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Molecular analyses indicate that both native and exotic pathogen populations serve as sources of novel outbreaks of Cypress Canker Disease

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Abstract Invasive pathogens may follow stepwise spread pathways in which novel disease outbreaks become themselves sources of genotypes initiating novel infestations. Due to its worldwide distribution, Cypress Canker Disease (CCD) provides an opportunity to understand patterns of global spread of a forest pathogen. A comparative genetic analysis was performed on 110 isolates of the causal agent of CCD, the fungus *Seiridium cardinale*, from Europe, North America, Africa and Oceania using β-tubulin gene sequences and repeat numbers of seven nuclear SSRs. SSR data were used to calculate genetic indices for each geographic population, including those calculating allele and private allele diversity; clonal genotype, haplotype, and gene diversity; expected heterozygosity; and presence of linkage disequilibrium. Additionally, SSR data were used to calculate migration rates between regions, and to draw a minimum spanning network to visualize genetic relatedness among genotypes. Results identified the California population as a sexually reproducing, highly diverse, native population, and the Mediterranean population as a large, clonally reproducing, and exotic population directly derived from the California population. The New Zealand population appeared to be non-native, with intermediate values of genetic diversity and an asexual reproductive mode. Unexpectedly, two sources were identified for the New Zealand population: an older source was identified in California, while a more recent one was identified in the Mediterranean. Results allowed to infer migration intensity between continents, and are among the first to show that an exotic outbreak of a forest disease can become a source of pathogen genotypes for novel outbreaks in disjunct geographic regions.

Keywords Ghost population · Intercontinental spread routes · Migrate · Minimum spanning network · *Seiridium cardinale* · Stepping-stone population

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Introduction

The list of exotic plant diseases caused by the long-distance movement of plant pathogens is large and expanding (Wingfield et al. 2001; Burgess and Wingfield 2017; Ghelardini et al. 2017), but our understanding of the source and spread pathways of invasive pathogens is often incomplete. Reconstructing the history of movement of plant pathogens is complicated by two, often co-occurring, factors: (1) the native range of novel plant-pathogens may be unknown, and, (2) the discovery of introduced plant pathogens occurs after a significant time lag since their introduction (Garbelotto 2008). These two factors are particularly relevant for forest diseases, characterized often by long disease cycles and occurring in ecosystems not as intensively manipulated and scrutinized as agricultural systems.

The literature on the long-distance human-mediated spread of forest pathogens encompasses at least two broad categories. The first includes single or rare introduction events associated with the occasional movement of an infected substrate directly from the source location into the new range (Coetzee et al. 2001; Engelbrecht et al. 2004; Gonthier et al. 2004). The second instead includes an important intermediate step through which an exotic forest pathogen first becomes prevalent in a certain commodity or industry (Geils et al. 2010; Grunwald et al. 2012; Danti and Della Rocca 2017). The trade and long-distance movement of the infected substrate by the industry in question are then responsible for the introduction of that pathogen in a novel range.

No matter how a pathogen has been moved, understanding its source and the pathways of its spread is of pivotal importance to prevent further introductions (Martinelli et al. 2015). Additionally, the identification of native populations of an exotic pathogen allows to better understand how native flora may tolerate or resist infection and to further our understanding of its biology and ecology (Simms 1996).

Recent studies have identified a significant adaptive evolution of forest pathogens introduced in a novel range compared to native populations of the same pathogen, if migration between the novel and native populations is hampered by isolation (Kasuga et al. 2012; Garbelotto et al. 2015; Robin et al. 2017; Della Rocca et al. 2018). Such evolution can occur at the genetic (Della Rocca et al. 2013; Kasuga et al. 2012), and phenotypic (Garbelotto et al. 2015) levels, and results in populations that are distinctively different from native populations. The importance of this last aspect becomes even more apparent when movement of an invasive pathogen follows a stepwise, or stepping-stone, pattern in which a novel disease outbreak may become itself a source of pathogen genotypes for further infestations (Kimura and Weiss, 1964). In such cases, in fact, divergent evolutionary processes are likely to occur in correspondence of each step of the spread route, so that a source population in location "A" and a derived population in location "C" may display higher divergence if the derived population had a spread route characterized by an intermediate step in location "B", and A B C are isolated from one another. It has also been suggested that the probability of a pathogen population acting as a source for novel further infestations may be directly and positively correlated with the scale and severity of extant outbreaks (Croucher et al. 2013).

The Cypress Canker Disease (CCD) global pandemic (Graniti 1998; Danti and Della Rocca 2017 and reference therein) caused by the fungal pathogen *Seiridium cardinale* (Wag.) Sutton and Gibson provides an excellent opportunity to understand patterns and mechanisms of global spread of a forest pathogen because of its worldwide and apparently stepwise spread, and because of the severity and scale of the outbreak in the Mediterranean basin, a region clearly isolated from its native range supposedly located in California.

The specific aims of this study were to (1) verify whether Californian populations may indeed be considered native as recently suggested, when broadening sample size (Della Rocca et al. 2011a, 2013), (2) determine whether the large outbreak in the Mediterranean region may itself have become the source of additional introductions elsewhere in the world, thus serving as a "bridge" or stepping-stone for the spread of the pathogen, and, (3) determine whether the New Zealand population may be native, and if not, attempt to identify its source(s). In order to achieve these three goals, we performed a series of genetic analyses similar to those previously performed on Californian, Mediterranean, and Moroccan populations (Della Rocca et al. 2011a) on a sample enlarged to include several genotypes isolated in New Zealand. New Zealand was selected because CCD was reported there

early on at the beginning of the epidemic (Birch 1933) and because an original source of CCD epidemics in the Pacific-rim, as well as an Oceania (and/or South Asian) origin of the pathogen could not be excluded a priori.

Materials and methods

Isolates used

A total of 110 isolates of *S. cardinale* from four different continents (Europe, North America, Africa, Oceania) were used in this study (Supplementary Table S1). The populations from California (23 isolates) and that from the Mediterranean (69 isolates, including 3 from Morocco) were the same used in Della Rocca et al. (2011a), however, this study included 18 additional New Zealand isolates. Note that, based on results presented in Della Rocca et al. (2011a), the Moroccan population is clearly distinct from and has a different source than all other Mediterranean populations, including the one from Algeria.

DNA extraction

The *S. cardinale* cultures from New Zealand were grown on cellophane disc placed on 2% Potato Dextrose Agar (PDA) (Potato Dextrose Broth (PDB) 20 g/l + Agar 20 g/l) in Petri dishes for 2 weeks in the dark at constant temperature of 25 °C. *S. cardinale* colonies were then scraped off the cellophane, placed in 2 ml Eppendorf tubes, and freeze-dried over-night. Genomic DNA was extracted from approximately 100 mg of grounded freeze-dried mycelium using the Plant Mini Kit (Qiagen, Hilden, Germany) and following the producer's instructions.

Sequencing of β-tubulin

The β-tubulin gene of the 18 New Zealand isolates was amplified according to Della Rocca et al. (2011a), and the sequence information obtained was used to determine their haplotype identity (A or B), through comparisons with sequence accessions deposited in GenBank, and using the NCBI BLAST function (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The primers used were Bt1a and Bt1b, and the resulting DNA amplicons were sequenced with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Editing and alignment of the amplicons were performed on MEGA, version 4. The β-tubulin gene sequences generated in the course of this study, were deposited in GenBank (Supplementary Table S1).

SSR amplification

Seven nuclear microsatellite (SSR) markers (Sc01, Sc03, Sc04, Sc05, Sc06, Sc07 and Sc08) specific for *S. cardinale*, were used for genotyping the New Zealand isolates (Della Rocca et al. 2009). Conditions for Polymerase Chain Reactions (PCRs), primers, and protocols were those described in Della Rocca et al. (2009, 2011a). The same dataset produced by Della Rocca et al. (2011a) amended with data from the New Zealand genotypes was used for all analyses.

Calculation of genetic indices and SSR analysis

The lengths of the regions flanking the microsatellites were subtracted from the total size of amplicons, and the resulting lengths were transformed into number of repeats for all analyses. Multilocus genotypes (MGs) were at first determined by identifying unique combinations of alleles within each *S. cardinale* population. The lack of amplification at SSR Sc8 for 9 isolates of New Zealand was likely the result of a significant mismatch caused by one or more SNPs in one of the priming sites. Although this type of result cannot technically be regarded as a null allele at the locus in questions, such SNPs do identify an allelic variation, and we opted to count lack of amplification as an additional and "null" allele for the calculation of the various indices described immediately below, except for the Ia. This allowed us to use the entire set of New Zealand genotypes, thus increasing our statistical power. The use of failed amplification as a null allele is a clear issue when interpreting SSR results, however there is a consensus that if amplification primers have been previously tested on a large sample of genotypes and can be regarded as robust, and SSR data is simply used to infer a genotype or halpotype, without the need to determine parentage or relationship, interpreting a failed amplification as a null allele is beneficial and should not significantly bias the results (Park et al. 2009), In fact, this approach

is currently been followed to maximize the discriminating power of a SSR analysis (Bernard et al. 2018). In our case, primers have been tested and found to reliably amplify the Sc8 locus in hundreds of samples from all provenances, excluding New Zealand, suggesting a single mutation or indel in the priming site may be responsible for the observed lack of amplification observed for many New Zealand isolates, thus we used information from locus Sc8 to genotype isolates. However, as suggested in Park et al. (2009), we prefer to omit information from this locus when relatedness of genotypes needed to be determined on a minimum spanning network, when migration rates between populations needed to be estimated, or when linkage disequilibrium needed to be calculated among SSR markers.

Calculations of presence of linkage disequilibrium (LD) were performed using the program MULTILOCUS v1.3b (Agapow and Burt 2001) within each of the three *S. cardinale* populations separately (California, Mediterranean, New Zealand). The index of association (I_a) was used to test the extent of multilocus linkage equilibrium within each population by quantifying the amount of recombination among the set of allelic profiles, and detecting association between alleles at different loci. If there are frequent recombination events (linkage equilibrium) the I_a value tends to zero, while in clonal populations the I_a value is higher and differs significantly from zero.

Using the identified MGs, we estimated the genotypic (clonal) diversity in each *S. cardinale* population using indices as described in Mascheretti et al. (2009) and Della Rocca et al. (2011a): (1) clonal genotype diversity (R), R = (G − 1)/(N − 1) where G is the number of MGs present in a population and N is the number of fungal isolates in the considered population; (2) gene diversity (expected heterozygosity) (He) (Nei 1987)

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

where p is the frequency of the ith of k alleles, averaged over each l of m loci; and (3) haplotype diversity (H) (Nei and Tajima 1981), a measure of the uniqueness of a particular haplotype in a given population,

$$H = \frac{N}{N-1}\left(1 - \sum_i x_i^2\right)$$

where x^2 is the (relative) haplotype frequency of each haplotype in the sample and N is the sample size, calculated with the program Arlequin v.3.5.2.2 (Excoffier and Lischer 2010); (4) Stoddart and Taylor index (G) (Stoddart and Taylor 1988), $G = 1/\Sigma p_i^2$, where p_i is the frequency of the ith MG (Table 1).

Allelic richness (no. of distinct alleles expected in random subsamples of different size selected from a population) and private allelic richness (number of private alleles expected in random and differently sized subsamples drawn from each population) were also calculated for each population as in Garbelotto et al. (2013), using individual multilocus genotypes data with the program ADZE v.1.0 (Szpiech et al. 2008). The program implements the rarefaction method for analyzing allelic diversity across populations while correcting for sample size differences.

A principal component analysis (PCA) of individual MGs belonging to the three populations of *S. cardinale* was performed using GenoDive, and the first two principal components were used to create a MG-level PCA graph.

The following two analyses, instead, relied heavily -both for theoretical purposes and for the sake of consistency with previous studies- on the variation in number of repeats at each microsatellite locus. For this reason, in these cases (see below), we opted to use only data from the 99 isolates that could be properly amplified with the entire set of 7 SSR primers.

A pairwise matrix of genotype distances among all MGs, using repeat number differences and the stepwise mutation model, was calculated according to the method of Bruvo et al. (2004) with the program GenoDive 2.0b27 (Meirmans and Van Tienderen 2004). Then, in order to depict the relationships among all MGs a minimum spanning network (MSN) was created on the basis of the Bruvo's distances using the program MINSPNET (Excoffier and Smouse 1994) and the results were visualised with NEATO in Graphviz 2.26.3 (Gansner and North 2000).

Finally, to define the possible route of spread of *S. cardinale* among continents, the software MIGRATE-N v3.6.11 (Beerli 2009) was used to perform coalescent analyses and determine the directional rate of migration among California, the Mediterranean basin

Table 1 Association index and indices of genetic diversity: I_a, association index; R, clonal genotype diversity; G, Stoddart and Taylor's index; H_E, gene diversity (expected heterozygosity); H, haplotype diversity

Population	Ia	He	R	G	H
California (CAL)	− 0.041	0.434 ± 0.256	0.864	17.065	0.984
Mediterranean (MED)	0.505	0.246 ± 0.160	0.379	4.730	0.807
New Zealand (NZ)	2.343	0.477 ± 0.279	0.294	3.767	0.781

(Morocco was not included), and New Zealand. To estimate direction and migration rates between these three populations we used a Bayesian inference method (Beerli 2005) and microsatellite evolution was modelled using the Brownian motion model. An assumption of a constant mutation rate for all loci, a burn in of 10,000, and a static heating scheme 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 Theta and M were assumed to be uniform for both, ranging between 0 and 0.1 (Mean 0.01, Delta 0.01, Bins 200) for Theta, and between 0 and 1000 (Mean 100, Delta 100, Bins 200) for M.

Results

All the isolates used in previous population genetics analyses and used again in this study (Della Rocca et al. 2011a, 2013) were confirmed to be true *S. cardinale* genotypes based on the identity of their ITS and β-tubulin sequences with Genbank accessions (Barnes et al. 2001). As an interesting control, several isolates collected at the same time and excluded from such studies, were instead found to be *S. unicorne* based on β-tubulin sequences (Della Rocca et al. 2011b). Sequences of the β-tubulin locus were obtained from all New Zealand isolates in the course of this study, and found to perfectly match either the A or B allele of *S. cardinale* sequences, as reported in Della Rocca et al. (2011a) (Supplementary Table S1). All sequences were clearly distinct from all other *Seiridium* spp. sequences (Bonthond et al. 2018).

Seven polymorphic microsatellites specifically developed for the fungal pathogen *S. cardinale*, agent of CCD (Della Rocca et al. 2009), were analyzed for 108 isolates belonging to four intercontinental populations (California, Mediterranean, Morocco, New Zealand, hereafter indicated respectively as CAL, MED, MOR, and NZ) (Supplementary Table S1). A total of 52 MGs were identified (Supplementary Table S2): 20 in CAL (over 23 isolates), 25 in the MED (over 66 isolates), 1 in MOR (3 isolates), and 6 in NZ (over 18 isolates). Interestingly, no MGs were shared among populations. Many MGs were singletons, i.e. they were represented by an individual isolate: 18 (78.3%) in CAL; 18 in MED (26.9%), and 3 (16.7%) in NZ. The most frequent MGs was MG31 from the Mediterranean (n = 29; 43.3%).

The association indices (I_a) of the three larger populations (California or CA:, Mediterranean or MED, New Zealand or NZ) are markedly different, very low in the CAL population ($I_a = -0.41$; $p = 0.553$), significantly higher in MED ($I_a = 0.505$; $p = 0.09$) and even higher in the NZ one ($I_a = 2.343$; $p < 0.001$) (Table 1).

The indices of genetic diversity are reported in Table 1. Both CAL and NZ populations had higher gene diversity/expected heterozygosity (He) (0.434 ± 0.256 and 0.477 ± 0.279, respectively) than that of the MED population (0.246 ± 0.160). Instead, the genotype diversity (R) and the Stoddart and Taylor index (G) were more than two fold higher in the CAL population than in the MED and NZ populations (R = 0.864 CAL, 0.379 MED, 0.294 NZ; G = 17.065 CAL, 4.730 MED, 3.767 NZ). Haplotype diversity (H) is quite similar in all three populations of *S. cardinale* (0.984 in CAL, 0.807 in MED, 3.767 in NZ) (Table 1).

The results of the rarefaction analysis indicated that both allelic richness and private allelic richness increased with sample size. Private allelic richness started to level with sample size for Mediterranean and New Zealand populations, indicating our sample size was adequate. The more diverse native California population was instead and expectedly undersampled. The highest overall allelic richness was found in the

CAL population, while the lowest overall value was found in the MED population. High private allelic richness with low sample size for the New Zealand population was also identified (Fig. 1).

Minimum spanning network

California and European populations were represented by two contiguous groups of genotypes, not overlapping, but closely related, as previously reported (Della Rocca et al. 2011a, 2013). The NZ population includes instead two disjunct groups of genotypes. One is closely related, but not overlapping, with the California population, while the second is represented by one genotype that cannot be differentiated from European genotypes, being positioned in between a Sicilian and a Portuguese genotype (Fig. 2).

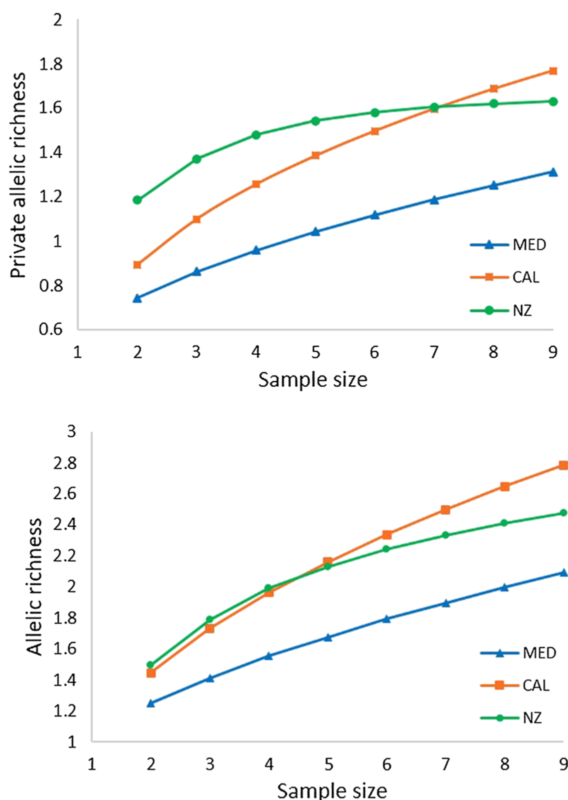

Fig. 1 Allelic richness (below), and private allelic richness (above) of *S. cardinale* populations (California, Mediterranean, New Zealand) as a function of standardized sample size

Migrate

Four significant migration routes were identified. The two largest numbers of migrants were identified from California into the Mediterranean, and vice versa. The third most significant number of migrants was identified between California and New Zealand. A fourth significant number of migrants was identified between the Mediterranean and New Zealand (Table 2).

The PCA based on the Bruvo's distances among MGs is shown in Fig. 3. The variance explained by the first component (Root 1) was 22.45%, whereas 13.54% of the variance was explained by the second (Root 2). The first function discriminates primarily the CAL population from the others, while the second function discriminates the NZ population from the other two main populations tested. At least three MGs from New Zealand clustered near (two MGs) or very near (one MG) the MED population.

Discussion

Cypress Canker Disease (CCD) is known to have a worldwide distribution and to cause significant dieback and mortality of cupressaceous host species (Wagener 1928, 1939; Graniti 1998; Danti and Della Rocca 2017). Several *Seiridium* species are known to cause disease on cupressaceous hosts, including the newly described S. *neocupressi*, *S. cancrinum* and *S. kenyanum* (Bonthond et al. 2018). However, surveys conducted in North America and Europe (Danti and Della Rocca 2017) have identified *S. cardinale* as the main causal agent of CCD, indicating that other two well known *Seiridium* species, namely *S. unicorne* (Cooke and Ellis) B. Sutton and *S. cupressi* (Guba) Boesew., are either marginal or prevalent only in individual regional outbreaks (i.e. East Africa and Australia, Wimbush 1944; Cunnington 2007), while the complete distribution of newly discovered species is still unknown (Bonthond et al. 2018). Spread of the disease within a region has been studied more intensively for the European outbreak. Spread in the Mediterranean is effected by mitospores known as conidia that are either windborne or vectored by insects (Danti et al. 2013). Conidia are also produced on cypress cones (Danti et al. 2013) and may have enhanced spread of the pathogen in the past, whenever seed was traded or moved long distance.

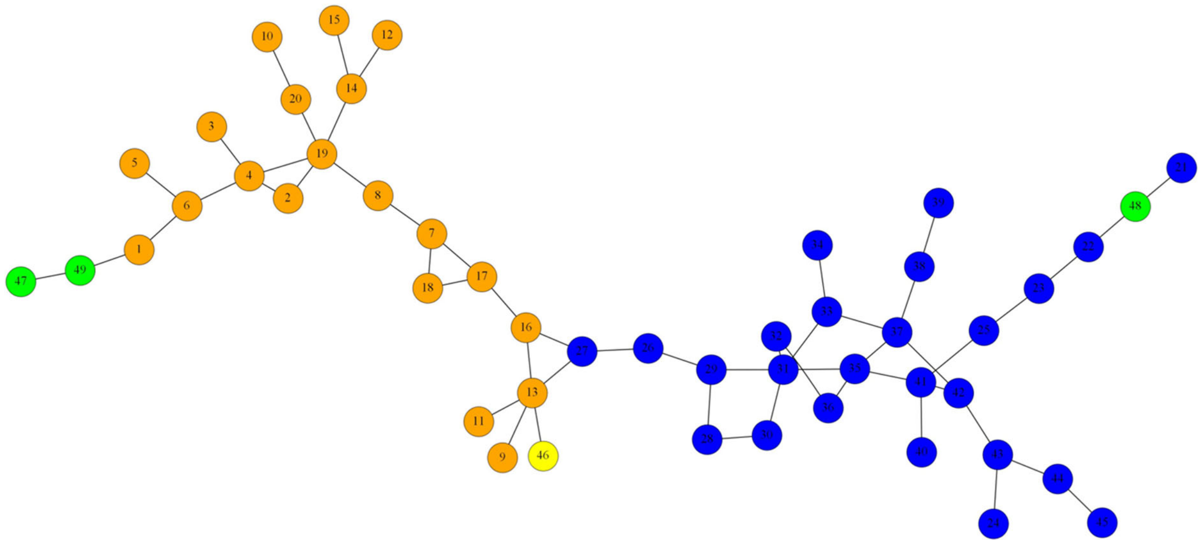

Fig. 2 Minimum spanning network (MSN) based on Bruvo's distances among multi locus genotypes (MGs) of Mediterranean (blue), California (orange), Morocco (yellow) and New Zealand (green) populations. Each node represents a single MGs

Table 2 Results of coalescent analysis for *S. cardinale*'s populations of California (CAL), Mediterranean (MED) and New Zealand (NZ) using Migrate-N

Population	Theta					Migration rate					
	2.50%	Mode	97.50%	Median	Mean	Direction	2.50%	Mode	97.50%	Median	Mean
1. CAL	0.0930	0.0983	0.1000	0.0983	0.0971	1 → 2	230.000	272.500	375.000	287.500	264.829
						1 → 3	35.000	67.500	140.000	82.500	99.147
2. MED	0.0950	0.0983	0.1000	0.0988	0.0976	2 → 1	20.000	42.500	65.000	52.500	129.967
						2 → 3	15.000	62.500	115.000	67.500	62.704
3. NZ	0.0000	0.0033	0.0065	0.0038	0.0036	3 → 1	0.000	12.500	35.000	22.500	15.372
						3 → 2	0.000	12.500	30.000	22.500	14.269

Successful infections by *S. cardinale* are associated with either wounds or with tunnels generated by insects burrowing under the bark (Scolitid beetles of the genus *Phloeosinus*) (Roques and Battisti 1999). Colonization results in lesions girdling the cambial layer of infected hosts and can be hampered by various degrees and types of disease resistance (Achotegui-Castells et al. 2015, 2016; Sparapano and Evidente, 1995). It is known that disease resistance is not universal, but it is a polygenic trait, mediated by a host genotype x pathogen genotype interaction (Ponchet and Andreoli 1990; Spanos et al. 1999), as recently documented in Della Rocca et al. 2018. Although not properly quantified, results presented by Della Rocca et al. (2018) suggested that frequency of failures in the disease resistant phenotype of hosts may be higher where pathogen populations are genetically more diverse, and/or when challenging putatively disease-resistant host genotypes with pathogen genotypes from a different world region. Success of invasive *S. cardinale* populations outside of the pathogen native's range is strongly correlated with intermediate (not high) pathogenicity ensuring prolonged sporulation, smaller conidia size resulting in greater dispersal, overall greater sporulation potential, and high plasticity of all traits ensuring fast adaptation to novel environments (Garbelotto et al. 2015). On the other hand, it appears that spread of *S. cardinale* in novel ranges may be hampered by high and low temperatures, in spite of the ability of native populations to

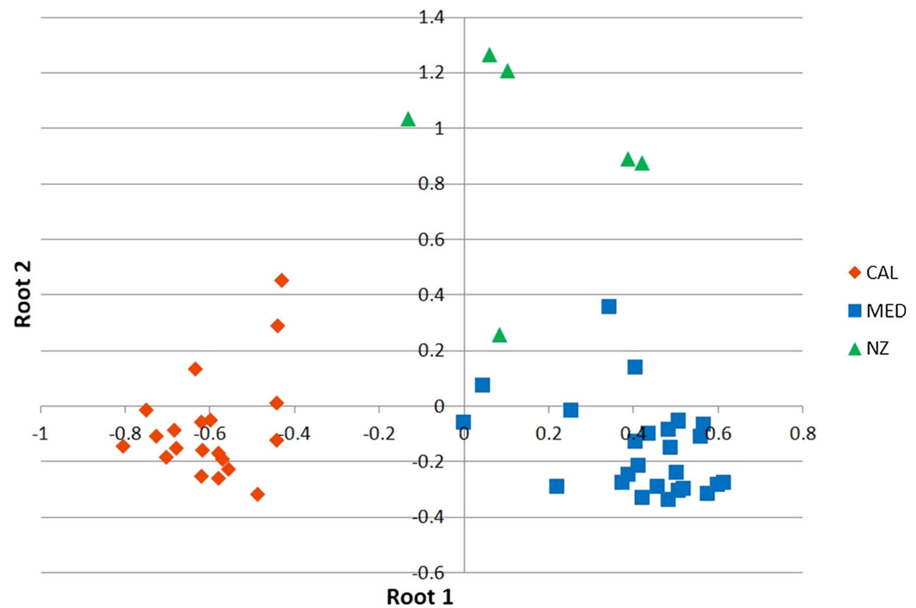

Fig. 3 Scatterplot of principal component analysis (PCA) based on Bruvo's distances among *S. cardinale*'s MGs belonging to California (orange), Mediterranean (blue), and New Zealand (green)

survive at such levels (Garbelotto et al. 2015). Because the combination of the above fitness traits is highly population dependent, understanding the source population for a novel outbreak has dramatic implications with regards to the frequency and durability of disease resistance, as well as with regards to our ability to predict the invasive potential of the pathogen.

Della Rocca et al. (2011a, 2013) have been able to identify the putative native pathogen population and to partially reconstruct the history of global and regional movement of *S. cardinale,* using a population genetic approach based on SSR markers and sequencing of the β-tubulin locus. The Californian population was genetically rather diverse, displayed no linkage disequilibrium (LD) as expected for a sexually reproducing population, and included two β-tubulin alleles. The Mediterranean population instead was less diverse, displayed LD typical of a clonally reproducing population, and included a single β-tubulin allele. A network analysis based on Bruvo's genetic distances identified a Mediterranean cluster of genotypes clearly distinct from the Californian cluster: the two were connected by a single link between two closely related genotypes (one Californian and one Mediterranean), suggesting the close relationship between the two populations and indicating a single introduction was responsible for the sizeable Mediterranean outbreak (Della Rocca et al. 2011a). Genotypes from an outbreak in Morocco (Danti et al. 2009) also appeared to be derived from a California genotype, but such genotype was different from the one identified as the founder for the Mediterranean outbreak. Strong and mostly unidirectional migration was identified between Italy and Greece, suggesting Italy was a stepping-stone for regional spread of the pathogen in the Mediterranean (Della Rocca et al. 2013). This conclusion appeared realistic based on the fact that Italian plant nurseries are, and long have been, the largest producers of Italian cypress (*Cupressus sempervirens* L.), the most common host for *S. cardinale* in the Mediterranean basin. Lack of significant MIGRATE results when comparing populations from California and Europe was interpreted as the result of a single migration event between the two continents.

The first aim of this study was to confirm the native nature of the Californian *S. cardinale* population by adding to the analysis genotypes from New Zealand, where an outbreak of CCD was putatively described as early as 1933 (Birch 1933). A series of genetic and genotypic indices were calculated to compare the Californian, Mediterranean, and New Zealand populations: New Zealand resulted to be in an intermediate position between California and the Mediterranean, by displaying a larger expected heterozygosity than the Mediterranean sample, similar to that of the Californian samples. Clonal genotype, haplotype, and gene diversity values for New Zealand were smaller than those for California and were instead similar or

slightly less than those of the Mediterranean sample. Additionally, the presence of LD in the New Zealand population indicated lack of sexual reproduction, a trait strongly suggesting *S. cardinale* may not be native to that region, while absence of LD confirms California populations are likely to be native.

Nonetheless, the presence of both β-tubulin alleles in New Zealand, the larger expected heterozygosity of its population, and the high value of private allelic richness at low sample size, suggested multiple introductions may be responsible for the New Zealand outbreak. The output of the MIGRATE analysis indicated migration was significant (e.g. different from zero) in four cases: from California to the Mediterranean and vice versa, from California to New Zealand, and, to a lesser degree, from the Mediterranean to New Zealand. The largest and second largest migration values were obtained for the California to the Mediterranean route, and vice versa, respectively. The third largest value was obtained for the California to New Zealand route, and a fourth relatively high and marginally significant migration value as identified between the Mediterranean and New Zealand. These results are interesting in more than one way: first, it appears that adding the New Zealand population (a population that could be regarded as a ghost population in Della Rocca et al. 2011a, 2013) allowed MIGRATE to confirm significant California to Mediterranean migration, and vice versa. This is consistent with the overall conclusions published in two papers by Della Rocca et al. (2011a, 2013), and is consistent with the overall general theory that states that better sampling of a population may significantly improve estimation of migration rates (Broquet et al. 2009), and that including ghost populations may also improve such estimation, although to a lesser degree than increasing representation of sampled populations (Beerli 2004). Better sampling in this study is the result of pooling all Mediterranean samples, except the genetically distinct Moroccan individuals, in a single population, rather than separating them in geographically distinct Italian, French, Greek or Algerian populations as done in Della Rocca et al. (2013). Additionally, the New Zealand population was by default omitted in previous studies, and thus it was a ghost population until it was included in the MIGRATE analysis presented in this study. The presence of reverse migration from the Mediterranean into California is also intriguing, and due to the documented differentiation between the derived Mediterranean and the Californian source population of this pathogen (Garbelotto et al. 2015), it has implications for worldwide policy.

The research presented here identifies two significant migration routes into New Zealand, one, prevalent and older, from California, and a secondary more recent one from the Mediterranean (see below). The possibility of two distinct introductions from two different sources would in part justify the relatively high diversity (β-tubulin haplotypes, expected heterozygosity) typical of the New Zealand *S. cardinale* population. Such diversity in fact was evident in spite of the fact that CCD outbreaks, and thus the size of *S. cardinale* populations, were, and still are reported to be much larger in the Mediterranean region than in New Zealand. This remains true even considering that CCD is the most significant limitation to cypress forestry in New Zealand, but outbreaks remain often unreported (Hood et al. 2009). Population size is important in clonally reproducing populations as it is likely to be directly and positively correlated with the amount of genetic diversity accumulated through mutations (Croucher et al. 2011). Hence, all things equal, and considering only clonally reproducing exotic populations, the diversity of *S. cardinale* should be greater in the Mediterranean basin, where outbreaks of CCD have been much more significant and pathogen populations have been reported as being extremely large (Danti and Della Rocca 2017).

The network of genotypes based on Bruvo's distances provides a visual rendition of the relative relatedness among individuals, with closely related genotypes being in the same portion of the network, possibly connected by a line indicating a recent shared ancestry. Populations that evolved in isolation but sharing a common ancestry are normally depicted as clusters of genotypes clearly distinct from clusters representing other populations. As previously reported, the Mediterranean and the Californian populations formed two interconnected but distinct and not-overlapping clusters. Unexpectedly, the New Zealand sample did not form a single continuous cluster of genotypes, rather, New Zealand genotypes were positioned in two distinct parts of the network. Two New Zealand MGs were placed at the edges of the California cluster, while one New Zealand genotype was placed at the edges of the Mediterranean cluster. This result strongly corroborates the dual

origin of New Zealand populations suggested by the MIGRATE analysis, and identifies the Mediterranean outbreak as a new source of pathogen genotypes responsible for further global-scale spread of a serious forest pathogen. Within this study there is no evidence of re-combination between these populations in New Zealand, but the genetic variation of New Zealand isolates should be monitored and considered for breeding, selection and disease management programs addressing CCD.

Because the New Zealand genotype originating from Europe is positioned in between two European genotypes, and because the PCA places the New Zealand population rather close to the Mediterranean population, it appears that the Mediterranean introduction pathway may be relatively recent, and not enough time may have occurred for significant drift-driven micro evolutionary divergence between such two populations. With the exception of the early report by Birch (1933) largely supported by corroborating evidence (Chou 1989, 1990), the actual early history of *S. cardinale* (synonym *Coryneum cardinale*) in New Zealand has not been well characterized over time, due to misidentifications with other *Seiridium* species (Fuller and Newhook 1954; Weston 1957; Newhook 1962; Gilmour 1966) and the official reconfirmation of its presence delayed until 1982 (Beresford and Mulholland, 1983). In New Zealand, the diffusion of *S. cardinale* may be limited by the wide presence of the congeneric species *S. cupressi* (van der Werff 1988; Hood 2007), preventing a reliable symptom-and species-specific chronology of the disease. Based on the findings of this study and on the disease reports available, it could be suggested that the first introduction of the pathogen from California predated 1933, and possibly occurred through the same pathway as that responsible for the introduction of the pathogen into the Mediterranean region. The second introduction from Europe, instead, may predate 1982 by a few years.

New Zealand's modest industry of Cypress forestry is currently only 10,000 ha of production and represents less than 0.7% of forestry production. This is a mixture of macrocarpa (*Hesperocyparis. macrocarpa*) and lusitanica (*C. lusitanica*), with minor plantings of Lawson cypress (*C. lawsoniana*) and Leyland cypress (X *C. leylandii*) clones. Expansion of the industry thus is currently limited by CCD. In New Zealand, cypress is currently consumed domestically as there is strong recognition of its wood properties and natural durability. Under current use and biosecurity regulation, exported wood is processed prior to shipment meaning that New Zealand presents minimal biosecurity risk internationally.

Understanding the exact pathway of spread of forest pathogens that disperse long distance following a stepping-stone model has long been shown to have important theoretical and applied implications, even if some uncertainty may exist on one or more legs of their spread. A recently discovered "stepping stone" pathway has been described for *Teratosphaeria nubilosa* sensu *strictu*, a serious pathogen of *Eucalyptus* spp. native to Eastern Australia and reportedly introduced multiple times in South Africa. In a second step of the worldwide spread of this pathogen, South African populations rather than Australian ones acted as a source of founder individuals for outbreaks in East Africa and Europe (Hunter et al. 2008; Pérez et al. 2012), resulting in populations progressively more simplified genetically as one goes from Australia to South Africa and finally to East Africa and Europe. Likewise, Eastern and Western North American outbreaks of white pine blister rust caused by the fungus *Cronartium ribicola* are genetically quite distinct (Hamelin et al. 2000). The reason behind this clear difference appears to be in the location (Holland vs. Germany vs. France) of the nurseries that provided infected *Pinus* seedlings to various North American buyers and that served as sources of founder individuals for the different outbreaks, even if it is still unknown how that diversity in inoculum among European nursery came to be (Geils et al. 2010). In other cases, the known consequences of a stepping-stone spread pathway for a plant pathogen are more complex than genetic diversity of invasive populations. For instance, in the case of the Chestnut blight fungus *Cryphonectria parasitica*, infected chestnut trees were first moved between Japan and China to the USA, and subsequently the US population acted as a bridge and became the most important source of European outbreaks of the disease (Milgroom et al. 1996). Because the European population is two steps removed from the native Asian population, it appears to be genetically oversimplified, it contains significantly fewer somatically incompatible VC groups than North American or Asian populations, and it's characterized by limited sexual reproduction. These three traits are strongly correlated with the successful

spread, both natural and assisted by humans, of hypoviruses that acted as biocontrols and have reduced the severity of European outbreaks, while failing in the USA (Dutech et al. 2012). Additionally, there is good evidence that the difference among dominant viral strains present in different European regions, may have to do with the different fungal founder populations that acted as sources of infestations located in different European regions (Breuillin et al. 2006). Likewise, it appears that the Pine Pitch Canker fungus *Fusarium circinatum* may have first been spread from its native Mexico to other parts of the world, including the USA and South Africa (Wikler and Gordon 2000). However, most data also seem to indicate that further spread of the disease into other parts of the world including Japan, a single shipment of plants to New Zealand, Spain and Portugal may have been effected by the exotic US population acting as a bridge (Wingfield et al. 2008; Vogler et al. 2004). Even so, the specific origin of the source of these further infestations may affect the nature of subsequent outbreaks. For instance, two dominant strains are present in Iberia: each seems to be associated with a different US source, and each is characterized by only one of the two possible mating types, with obvious implications for disease epidemiology, especially when comparing regions infested only by one such strain versus regions infested by both (Berbegal et al. 2013). Finally, the Sudden Oak Death pathogen, *Phytophthora ramorum*, became prevalent in North American nurseries during the 1990s, but it did so following clearly distinct, even if not fully understood, spread pathways (Garbelotto and Rizzo 2005). The two lineages NA1 and NA2 carrying the A2 mating type may have arrived to the USA directly from the putative East Asian native range of the pathogen, while the EU1 lineage, carrying the A1 mating type, is known to have arrived to the US from Europe through the trade of infested plant stock (Grunwald et al. 2012). Besides mating type, the three lineages differ in temperature optima, virulence on plant hosts, and resistance to fungicides (Eyre et al. 2014; Hunter et al. 2018), thus, whether an invasive population came through a stepping stone (Europe) or not, has extremely relevant consequences for disease spread and control.

In summary, there are at least three important implications associated with the finding that outbreaks outside the native range of a plant pathogen may become the source of further outbreaks. The most important one has to do with the fact that exotic populations of a pathogen, as specifically demonstrated for *S. cardinale*, differ both genetically and phenotypically from native source populations (Garbelotto et al. 2015). Hence, the outcomes of introductions linked to different sources may be more difficult to predict, given that higher diversity of founder populations may confer a greater adaptive ability to an exotic pathogen. For instance, it has been recently shown that Italian cypress clones selected for resistance against Italian populations of *S. cardinale*, may actually be less resistant when challenged with Californian pathogen genotypes (Della Rocca et al. 2018). Likewise, it may be interesting to compare overall virulence and transmission potential of New Zealand genotypes with different sources (Mediterranean vs. Californian).

The second implication has to do with the understanding that uncontrolled disease outbreaks caused by infectious organisms not only have serious ecological, social, and economic implications for the region directly affected by the outbreak, but by becoming themselves a source of the pathogen, they may be responsible for novel outbreaks in other parts of the world with implications for biosecurity and trade. This additional consequence of unchecked outbreaks may justify sustaining higher costs to locally mitigate and reduce the severity of outbreaks caused by infectious tree diseases (Cunniffe et al. 2016).

The third implication stems from the fact that, with distinct populations established in Europe, California and New Zealand, each established population is a potential source of genotypes of the pathogen still absent in many world regions, but possibly also a source of "exotic" genotypes for one another. These demonstrable differences among established plant pathogen populations in different world regions should be taken into account by regulatory policies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no direct or indirect conflicts of interest in this research.

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