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An increase in transmission-related traits and in phenotypic plasticity is documented during a fungal invasion

MATTEO GARBELOTTO,[1] GIANNI DELLA ROCCA,[2,]† TODD OSMUNDSON,[1,3] VINCENZO DI LONARDO,[2] AND ROBERTO DANTI[2]

[1]*Department of ESPM, 54 Hilgard Hall, University of California, Berkeley, California 94720 USA*
[2]*Institute for Sustainable Plant Protection, CNR, Via Madonna del Piano 10, 50019 Sesto Fiorentino (FI), Italy*

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Abstract. The adaptive rapid evolution of phenotypic traits is potentially a key contributor to invasiveness, but has been relatively little studied for the fungi, even though these organisms are responsible for devastating losses in agriculture and natural resources. In this study, we compare biologically relevant phenotypic characters of spore-generated individuals from two native and two invasive populations of the fungal pathogen *Seiridium cardinale* to infer which traits may be adaptive and rapidly evolving during an ongoing biological invasion. Results show that: (1) lower growth rate and smaller spore size are selected for in invasive populations, independent of the stage of invasion; (2) there is no selection evident towards increased rapid sporulation, but overall reproductive potential increases in later stages of the invasions; and (3) demographic plasticity of most traits increases during the initial stages of invasion, but decreases in a later phase. Comparisons against levels of neutral genetic variation (Qst-Fst comparisons) showed that the decrease in spore size is strongly adaptive, despite the trade-off of reduced viability. Lesion size of isolates inoculated on the naïve Italian cypress host was not correlated with their growth rate, and was significantly lower in invasive than in native populations. This last result indicates that rate of host colonization is a complex trait affected both by host and pathogen, which may not be necessarily adaptive and/or which may not easily evolve. In summary, the success of *S. cardinale* as an invasive in the Mediterranean basin is associated with reduced spore size and increased plasticity of almost all traits in initial phases, followed by further decreased spore size, increased overall sporulation, and decreased plasticity in a second phase of the invasion. Interestingly, growth rate by population results show that invasive populations are well adapted only to moderate temperatures, while native populations fare well also when exposed to relative extremes in temperature. This different adaptation suggests a "master-of-some" specialization scenario for the invasion by *S. cardinale* in the Mediterranean.

Key words: adaptation; canker; co-evolution; cypress; disease; epidemic; genetic variability; pathogenicity; selection.

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[3] Present address: Department of Biology, University of Wisconsin, La Crosse, Wisconsin 54601 USA.
† **E-mail:** gianni.dellaroca@ipsp.cnr.it

INTRODUCTION

As they expand their ranges and encounter new environments, invasive species provide an invaluable opportunity to understand the evolution of adaptation in organisms in real-time (Suarez and Tsutui 2008). In particular, they allow for the observation of how trait variation

may be correlated to adaptation within the manageable timeframe of an invasion process. Useful observations include not only the measurement of changes in phenotypic traits, but also of the range of their variability during an invasion, a response that may be driven in part by plasticity (Pigliucci 2001). Because invasions are often initiated by the long-distance movement of a few individuals, the extent of phenotypic variability in the presence of limited genetic variability may provide insights into the role plasticity may play in the invasion process, a concept that has been often theoretically suggested, but little studied (Richards et al. 2006). This is particularly true for clonally reproducing organisms, in which the genetic make up of founder individuals has the potential to influence invasive populations for periods of considerable length, as mutation-derived adaptation occurs at much lower frequency than that driven by recombination in sexually reproducing species (Fisher 1930, Muller 1932). Although the literature on phenotypic adaptation of invasive organisms is ample, there is often no clear consensus on what traits may be driving invasions (reviewed in van Kleunen et al. 2010). In many cases, the inability to draw solid conclusions is derived from the fact that "soft" traits, more easily measurable and hence more frequently analysed, are not a perfect proxy for the "hard" traits directly involved in the determination of invasiveness (Violle et al. 2007). The limited amount of published research focusing on trait selection for fungi is also noteworthy. The scant publication record on this subject for an entire Kingdom has been determined not only by the difficulty in performing analysis of phenotypic traits for microorganisms, but also by an excessive focus on the presumed role played by high virulence levels in non-coevolved host-fungus interactions, neglecting other ecological and transmission factors (Garbelotto et al. 2010). In recent times, a new impetus has been placed in providing a more comprehensive framework to understand the invasive potential of fungi, which can be better modelled and explained by determining their transmission potential as a function of their establishment success, survival, reproductive rate and dispersal, rather than just as a function of their pathogenicity (Gonthier and Garbelotto 2013). A further limitation—this one common to the study of all invasive species—is the imperfect knowledge of the exact geographic origin of most invasive fungi. The inclusion of the correct source population is critical for studying trait variation of an invasive population through comparison with native populations (Keller and Taylor 2008).

In a simulation study, Burton et al. (2010) showed that during range expansion, dispersive traits are selected for at the front of the expanding population, whereas traits associated with fitness at equilibrium density (i.e., competitive ability) show strong declines. However, hard data in support of these predictions for fungi are still scant.

In this study, we analyze and compare traits of two native and sexually outcrossing California populations of the pathogenic fungus *Seiridium cardinale* (Wagener 1928) Sutton & Gibson (*Ascomycota*; class: *Coelomycetes*; order: *Melanconiales*) with those of two populations sequentially derived from them after the successful movement of the pathogen to southern Europe (Della Rocca et al. 2011, 2013). The two native populations can be differentiated by sequence differences at the β-tubulin locus, and thus they have been called β-tubulin A and B or, in short, Cal A and Cal B (Della Rocca et al. 2011). The two European populations are sequentially derived from the Cal A population but can be differentiated using Bayesian analysis of population structure (STRUCTURE; Pritchard et al. 2000) based on AFLP loci. Structure group 1 includes the founder Mediterranean population (i.e., that directly derived from Cal A) and has been called Med 1, while Structure group 2—the population derived from Med 1 in Europe—has been called Med 2 (Della Rocca et al. 2013). Structure group 1 also includes some California individuals (referred to as Cal A1), presumably related to the founder individuals that were originally transported from California to Southern Europe (see Fig. 1 for a schematic representation of populations in this study). All studies thus far have shown Mediterranean populations to be genetically rather uniform (Viljoen et al. 1993, Moricca et al. 2000, Barnes et al. 2001, Krokene et al. 2004, Pedron et al. 2007) and reproducing clonally (Della Rocca et al. 2011, 2013).

S. cardinale is the main causal agent of a pandemic tree disease called cypress canker

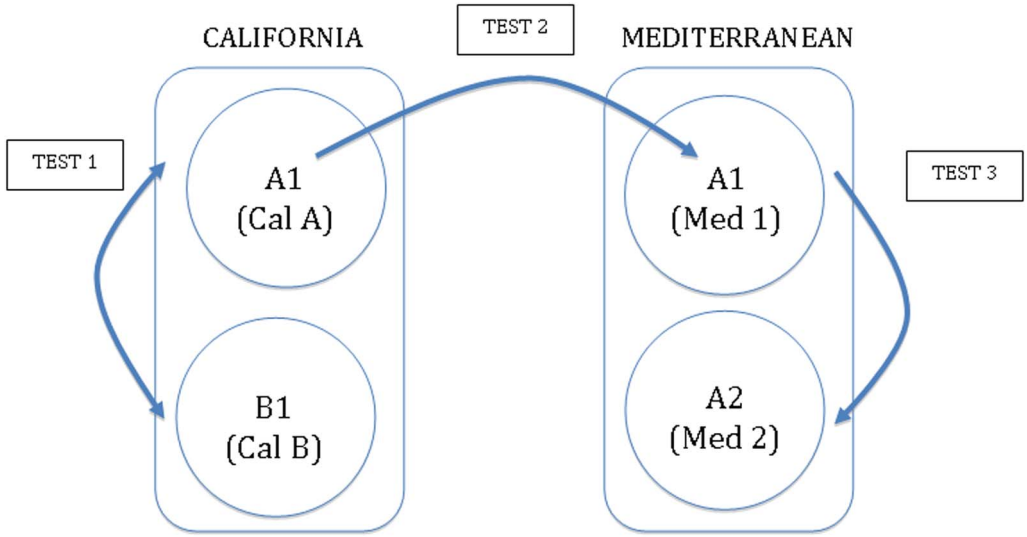

Fig. 1. Migration pathways of *Seiridium cardinale* inferred from genotype data (Della Rocca et al. 2011, 2013), with tests of genotypic and phenotypic divergence noted. Tests were conducted between (1) the two β-tubulin haplotypes found within California, the presumed host range of *S. cardinale*; (2) California population A1 (the presumed source of the California epidemic) and the initial Mediterranean invasive population A1; and (3) Mediterranean population A1 and the secondarily derived Mediterranean population A2. Beta-tubulin haplotypes (Della Rocca et al. 2011) are denoted by letters A and B; clusters defined by STRUCTURE analyses (Della Rocca et al. 2013) are denoted by numbers 1 and 2.

(Panconesi 1990, Graniti 1998, Danti et al. 2013*a*) causing significant mortality in many species of the plant family *Cupressaceae* worldwide, with reports from the USA, New Zealand, France, Chile, Italy, Argentina and Greece (Birch 1933, Barthelet and Vinot 1944, Grasso 1951, Saravì Cisneros 1953, Anastassiades 1963), as well as from the rest of the Mediterranean region and from other countries in central-northern Europe, to Canada, North and South Africa and Australia (Torres 1969, Strouts 1970, Sutton and Gibson 1972, Funk 1974, Caetano 1980, Solel et al. 1983, Wingfield and Swart 1988, Danti et al. 2009, 2013*a*). Although two other congeneric species, namely *S. cupressi* (Guba) Boesew. (teleomorph: *Lepteutypa cupressi* (Nattrass, Booth & Sutton) Swart and *S. unicorne* (Cooke & Ellis) Sutton, can cause the same disease, their abundance is extremely limited in most regions of the world (Graniti 1998). While long distance spread of *S. cardinale* is presumably mediated by the movement of infected plant material (Della Rocca et al. 2011, 2013), short and medium range spread of the pathogen occurs through spores that may either be windborne in warm and wet spells, or carried by insect vectors (Panconesi 1990, Roques and Battisti 1999).

The aims of this study were to compare the variation in measurable functional traits that are considered important for establishment, plant infection, and transmission of fungal pathogens, in order to determine which traits may favor an invasion and which traits may be rapidly evolving. In addition, we studied whether invasive populations may undergo measurable changes in the plasticity of such traits, and we assessed whether these changes may be adaptive (Keller and Taylor 2008). In order to achieve the above goals, traits were measured in common garden experiments for discrete populations to provide comparative demographic parameters (Violle et al. 2007). Comparisons between levels of trait differentiation and neutral genetic differentiation between populations (Q_{ST}-F_{ST} comparisons; Prout and Barker 1993, Spitze 1993) were used to assess the possible role of selection in the evolution of traits (reviewed in Leinonen et al. 2013). Such comparisons draw upon the level of neutral genetic variation between populations relative to total genetic variance, as a null

expectation for the degree of between-population variance in additive genetic traits.

Our specific hypotheses were as follows: (1) Range expansion should be strongly mediated by traits facilitating transmission (such as overall fruiting body production) and long-distance spread (e.g., smaller spore sizes), while traits linked to fitness and virulence (e.g, growth rate *in planta* and *in vitro*) should not necessarily be favored. In addition, assuming *S. cardinale* invaded a niche unoccupied by other competitors, there should be no measurable shift towards increased sporulation in the short term and towards increased germinability, two traits that may confer an advantage to the invasive species in the presence of competitors. (2) Demographic plasticity should be favored and should increase in the early stages of invasion, thus allowing the exotic species to adapt to a variety of novel environments, but establishment of the exotic organism in the most favorable habitats should reduce plasticity in the second stages of an invasion. (3) Although measurable shifts may be identified in various traits, only some will be adaptive, i.e., driven by selection, while others may simply shift due to genetic drift. In addition, a delay in the change of a trait (i.e., a trait appears to change only in the second stage of an invasion) may be an indication that genetic regulation of the trait is complex and that the trait may be only slowly evolvable. (4) Due to the genetic bottleneck experienced by the invasive species, naturalized invasive populations may be phenotypically distinct from the native ones they derive from, even after their demographic expansion in the novel region may have increased their genetic variability. As a result, traits such as growth *in planta* and the ability to grow at different temperatures may differ markedly between source and invasive populations, with important implications related to possible additional introductions of individuals belonging to an exotic species.

Materials and Methods

Isolation

Seiridium cardinale isolates were obtained in 2007–08 from symptomatic trees across the Mediterranean region and California. Diseased stems, branches, or twigs were excised, put in plastic bags, and processed in the laboratory within 24 hours. The outer bark was carefully scraped with a sterile scalpel to expose the margin of cankers. Small fragments cut from the margin of the necrotic tissues were placed onto PDA (Potato Dextrose Broth 20 g/L + 20 g/L Agar) plates maintained at 25°C in the dark.

White-greyish, cottony colonies (with olive-green shades) were transferred within 3–5 days on 1% MEA (Malt Extract Agar 10 g/L + 20 g/L Agar) supplemented with autoclaved (120°C, 20 min) cypress seeds in 60-mm Petri dishes. Plates were then incubated at 18°C under mixed visible and near-ultraviolet light (NUV, 400–200 nm), set to provide 12-h light/dark cycles to induce sporulation. After 3–4 weeks, acervuli (i.e., asexual fruiting bodies) of the fungus developed on the seeds and on the agar surface.

The identity of the fungus was confirmed based on the morphology of the six-celled fusiform conidia under a compound microscope at 400× magnification (Wagener 1939, Sutton and Gibson 1972, Mordue 1976, Chou 1989) and by sequence analysis of a portion of the β-tubulin locus (Della Rocca et al. 2011, 2013).

Cultures from single *S. cardinale* conidia were obtained for each isolate as follows: 2–3 mature acervuli were collected and placed in 1.5-mL microcentrifuge tubes where they were gently crushed in sterile distilled water using a sterile plastic pestle. Aliquots of 100 μl of conidial suspension were spread onto the surface of 2% Water Agar (WA) in 90-mm Petri dishes which were then maintained at 25°C in the dark. After 12 h of incubation, germinating conidia were singly transferred to 1% MEA Petri dishes containing autoclaved cypress seeds and were incubated at 18°C under mixed white and NUV light as reported above. For each isolate, seeds with acervuli were collected and transferred in plastic vials and stored at −20°C. All isolates were maintained in the culture collection of the Institute for Sustainable Plant Protection, IPSP-CNR, Florence, Italy.

Morphological and cultural observations

Observations of morphological and cultural traits as well as of size of induced cankers on inoculated trees were conducted on 22 *S. cardinale* isolates from different Mediterranean countries and 23 isolates from several California

counties (USA). Isolates were chosen as representatives of the two β-tubulin haplotypes and of the two Structure groups that were evidenced in previous studies on genetic diversity of the fungus (Della Rocca et al. 2011, 2013; Appendix A).

Radial growth of colonies.—Radial growth of colonies was determined on 9 cm diameter Petri dishes containing 18 mL 2% MEA, by placing a 4-mm plug taken from the margin of a colony grown on MEA 2% for 10 d at 25°C at the center of the plate. Plates were then incubated for 14 d in the dark at 15°, 20°, 25° and 30°C, before measuring the two perpendicular diameters for each colony. Five replicates were used for each isolate and temperature.

Production of acervuli in vitro.—The production of acervuli, i.e., structures bearing the asexual spores (conidia), was evaluated by obtaining a 4-mm plug of mycelium taken from the margin of a colony grown for 10 d at 25°C on 2% MEA and placing it in the center of a 6 cm diameter Petri dish containing 5 mL of 2% Water Agar and 10 autoclaved cypress seeds evenly distributed on the surface. Four replicates were used for each isolate. Plates were incubated at 18°C under mixed white and NUV light, as reported above. After 30 and 90 d, acervuli on the surface of the 10 seeds were counted with a dissecting scope.

Conidial size.—For each isolate, conidia extruding from mature acervuli were suspended in sterile water in 1.5-mL microcentrifuge tubes. After 10 minutes, the conidial suspension was placed on a microscope slide to allow for the measurement of the length and width of 100 conidia using a light microscope (400×; Axioskop Zeiss) equipped with a digital camera.

Germination of conidia.—For each isolate, conidial suspensions (see above) were diluted with sterile water in 1.5-mL microcentrifuge tubes water to a concentration of 5×10^3 conidia mL^{-1}. Suspensions were incubated at 25°C in the dark. After 24 h, 100 μl of suspension were placed on a microscope slide and the number of germinated conidia and mean number of germination tubes per conidium for 100 random conidia were counted at 400× using a light microscope (Axioskop Zeiss).

Artificial inoculations

Italian cypress (*Cupressus sempervirens* L. var. *horizontalis*) plants obtained from seed and grown in 30 L pots containing a mixture of peat, compost and perlite (3:1:1 v/v/v) were placed in a greenhouse at the IPSP experimental farm located at Antella, Bagno a Ripoli, Florence, Italy (43°44.2′ N, 11°19.4′ E, 139 m a.s.l.). The average maximum temperature was 25°C ± 4°C and average relative humidity was 65% ± 5%. Five-year-old plants with a mean height of 1.9 m and a mean basal stem diameter of 4.7 cm were inoculated in April 2010. Inoculations were made at a point where the stem had a 1–2 cm diameter. A 4 mm circular plug of bark extending into the cambium was removed with a cork borer and replaced with an equally sized plug of mycelium (top-side-down) taken from the margin of a fungal colony grown in the dark for 10 d at 25°C on 2% MEA. Inoculations were covered with cotton and wrapped with tape for one week. Each *S. cardinale* isolate was inoculated on 10 cypress plants, for a total of 450 plants. On 10 control cypress plants a sterile 2% MEA plug was inserted onto the wound. Plants were arranged in a completely randomized design inside the greenhouse. The entire experiment was repeated twice.

Development of cankers was evaluated 6 months after the artificial inoculations. The outer bark of inoculated stems was carefully scraped off with a sterile scalpel blade near the inoculation site in order to expose the margins of necrotic lesions. The number of successfully induced cankers and length and width of necrotic lesions were measured for each isolate. In order to fulfil Koch's postulates, small pieces of necrotic tissues cut from the edge of lesions were plated onto 2% PDA plates to recover the inoculated fungus.

Statistical analyses

Assay results were analyzed using STATISTICA 10.0 software. Differences among isolates within groups or between paired groups or populations of the fungus and effects of factors were examined using analysis of variance (ANOVA). Tukey's HSD (honestly significant difference) was performed for post-hoc pairwise comparisons. Percentages were converted using the formula $y = \arcsin\sqrt{p/100}$ where p is the percentage value, prior to analysis.

Pearson correlation coefficients were calculated to explore relationships between the mean values

of the following traits: size of induced cankers and radial growth of colonies at various temperatures; size of cankers and number of acervuli produced in vitro by isolates. Size of the necrotic lesions was approximated to the area of an ellipse and calculated using the formula $(D/2 \times d/2)\pi$, where D is the length and d the width of cankers, respectively. Correlation between conidial size and percentage of germinated conidia and number of germination tubes per 100 conidia were also studied.

A discriminant analysis was performed on a matrix containing the mean values calculated for each isolate for all of the assayed traits to explore the possibility of a relationship between clusters based on phenotypic traits and those previously determined based on genetic markers and geographic provenance, e.g., the two β-tubulin haplotypes and the two AFLP Structure groups.

Phenotypic plasticity of all examined traits was evaluated for the California and Mediterranean populations and for the genetic sub-populations of the fungus using two quantitative estimators: the phenotypic plasticity index (PIv) as (maximum mean − minimum mean)/maximum mean, and the coefficient of variation (CV) as standard deviation/mean (Valladares et al. 2006).

The following indices of genetic diversity were also calculated based on the number and frequency of multilocus genotypes (haplotypes) determined from the SSR analysis of *S. cardinale* populations from the Mediterranean and California reported by Della Rocca et al. (2011), including the isolates used in the present work: (1) Haplotype diversity, $H = k/n$ where k is the number of haplotypes, i.e., individuals sharing identical alleles for all the loci analyzed, and n is the number of individuals analyzed; (2) gene diversity

$$\hat{H} = \frac{n}{n-1}(1 - \sum_{i=1}^{k} p_i^2)$$

where k is the number of haplotypes, n is the number of individuals analyzed and p_i is the frequency of the ith haplotype; and (3) gene diversity (expected heterozygosity), estimated as

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

where p is the frequency of the ith of k alleles, averaged over each of m loci.

Q_{ST}-F_{ST} comparisons

Traits exhibiting Pearson product-moment correlations greater than 0.6 were removed from subsequent analyses. Traits analysed were number of acervuli at 1 month, canker area, germination percentage, number of germination tubes, conidial length, growth at 15°C, growth at 20°C, and growth at 30°C. Q_{ST} values were calculated for the phenotypic data collected from the common garden experiments using the following formula:

$$Q_{ST} = \frac{\sigma_B^2}{\sigma_W^2 + \sigma_B^2}$$

where σ_B^2 is the between-group trait variance and σ_W^2 is the within-group trait variance (Spitze 1993). Variance components were determined using a single-factor analysis of variance. Mean and single-locus F_{ST} values were calculated using the hierfstat package (Goudet 2005) for the R statistical computing environment (www.r-project.org). Observed Q_{ST} values were compared to a null distribution of Q_{ST}-F_{ST} values using the simulation method of Whitlock and Guillaume (2009) as implemented by Lind et al. (2011; R script provided in Supplemental Information therein). Values of Q_{ST}-$F_{ST} \approx 0$ suggest that observed trait variance between populations is the result of genetic drift; Q_{ST}-$F_{ST} > 0$ suggests directional selection on the trait, and Q_{ST}-$F_{ST} < 0$ suggests stabilizing selection on the trait (Leinonen et al. 2013).

Results

Morphological observations

Definition of populations.—Although the populations studied here are either outcrossing (Cal A and Cal B), nested within one another (California and Mediterranean populations in the Structure 1 group, or Med 1 and Med 2 both within the β-tubulin A group), or sequentially derived from one another (Med 2 from Med 1), and results are presented for all possible genetically distinct demographic units, major inferences are drawn for the following four demes: the Cal A and Cal B California populations, and the Med 1 and Med 2 Mediterranean populations (see Fig. 1). Boxplots showing trait differences between groups are

Table 1. Mean diameter of colonies (in cm) of *S. cardinale* isolates developed on 2% MEA after 14-d incubation in the dark at different temperatures. Isolates were grouped according to their geographic origin or genetic subpopulations. Means not sharing same letters between paired sub-populations within each row were significantly different for $P = 0.01$ or * $P = 0.05$.

	Geographic population		β-tubulin haplotype A		Structure group 1		β-tubulin haplotype (California isolates)		Structure group (Mediterranean isolates)	
T (°C)	MED	CAL	Med A	Cal A	Med 1	Cal 1	Cal A	Cal B	Med 1	Med 2
15°	3.18 a	4.39 b	3.18 a	4.19 b	3.33 a	4.39 b	4.19 *a**	4.54 *b**	3.33 b	2.77 a
20°	5.10 a	5.94 b	5.10 a	5.72 b	5.08 a	5.94 b	5.72 a	6.10 b	5.08 a	5.16 a
25°	5.61 a	6.92 b	5.61 a	7.01 b	5.64 a	6.91 b	7.01 a	6.86 a	5.64 a	5.64 a
30°	2.04 a	3.39 b	2.04 a	3.46 b	2.08 a	3.39 b	3.46 a	3.34 a	2.08 a	2.11 a

provided in Appendix B.

Radial growth of colonies.—For both geographic populations of the pathogen (Californian and Mediterranean, respectively CAL and MED) the mean radial growth rate on 2% MEA was higher at 25°C and progressively lower at 20°, 15° and 30°C (Table 1). At each assayed temperature, the growth rate of CAL isolates was consistently higher than that of MED isolates (15°C: $F_{1, 204} = 105.4$, $P < 0.001$; 20°C: $F_{1, 197} = 53.6$, $P < 0.001$; 25°C: $F_{1, 192} = 115.4$, $P < 0.001$; 30°C: $F_{1, 201} = 105.5$, $P < 0.001$). Within the California population, the radial growth rate of isolates belonging to β-tubulin haplotype B was higher than that of haplotype A at 15°C ($F_{1,90} = 5.71$, $P < 0.05$) and 20°C ($F_{1,90} = 9.76$, $P < 0.01$) but not at 25°C and 30°C (Table 1).

Within the Mediterranean isolates, those belonging to the Structure group Med 1 showed a significantly higher growth rate than those of Structure group Med 2 only at 15°C ($F_{1,98} = 8.03$, $P < 0.01$; Table 1).

No significant correlation was found between radial growth of isolates for any pairs of temperatures (data not shown) while interaction between isolate and temperature was highly significant both for the MED ($F_{63, 350} = 21.1$, $P < 0.001$) and the CAL ($F_{66, 276} = 47.0$, $P < 0.001$) populations. The difference between radial growth at 15° and 30°C was rather marked for some isolates that showed a preference for one of the two, while others showed similar radial growth at these two temperatures.

Production of acervuli in vitro.—The mean number of acervuli produced in vitro was significantly higher for CAL than for MED isolates both after 30- and 90-d incubation ($F_{1, 203} = 28.4$, $P < 0.001$ and $F_{1, 203} = 6.96$, $P < 0.01$, respectively; Table 2). Within the β-tubulin haplotype group A, isolates from California produced a higher number of fruit bodies both after 30- and 90-d incubation than isolates from the Mediterranean ($F_{1, 135} = 13.9$, $P < 0.001$ and $F_{1, 135} = 3.96$, $P < 0.05$). No significant difference in production of acervuli was observed between isolates belonging to the two β-tubulin haplotypes within the CAL population; however, within the MED population, isolates of Structure group 2 produced a higher number of acervuli than Structure group 1 isolates ($P < 0.05$) after 90 days of incubation (Table 2). In both CAL and MED populations, significant differences among isolates were observed with regards to the number of acervuli developed 30

Table 2. Mean number of acervuli produced in vitro on 2% WA after 30-d and 90-d incubation at 18°C under 12 h cycles under mixed white and NUV light by isolates of the two geographic populations (MED and CAL) and the genetic subpopulations of *S. cardinale*. Paired means not sharing same letters within a same line were different for $P = 0.01$ or * $P = 0.05$.

	No. acervuli	
Population	30 d	90 d
MED	2.9 a	8.6 a
CAL	5.1 b	10.3 b
Haplotype A		
MED	2.9 a	8.5 *a**
CAL	4.6 b	10.3 *b**
Structure group 1		
MED	3.1 b	7.9 a
CAL	5.1 a	10.3 a
CAL haplotype		
A	4.6 a	10.3 a
B	5.4 a	10.4 a
MED structure group		
Med 1	3.1 a	8.0 *a**
Med 2	2.7 a	9.9 *b**

Table 3. Mean percentage of germinated conidia and mean number of germinated tubes per 100 conidia of *S. cardinale* isolates after 24 h incubation in water, at 25°C in the dark. Isolates were grouped in geographic populations (MED and CAL) and genetic sub-populations. Means not sharing same letters between paired subpopulations within a same row were different for $P = 0.01$ or * $P = 0.05$.

Measure of germinating conidia	Geographic population		β-tubulin haplotype A		β-tubulin haplotypes (only California)		Structure group 1		Structure group (Mediterranean isolates)	
	MED	CAL	Med A	Cal A	Cal A	Cal B	Med 1	Cal 1	Med1	Med2
Germinated conidia (%)	88.9 a	97.3 b	88.6 *a**	96.9 *b**	96.9 a	97.5 a	88.0a	97.3 b	88.0 a	86.8 a
No. germinating tubes for 100 conidia	165.8 a	185.0 b	165.8 a	189.8b	189.8 a	181.3 a	166.8 *a**	185.0 *b**	166.8 a	148.8 a

and 90 d after incubation. Nine out of 10 isolates that produced the highest number of acervuli were from California, while 8 out of 10 isolates exhibiting the lowest number of acervuli after 30-d incubation were from Mediterranean (data not shown). With few exceptions, isolates showed a more or less marked increase in the number of developed acervuli between 30-d and 90-d incubation periods.

Percentage of germinated conidia.—The mean percentage of conidia germinated in water after 24 h incubation at 25°C in the dark was high for both CAL (97.3%) and MED (88.9%) isolates, though the difference between mean germination was statistically significant between the two populations ($F_{1,43} = 16.8$, $P < 0.001$; Table 3).

Within β-tubulin haplotype A and Structure group 1, CAL isolates showed a significantly higher percentage of germinated conidia than MED isolates ($F_{1,30} = 6.84$, $P < 0.02$ and $F_{1,35} = 14.8$, $P < 0.001$, respectively). The difference between the two β-tubulin haplotype groups was not significant if only isolates from California were considered ($F_{1,21} = 0.37$, $P = 0.54$; Table 3). Isolates that showed germination percentages below 92% were all from the Mediterranean, while among the 9 isolates with 100% germination, 6 were from California (data not shown).

The mean sum of germinating tubes for 100 conidia was higher for CAL than for MED isolates ($F_{1,43} = 5.16$, $P = 0.02$), as it was for isolates from California compared to those from the Mediterranean within Structure group 1 ($F_{1,35} = 4.07$, $P = 0.05$; Table 3). No significant differences were observed between isolates of the two California β-tubulin haplotypes and between isolates of the two Mediterranean Structure groups (Table 3).

Conidial size.—Both mean length and width of conidia were significantly higher for CAL isolates compared to MED isolates (length: $F_{1,2348} = 347.9$, $P < 0.001$; width: $F_{1,2348} = 469.4$, $P < 0.001$; Table 4). Within β-tubulin haplotype A and Structure group 1, isolates from California had longer and wider conidia ($P < 0.01$) than those produced by Mediterranean isolates (comparison CAL – MED within β-tubulin haplotype A, length: $F_{1,1698} = 315.5$, $P < 0.001$; width: $F_{1,1698} = 298.9$, $P < 0.001$. Comparison CAL – MED within Structure group 1, Length: $F_{1,\ 2048} = 288.5$, $P < 0.001$; Width: $F_{1,\ 2048} = 369.9$, $P < 0.001$) (Table 4). Within CAL isolates, only mean length of conidia differed significantly between β-tubulin haplotype groups (A > B, $F_{1,1148} = 26.4$, $P < 0.001$). Between MED isolates, both length and width of conidia differed (Structure group Med 1 > Structure

Table 4. Mean length and width of 50 conidia of *S. cardinale* isolates randomly measured under light microscope at 400× magnification. Isolates were grouped in geographic populations and genetic sub-populations. Means not sharing same letters between paired subpopulations within rows were significantly different for $P = 0.01$.

Size of conidia	Geographic population		β-tubulin haplotype A		β-tubulin haplotypes (California isolates)		Structure group 1		Structure group (Mediterranean isolates)	
	MED	CAL	Med A	Cal A	Cal A	Cal B	Med 1	Cal 1	Med 1	Med 2
Length (μm)	23.6 a	25.1 b	23.6 a	25.4 b	25.4 b	24.8 a	23.5 a	25.0 b	23.5 b	22.8 a
Width (μm)	8.3 a	9.1 b	8.3 a	9.12 b	9.10 a	9.16 a	8.3 a	9.1 b	8.4 b	8.0 a

Table 5. Mean length and width of necrotic lesions developed 6 months after inoculation with *S. cardinale* isolates on stems of 5-year-old cypress trees growing in pots. Isolates were grouped in geographic populations and genetic sub-populations. Means not sharing same letters between paired subpopulations within a row were significantly different for $P = 0.01$.

Size of necrotic lesion	Geographic population		β-tubulin haplotype A		β-tubulin haplotypes (California isolates)		Structure group 1		Structure group (Mediterranean isolates)	
	MED	CAL	Med A	Cal A	Cal A	Cal B	Med 1	Cal 1	Med1	Med2
Length	77.1 a	85.9 b	77.1 a	86.5 b	86.5a	85.3 a	75.3 a	85.9 b	76.7 a	75.3 a
Width	24.8 a	25.9 a	24.8 a	25.1 a	25.1a	26.5 a	23.5 a	26.0 b	23.5 a	24.9 a

group Med 2; length: $F_{1,1148} = 37.8$, $P < 0.001$; width: $F_{1,1148} = 30.2$, $P < 0.001$).

Overall, mean length and width of conidia of isolates were significantly correlated ($r = 0.55$, $P < 0.01$). The conidial length/width ratio differed significantly between the CAL (2.76) and the MED (2.87) populations of the fungus ($F_{1,2348} = 58.8$, $P < 0.001$).

Artificial inoculations

Six months after inoculation, 100% of inoculated trees showed typical spindle-like necroses. CAL isolates produced lesions significantly longer than those generated by the MED isolates ($F_{1,459} = 13.1$, $P < 0.001$). Mean width of necrotic lesions was not significantly different between CAL and MED isolates ($F_{1,459} = 2.43$, $P = 0.11$; Table 5). Within the β-tubulin haplotype group A, isolates from California generated longer necroses ($P < 0.01$) than Mediterranean isolates ($F_{1,331} = 9.94$, $P < 0.01$). When considering isolates from California only, differences between the two β-tubulin haplotype groups were significant neither for length nor width of cankers (Table 5). No significant differences were found between the two Structure groups within the MED isolates; however, significant differences among isolates were found for both length and width of necrotic lesions within the MED (length: $F_{21,202} = 10.9$; $P < 0.001$; width: $F_{21,202} = 11.6$; $P < 0.001$) and within the CAL (length: $F_{22,214} = 6.53$; $P < 0.001$; width: $F_{22,214} = 6.97$; $P < 0.001$) populations.

Overall, a highly significant positive correlation was found between mean length and width of cankers ($r = 0.61$; $P < 0.001$).

Q_{ST}-F_{ST} comparisons

Between California β-tubulin haplotype groups, acervuli production, germination percentage, germination tube production, conidial length, and growth at 15° and 20°C exhibited significant differences from neutral expectations (Fig. 1, Test 1; Appendix C: Fig. C1). Traits differing significantly between Cal A1 and Med A1 populations were conidial length, growth at 15°C and 30°C (Fig. 1, Test 2; Appendix C: Fig. C2). Conidial length was the only trait for which variation between Mediterranean populations A1 and A2 differed significantly from neutral expectations (Fig. 1, Test 3; Appendix C: Fig. C3). In each case of a significant difference, the level of phenotypic differentiation (Q_{ST}) exceeded the level of genotypic variance (F_{ST}).

Relationships between traits

No significant correlation was found between canker size (length, width or area of necrosis) and radial growth rate of colonies in vitro at any of the considered temperatures (15°, 20°, 25° and 30°C) either for the set of all isolates or when considering CAL and MED isolates separately. Canker area was significantly correlated with the number of acervuli produced after 30- or 90-d incubation ($r = 0.63$, $P < 0.01$ and $r = 0.49$, $P < 0.05$, respectively) for the MED isolates, but not for the CAL isolates.

Conidial size (1/2 length × 1/2 width) was highly correlated with the percentage of germinated conidia ($r = 0.44$; $P < 0.01$; Fig. 2) and with the number of germination tubes per 100 conidia ($r = 0.36$; $P < 0.02$). The total number of germination tubes of 100 conidia was also correlated to the percentage of germinating conidia ($r = 0.50$; $P < 0.001$).

Phenotypic variability

The phenotypic plasticity index (PIv) and the coefficient of variation (CV) of the examined traits calculated for the geographic and the

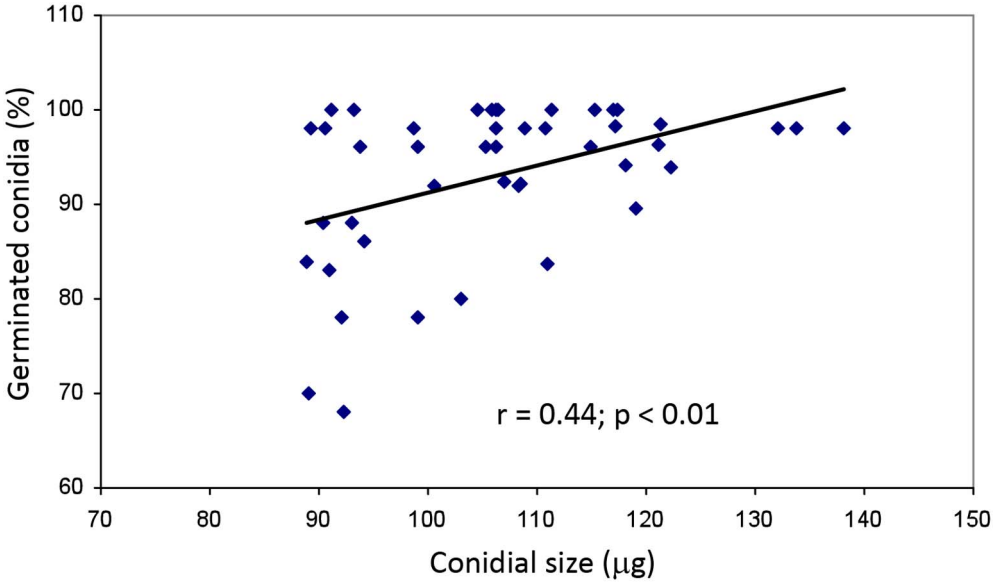

Fig. 2. Graph displaying the correlation between the mean size of conidia of all the assayed isolates (meant as length/2 × width/2, expressed in μ^2) and the percentage of germinated conidia obtained for each of them. Degrees of freedom N-2 = 43.

Table 6. Phenotypic plasticity index (PIv = max mean−min mean/max mean) and coefficient of variation (in brackets) of all the assayed traits calculated for the geographic *S. cardinale* populations and the genetic sub-populations. Genetic diversity was evaluated for the populations and sub-populations of the fungus using three different indices: Haplotype diversity; gene diversity and gene diversity as expected heterozygosity.

	Geographic population		β-tubulin haplotype (California isolates)		Structure group (Mediterranean isolates)	
Trait	CAL	MED	Cal A	Cal B	Med 1	Med 2
Radial growth (cm)						
15°C	0.52 (0.16)	0.66 (0.29)	0.49 (0.15)	0.22 (0.16)	0.62 (0.23)	0.64 (0.39)
20°C	0.36 (0.10)	0.49 (0.18)	0.31 (0.11)	0.29 (0.08)	0.37 (0.16)	0.45 (0.21)
25°C	0.26 (0.07)	0.48 (0.18)	0.15 (0.05)	0.26 (0.07)	0.47 (0.19)	0.39 (0.16)
30°C	0.75 (0.34)	0.70 (0.32)	0.54 (0.31)	0.74 (0.37)	0.71 (0.34)	0.62 (0.30)
Acervuli production						
30 d	1.00 (0.67)	1 (0.72)	1.00 (0.70)	0.77 (0.64)	1.00 (0.72)	0.50 (0.56)
90 d	0.81 (0.46)	0.86 (0.55)	0.79 (0.49)	0.73 (0.44)	0.85 (0.61)	0.37 (0.24)
Germinated conidia (%)	0.08 (0.02)	0.30 (0.11)	0.07 (0.02)	0.07 (0.02)	0.32 (0.12)	0.20 (0.08)
No. germinated cells/conidium	0.27 (0.09)	0.51 (0.19)	0.17 (0.07)	0.26 (0.09)	0.51 (0.17)	0.38 (0.21)
No. germinating tubes (50 conidia)	0.28 (0.09)	0.63 (0.21)	0.22 (0.08)	0.26 (0.10)	0.58 (0.21)	0.40 (0.22)
Size of conidia						
Length	0.13 (0.07)	0.63 (0.30)	0.08 (0.07)	0.13 (0.07)	0.17 (0.08)	0.03 (0.06)
Width	0.22 (0.11)	0.61 (0.29)	0.12 (0.09)	0.22 (0.12)	0.18 (0.09)	0.09 (0.16)
Canker size						
Length (mm)	0.52 (0.33)	0.63 (0.30)	0.52 (0.38)	0.48 (0.31)	0.62 (0.32)	0.30 (0.22)
Width (mm)	0.44 (0.25)	0.61 (0.29)	0.35 (0.27)	0.35 (0.25)	0.61 (0.31)	0.29 (0.23)
Genetic diversity						
Haplotype diversity	0.91	0.65	1.00	0.92	0.58	1.00
Gene diversity	0.98	0.88	1.00	0.98	0.87	1.00
Expected heterozygosity	0.41	0.30	0.35	0.41	0.31	0.20
Nucleotide diversity	0.076	0.065	0.075	0.113	0.082	0.027

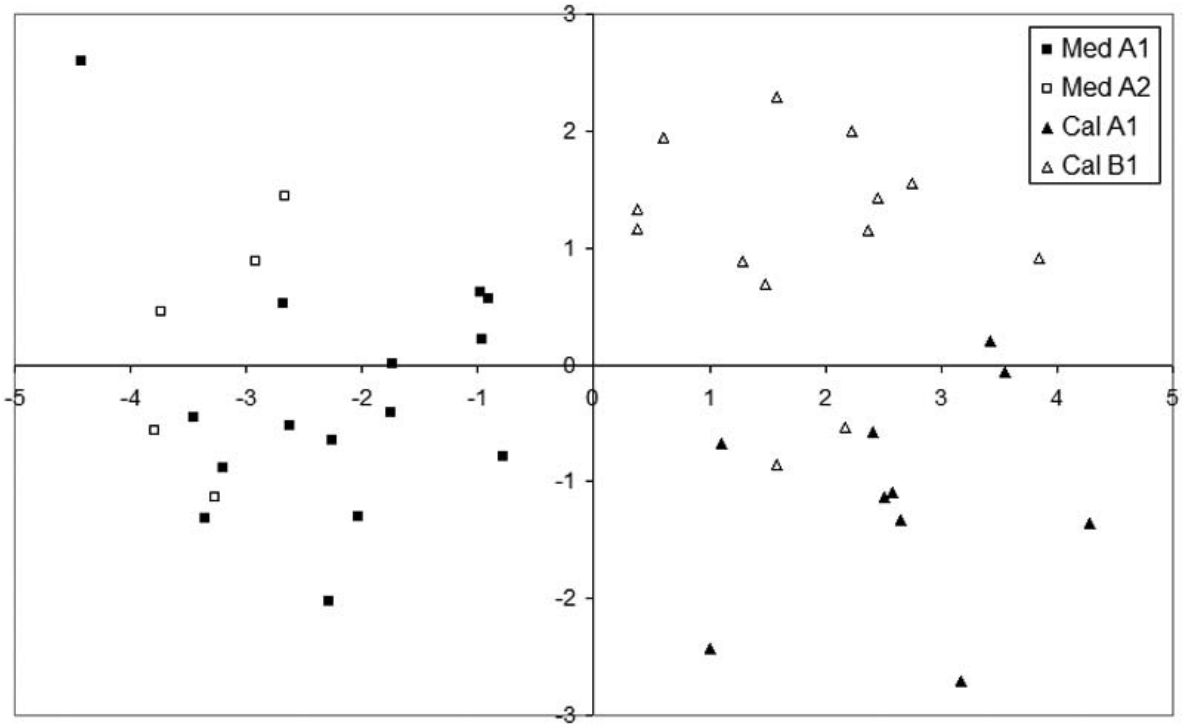

Fig. 3. A discriminant analysis performed on a matrix containing the mean values calculated for each *S. cardinale* isolates for all the assayed traits. Isolates were subdivided in four groups according to their geographical origin; their β-tubulin haplotype group (A or B, see text) and Structure AFLP group (1 or 2, see text).

genetic sub-populations of the fungus are reported in Table 6. Generally, both PIv and CV of all evaluated traits were higher for MED isolates than for CAL isolates. Within the MED isolates, those belonging to Structure group Med 1 showed higher PIv values than isolates of Structure group Med 2 for all of the evaluated traits except radial growth of colonies at 15° and 20°C. Values of CV followed the same trend as PIv except for the number of germinating tubes per 100 conidia and width of conidia, which were higher for the Structure group Med 2 isolates. Within California, β-tubulin haplotype A isolates (Cal A) showed higher PIv and CV values than haplotype B isolates (Cal B) for most of the evaluated traits except for radial growth of colonies at 25° and 30°C, number of germinating tubes per 100 conidia, and the length and width of conidia, which were higher for the β-tubulin haplotype B isolates. All three calculated indices of genetic diversity were higher for the CAL compared to the MED isolates, for the Cal A compared to Cal B, and for Med 2 compared to Med 1 within the Mediterranean (Table 6).

Discriminant analysis

Discriminant analysis of the overall phenotypic data depicted a clear separation between CAL and MED isolates based on axis 1 (Fig. 3). Among the CAL isolates, axis 2 discriminated fairly well between β-tubulin haplotypes Cal A and Cal B. A clear separation was not observed within the MED population, between Structure groups Med 1 and Med 2.

Discussion

The success of biological invasions is mediated by the ability of invasive organisms to survive long distance movement, to establish themselves in a novel environment, and disperse at a rate greater than their mortality (McCallum 2008). It is theorized that traits facilitating reproduction and dispersal should be favored at the front of an expanding range, while traits enhancing intra-specific competitive ability should be selected in

the area behind the expanding range where densities of invasive populations may increase (Burton et al. 2010).

In the case of pathogens, a further complication is that their dispersal is linked to successful infection, a process mediated by responses and traits of both pathogen and host populations. Until recently, literature on the subject of invasions by plant pathogens has mostly focused on so-called qualitative aspects of successful infection, and in particular on the effects that presence/absence of compatible resistance and virulence genes in hosts and pathogens, respectively, may have on success of the invasion. This has resulted in an excessive use of the "lack of co-evolution" hypothesis to interpret invasions by plant pathogens, while more inclusive studies on factors such as infection efficiency, latent period, spore production rate, spore size, infectious period, lesion size, and toxin production are scant (Lannou 2012). The roles of these quantitative traits and their trade-offs have been well studied in several pathosystems (Roff 2002, Lannou 2012), but this knowledge has rarely been transferred to the study of invasive pathogens (Brasier 2001, Cobb et al. 2010, Robin et al. 2010, Giordano et al. 2014).

With regard to our first hypothesis, results show traits associated with enhanced transmission to be favored over those associated with increased virulence and greater competitive ability. Our results confirm that invasion by *S. cardinale* is associated with decreasing spore size during both stages of its range expansion. This trait is known to be strongly and positively correlated with increased dispersal range even for microscopical propagules (Norros et al. 2014), and in our study it appears to confer an advantage, in spite of the documented trade-off due to the reduced germinability of smaller spores (Fig. 2). On the other hand, lesion size on cypress, a quantitative trait that is the result of host-pathogen interactions and that can be used as a proxy of the aggressiveness of the invasive organism, was significantly lower in invasive than in native populations. Because lesions are responsible for the girdling and death of the infected portion of the plant, leading to a complete arrest of sporulation, it is reasonable to assume that intermediate virulence may be selected for in an epidemic mode such as that recorded in the Mediterranean. In fact, highly virulent genotypes may actually lead to overall lower sporulation loads (Violle et al. 2007), while within the intermediate range, higher levels of virulence may be beneficial to transmission. In our study, this benefit was confirmed by the positive correlation observed in the Mediterranean populations between lesion size on cypress and production of acervuli.

Acervuli production at 90 days was unchanged in the founder Med 1 population compared to California populations, but it increased significantly in the derived Med 2 population, suggesting that an overall higher spore production may be favored. The "delay" in the change of this trait may indicate its evolvability may be lower, e.g., it may require a longer period of time. Interestingly, acervuli production in the short term was not increased. Likewise, higher spore germinability was not favored either; to the contrary, lower germinability was observed in invasive populations. These two observations suggest that *S. cardinale* is not encountering significant interspecific competition requiring quicker establishment; this finding is in agreement with the observation that other pathogens causing a similar disease are rather infrequent (Graniti 1998, Danti et al. 2013*a*). In summary, our results suggest that traits affecting transmission and long-range dispersal, such as overall spore production and smaller spore size, are favored during the invasion process, while traits favoring virulence and competitive establishment, such as rapid growth *in planta*, quick production of spores, and high spore germinability, are not.

With regard to our second hypothesis, our data indicated demographic plasticity of most traits to be greatly increased in the first stages of invasion. Notably, despite the reduced genetic variability in the invasive population Med 1, the amplitude of responses of all traits as indicated by the indices PIv and CV is greater than in the source CAL population. On the contrary, when comparing the two Mediterranean populations, in spite of an increase in genetic variability in Med 2 (likely to be due to an increase in population size), we recorded a significant decrease in the amplitude of responses of all traits measured. The only exceptions were growth rates at cold and hot temperatures, 15° and 30°C respectively, in which cases lack of adaptation justifies the

maintenance of variable responses at the population level. We suggest that the observed patterns imply a significant role played by plasticity in the first stages of the invasion process (plasticity Med 1 > CAL) followed by a decrease of such a role as invasive populations find their most suitable niche in the novel geographic range (plasticity Med 2 < Med 1).

The expectations of our third hypothesis were also met; i.e., only some of the measured traits appeared to be under selection. It should, however, be pointed out that lack of support for selection in Q_{ST}-F_{ST} analyses does not necessarily mean selection is not at play, but rather that its role cannot be discerned given the level of neutral variation for the genetic markers employed. The genetic markers employed in this and in previous studies are not necessarily linked to the phenotypic traits here analyzed; nonetheless, some generalized conclusions can be drawn from a comparative analysis of all traits. The discriminant analysis based on phenotypic traits shows clear distinction of all four populations, with overall statistically significant differences between CAL and MED populations. Among traits under adaptive selection, decreasing spore size is the trait that appears to be continuously favored in both stages of the invasion, while growth at low and high temperatures (15° and 30°C, respectively) appears to rapidly decrease during the first stages of the invasion, possibly suggesting a maladaptation of introduced populations to temperatures extremes. When comparing the two native populations Cal A and Cal B, evidence for significant selection was found once again for conidial size, but also for germinability (which may confer an intraspecific competitive advantage) and for growth at low and intermediate temperatures (15° and 20°C, respectively), suggesting the two populations are competitively interacting as suggested by spore germinability data, but may be differently adapted to coastal and interior habitats, favored by higher growth at low/medium or high temperatures, respectively.

Finally, our last hypothesis stated that some individuals in native populations may have traits—or, more properly, may have responses in various traits—that differ significantly from responses measured for individuals in invasive populations. The discriminant analysis presented in this study indicates that California populations are overall phenotypically distinct from the Mediterranean populations. Our analyses identify spore size, growth rates, growth rates × temperature, and pathogenicity as traits clearly distinct among populations from the two regions. We believe some of these traits may be detrimental to naïve hosts in the zone of invasion (ZOI), if acquired by invasive populations. For instance, while our study points to virulence as a trait under stabilizing selection in the ZOI (Leonard and Czochor 1980), it also indicates that additional introductions of *S. cardinale* from California into the Mediterranean basin could have serious consequences on the survival of native host populations, resulting in higher mortality rates. Furthermore, a further expansion in areas both warmer and colder than those currently occupied by the invasive pathogen could be an obvious outcome of the introduction of genotypes with different thermal optima. Because control regulations are relaxed when the same species is reported in two different countries, this finding underlines a considerable weakness of current international policies aimed at limiting introduction of invasive species.

To our knowledge, this is one of the first studies presenting a comparative analysis between source and invasive fungal populations focusing on a broad array of phenotypic traits and including a comparison with genetic data. Our findings indicate the presence of a measurable phenotypic differentiation between native and invasive populations belonging to the same species. Furthermore, it appears from Q_{ST}-F_{ST} comparisons that selection on traits related to dispersal is stronger than that for traits related to virulence, at least when comparing invasive to source populations. Our data further suggest that in the absence of significant competition, traits enhancing competitive fitness—such as rapid sporulation—may not be selected for. We also show that plasticity increases in the first phases of the invasion, possibly assisting the invasive population in surviving in novel habitats, and then it decreases, possibly as the invasive population adapts and expands in the most suitable niches. Finally, we show that quantitative pathogenicity does not increase between source and invasive populations, either because it is not easily evolvable, as it may be determined

by multiple loci of both host and pathogen, and/or because intermediate pathogenicity levels may be ideal to maximize transmission. Nonetheless, native source populations include genotypes that are genetically distinct and display higher pathogenicity than genotypes already introduced: their introduction could lead to increased mortality rates of hosts in the exotic range, even of cultivars currently deemed resistant (Danti et al. 2006, 2013*b*). Based on these results, we conclude that regulations should prevent any further introductions of *S. cardinale* into the Mediterranean region: this conclusion is also supported by the fact that introductions of a compatible mating type postulated to exist in California (Della Rocca et al. 2011) could lead to sexual reproduction among individuals from different provenances, a mechanism known to increase the adaptive potential of invasive species (Facon et al. 2008).

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Supplemental Material

Ecological Archives

Appendices A–C are available online: http://dx.doi.org/10.1890/ES14-00426.1.sm