Recent episodes of mass mortalities in the Mediterranean Sea have been reported for the closely related marine sponges *Ircinia fasciculata* and *Ircinia variabilis* that live in sympatry. In this context, the assessment of the genetic diversity, bottlenecks and connectivity of these sponges has become urgent in order to evaluate the potential effects of mass mortalities on their latitudinal range. Our study aims to establish (1) the genetic structure, connectivity and signs of bottlenecks across the populations of *I. fasciculata* and (2) the hybridization levels between *I. fasciculata* and *I. variabilis*. To accomplish the first objective, 194 individuals of *I. fasciculata* from 12 locations across the Mediterranean were genotyped at 14 microsatellite loci. For the second objective, mitochondrial *cytochrome c oxidase subunit I* sequences of 16 individuals from both species were analyzed along with genotypes at 12 microsatellite loci of 40 individuals coexisting in 3 Mediterranean populations. We detected strong genetic structure along the Mediterranean for *I. fasciculata*, with high levels of inbreeding in all locations and bottleneck signs in most locations. Oceanographic barriers like the Almeria-Oran front, North-Balearic front and the Ligurian-Thyrrenian barrier seem to be impeding gene flow for *I. fasciculata*, adding population divergence to the pattern of isolation by distance derived from the low dispersal abilities of sponge larvae. Hybridization between both species occurred in some populations that might be increasing genetic diversity and somewhat palliating the genetic loss caused by population decimation in *I. fasciculata*.

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Sessile invertebrates, like sponges, are also particularly susceptible to temperature anomalies and continuous exposure to waterborne pathogens and pollutants because of their immobility (see, for example, Cebrian et al., 2006; Garrabou et al., 2009).

Reports of sponge disease have increased dramatically in recent years, with sponge populations decimated at some sites in the Mediterranean and Caribbean (see Webster, 2007 for a review). In particular, Mediterranean and Adriatic dictyoceratids (especially *Ircinia* spp.) have been consistently reported as affected by episodes of mass mortalities since the 1990s (see, for example, Garrabou et al., 2009; Maldonado et al., 2010b; Cebrian et al., 2011; Stabili et al., 2012). Three species of the genus *Ircinia* are widely distributed in the Mediterranean Sea, with populations occurring in shallow rocky coasts: *Ircinia fasciculata*, *Ircinia variabilis* and *Ircinia oros*. *Ircinia* species are aspiculate sponges, chemically diverse and harbor complex host-specific communities of bacterial and cyanobacterial symbionts (Erwin et al., 2012a,b; Pita et al., 2013). Both *I. fasciculata* and *I. variabilis* are affected by a disease-like condition (Stabili et al., 2012) reported by several authors in 2008 in the North and South Western Mediterranean Sea (Maldonado et al., 2010b; Cebrian et al., 2011). Whereas a monitored population of *I. fasciculata* in the southern coast of Spain (Granada) experienced 30% of mortality (Maldonado et al., 2010b), two monitored populations of *I. fasciculata* in Cabrera and Scandola Marine Reserves were decimated with ~90% mortality (Cebrian et al., 2011). This disease-like condition, which was characterized by small round pustules that developed into large areas of necrotic tissue and eventually resulted in the death of the sponges, was linked to abnormally high seawater temperatures (>24 °C; Cebrian et al., 2011). Moreover, *I. fasciculata* disease probably resulted from a proliferation of several bacterial pathogens (Maldonado et al., 2010b). Given the large impact of the disease-like condition on some populations, Cebrian et al. (2011) predicted local extinctions of the species in the studied areas. In situations under environmental stress, extinction rates could also be increased substantially by high inbreeding levels that are predicted to decrease species resilience to environmental stressors and therefore reduce the adaptive evolutionary potential of the species (Spelman et al., 2004; Frankham, 2005). Thus, determining the genetic diversity and level of connectivity in sponge populations of this species is crucial when designing management strategies to prevent its extinction. Sponge populations are usually highly structured in 'stable conditions' (that is, healthy populations), with relatively low gene flow and high levels of inbreeding (see, for example, Duran et al., 2004; Dailianis et al., 2011; Chaves-Fonnegra et al., 2015; Giles et al., 2015; Pérez-Portela et al., 2015). Therefore, even in 'stable conditions' the recovery of sponge populations after massive reduction of the effective population size can be limited by their own biology, dispersal potential and oceanographic currents, and one could even predict lower recovery rates in stressed populations.

The two target species of the present study are *I. fasciculata* (Pallas, 1766) and *I. variabilis* (Schmidt, 1862). These species are phylogenetically closely related (see Erwin et al., 2012b) with an external appearance so similar and plastic that it can lead to uncertainties in identification, even though they are generally considered different species (Pronzato et al., 2004). From a morphological point of view, *I. variabilis* contains inorganic inclusions in its skeletal fibers, whereas *I. fasciculata* lacks such inclusions, prompting Pronzato et al. (2004) to transfer *I. fasciculata* to the genus *Sarcotragus* and rename the species, *S. fasciculatus* (Pallas, 1766). From a molecular point of view, a recent analysis using the nuclear ribosomal gene *internal transcribed spacer 2* (ITS-2) indicated that *I. variabilis* and *I. fasciculata* were indeed different but closely related species with a divergence of 2.97% ± 0.38 (Erwin et al., 2012b), even though analyses of mitochondrial sequences lacked enough resolution to differentiate among them. In light of these results, we decided to maintain the name *I. fasciculata* in this study until a thorough analysis is performed to solve its true generic status. Each species (*I. variabilis* and *I. fasciculata*) harbors several specific microbes within their symbiotic content (Erwin et al., 2012b), and interestingly, even though these species live in sympathy, they present slightly different habitat preferences and large differences in growth rates (Turon et al., 2013). In marine sperm broadcasters, even when species show large phylogenetic divergences, hybridization is relatively common in areas of secondary contact (see, for example, Bierne et al., 2003; Willis et al., 2006; Harper and Hart, 2007; Nydam and Harrison, 2011). In particular, hybridization/introgression among sponges has long been suspected and previously suggested in closely related species with sympatric distributions (Dailianis et al., 2011; Escobar et al., 2012). In this context, assessing the effect of potential hybridization on overall genetic diversity of both *Ircinia* species is fundamental to understanding their genetic structure and connectivity.

The main objectives of this study are twofold: (1) to describe the genetic structure of *I. fasciculata* across the Mediterranean and Adriatic seas in order to reveal connectivity patterns among populations and detect the potential effects of mass mortalities on the effective population size and (2) to identify whether hybridization occurs between *I. fasciculata* and *I. variabilis* and, if it does, to which extent.

**MATERIALS AND METHODS**

**Sampling and DNA extraction**

We collected ~2 cm$^2$ of tissue from 194 specimens of *I. fasciculata* of similar size (>500 cm$^3$) between 2010 and 2013 in 12 locations across the Mediterranean and Adriatic seas (see details in Table 1). Similar sizes were selected in an effort to avoid sampling sponges from different cohorts or sponges that had recently been diseased (small fragments sometimes remained attached to the rock if the disease did not kill the whole sponge and could be confused with juveniles). Sampling was performed by SCUBA diving within the first 10 m of each location. Only six healthy individuals were sampled from Scandola Marine Reserve (Table 1) given the extreme decimation this population experienced from 2008 to 2010 (Cebrian et al., 2011). Variation in geographical distance ranged from 21 to 3006 km for the studied area. Sponge fragments were preserved in absolute ethanol that was replaced with fresh ethanol at least three times within 48 h and stored at ~20 °C until further processed. DNA was extracted with the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions with a minor modification concerning overall cell lysis time (that is, incubation was conducted overnight) and the final DNA elution step (performed twice using 50 μl of buffer EB each time).

**Microsatellite amplification and analysis**

We genotyped all individuals of *I. fasciculata* from all locations at 14 microsatellite loci (3IFAS, 7IFAS, 8IFAS, 10IFAS, 22IFAS, 25IFAS, 36IFAS, 40IFAS, 41IFAS, 42IFAS, 44IFAS, 47IFAS, 48IFAS and 49IFAS) previously described elsewhere (Riego et al., 2014), using the PCR conditions described therein. The sizes of the fluorescently labeled PCR products were estimated using an internal size marker (GeneScan 500 LIZ, Applied Biosystems, Foster City, CA, USA) on an ABI Prism 7700 Sequencer (Applied Biosystems) and analyzed with PeakScanner v1.0.

**Genetic diversity in *I. fasciculata* populations**

Estimations for the observed (H$\text{O}$) and expected (H$\text{E}$) heterozygosity, and the fixation index ($F$_{IS}$), commonly used as an inbreeding coefficient, were performed using GenAlEx 6.5 (Peakall and Smouse, 2006). We used Genepop version 4.0.10 (Raymond and Rousset, 1995) to obtain values for departure of Hardy–Weinberg equilibrium (HWE) by locus and population (sampling site) using a probability test with level of significance determined by the following Markov chain parameters: 5000 dememorization steps, 1000 batches
and 5000 iterations per batch. The total number of alleles per locus and population, allele richness, number of private alleles and genetic (gene) diversity (expected frequency of heterozygotes within subpopulations, including a correction for sampling bias stemming from sampling a limited number of individuals per population) were calculated with GENODIVE (Meirmans and Van Tienderen, 2004) and FSTAT 2.9.3.2 (Goudet, 1995). Tests for linkage disequilibrium for each locus were previously performed elsewhere (Riesgo et al., 2014).

**Population differentiation in *I. fasciculata***

We performed six different methods to assess population structure and differentiation in *I. fasciculata*. Four methods were based on distances: $F_{ST}$ estimations, isolation by distance (IBD), BARRIER analyses and the analysis of the molecular variance (AMOVA), and two used a clustering approach: STRUCTURE and the discriminant analysis of principal components (DAPC). Given that the distance methods for measuring population dissimilarity are largely affected by the violation of the HWE (Waples, 2015), we removed five loci from the final data matrix that displayed clear HWE deviation in more than half of the analyzed locations (3IFAS, 10IFAS, 8IFAS, 47IFAS and 49IFAS; see Riesgo et al., 2014). Thus, all distance-based analyses were computed on a subset of data with the remaining nine microsatellites that did not show deviation from the HWE.

We first assessed the occurrence of population subdivision in large clusters using a Bayesian clustering approach in STRUCTURE (Pritchard et al., 2000), a software that assigns individuals to clusters probabilistically, under an admixture model and with allele frequencies correlated between samples. The program was run with a burn-in time of 50,000 repetitions and 150,000 iterations (Markov chain Monte Carlo), setting the putative $K$ (predicted number of populations) from 1 to 15 and 10 replicates for each run. The estimation of log probabilities of data Pr(X|K) for each value of $K$ was evaluated by calculating $\Delta K$ that accounts for the rate of change in the log probability of data between successive $K$ values (Evanno et al., 2005). $\Delta K$ is currently considered a more reliable predictor of the true number of populations (Evanno et al., 2005). Calculations and evaluation of $\Delta K$ were performed with Structure Harvester (Pritchard et al., 2000) and graphs were visualized in STRUCTURE (Pritchard et al., 2000). The CLUMPAK web server (Kopelman et al., 2015) was then used to find the best alignment of the results from STRUCTURE across the range of $K$ values by averaging the probabilities of each $K$ cluster, enabling the production of a figure with the entire range of $K$ values.

Population differentiation was estimated with $F_{ST}$ statistic between pairwise sampling sites using an infinite allele model and the software Arlequin (Excoffier et al., 2005). We only calculated the $F_{ST}$ statistic and not $R_{ST}$ because the former performs better when sample size is $50'$ individuals per site or $20$ loci are analyzed (Gaggiotti et al., 1999). Significance of $F_{ST}$ values was evaluated by performing 20,000 permutations in Arlequin and corrected based on the false discovery rate approach described in Narum (2006). The frequency of null alleles was estimated using the expectation maximization algorithm (Dempster et al., 1977) in FreeNA (Chapuis and Estoup, 2007).

As the presence of null alleles in well-differentiated populations is known to yield an overestimation of population differentiation (Chapuis and Estoup, 2007), we repeated our analysis Excluding Null Alleles (Chapuis and Estoup, 2007). In all cases, the corrections only affected the fourth or fifth decimal place in the $F_{ST}$ value and consequently the effect of presence of null alleles was disregarded.

To determine the spatial patterns driving differentiation among populations, linearized pairwise $F_{ST}$ estimates ($F_{ST}/(1−F_{ST})$) obtained for nine microsatellite loci (see above) were correlated against log-transformed geographical distances between samples and IBM using a Mantel test in Arlequin (Excoffier et al., 2005). Geographical distances were estimated as the minimum linear distance between pairs of locations by sea. We tested IBD for the whole data set, and also separately for a data set containing the locations on the Western Mediterranean (CAI, ESC, TOS, BLA and ALI) and the Pre-Balear island locations (CAB and COR), between which potential dispersal barriers exist (that is, oceanic currents). The effect of barriers in determining the genetic structure of *I. fasciculata* populations was further evaluated using pairwise $F_{ST}$ values and visualized with the software BARRIER v2.2 (Manni et al., 2004). This method links a matrix of geographical coordinates with their corresponding distance matrix ($F_{ST}$), and applies the Monmonier’s maximum distance algorithm to identify ‘barriers’ to gene flow among sites, namely the zones where differences between pairs of sites are the largest.

We also performed DAPC with the adegenet package (Jombart, 2008) implemented in R (https://www.r-project.org/). DAPC defines clusters using the clustering algorithm k-means on transformed data with principal component analysis. The algorithm k-means is then run sequentially with increasing values of $k$, and different clustering solutions are compared using Bayesian information criterion. The optimal cluster solution should correspond then to the lowest value of Bayesian information criterion. We applied the DAPC analysis for both the complete matrix of all sampling sites and a reduced set containing only those from the Western Mediterranean (CAI, ESC, TOS, BLA, CAL and ALI) and the Pre-Balear island (CAB and COR) groups. AMOVA was used to determine the hierarchical distribution of genetic variation. To run this analysis, we a priori defined five groups according to biogeographical areas previously defined by Spalding et al. (2007) and partially isolated by oceanographic fronts as found by Schunter et al. (2011) and Villamor et al. (2014): Alboran Sea, Western Mediterranean, Pre-Balear islands, Thyrrenian Sea and Adriatic sea (see Table 1). The significance of the AMOVAs was calculated with 16,000 permutations of the original data.

**Population assignment and migration in *I. fasciculata***

In addition, we performed a population assignment analysis calculating the likelihood ratio thresholds for all 12 populations based on the Monte Carlo test with an $\alpha$ of 0.002 and 1000 replicated data sets using GENODIVE version 2.0b23 (Meirmans and Van Tienderen, 2004). This method assigns or excludes reference populations as possible origins of individuals on the basis of

| Table 1 Details on collection sites and number of individuals per location for *Ircinia fasciculata* |
|---------------------------------|---------------------------------|-----------------|-----------------|-----------------|
| Label  | Location                        | Coordinates         | N    | Group          |
| CAI    | Es Caials, Cap de Creus, Spain  | 42° 17’ 06.99” N, 3° 17’ 48.57” E | 14   | Western Mediterranean |
| ESC    | L’Illa Mateua, L’Escala, Spain  | 42° 6’ 43.45” N, 3° 10’ 23.68” E | 16   | Western Mediterranean |
| TOS    | Mar Menuda, Tossa de Mar, Spain | 41° 43’ 17.81” N, 2° 56’ 23.09” E | 17   | Western Mediterranean |
| BLA    | S’Agulla, Blanes, Spain         | 41° 40’ 54.16” N, 2° 48’ 57.42” E | 24   | Western Mediterranean |
| CAL    | Calafat, Tarragona, Spain      | 40° 55’ 16.93” N, 0° 50’ 30.61” E | 12   | Western Mediterranean |
| ALI    | Benidorm Island, Vilajoiosa, Alicante, Spain | 38° 30’ 00.37” N, 0° 08’ 12.38” E | 10   | Western Mediterranean |
| CAB    | Illoit L’Imperial, Cabrera National Park, Spain | 39° 07’ 33.14” N, 2° 57’ 34.32” E | 18   | Pre-Balear |
| COR    | Reserve Naturelle de Scandola, Corsica, France | 42° 21’ 31.34” N, 8° 33’ 16.42” E | 6    | Pre-Balear |
| TAR    | Las Palomas, Tarifa, Spain     | 36° 0’ 42.74” N, 5° 35’ 48.17” W | 20   | Alboran Sea |
| GRA    | Piedra del Nombre, Almuñécar, Granada, Spain | 36° 43’ 16.00” N, 3° 44’ 13” W | 20   | Alboran Sea |
| NAP    | Bacoli, Napoli, Italy          | 40° 47’ 39.84” N, 14° 5’ 16.16” E | 17   | Thyrrenian Sea |
| CRO    | Hvar Island, Croatia           | 43°78’ 22.92” N, 16° 43’ 38.42” E | 20   | Adriatic Sea |
multilocus genotypes. Genetic assignment methods allow inferring where individuals originated, providing estimates of real-time dispersal through the detection of immigrant individuals. These analyses are useful in addressing relationships and structure when genetic differentiation at the population level is low. In addition, the detection of last-generation migrants was performed based on the calculations of the likelihood of an individual belonging to a given population that was then done replacing the zero frequencies by a random 0.005 frequency (estimated to outperform tests) in 4000 permutations in GENODIVE.

Demographic effects of mass mortalities in *I. fasciculata*
In order to detect recent effective population size reductions (bottlenecks) from allele data frequencies in our localities because of mass mortalities we used the software BOTTLENECK (Cornuet and Luikart, 1996). This program is based on the assumption that populations that have gone through a recent reduction of their effective population size show a reduction of the allelic diversity and heterozygosity, even though the allele frequencies are reduced faster than the heterozygosity. The software computes the distribution of the heterozygosity expected from the number of alleles for each population and locus, and it takes into account the sample size under the assumption of mutation–drift equilibrium using three different models: infinite allele model, two-phase model and the stepwise mutation model. The analysis is performed using three tests: a ‘sign test’, a ’standardized differences test’ (Cornuet and Luikart, 1996) and ’Wilcoxon sign-rank test’ (Luikart and Cornuet, 1998).

Phylogenetic relationships between *I. variabilis* and *I. fasciculata*
In order to shed light into the phylogenetic relationships between *I. variabilis* and *I. fasciculata*, we amplified a fragment of the mitochondrial cytochrome c oxidase I (COI) gene, increasing the taxon sampling from only 3 specimens of *I. fasciculata* (Erwin et al., 2012b) to 15, and including all sequenced species of the family Irciniidae (see below). A fragment of 1093 bp of COI was amplified for 12 specimens of *I. fasciculata* from the most distant sites (CAL, n = 1; ESC, n = 2; TAR, n = 1, LOS, n = 2; NAP, n = 4; CAB, n = 2) using the primers dglCO1490 (Meyer et al., 2005) and COXI-1 (Rot et al., 2006). COI sequences of *I. variabilis* and *I. fasciculata* from BLA were obtained from GenBank (see accession numbers in Figure 1a). Reactions were performed using 0.5 μl of total DNA (~0.5–1 ng of DNA), 5.1 μl of pure distilled water, 3.3 μl of Extract-N-Amp PCR Reaction Mix (Sigma-Aldrich, St Louis, MO, USA) and 0.3 μl of each primer (10 μM). The amplification program was as follows: denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 60 s and extension at 72 °C for 60 s; with a final extension step at 72 °C for 7 min. PCR products were purified and sequenced using the BigDye Terminator v3.1 cycle sequencing kit (ABI, Foster City, CA, USA), an annealing temperature of 50 °C and using the same primers as for the amplification step. Samples were analyzed on an Applied Biosystems 3730xl Genetic Analyzer available at the University of Barcelona Scientific and Technological Centers. Sequences were edited using Geneious 8.0 (Kearse et al., 2012) and aligned with MAFFT (Katoh et al., 2012). Additional sequences of species from the family Irciniidae were obtained from GenBank: *I. oros* (JN653186, JN653189), *I. strobilina* (JX306089), *I. fexi* (JX306085), *I. ramodiguita* (F552859), *I. irregularis* (F552858), *Ircinia* sp. (LN828727), *Sarcotragus* sp. (HG816026, HG16027, HE591460), *Psammocinia* sp. (F552812). We included as outgroups Spongia officinalis (HQ383064) and *Spongiosis* sp. (F553287) that are also members of the order Dictyoceratida, but not from the family Irciniidae.

The evolutionary model that best fitted our data was obtained with jModeltest2 (Darriba et al., 2012), and evaluated using the Akaike information criterion. The phylogenetic tree was reconstructed using maximum likelihood in PhyML (Guindon and Gascuel, 2003) with 500 bootstrap replicates, 10 initial trees and the evolutionary model HKY85 (see above). Bayesian inference analysis was performed in MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) with two runs, each with three hot chains and one cold chain with 20 million generations, sampling every 2500th generation, using random starting trees. The remaining trees were combined to find the maximum a posteriori probability estimate of phylogeny. This analysis was conducted twice and convergence was tested using Tracer v1.5 (Rambaut and Drummond, 2007). The relationships among the COI sequence haplotypes of both *I. fasciculata* and *I. variabilis* were inferred using the TCS method in the program TCS (Clement et al., 2000) as implemented in Popart 1.7 (http://popart.otago.ac.nz).

Hybridization between *I. variabilis* and *I. fasciculata*: microsatellite analyses
We performed a fine-scale analysis of the introgression processes between our two target *Ircinia* species by genotyping 14 individuals from Tossa de Mar, Corsica and Cabrera of *I. variabilis* (TOS: n = 5, COR: n = 6 and CAB: n = 3, respectively), and randomly selected 36 individuals from the same sampling sites of *I. fasciculata* that were already genotyped (TOS: n = 10, COR: n = 6, and CAB: n = 10) using 12 microsatellite loci (all listed above except for 7IFAS and 40IFAS, as we were not able to amplify them in *I. variabilis*). We subsampled TOS and CAB for *I. fasciculata* in order to maintain the population sizes as even as possible, given that unbalanced sample sizes could hamper the recovery of population structure (Puechmaille, 2016). These three localities (TOS, COR and CAB) were selected because both species live in sympathy. Hybrid detection was achieved by performing an individual assignment to each cluster using STRUCTURE (Pritchard et al., 2000), running the program with a burn-in time of 50,000 repetitions and 150,000 iterations (Markov chain Monte Carlo), and setting the putative K (predicted number of genetic units) from 1 to 6, with 10 replicates for each K. The estimation of log probabilities of data and evaluation of ΔK were calculated as described above. We then used CLUMPPACK (Kopelman et al., 2015) to find the best alignment of the results across the range of K values. Genetic differentiation between species and sites was visualized with a DAPC and the adegenet package (Jombart, 2008) implemented in R.

RESULTS
Population structure and connectivity of *I. fasciculata*
None of the 194 individuals analyzed showed identical genotypes, and therefore clonality can be regarded as negligible in the studied populations. The total number of alleles ranged from 2 to 17 per population, and the average number of alleles per population ranged from 3.857 (COR) to 8.000 (CRO) (Supplementary Table 1). Similarly, the average frequency of private alleles differed from 0.143 (COR) to 1.500 (CRO). Private alleles were present in all populations varying from 1 (ALL, average pA frequency = 0.429) to a maximum of 4 in CRO (average pA frequency = 1.5; Table 2).

Genetic (gene) diversity values were very similar across populations, ranging from 0.626 in TOS to 0.728 in CAL (Supplementary Table 1). Expected heterozygosity values (H<sub>e</sub>) showed the same trend as the genetic diversity values in all populations when using all 14 loci and the subset of 9 loci (Table 2). When the matrix containing all 14 loci was analyzed, fixation indexes (F<sub>is</sub>) were positive in all cases except for CAL and TOS. When considering the 14 microsatellite markers, all populations except for BLA, COR and CRO deviated from HWE, whereas with the 9 microsatellites subset all populations remained in HWE (Table 2 and Supplementary Table 1).

Population differentiation in *I. fasciculata*
The optimal number of clusters for the whole data set by the method of Evanno et al. (2005) in the Bayesian analysis with STRUCTURE was two genetically homogenous groups (K = 2) being followed by five (K = 5; Figures 1a and b; Supplementary Figure 1). Results for K = 2 revealed the division into two differentiated clusters without a clear pattern of subdivision: one cluster corresponded to the populations TAR and GRA (Alboran Sea), as well as some individuals from CAL, CAB, CAR, NAP and CRO (Table 1), and another cluster containing most individuals sampled from Western Mediterranean.
locations (CAI, ESC, TOS, BLA, CAL and ALI) and the Pre-Balear island locations (CAB and COR; Figure 1a). When the number of clusters $K$ was selected by the highest likelihood ($K=5$; Supplementary Figure 1) the populations revealed inner substructuring (Figures 1b and c): (1) the first group (green) was formed mostly by specimens from the east Mediterranean and half of the individuals of NAP, and CAL populations; (2) the second group (yellow) was formed by several individuals from three different geographical areas, half of the populations of TAR, TOS, NAP and CRO; (3) the third group (pink) was formed by half population of TAR, the entire GRA population and some individuals in NAP; (4) the fourth group (blue) comprised CAI, BLA, COR and half of the populations of ESC, TOS, CAL and CAB; and finally (5) the fifth group (red) comprised half ESC and CAB populations (Figures 1b and c). It is noteworthy that in most populations individual genotypes were assigned to a specific cluster, and ‘mixed’ genotypes from different clusters were detected at low frequency.

$F_{ST}$ pairwise comparisons using 9 loci were always significant (Supplementary Table 2), except for comparisons between CAI and ESC ($F_{ST}=0.01905$), ESC and ALI ($F_{ST}=0.06504$), TAR and CAB ($F_{ST}=0.00336$) and ESC and CRO ($F_{ST}=0.01581$), indicating significant differentiation in the genetic structure among most of the studied populations.

The Mantel tests (Figure 2) revealed IBD only when the Western Mediterranean and the Pre-Balear island locations were analyzed separately (Figure 2b), but it was not significant when all populations

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**Figure 1** Assignment of individual genotypes of *I. fasciculata* to genetically similar clusters ($K$) as inferred by STRUCTURE for all studied locations with (a) $K=2$ and (b) $K=5$. (c) Pie frequency charts showing percentage of populations assigned to each of the five clusters represented in (b) for all locations. Gray color is applied to the percentage of individuals that were assigned to more than one cluster. Pre-Bal, Pre-Balear islands; Thyrre, Thyrrenian Sea; Adria, Adriatic Sea. A full color version of this figure is available at the *Heredity* journal online.
In our case, three barriers were set a priori for population differentiation more than distance (Figure 3a). In our case, three barriers were set a priori given the three major oceanographic waterfronts known in the Mediterranean (Figure 3b). The Monmonier’s maximum distance algorithm detected these three barriers and denoted their rank of importance (Figure 3a): first the Almeria-Oran Front (a) separating the Alboran Sea (TAR and GRA) locations from the rest, then the Ligurian-Thyrrenian barrier (b) separating Thyrrenian and Adriatic locations (NAP and CRO) from the western ones, and finally the North-Balearic Front (c) that also separated BLA and CAL from the rest of the Western Mediterranean populations.

The DAPC showed separation between the Alboran Sea (TAR and GRA), the Pre-Balear islands (COR and CAB), the Thyrrenian and Adriatic locations (NAP and CRO) and the remaining locations of the Western Mediterranean (Figure 4a). Indeed, the northern locations of the Western Mediterranean (CAI and ESC) and the Pre-Balear islands (COR and CAB) grouped more closely together, being slightly separated from the rest of populations of the Western Mediterranean and Thyrrenian and Adriatic Sea group (Figure 4a). In addition, when analyzing the spatial structure of Western Mediterranean and Pre-Balear island locations, only CAL was clearly separated from the rest of populations (Figure 4b).

When analyzing population differentiation in the different regions using AMOVA, we found significant genetic differentiation among the groups containing Alboran Sea, Western Mediterranean, Pre-Balear islands and Adriatic Sea locations (Tables 1 and 3). In addition, significant genetic differentiation among the populations within these groups was also observed (Table 3).

### Table 2 Descriptors of genetic diversity for all 12 locations of *Ircinia fasciculata* using the data set containing 14 loci and the corrected data set with only 9 loci

| Pop. | N    | A    | rA   | pA   | H0   | H0   | FIS  | HWE |
|------|------|------|------|------|------|------|------|-----|
| TAR  | 17   | 5.857| 7.111| 4.683| 4.666| 1.071| 0.778|     |
| GRA  | 20   | 5.857| 5.556| 4.129| 3.918| 0.786| 0.778|     |
| CAI  | 14   | 5.928| 5.667| 4.683| 4.255| 1.071| 0.778|     |
| ESC  | 16   | 5.714| 5.778| 4.228| 4.183| 0.786| 0.778|     |
| TOS  | 17   | 6.357| 5.889| 4.192| 3.944| 0.857| 0.778|     |
| BLA  | 24   | 6.857| 6.444| 4.169| 4.055| 1.071| 0.778|     |
| CAL  | 12   | 7.285| 7.556| 5.315| 5.424| 1.071| 0.778|     |
| ALI  | 10   | 5.428| 5.444| 4.507| 4.506| 0.857| 0.778|     |
| CAB  | 18   | 6.571| 5.778| 4.537| 4.116| 0.643| 0.444|     |
| COR  | 6    | 3.857| 3.889| 3.857| 3.889| 0.143| 0.222|     |
| NAP  | 20   | 7.928| 8.333| 4.876| 5.069| 0.786| 0.889|     |
| CRO  | 20   | 8.000| 8.111| 4.737| 4.735| 1.500| 1.778|     |

**Figure 2** IBD analyses in *I. fasciculata* for (a) all sites and (b) only Western Mediterranean and Pre-Balear sites.
Demographic effects of mass mortalities in *Ircinia fasciculata*

All three BOTTLENECK tests identified a population reduction in COR under the infinite allele model (Table 4), but also detected population reductions in other locations depending on the model used. Using two-phase model, the sign test did not identify any bottleneck in any of the studied locations using the Wilcoxon rank test.

**Figure 3** Detection of barriers to gene flow in *I. fasciculata* across the Mediterranean Sea. (a) Barriers detected by software BARRIER ranked a to c in order of magnitude. (b) Map of the Mediterranean seawater circulation indicating major fronts and oceanographic barriers previously known in the Mediterranean. Note that sampling locations for *I. fasciculata* are indicated in the map.

**Figure 4** Subdivision of the *I. fasciculata* sites according to DAPC analysis for (a) all areas and (b) only Western Mediterranean and Pre-Balear sites.
Table 3 Details on the results of the analysis of the molecular variance (AMOVA) performed using three groups for the collection sites of Ircinia fasciculata: Alboran Sea (TAR and GRA), Western Mediterranean (CAI, ESC, TOS, BLA, CAL and ALI), Pre-Balear islands (CAB and COR), Thyrrenan Sea (NAP) and Adriatic Sea (CRO)

| Source of variation                  | Sum of squares | Variance components | Percentage variation | Fixation indices | P-value |
|--------------------------------------|----------------|---------------------|----------------------|------------------|---------|
| Among groups                         | 18.7940        | 0.0427              | 1.9200               | 0.01923          | 0.01214 |
| Among populations within groups      | 38.5160        | 0.0765              | 3.4500               | 0.03516          | 0.00000 |
| Among individuals within populations | 348.9400       | −0.1818             | −8.2000              | −0.08663         | 1.00000 |
| Within individuals                   | 442.5000       | 2.2809              | 102.8300             | 2.28093          | 0.99496 |

See Table 1 for full name of locations. Significant P-values are in bold.

Figure 5 Population assignment of individuals and last-generation migrants in I. fasciculata. (a) Number of individuals assigned to each population for all sampling locations. (b) Number of last-generation migrants (indicated in percentage of population). The original location of migrants (origin) is color coded following the same scheme as in DAPC of Figure 4a. A full color version of this figure is available at the Heredity journal online.

Phylogenetic relationship between I. variabilis and I. fasciculata
The 19 sequences of the COI marker analyzed from 8 different locations of I. variabilis and I. fasciculata were 1093 base pairs long and, among them, 28 sites showed polymorphism (5.004%). Within the reduced phylogeny of the genus Ircinia, both the maximum likelihood and Bayesian inference analyses showed a large, well-supported clade containing sequences of both I. variabilis and I. fasciculata (Figure 6a). Within this clade, sequences showed no clear grouping pattern between species or among locations (Figure 6a). The TCS haplotype network showed that the main haplotype for I. fasciculata was recovered in three locations (BLA, CAL and NAP) and this same haplotype was shared with three individuals of I. variabilis from BLA (Figure 6b). In addition, seven more haplotypes were recovered for I. fasciculata (Figure 6b).

Hybridization patterns in I. variabilis and I. fasciculata
The DAPC analysis using microsatellite data of both species collected in the same three sites showed a clear group containing all locations of I. variabilis (IVTOS, IVCAB and IVCOR) and those individuals of I. fasciculata collected in Tossa de Mar (IFTOS), whereas the rest of locations of I. fasciculata appeared separated from them (IFCAB and IFCOR, Figure 6c). In addition, the individual assignment analysis performed in STRUCTURE showed that individuals from the locations TOS and CAB of I. variabilis belonged to the same group (blue) as some individuals of I. fasciculata in the sites TOS, COR and CAB (Figure 6d). Similarly, two individuals in the location of COR of I. variabilis appeared as a ‘mixture’ of I. variabilis clusters (Figure 6d). The crimson cluster mostly belonged to individuals of I. fasciculata at the locations of TOS and CAB (Figure 6d). All our results indicate some degree of hybridization between I. variabilis and I. fasciculata, at least in some locations, and especially in TOS.

DISCUSSION
Genetic diversity in I. fasciculata
Genetic diversity (calculated with the parameters gene diversity in GENODIVE and Ht in GenAlex) was ~0.6, a value that is similar to those observed in other Mediterranean sponges, including Crambe crambe (~0.6, Duran et al., 2004), the endangered species Spongia lamella (~0.5, Pérez-Portela et al., 2015), the bath sponge S. officinalis (~0.8, Dailianis et al., 2011) and Scopalia lophiropoda (~0.4; Blanquer and Uriz, 2010). Inbreeding analysis detected a significant heterozygosity deficit in most localities, indicating that they were not in HWE, a situation widespread among most studied sponges (Duran et al., 2004; Blanquer and Uriz, 2010; Dailianis et al., 2011;...
Table 4 Results of the Bottleneck analysis for the 12 locations (Loc) of *Ircinia fasciculata*

| Loc | IAM | TPM | SMM | P | P1 | P2 | P3 | P4 | P5 | P6 |
|-----|-----|-----|-----|---|----|----|----|----|----|----|
| CAI | 5.93 | 9.77 | 7.13 | 0.00258 | 0.000789 | 0.000857 | 0.000857 | 0.000857 | 0.000857 | 0.000857 |
| ALI | 19.57 | 5.43 | 6.83 | 0.000209 | 0.000209 | 0.000209 | 0.000209 | 0.000209 | 0.000209 | 0.000209 |
| CAR | 12.00 | 3.86 | 6.67 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| CAB | 34.14 | 6.57 | 6.83 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| ESC | 29.29 | 5.71 | 6.42 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| CAL | 23.86 | 7.29 | 6.25 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| TAR | 32.43 | 7.07 | 6.42 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| TOS | 33.43 | 7.29 | 6.35 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| NAP | 37.00 | 7.93 | 6.71 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |
| CRO | 38.14 | 8.00 | 6.71 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 | 0.000000 |

**Abbreviations:** IAM, infinite allele model; TPM, two-phase model; SMM, stepwise mutation model. Significant values are in bold.

In summary, both inbreeding and the existence of substructure might be the factors that most likely are causing the deviation of HWE in our *I. fasciculata* populations.

**Population structure and connectivity in *I. fasciculata***

The populations of *I. fasciculata* showed genetic structure at both large and small geographical scales. First, two major clusters were detected across the testing site in this study, one grouping the Alboran Sea locations and the Thyrrenian and Adriatic Sea locations, and the other one the locations along the Western Mediterranean coast of Spain and the Balearic and Corsica Islands (Figure 2a). However, the Mantel test performed along the entire range of our study did not reveal a pattern of stepping stone gene flow (derived from a process of IBD), suggesting that other major processes such as the existence of oceanographic barriers (for example, major currents) may be driving population differentiation patterns in this area. In fact, three barriers to gene flow were detected in our data set: the strongest barrier appeared to separate the Alboran Sea locations from the rest of the Mediterranean Sea (presumably the Almería-Oran Front, Figure 4), and this was also correlated with the limited gene flow detected between these areas and the high population differentiation observed between both regions. For many marine organisms, the Almería-Oran Front acts as a strong barrier for gene flow between Atlantic and Mediterranean populations (see review in Patarnello et al., 2007), including sponges (see, for example, Pérez-Portela et al., 2015). In contrast, stepping stone gene flow was observed when analyzing the spatial distribution of genetic divergence exclusively between the Western and Thyrrenian and Adriatic locations. The other two barriers detected corresponded to the North-Balearic Front and the Ligurian-Thyrrenian barrier (Figure 4). As found for other benthic organisms (Villamor et al., 2014),
the Ligurian-Thyrrenian barrier appeared to be a seasonal barrier, and it did not completely prevent the gene flow between populations across it.

Population differentiation was also detected at smaller scales: significant differences were detected between all pairwise comparisons except for the closely located sites of CAI and ESC in the North-Western coast of Spain, and the distantly located pairs of ESC and ALI, TAR and CAB and ESC and CRO (Figure 2c). Although high gene flow may be expected between CAI and ESC (only located 50 km apart), the extensive gene flow detected among the other 3 site pairs (located between 550 and 2250 km apart) does not have a straightforward explanation. In general, populations of sessile invertebrates with relatively low dispersal capabilities in the Mediterranean are highly structured like those of *I. fasciculata*, including not only sponges (Duran et al., 2004; Blanquer and Uriz, 2010; Dailianis et al., 2011; Pérez-Portela et al., 2015), but also cnidarians and ascidians (Goffredo et al., 2004; López-Legentil and Turon, 2006; Pérez-Portela and Turon, 2008; Mokhtar-Jamaï et al., 2011). Even sponges from other distant regions show high values of population differentiation (Chaves-Fonnegra et al., 2015; Giles et al., 2015). The overall $F_{ST}$ value of genetic differentiation found for *I. fasciculata* ($F_{ST} = 0.221$) was higher than that reported for the closely related dicycletid *S. officinalis* ($F_{ST} = 0.061$, Dailianis et al., 2011), but similar to other Mediterranean demosponges studied using microsatellites (*C. crambe* $F_{ST} = 0.18$, Duran et al., 2004; *S. lophyropoda* $F_{ST} = 0.122$, Blanquer and Uriz, 2010; *S. lamella* $F_{ST} = 0.236$, Pérez-Portela et al., 2015) and even corals, such as *Balanophyllia europaea* (0.202; Goffredo et al., 2004), indicating that the differentiation patterns in these Mediterranean benthic invertebrates might be equally driven by the inbreeding caused by the low dispersal abilities of their larvae, and the occurrence of oceanographic barriers to their gene flow.

Demographic effects of mass mortalities in *I. fasciculata*

Recent reductions in the effective population size (or bottlenecks) have been detected in almost all the studied sites of *I. fasciculata* except for CAI, ALI and CAL depending on the model used. During a bottleneck and under the infinite allele model, allelic diversity is reduced faster than the heterozygosity. However, under the strict stepwise mutation model and probably under the two-phase model, heterozygosity excess may not be observed (Cornuet and Luikart, 1996). In our case, the different results in the detection of bottlenecks under different models may be reflecting differences in the resolution of the analyses rather than inconclusive results. In fact, these reductions in population size can well be related to recent episodes of massive individual losses reported for *I. fasciculata* (see, for example, Garrabou et al., 2009; Maldonado et al., 2010b; Cebrian et al., 2011). Even though a massive episode of mortality was observed in ALI in 2008 (Maldonado et al., 2010b), a bottleneck was not detected with our genetic analysis. The most parsimonious explanations for our observation are that (1) ALI was unaffected by genetic drift after massive dye-offs and/or (2) that this location is well connected (by means of gene flow) with other nearby *I. fasciculata* populations. Indeed, the largest number of migrants from last generation was detected in ALI, suggesting higher migration rates toward this location.

Phylogenetic analyses and hybridization between *I. variabilis* and *I. fasciculata*

Previous analysis using COI sequences did not provide enough resolution to delimit *I. variabilis* and *I. fasciculata* species, but analyses of the nuclear ribosomal gene *ITS-2* showed that they formed different clades with a divergence of 2.97% ± 0.38 (Erwin et al., 2012b). Similarly, our results show that COI did not separate *I. variabilis* and *I. fasciculata* because they form together a well-supported but
unresolved clade, with shared haplotypes between both species. This was not surprising, as nucleotide substitution rates in the mitochondrial genome in sponges and other lower metazoans are up to 100 times slower than other animal lineages (Hellberg, 2006; Shearer et al., 2002). In this sense, although COI has been previously found to be useful to explain speciation and phylogeographic patterns in some haplosclerid sponges (see, for example, López-Legentil and Pawlik, 2009; DeBlaise and Hellberg, 2015), it seems to be unsuitable for resolving phylogenetic relationships in other sponges (see, for example, Duran et al., 2004; Dailianis et al., 2011). In light of these results, two possible explanations arise: the first one is that they are separate but closely related species that have undergone secondary contact favoring introgression, and a second explanation may be that \textit{I. variabilis} and \textit{I. fasciculata} are the same species but are currently undergoing incipient speciation without reproductive isolation yet occurring, and therefore a certain level of mixing still exists. Both the reciprocal monophyly of ITS-2 sequence clades and the existence of some morphological differences between these two species lend support to our first hypothesis. In addition, it is important to note here that our molecular results do not support the reassignment of \textit{I. fasciculata} to the genus \textit{Sarcothoa}, as previously proposed by Pronzato et al. (2004), as \textit{I. fasciculata} and the type species for \textit{Sarcothoa} (\textit{S. spinulosus}) appear in two separated and clearly divergent clades (Figure 6a).

Instead of using COI, several authors have suggested multilocus (for example, nuclear coding genes, amplified fragment length polymorphisms, microsatellites and single-nucleotide polymorphisms) approaches as potentially suitable for resolving species boundaries (Pettit and Excoffier, 2009; Hausdorf and Henning, 2010). In particular, microsatellite loci are characterized by relatively high mutation rates, an assumed neutral selection rate and expected lower introgression potential than mitochondrial markers for at least some organisms (Pettit and Excoffier, 2009). Our results using microsatellite loci suggested that some degree of hybridization and introgression occur in at least some populations where both species of \textit{Ircinia} appear in sympathy (Figures 6b and c). Introduction between closely related species with overlapping ranges and incomplete reproductive barriers (such as the \textit{Ircinia} species here) is common in marine invertebrates (see, for example, Willis et al., 2006) even when the divergence between species is relatively large (Nydam and Harrison, 2011). Interestingly, introgression could be increased when intraspecific gene flow decreases (Pettit and Excoffier, 2009). Thus, as the number and densities of \textit{Ircinia} populations decrease in the Mediterranean Sea, a concomitant decrease in gene flow among affected populations may be accompanied by higher hybridization rates between \textit{I. variabilis} and \textit{I. fasciculata} populations, further blurring these two species boundaries. Although the relationship between intra- and interspecific gene flow is mostly documented for human populations (Currait et al., 2008), the hybridization patterns of \textit{I. fasciculata} reported here might be a first step toward understanding the impact of introgression in the genetic diversity and structure of marine invertebrates after population reductions.

In conclusion, our results agree with previous studies on the genetic differentiation of sessile invertebrates with lecithotrophic larvae and limited dispersal potential in the Mediterranean Sea (Duran et al., 2004; Goffredo et al., 2004; Pérez-Portela and Turon, 2008; Blanquer and Uriz, 2010; Dailianis et al., 2011; Mokhtar-Jamati et al., 2011; Pérez-Portela et al., 2015), highlighting the important role of oceanographic barriers in shaping the genetic structure across their distribution (Patarnello et al., 2007; Villamor et al., 2014). In particular, we found that most locations of \textit{I. fasciculata} are strongly genetically differentiated, with barriers preventing gene flow between many population pairs that correlate with well-known oceanographic barriers. Importantly, our results indicate that the genetic diversity of \textit{I. fasciculata} is threatened by massive demographic losses in the Mediterranean Sea, and that its recovery is hindered by limited connectivity between populations. Demographic reductions of \textit{I. fasciculata} in the Alboran sites might be especially worrisome, as these locations are genetically isolated from the rest. Interestingly, the hybridization potential observed between \textit{I. fasciculata} and \textit{I. variabilis} may allow the former to maintain a genetic pool large enough to palliate genetic diversity losses. Notably, increased introgression events among these species may offset reductions in intraspecific gene flow resulting from stress and mass mortality events.

**DATA ARCHIVING**

All data sets have been archived in PANGAEA [https://doi.pangaea.de/10.1594/PANGAEA.860018](https://doi.pangaea.de/10.1594/PANGAEA.860018).

**CONFLICT OF INTEREST**

The authors declare no conflict of interest.

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