not a multiple introduction of *S. cardinale* from California into central Italy. An alternative explanation of the data could be provided by convergence of genotypes from two distinct introductions from a third region leading through random genetic drift processes to two extremely closely related genotypes, one in California and one in central Italy. In order for this hypothetical convergent microevolutionary process to have occurred, convergent evolution would have to have occurred independently at all loci employed in this analysis. We decided to compare the probability (*P*) of these two alternative hypotheses; namely, the direct emergence of the basal Mediterranean genotype from a California genotype (H_1) and the convergent evolution of two genotypes from two independent founder genotypes (H_2). We opted to actually favor H_2 by not considering independent evolution of each SSR locus but assuming that the only ecologically possible genotypes were the ones depicted in our network (Fig. 2). We assumed the founder MG of each infestation would be (i) very common, (ii) the progenitor of a large number of other genotypes, and (iii) situated somewhere in the center of the network for each region (i.e., MG20 for California and MG30 for the Mediterranean). We then calculated the probability (*P*) that from those two founder genotypes, MG10 and MG26 (i.e., the two closely related genotypes in the two regions of the world), would arise independently for California and the Mediterranean, respectively, by multiplying the *P* of generating the next MG in the chain of genotypes between MG20 and MG10 for California and between MG30 and MG26 for the Mediterranean. At each step, the *P* of generating the "correct" next MG was calculated as $P_{next} = 1/n$ MG derived from progenitor.

For each region, the overall P_{reg} was calculated by multiplying the *P* of one MG by the *P* of the next, and so on, until MG10 and MG26 were generated, starting from the founder genotypes for each region, as follows: $P_{cal} = 0.2 \times 0.33 \times 0.5 \times 0.2 \times 0.5 = 0.0033$ and $P_{med} = 0.2 \times 0.33 \times 0.5 = 0.033$.

The probability (*P*) of convergent evolution of two closely related genotypes from two independent sources is $P_{H2} = P_{cal} \times P_{med} = 0.0033 \times 0.33 = 0.0001$.

Alternatively, if we accept that MG26 in Italy is a direct descendant of MG10 in California, then $P_{H1} = 0.5$.

Of course, if convergent evolution at each of the seven loci were to be considered, the difference between probabilities associated with H_1 and H_2 would increase by several orders of magnitude. We conclude that, at this point, an introduction from California into Italy is the best explanation of the data. Additionally, preliminary analysis of >200 amplified fragment length polymorphism markers (G. Della Rocca, *unpublished data*) shows an extremely close relatedness of isolates from California and central Italy, with Californian isolates being basal to Italian isolates, further supporting our interpretation of the SSR data here presented.

In the case of the sudden oak death pathogen, *Phytophthora ramorum*, putative source genotypes were the most abundant and were additionally identified as direct progenitors of multiple new MGs, generated through mitotic recombinations or mutations (38,39). In the case of *S. cardinale*, it appears that epidemic expansion of the pathogen started with the emergence of MG28 and not with the original founder genotype, MG26 (Fig. 2). This difference may be due to the introduction of a genotype already preadapted to the ecological conditions of the novel environment and to favorable weather conditions in the case of *P. ramorum*, whereas a longer lag phase may have occurred for *S. cardinale* due to either the need for adaptation or the presence of unfavorable weather conditions immediately after the introduction of the pathogen in the Mediterranean region.

Network analysis suggests that introduction of the pathogen into the Mediterranean did not occur multiple times whereas movement within the Mediterranean may have occurred repeatedly, given the frequent retrieval of the same genotype in distant locations. We performed coalescent analysis to estimate migration among regions and potentially validate the results suggested by the network analysis. Only the three well-sampled populations from California, Italy, and Greece were included in this analysis, to avoid biases caused by small population size. Results of MIGRATE-N indicated a lack of significant migration between California and Italy or Greece, as expected of an epidemic origi-

TABLE 3. Results of coalescent analysis for California, Greece, and Italy populations using Migrate-N

	θ					Migration rate					
Location	2.50%	Mode	97.50%	Median	Mean	Direction	2.50%	Mode	97.50%	Median	Mean
1, Greece	0.073	0.098	0.100	0.091	0.088	1→2	0	22.5	45	27.5	23.16
	…	…	…	…	…	1→3	0	2.5	20	12.5	5.142
2, Italy	0.094	0.098	0.100	0.098	0.097	2→1	0	42.5	135	462.5	347.45
	…	…	…	…	…	2→3	0	2.5	25	12.5	6.18
3, California	0.094	0.098	0.100	0.098	0.097	3→1	0	2.5	25	12.5	6.392
	…	…	…	…	…	3→2	0	2.5	25	12.5	6.886

nated by a single introduction event. However, high migration was detected from Italy into Greece and lower but significant migration was also detected from Greece back into Italy. These high migration values are likely to be justified by repeated movement of infected plant material within the Mediterranean. An asymmetry in migration levels is expected during the initial phases of an invasion characterized by movement mainly from the area of original establishment (e.g., Italy) into areas being subsequently colonized (e.g., Greece).

We believe that data overall indicate that California is the most likely source of the epidemic in the Mediterranean, with two distinct introduction events, one in Italy and one in Morocco. After a lag phase in central Italy, populations of the pathogen expanded in size and started being moved across southern Europe, most likely in association with the movement of plants by humans. The magnitude of migration from Italy into Greece is in agreement with central Italy being at the source of the Mediterranean epidemic: pathogen populations are likely to have been greatly favored by the high density of susceptible *C. sempervirens* in central Italy. In contrast, the low density of hosts in Morocco (and perhaps a later date of introduction) may have slowed the epidemic linked to that introduction. Incidentally, our genetic reconstruction based on a variety of approaches depicts a scenario that well matches the history of the disease, which was first reported in California, then in central Italy, and then in other parts of Italy and the Mediterranean (2,15,17,18,25,31,65,67,69,70,75).

The presence of higher genetic diversity in California, including two clearly distinct β-tubulin alleles, and the finding that genotypes from the Southern Hemisphere display at least some genetic differences with European isolates, suggests that care should be taken to avoid further introductions of *S. cardinale* into the Mediterranean basin from either California or the Southern Hemisphere. Likewise, further introductions of *S. cardinale* from California and the Mediterranean into countries of the Southern Hemisphere should be avoided, if broader sampling confirms the homogeneity of pathogen populations in those countries. Although the exact relationship between genotypic and phenotypic variability is currently unknown, ecological fitness, pathogenicity, and potentially mating type (given the lack of LD reported in this study for California) are all likely to be correlated to genotypic variability. Consequently, the introduction of genetically different individuals in areas where the pathogen was previously introduced may result in a shift in the course of the epidemic, with dramatic impacts on host populations. Such shifts have been reported in other pathosystems in both the presence or absence of sexual reproduction (13,29,52). Unfortunately, regulatory systems of most countries often do not easily allow for a distinction between groups of genotypes of the same species, even when genetic information points to a significant isolation and independent evolution of such groups. In the case of cypress canker in the Mediterranean region, the introduction and establishment of significantly different genotypes or of genotypes bearing a different mating allele would also nullify the utility of resistant cypress lines selected to tolerate the closely related genotypes currently established in the Mediterranean. The discovery of California as a center of diversity for *S. cardinale* allows for the design of further selection trials using genotypes that are clearly distinct from the ones currently present in the Mediterranean. However, in order to ensure the durability of resistant host lines, it is imperative to minimize both the introduction of different genotypes and the potential for sexual recombination. Prevention of the introduction of novel mating alleles requires regulations to minimize or halt any further introductions of genetically distinct genotypes.

Given the good spatial coverage of the sampling conducted in the Mediterranean basin, the presence of identical genotypes in locations separated by thousands of kilometers and by large bodies of water is unlikely to be justified by natural spread of the pathogen and is most likely associated with movement of infected plant material by humans. Airborne spread of multicelled conidia at large distances is unreported for this type of fungal pathogen and, although movement of infected plant parts on migratory birds cannot be excluded, it is extremely less likely than human induced movement, given that Cypress trees are not known as significant foraging or nesting species for any migratory species. Although spread through human trade is not unexpected, the large number of findings ($n = 50$) of identical but geographically distant genotypes indicates that humans are moving the pathogen quite frequently. It is important to fully understand how and through which routes human activities are facilitating the long-range dispersal of this serious pathogen to prevent further introductions. The fact that the establishment of a single genotype may have caused the entire Mediterranean infestation highlights the absolute need and the challenge of preventing any further introduction. Although the genetic relatedness of Californian and Mediterranean populations is well supported by our data, sampling from other regions of the world was only minimal. Hence, although California appears to be the most likely source of the epidemic in the Mediterranean, whether it is the worldwide source of *S. cardinale* remains a distinct possibility but needs to be verified by further comparison with populations from other regions of the world. In the meanwhile, a closer look at the genotypic and phenotypic variability of Californian populations may help better understand the Mediterranean epidemic. Finally, additional research needs to confirm whether and where sexual reproduction may be occurring in California. Certainly, the absence of epidemics in the natural range of some Californian *Cupressus* spp., including *C. macrocarpa*, *C. bakeri*, *C. macnabiana*, *C. forbesii*, and *C. pygmaea*, is suggestive of a long co-evolutionary process between hosts and pathogen.

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