MOLECULAR ECOLOGY

Molecular Ecology (2012) **21**, 3671–3685 doi: 10.1111/j.1365-294X.2012.05655.x

Genetic assessment of population structure and connectivity in the threatened Mediterranean coral *Astroides calycularis* (Scleractinia, Dendrophylliidae) at different spatial scales

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Abstract

Understanding dispersal patterns, population structure and connectivity among populations is helpful in the management and conservation of threatened species. Molecular markers are useful tools as indirect estimators of these characteristics. In this study, we assess the population genetic structure of the orange coral *Astroides calycularis* in the Alboran Sea at local and regional scale, and at three localities outside of this basin. Bayesian clustering methods, traditional *F*-statistics and D_{est} statistics were used to determine the patterns of genetic structure. Likelihood and coalescence approaches were used to infer migration patterns and effective population sizes. The results obtained reveal a high level of connectivity among localities separated by as much as 1 km and moderate levels of genetic differentiation among more distant localities, somewhat corresponding with a stepping-stone model of gene flow and connectivity. These data suggest that connectivity among populations of this coral is mainly driven by the biology of the species, with low dispersal abilities; in addition, hydrodynamic processes, oceanographic fronts and the distribution of rocky substrate along the coastline may influence larval dispersal.

Keywords: *Astroides calycularis*, genetic connectivity, genetic structure, microsatellites, Scleractinia, threatened species

Received 3 February 2012; revision received 7 April 2012; accepted 18 April 2012

Introduction

Population dynamics and structure of marine organisms reflect the historical and contemporary interaction at different spatial and temporal scales among a complex set of ecological, demographic, behavioural, oceanographic, climatic and geological processes (Grosberg & Cunningham 2001). Within this general framework, it is worth mentioning that population connectivity plays a fundamental role from local to metapopulation dynamics and in structure, genetic diversity, demographic structure and the resilience of populations (Botsford *et al.* 2001). Therein, all those features are linked with species life history traits, such as species dispersal capacity at any stage of its life cycle, and its interaction with associated biotic and abiotic factors (DiBacco *et al.* 2006). Among the latter, dispersal among populations can be affected by physical barriers to larval dispersal (hydrographic fronts, upwelling systems, eddies or counter currents) (Pineda *et al.* 2007), or enhanced by oceanographic features such as global and local water currents (Thiel & Gutow 2005) or drafting events (Fraser *et al.* 2011). Most marine benthic invertebrates have restricted adult movement, so their larval stages represent their main, and likely their only, opportunity for dispersal (Hellberg 2009). As, in general, the larval phase of marine invertebrates is difficult to track

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molecular tools provide an ideal technique for indirect estimation of population structure and connectivity (Cowen & Sponaugle 2009). Besides, defining the scale and processes that affect connectivity among marine populations and the identification of barriers to gene flow are fundamental tasks for understanding their genetic structure and establishing appropriate management and conservation plans for threatened species (Botsford *et al.* 2001).

Within the generally high diversity found throughout the Mediterranean Sea (Coll *et al.* 2010), some studies suggest that the Alboran Sea, the westernmost subbasin of the Mediterranean Sea, harbours one or more hotspots of biodiversity because of the coexistence of species from three marine biogeographic provinces (Mediterranean, Lusitanean and Mauritanean regions) and to the endemic species restricted to this zone (Coll *et al.* 2010; Aguilar *et al.* 2011). The eastern boundary of the Alboran Sea is delimited by the Almeria-Oran Front (AOF). The AOF is a strong, large-scale density front extending between Cabo de Gata (SE corner of Spain) and Oran (Algeria), formed by the convergence of two distinct water masses and controlled by the geographic position and strength of the Eastern Alboran Gyre (Tintoré *et al.* 1988). Physical and biochemical data indicate that this front is limited to the upper 300 m, with a strong southward baroclinic jet. This front acts as an effective barrier for many planktonic organisms and larvae likely due to the presence of a zone of turbulences and to pronounced changes in salinity and temperature gradients associated with water currents. Some molecular studies have shown noticeable genetic differences between populations on either side of the front (see Patarnello *et al.* 2007 for a review).

The scleractinian coral *Astroides calycularis* (Pallas, 1766) is an azooxanthellate colonial species, characterized by the bright orange colour of its coenosarc and polyps (Zibrowius 1995). It is a typical shallow water species that usually lives close to sea level, from the intertidal fringe to a depth of 40 m, with lower abundances of the species below this level (Zibrowius 1995; Kruzic *et al.* 2002). This coral is considered a warm-water species with a narrow temperature tolerance (Bianchi 2007). Even though it was widely distributed in the western Mediterranean during the Pleistocene, climatic fluctuations occurred during that period leading to a regression of the species (Zibrowius 1995). Its current distribution range is limited to rocky coastal areas of the south-western basin of the Mediterranean, presently undergoing a net regression because of several anthropogenic factors (Moreno *et al.* 2008). Because of its limited geographic distribution, and the historical and current regression of the species, *A. calycularis* is considered under protection and catalogued as 'vulnerable' under different national and international legislative assessments (Templado *et al.* 2004).

Astroides calycularis has been characterized as a brooder coral (Lacaze-Duthiers 1873; Goffredo *et al.* 2010). Once released, its planula larvae show negative buoyancy and demersal behaviour, crawling along the vertical rocky wall before finding a substrate on which to settle (Goffredo *et al.* 2010). Hence, it is predicted that the species' larvae will show low dispersal capabilities. Limited larval dispersal, and the subsequent restricted gene flow and connectivity, can lead to a low degree of local genetic variability and increased differentiation among populations as a result of isolation by distance (Goldson *et al.* 2001). The relationship between the dispersal ability of organisms and the genetic differentiation of their populations at multiple scales provides a fundamental link between ecology and evolution (Bohonak 1999). These features of *A. calycularis* may reveal different historical patterns and migration routes than populations of high dispersal species, in which local genetic variability may be enhanced by random mixing of alleles from other populations (Slatkin 1993), thus, contributing to a more complete knowledge of the relationships between evolutionary history and contemporary distribution of genetic variation (Marshall & Morgan 2011).

Taking into account all of the above, the main aims of this study were to assess the genetic structure and connectivity patterns among populations of *A. calycularis* in the Alboran Sea at local and regional scales. In addition, we aimed to compare structure and connectivity with more distant localities through the inclusion of three populations outside of this basin, which correspond to two distal limits within its overall range.

Material and methods

Sample collection

We focused our study on the Alboran Sea, the westernmost basin of the Mediterranean Sea. Moreover, to get a more complete picture of the population genetics of *A. calycularis* throughout its distribution range, we included three additional localities outside of the Alboran Sea: one in the Algerian Basin and two more in the Tyrrhenian Sea. These three localities correspond to the known limits of the distribution range of *A. calycularis* (Fig. 1; Table 1). Therefore, the sampling design followed a hierarchical approach, allowing the study of genetic structure, connectivity and gene flow patterns of the species considering a local scale with six sites at the Chafarinas Islands and two sites at Alboran Island (distance among localities range from 0.6 to 1.4 km); a regional scale that is within all the localities of the

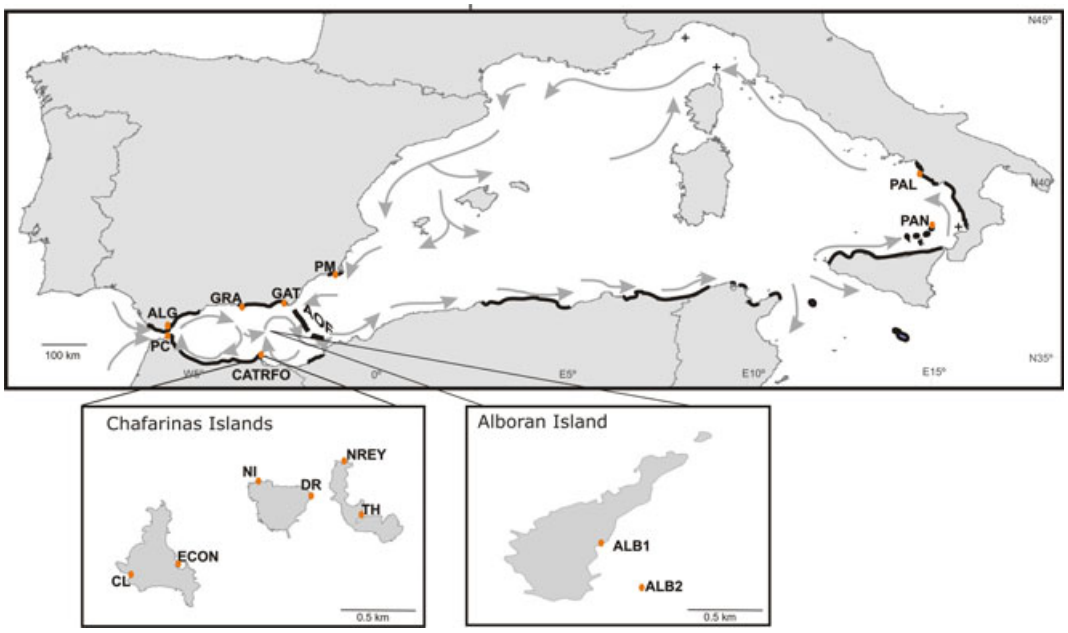

Fig. 1 Geographic location of the sixteen localities where *Astroides calycularis* was sampled. The black line indicates the coasts and nearby islands where the species is known. (+) symbols represent the places where fossils of the species have been found (modified from Goffredo *et al*. 2010). Sea surface water currents are included in the map (modified from Millot 1999) (for locality codes see Table 1).

Table 1 Sampling localities of *Astroides calycularis*

Basin	Locality	Code	Geographical coordinates		Sample size
			Latitude	Longitude	
Alboran Sea	Algeciras	ALG	36°06′42.18″N	05°24′52.56″W	25
Alboran Sea	Ceuta	PC	35°53′45.13″N	5°16′47.71″W	25
Alboran Sea	Granada	GRA	36°43′40.50″N	3°41′38.10″W	20
Alboran Sea	Cape Tres Forcas	CATRFO	35°25′56.50″N	2°59′34.61″W	23
Alboran Sea	*Alborán Island*				
	Limpets Cave	ALB1	35°56′59.11″N	3°2′17.72″W	18
	South of Alboran Island	ALB2	35°56′53.84″N	3°2′25.59″W	24
Alboran Sea	*Chafarinas Islands*				
	Congreso Island. Cuevas del Lobo	CL	35°10′40.67″N	2°26′37.03″W	22
	Congreso Island. East face of the island	ECON	35°10′40.73″N	2°26′21.72″W	22
	Isabel II Island. North face of the island	NI	35°11′0.66″N	2°25′45.26″W	20
	Isabel II Island. Dique Roto	DR	35°10′53.63″N	2°25′34.98″W	24
	Rey Francisco Island. North face of the island	NREY	35°11′5.78″N	2°25′23.15″W	22
	Rey Francisco Island. Tajo del Halcón	TH	35°10′53.84″N	2°25′23.75″W	22
Alboran Sea	Cabo de Gata	GAT	36°43′34.08″N	2°11′45.60″W	25
Algerian Basin	Portman Bay	PM	37°34′42.96″N	0°50′31.77″W	23
Tyrrhenian Basin	Panarea Island	PAN	38°38′10.67″N	15°04′15.21″E	31
Tyrrhenian Basin	Palinuro	PAL	40° 1′43.58″N	15°16′4.36″E	24

Alboran Sea (distance between localities range from 18.5 to 471.7 km); and three distant localities outside of the Alboran Basin distant up to 1500 km from its limit, the AOF: Portman Bay (Algerian Basin), Panarea Island and Palinuro (Tyrrhenian Basin) allowing the study the above-mentioned parameters at more distant localities and the effect of putative barriers among the different marine areas involved in the study.

Three of the sampled localities corresponded with Marine Protected Areas (MPAs): Cabo de Gata, Chafarinas Islands and Alboran Island (see Table 1) that allowed us to establish an initial survey on the genetic condition of this areas and its relation to nearby localities.

At each of the sampled localities, individual polyps from 18 to 31 adult colonies were randomly collected

by SCUBA diving. Sampled colonies were randomly collected, but a minimum distance of 1–2 m was considered to avoid sampling the same colony twice. To minimize the damage to the sampled colonies and taking into account the 'vulnerable' status of the coral and the scarcity of colonies in several localities, 1–2 polyps were cut with scissors from each colony. Samples were immediately stored in a vial in absolute ethanol until laboratory analyses.

DNA extraction, microsatellite amplification and genotyping

Total DNA was extracted from a total of 381 polyps, using Qiagen BioSprint IT 15DNA Blood Kit (45). Thirteen microsatellite loci specifically developed for *A. calycularis* (Molecular Ecology Resources Primer Development Consortium *et al.* 2010) were amplified with fluorescently labelled primers following the PCR conditions described in Molecular Ecology Resources Primer Development Consortium *et al.* (2010). PCR products were visualized with an automated sequencer (ABI PRISM 3730 DNA Sequencer, Applied Biosystems) with the GeneScan-500 (LIZ) internal size standard. Electropherograms were analysed for allele scoring with GeneMapper software 3.0 (Applied Biosystems).

Data analysis

Genetic diversity. We quantified genetic diversity as allelic richness (N_a, number of alleles) per sample for each locus and over all loci and private allele richness (P_a) per locality and overall loci. Both analyses were performed using GENALEX 6.0 (Peakall & Smouse 2006). The number of alleles standardized to those of the population sample with smallest size ($N = 18$) was calculated with the rarefaction method implemented in FSTAT ver. 2.9.3.2 (Goudet 1995).

Analyses of departures from Hardy–Weinberg equilibrium (HWE) within populations for each locus and over all loci were quantified as the observed (H_o) and expected (H_e) heterozygosities using GENALEX 6.0 software (Peakall & Smouse 2006). Estimations of the inbreeding coefficient, F_{IS}, an estimate of the deficit or excess of heterozygotes, within each population for each locus and over all loci were computed with Genetix software (Belkhir *et al.* 2004). Significance of the estimation analysis was tested with 10 000 permutations.

MICRO-CHECKER v.2.2.3 software (Van Oosterhout *et al.* 2004) was used to check for scoring errors because of stuttering, large allele dropout and to estimate null allele frequencies.

Linkage disequilibrium (LD) was tested among all pairs of loci at each of the sampled locations with a permutation test using Genepop version 3.4 (Raymond & Rousset 1995a). Analysis of significance was tested with Markov chain Monte Carlo (MCMC that was run using 1000 dememorizations with 100 batches and 1000 iterations per batch). Step-down Bonferroni (Holm 1979) correction was applied to P values in all the statistical analyses that included multiple comparisons.

Neutrality of the markers was tested using LOSITAN software (Antao *et al.* 2008), which performs an F_{ST}-outlier analysis (Beaumont & Nichols 1996) to identify microsatellite loci under selection. 50 000 simulations of the software were generated. Significance was estimated with a 95% confidence interval. Two rounds of the analysis were performed, one across the whole data set and a second one removing from the analysis the two more distant localities (PAN and PAL, for these and the following localities codes, see Table 1) to avoid the effect of random drift and historical events that can affect gene flow and drift, which could potentially bias the relative influence of natural selection on the studied markers (Hellberg 1995; Luikart *et al.* 2003). Both rounds were conducted for both the infinite alleles (IAM) and the stepwise mutation model (SMM) (Kimura & Crow 1964).

Sampling benthic sessile invertebrates may result in the collection of closely related individuals. That would lead to nonindependent genotypes and violation of HWE and LD assumptions. Therefore, the software ML-RELATE (Kalinowski *et al.* 2006) was used to calculate which of four levels of relatedness (unrelated, half-siblings, full-siblings and parent-offspring) had the highest likelihood for each pair of individuals within populations. Also, to ensure that duplicate samples (e.g. polyps sampled twice) were not included in the analyses, the software Cervus (Kalinowski *et al.* 2007) was used for detecting duplicated genotypes.

Population genetic structure analysis. We estimated population genetic structure using three different approaches: (i) a Bayesian clustering method; (ii) a hierarchical analysis of molecular variance (AMOVA); and (iii) traditional measures of genetic differentiation (F_{ST}) among a priori defined populations (indicating the genetic subdivision among populations) and D_{est}, an estimator of actual differentiation (Jost 2008).

The number of genetically differentiated *A. calycularis* populations, K, was estimated by employing the Bayesian approach implemented in the software STRUCTURE (Pritchard *et al.* 2000), without a priori information on the geographical location of each sample. A hierarchical approach was used for this analysis, running the 'admixture ancestry model' (because even though the planula stage of *A. calycularis* has been characterized as demersal, connected populations could occur through

surface currents) under the assumption of 'correlated allele frequencies' to improve clustering of closely related populations. MCMC consisted of 50 000 burn-in iterations followed by 500 000 sampled iterations. To check the consistency of the results, 20 replicated runs were launched for each K. The K value that better fit the data set was inferred by plotting the log probability of the data (Log P($X|K$)) as a function of K across the 20 runs, corresponding to the selected K value with the point at which the curves plateau (Pritchard *et al.* 2000). The software was run three times. On a first run, the likelihood of the data and following probabilities for the different number of populations were calculated with K (1–16), where K is the different number of populations. After plotting Log P ($X|K$) as a function of K, there was no clear plateau but a weak increase in Log P ($X|K$); thus, as no single K value provided a full description of population structure because of the additional substructure (Mokhtar-Jamaï *et al.* 2011), the values between 2 and 14 were discussed. As $K = 2$ corresponded to the two localities in the Tyrrhenian Basin *versus* those in the Alboran Sea-Algerian Basin, on a second run, only the localities across the Alboran and Algerian Basins were taken into account for the analysis K (1–14). In this second run, the peninsular locations north of Morocco, PC and CATRFO (Table 1; Fig. 1) were found to have high degrees of admixture within two of the identified clusters. Therefore, samples from both localities were designated as 'unknown', and STRUCTURE was a third time to assign these genotypes to their place of origin with the PopInfo option.

ARLEQUIN 3.1.1 (Excoffier *et al.* 2005) was used to perform hierarchical spatial analysis of molecular variance (AMOVA, $n = 1000$ permutations), using the groups defined by STRUCTURE in each of the runs.

For the measures of genetic differentiation, sample sites were used as a population unit. We estimated F_{ST} values and their statistical significance with 10 000 bootstrap replicates (Weir & Cockerham 1984) using ARLEQUIN 3.1.1 software (Excoffier *et al.* 2005). As null alleles can induce overestimation of genetic distances (Chapuis & Estoup 2007), pairwise estimates were computed with and without correction for null alleles (Brookfield 1996).

Recently, the use of F_{ST} statistics to assess differentiation with hypervariable markers has been questioned as it depends on within-population heterozygosity tending to underestimate the differentiation between populations as variation increases (Jost 2008). Therefore, the estimator of actual differentiation (D_{est}) was also calculated. The package DEMEtics (Gerlach *et al.* 2010) within the statistical package R v2.12.2 (R Development Core Team 2009) was used to estimate pairwise population D_{est} values, and bootstrapping (1000 iterations) was used to calculate P values. Nevertheless, as Whitlock (2011) recently maintain, F_{ST} estimations should still be reported as they better convey the evolutionary and demographic processes that lead to differentiation among populations.

We calculated the influence of geographic distance on population genetic differentiation with two models: Rousset (1997) model for two-dimensional habitats with the correlation between pairwise population linearized F_{ST} ($F_{ST}/(1 - F_{ST})$) and the logarithm of the geographical distance (in metres); and Slatkin (1993), which used the logarithm of $\hat{M}$ calculated separately for pairs of populations, (where $\hat{M} = (1 - F_{ST})/4F_{ST}$) as a measure of similarity, versus the logarithm of the geographical distance. This parameter corresponds to the number of migrants required to account for observed genetic differences if migrants could move directly between populations. In both cases, Mantel tests (10 000 permutations) were used to assess the statistical significance. The strength of the isolation by distance (IBD) relationship was quantified with the slope and intercept of genetic similarity ($\hat{M}$) or distance (linearized F_{ST}) against geographic distance. Both parameters were calculated using reduced major-axis regression (RMA) (Sokal & Rohlf 1981). Asymmetric 95% confidence intervals around the RMA regression coefficient were calculated with 10 000 bootstraps around individual population pairs. All the analyses were performed with IBDWS software (Jensen *et al.* 2005).

We used PopTools software (Hood 2010) with $n = 1000$ randomizations to perform a Mantel test with the D_{est} parameter and the logarithm of the geographical distance.

At first instance, the IBD patterns among the different parameters for measuring genetic distance and the geographical distance were assessed on the whole data set. As a second step, to ensure that the three most distantly localities outside of the Alboran Basin did not impact the significance of the IBD analysis, the analysis was repeated taking into account only the thirteen sampling localities in the Alboran Basin. Geographical distances among localities along the same coast were measured as the minimum coastal distance, generalized at the scale of 1:25 000, and distances between sampling sites of different coastal areas were determined by means of dead-reckoning distances.

Detection of migrants, migration rates and effective population sizes. We estimated first-generation migrants, individuals not born in the corresponding sampled population, with GENECLASS 2.0. (Cornuet *et al.* 1999). The detection of migrants was estimated under the option 'L_home', the likelihood of finding a given individual in the population in which it was sampled, which is the most appropriate estimation to use when not all potential source populations have been sampled

(Paetkau *et al.* 2004). The method used was a Bayesian approach (Rannala & Mountain 1997) and Monte Carlo resampling of 10 000 individuals per locality (Paetkau *et al.* 2004). The analysis was run between pairwise sampling localities along the entire data set.

We used the software MIGRATE 2.1.3. (Beerli & Felsenstein 2001) to infer the population size parameter θ ($4N_e\mu$) (where N_e is the effective population size and μ is the mutation rate per site) and migration rates (m/μ) (where m is the immigration rate per generation). We used the software under the maximum likelihood strategy to build a full migration matrix model with a Brownian motion approximation to the stepwise mutation model. As suggested by the program author, Markov chain parameters were set as short chains = 10 (10 000 genealogies sampled) and long chains = 3 (100 000 genealogies sampled), with a burn-in of 10 000 genealogies for each chain. We ran the software twice to verify consistency of results. The program was run on a set of data structured according to the five clusters inferred by the Bayesian STRUCTURE analysis.

Results

Genetic diversity

Overall allelic richness ranged from 2.1 (Ac-L22) to 10.6 (Ac-L20) (Table S1, Supporting Information). At each locality, allelic richness ranged from 2.92 (PM) to 7.15 (PAL). Mean value was 4.70 ± 0.22 (mean ± SD, here and hereafter) (Table 2). Private allelic richness ranged from 0 (ALG) to 1.92 (PAN) (mean value of 0.35 ± 0.60). Allelic richness after rarefaction ranged from 2.55 (PM) to 6.15 (PAN), with a mean value of 4.54 ± 1.13 (Table 2). Over all loci, significant heterozygote deficiencies were found in six localities of sixteen (Table 2). Multilocus F_{IS} values ranged from −0.028 (DR) to 0.188 (PM). Observed and unbiased expected heterozygosities ranged from 0.21 to 0.59 and from 0.27 to 0.63 for PM and PAL, respectively (mean value of 0.47 ± 0.02 and 0.49 ± 0.02, respectively) (Table 2). Examining each locus separately, F_{IS} values ranged from −0.533 for Ac-L11 (PAN) to 1.000 for Ac-L31 (PM) (Table S1, Supporting Information).

Departures from HWE equilibrium were not generalized in all loci for each sampled site. In cases of heterozygote deficiencies, evidence for null alleles was checked and their frequencies were computed at each locus for each sampled site (Table S1, Supporting Information). No evidence of allele dropout or scoring errors because of stuttering was found. The analysis detected the possibility of null alleles in several loci across different localities (Table S1, Supporting Information). Over all, the localities and loci analysed, 7.2% of the F_{IS} values were statistically significant because of a deficit of heterozygotes, and all showed different estimated frequencies of null alelles, from 0.03 for Ac-L25 (PM) to 0.19 for Ac-L23 (PAN). However, applying the corresponding correction for null alleles (Brookfield 1996, in all cases) did not qualitatively affect the results, as 4.3% of the F_{IS} values were still significant.

Overall, LD among loci was found ($P < 0.05$ after step-down Bonferroni correction) in only four of the 78 pairwise comparisons per sampling locality, involving different loci. Only the LD analyses of loci Ac-L10 and AcL-37 were significant for two localities at the same time (ALB2 and PAN). Physical linkage can therefore be discarded.

On the first run, a neutrality test carried out under the IAM model, and the analysis showed loci Ac-7E-AC, Ac-L18 and Ac-L20 to be candidates for exhibiting balancing selection. Under the SMM model, only locus Ac-7E-AC appeared as an outlier. On the second run, after removing the most distant localities (PAN and

Table 2 Summary statistics for each sampled site of *Astroides calycularis*

	Population															
	ALG	PC	GRA	ALB1	ALB2	CATRFO	CL	ECON	NI	DR	NREY	TH	GAT	PM	PAN	PAL
N	25	25	20	18	24	29	22	22	20	24	22	22	23	31	24	30
N_a	3.92	4.92	3.77	4.08	4.31	5.92	4.62	5.08	4.23	5.15	4.54	4.92	3.08	2.92	6.62	7.15
N_s	3.75	4.68	3.69	4.08	4.08	5.31	4.32	4.74	4.08	4.67	4.25	4.67	2.98	2.55	6.15	6.13
P_a	0.00	0.08	0.08	0.15	0.08	0.15	0.23	0.08	0.15	0.08	0.08	0.23	0.15	0.23	1.92	1.85
H_o	0.57	0.55	0.50	0.47	0.45	0.54	0.41	0.43	0.49	0.48	0.41	0.44	0.41	0.21	0.55	0.59
H_e	0.56	0.59	0.51	0.48	0.52	0.58	0.43	0.47	0.45	0.47	0.47	0.45	0.42	0.27	0.63	0.63
F_{IS}	−0.027	0.065	0.021	0.008	**0.146**	**0.068**	0.059	0.075	−0.087	−0.028	**0.133**	0.019	0.025	**0.188**	**0.131**	**0.066**

N, number of collected individuals; N_a, number of alleles per locality; N_s, mean allelic richness standardized to the smallest sample size (18) using the rarefaction method of FSTAT 2.9.3; P_a, number of private alleles per site; H_o, observed heterozygosity; H_e, expected heterozygosity; F_{IS}, inbreeding coefficient; Bold F_{IS} values are significant probability estimates. (for locality codes see Table 1).

PAL), no loci were found to be under selection for both the IAM and the SMM models. Therefore, it is possible to assume that no loci used deviate from neutrality.

An average number of 85.9 ± 4.7% of paired individuals of all the collected polyps were assigned as unrelated in the likelihood analysis. Parent–offspring relations were found in 0.7 (PAN and PAL) to 11.6% (PM) of the pairwise comparisons. Full-siblings were found in 0.7% (ALB1) to 6.0% (PM), and half-siblings were found in 7.3% (PAN and PAL) to 12.6% (NREY) of the individual pairwise comparisons (Fig. S1, Supporting Information). All individuals examined had a distinct genetic identity, and none were clone mates, indicating that individuals were not sampled twice even though all reproduce sexually.

Population genetic structure

On the first STRUCTURE run after plotting Log P ($X|K$) as a function of K, we did not find a clear plateau but rather a weak increase in Log P ($X|K$) values from K = 2–16 (Fig. S3a, Supporting Information). For K = 2, a clear separation appeared between samples from the Tyrrhenian Sea (PAN and PA, hereafter 'Tyrrhenian Sea cluster') and the Alboran Sea-Algerian Basin localities (Fig. 2a). On the second STRUCTURE run, eliminating populations from the 'Tyrrhenian Sea cluster', a plot of Log P ($X|K$) against the number of K, revealed clear Log P ($X|K$) increments in K = 4, K = 6 and 8. K = 4 had the highest likelihood mean (Log P ($X|K$) = −8665.05 ± 1.5) (Fig. S3b, Supporting Information) with less standard

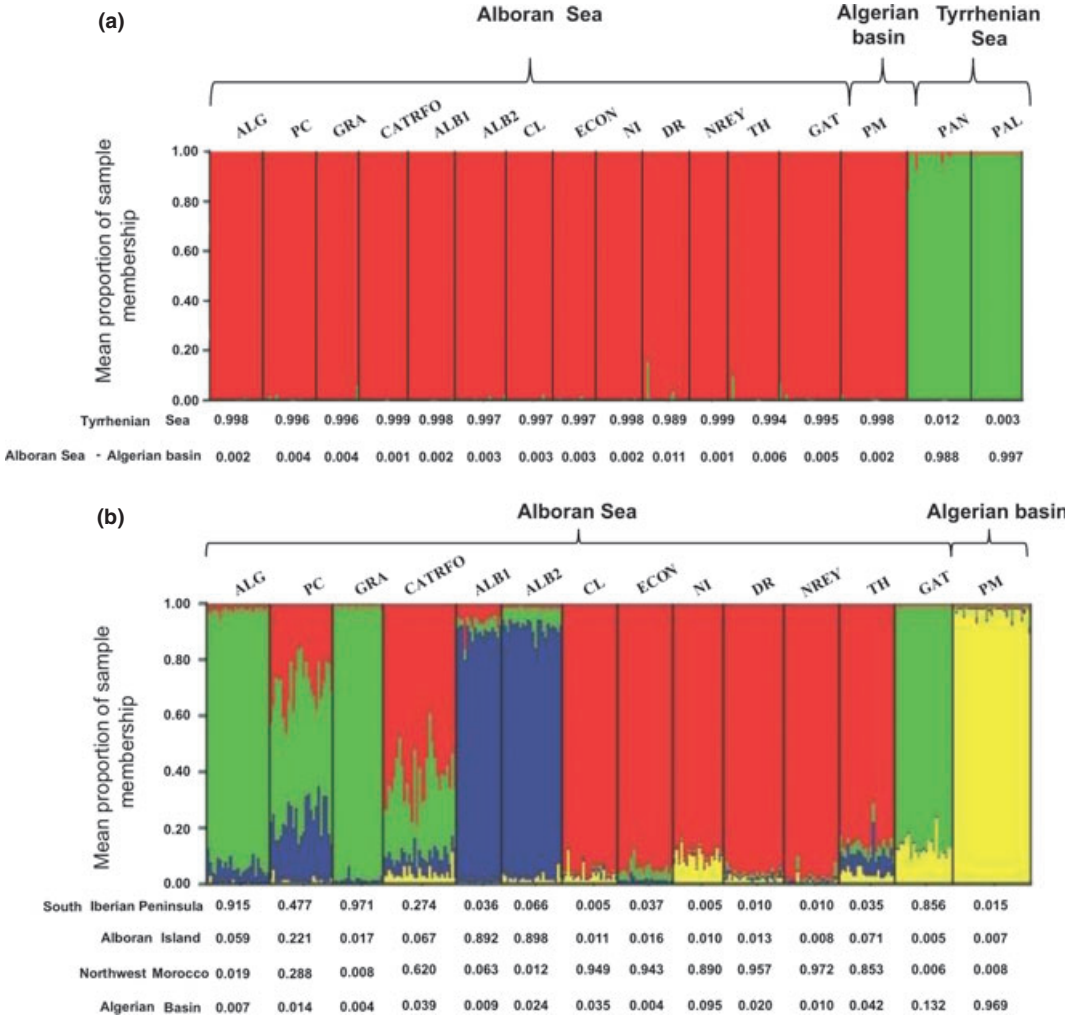

Fig. 2 Bayesian assignment probabilities of individuals of *Astroides calycularis* to clusters estimated using STRUCTURE 2.3.3. (a) Results for the first run of the software, separating the localities of the Tyrrhenian Sea cluster from the Alboran Sea-Algerian Basin. (green: 'Tyrrhenian Sea cluster'; red: 'Alboran Sea-Algerian Basin cluster') (b) Results after the third run, designating the samples collected in PC and CATRFO as 'unknown' and all other samples assigned a priori. (green: 'South Iberian Peninsula cluster'; blue: 'Alboran Island cluster'; red: 'Northwest Morocco cluster'; yellow: 'Algerian Basin cluster') (for locality codes see Table 1).

error throughout the 20 replicates, being considered the most consistent analysis. We then took into consideration parameter $K = 4$, where samples from ALG, GRA and GAT were grouped in cluster 1 (hereafter 'South Iberian Peninsula cluster'), ALB1 and ALB2 in cluster 2 (hereafter 'Alboran Island cluster'), the six localities of Chafarinas Islands in cluster 3 (hereafter 'Northwest Morocco cluster') and the locality in the Algerian Basin, PM, was separated in a single cluster (cluster 4) (hereafter 'Algerian Basin cluster'). On the third run, using the PopInfo parameter, designating PC and CATRFO localities as 'unknown', both localities showed mixed ancestry, with unequal likelihood of both localities even though they were located on the same coast. Membership coefficients for PC were common between three different clusters with proportions of 0.48, 0.22 and 0.29 for clusters 'South Iberian Peninsula', 'Alboran Island' and 'Northwest Morocco', respectively. In the case of admixture patterns for CATRFO, membership coefficients were mainly shared between the South Iberian Peninsula and Northwest Morocco clusters with proportions of 0.27 and 0.62, respectively (Fig. 2b).

The AMOVA analysis revealed a highly significant genetic structure among the clusters explained by the first and second STRUCTURE runs ($P < 0.01$; $K = 2$, Table 3a). In the first instance, 30.58% of the variance was explained by genetic differences among clusters. When the analysis was performed within the clusters inferred by the second run of STRUCTURE, this value was 12.49% ($P < 0.01$; $K = 4$, Table 3b).

The global F_{ST} value was high (0.236 ± 0.020), with a highly significant differentiation. Pairwise F_{ST} values (Table S2, Supporting Information) ranged from 0.004 (ECON-DR distant 1.2 km) to 0.526 (PM-PAN distant 1395 km). After correction for null alleles, no significant differences between pairwise F_{ST} values and pairwise F_{ST} values corrected for null alleles were observed (*t*-test, $P = 0.348$). Therefore, it is possible to assume that the presence of null alleles did not affect the analyses. Pairwise D_{est} values ranged from 0.006 (ECON-DR) to 0.835 (ALB1-PAN) (Table S2, Supporting Information). Population pairwise F_{ST} estimates were lower than D_{est}. However, in both cases, most of the values were statistically significant, except for F_{ST} and D_{est} values corresponding to pairwise comparisons among three of the Chafarinas Island localities at distances of 0.6 km (DR-NREY), 0.8 km (NI-DR) and 1.2 km (DR-ECON). Other pairwise comparisons from distances ranging from 0.6 to 0.8 km showed slight but significant F_{ST} values in the range of 0.027–0.042; however, these were the lower values across the whole data set.

When considering the whole data set, the IBD analysis provided positive and significant relationships between $F_{ST}/(1 - F_{ST})$ values and the logarithm of the geographical distance (computed as the minimum coastal distance between localities located on the same coast and by means of dead-reckoning distances between sites on different coasts) ($R^2 = 0.45$, $P < 0.0001$; $F_{ST}/(1 - F_{ST}) = -1.07 + 0.26 \log(\text{dist})$) (data not shown). The correlation remained significant when only the localities in the Alboran Sea were taken into account ($R^2 = 0.37$, $P = 0.001$) (Fig. 3a), indicating that the correlation between geographical and genetic distance was not an artefact caused by the strong differentiation of the samples from the Tyrrhenian and Algerian Basins that were the most distant. Slatkin's (1993) model correlation between the genetic similarity ($\hat{M}$) and the logarithm of the geographic distance was also significant but negative, when considering both approaches; in the first instance, $R^2 = 0.64$, $P < 0.001$; $\log(M) = 3.14–0.58 \log(\text{dist})$ (data not shown); at the regional scale (Fig. 3b), $R^2 = 0.46$, $P = 0.006$. The slope of the RMA regression between $\hat{M}$ and the geographical distance was −0.58 when considering the whole data set, and −0.57 when considering only the localities in the Alboran Basin (Fig. 3b). Asymmetric 95% confidence intervals around the RMA regression coefficient ranged from −1.09 to −0.36 in the former case and −1.03 to 1.25, thus including both times −0.5, falling within the range of values for simulated two-dimensional stepping-stone dispersal models. Considering distances measured by way of dead-reckoning distances among localities, correlation analysis gave similar values in both analyses (data not shown).

When considering D_{est} estimates, the Mantel test analysis showed a similar pattern; there was a significant correlation ($R^2 = 0.54$, $P < 0.001$) among all the sampled localities (data not shown). For the Alboran Sea localities, this correlation was also significant

Table 3 (a) Analysis of molecular variance (AMOVA) of spatial genetic variation between the clusters 1 (Tyrrhenian Sea cluster) and 2 (Alboran Sea-Algerian Basin cluster), for thirteen microsatellite markers. (b) AMOVA of spatial genetic variation between the four clusters defined by STRUCTURE after the second and third runs ($K = 4$) among Alboran Sea-Algerian Basin samples

Source of variation	d.f.	Percent of variance	*P* value
(a)			
Among clusters	1	30.58	<0.01
Among sampling sites within cluster	14	9.91	<0.01
Within sampling sites	746	59.61	<0.01
(b)			
Among clusters	3	12.49	<0.01
Among sampling sites within cluster	10	4.67	<0.01
Within sampling sites	640	82.84	<0.01

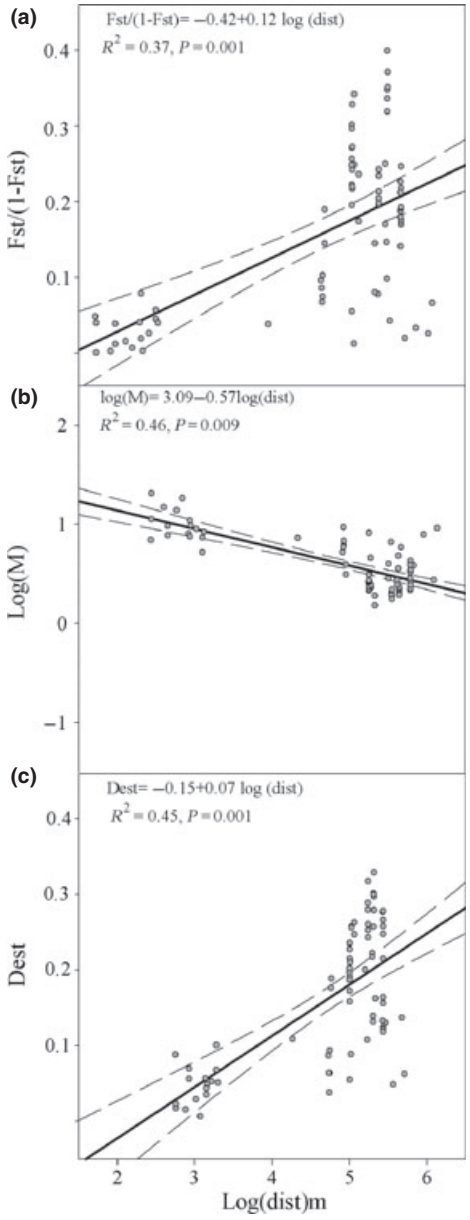

Fig. 3 Correlation between genetic distances at the regional scale measured computed as: (a) M (genetic similarity), (b) $F_{ST}/(1 - F_{ST})$, and (c) D_{est}; and the logarithm of geographical distances (m). 95% Confidence Intervals are presented by dashed lines.

($R^2 = 0.42$, $P < 0.001$, Fig. 3c). This was also true when considering distances determined by way of dead-reckoning among the whole data set and Alboran Sea localities (data not shown).

Detection of first-generation migrants, migration rates and effective population sizes

Assignment tests for first-generation migrants showed a very low number of individuals assigned to sampled localities different from their corresponding locality within the whole data set (Table S3, Supporting Information); 79.17–96.00% of the individuals were assigned to their actual sampling location. In general, first-generation migrants were only found within STRUCTURE-defined clusters. At the local scale, among each sampling site located on each island of the Chafarinas archipelago, between islands and among Alboran Island locations, first-generation migrants were found but at very low percentages. Among these localities, from 3.45% to 9.09% of the individuals were considered to belong to sites different from their sampling locality. The localities with higher percentages of individuals belonging to a different locality than the sampled one were ECON and TH with 9.09% of the individuals from DR. Regarding Alboran Island sampling localities, gene flow between ALB1 and ALB2 was detected by the analysis, while both localities showed migrants from localities that had not been sampled for this study. In the case of first-generation migrants coming from unsampled, localities from 3.23% to 13.33% of migrants were found in the localities PM and PAL, respectively.

Maximum likelihood estimates of the values of θ made with Migrate software ranged from 0.67 ('Alboran Island cluster') to 1.51 ('South Iberian Peninsula cluster') (Table S4, Supporting Information); these values were translated to effective population size values ($N_e = \theta/4\ \mu$) ranging from 1875 ('Algerian Basin cluster') to 3775 ('South Iberian Peninsula cluster'), assuming a microsatellite mutation rate of 10^{-4} per locus per generation (González & Zardoya 2007). In general, estimated migration rates were small, with values ranging from 0.74 to 1.37. We applied a one-way ANOVA to test the null hypothesis of equal rate of immigrants between pairwise samples. The analysis showed nonsignificant differentiation among pairwise samples ($F_{1,5} = 0.84$, $P = 0.67$), therefore indicating little immigration among sampling sites ordered according to STRUCTURE analysis.

Discussion

With the analysis of thirteen microsatellite loci, we found high levels of connectivity among localities placed no more than 1 km apart, while at a greater distance, a clear differentiation was found. We found significant heterozygote deficits in six of 16 localities. Other studies on tropical corals (e.g. Ridgway *et al.* 2008; Polato *et al.* 2010), as well as Dendrophylliidae species in the Mediterranean (Goffredo *et al.* 2004, 2009) and in other geographical areas (Hellberg 1994), suggest that heterozygote deficits are common in populations of these organisms. Though, we did not find this as abundant across the localities analysed. Heterozygosity deficiencies may be caused by methodological or technical

problems (e.g. presence of null alleles, Brookfield 1996), or genetic, historical and demographic events such as selection, population mixing and nonrandom mating (Raymond & Rousset 1995b; Luikart *et al.* 2003). In all cases in which populations showed positive and statistically significant values of F_{IS}, null alleles were estimated from low to moderate levels, but this presence of null alleles was not generalized over all loci within these localities. The neutrality test did not find selection against the loci used. Biological factors may also contribute to explaining the observed heterozygote deficiencies. Partial inbreeding may occur in border populations with low population density (Astanei *et al.* 2005). PAL is located in the region close to the eastern limit of the distribution range of the coral. PM corresponds to the northern limit of the species range in the Iberian Peninsula. On the other hand, restricted gametes or larval dispersal, together with certain habitat conditions (e. g. instability, closed environment), and low colony density could cause mating among closely related individuals (Knowlton & Jackson 1993). The demersal and low dispersant character of *A. calycularis* planula may affect the degree of matting among relatives. In the case of PAL and NREY, samples were taken from the entrance of a cave in the former and from the inner part of a crevice in the latter. Samples from PM were taken from a closed bay, and its colonies present the highest likelihood of offspring-parent relatedness. Such is the case of other anthozoan species commonly found in overhangs, caves and small crevices [e.g. the scleractinian coral *Leptosammia pruvotti* (Goffredo *et al.* 2009) and the red coral *Corallium rubrum* (Costantini *et al.* 2007)]. The instability of the habitat such as in the case of PAN, an active volcanic island characterized by the presence of hydrothermal vents eruptions (Anzidei *et al.* 2005; Capaccioni *et al.* 2007), and ALB2, a rocky seabed continuously affected by local eddies (Templado *et al.* 2006), suggests that population abundance and therefore genetic diversity in both cases may have been historically affected.

Genetic structure and connectivity at different spatial scales

Genetic structure analyses using the Bayesian clustering approach showed discontinuities in the spatial distribution of genetic diversity congruent with the geographical locations of the samples. On the first run, dissimilarities among localities from the eastern and western limits of the distribution range were revealed, separating two distinct clusters one including the localities from the Tyrrhenian Sea, and the other including those samples from the Alboran Sea-Algerian Basin. This genetic differentiation between the regions was previously noted in an earlier study (Merino-Serrais *et al.* in press), which used nuclear (ribosomal internal transcribed spacers, ITS) and mitochondrial (cytochrome oxidase subunit I, COI) markers, with slight differences between the regions. Within the Alboran Sea-Algerian Basin cluster, four different assemblages were highlighted in a second analysis. The 'Algerian Basin cluster' hosted the locality PM, which is separated from the Alboran Basin by the Almeria-Oran Front (AOF). This front may act as a barrier to gene flow in synergy with the low dispersal behaviour of the species, as is the case of the brooder gorgonian *Paramuricea clavata* (Mokhtar-Jamaï *et al.* 2011).

At a regional scale, within the Alboran Sea, the genetic structure of *A. calycularis* tended somewhat towards a stepping-stone model, in which localities are more likely to exchange individuals with adjacent localities rather than with more distant ones (Kimura & Weiss 1964; Kenchington *et al.* 2006). The Bayesian analyses at this scale shaped three clusters: the 'South Iberian Peninsula cluster', the 'Alboran Island cluster' and the 'Northwest Morocco cluster', therefore, showing certain homogeneity over all sampling localities, except for PC and CATRFO. These localities, despite their location along the North African coast, showed different degrees of admixture from the three clusters delimited in the Alboran Sea. Whereas PC was seen to be admixed from the three above-mentioned Alboran Sea clusters, with the highest membership proportion from the 'Southern Iberian Peninsula' cluster, CATRFO had the highest membership proportion from the 'Northwest Morocco cluster'. Even though, in general, first-generation migrants were found among sampling localities assigned to the same cluster, exceptions to this included the detection of migrants assigned to Alboran Island in the PC and GRA localities. As pointed out by Skliris & Beckers (2009), the Alboran Sea is characterized by particularly complex hydrological and oceanographic patterns, such as two large-scale quasi-permanent gyres, tidal motions, mesoscale eddies and upwelling, which might limit or enhance gene flow in a nonlinear pathway. That could also explain the degree of admixture of PC with the 'South Iberian Peninsula' and 'Alboran Island' clusters. PC is located in the Strait of Gibraltar, an area subjects to a strong incoming Atlantic current, which is first directed towards the south coast of the Iberian Peninsula, and then turns towards the north of Morocco (Millot 1999). This gyre, called the Western Alboran Gyre, is suggested to be the cause of some dispersal events in which planulae of this coral are dragged through it and eventually rafted along this route, linking the dispersal patterns of the species at a regional scale in the Alboran Sea. A similar case was found in the marine isopod *Stenosoma nadejda*

(Xavier *et al.* 2011). Thus, this relationship could be the result of a historical connection or a consequence of contemporary water circulation patterns (Viudez & Tintore 1995; Xavier *et al.* 2011). Nevertheless, in the case of the Alboran Island, its biogeographical affinities are far from understood as, in many cases, its fauna does not resemble the nearby localities on the Spanish or African shores (Templado *et al.* 2006).

The highest gene flow, measured through either F_{ST} or D_{est}, was observed at the local spatial scale corresponding with three localities in the Chafarinas Islands as much as 1 km apart. At the nearby localities (e.g. CL, TH and NI), some of them at distances of <1 km, small but statistically significant genetic differentiation was found. This fact is confirmed by the outliers present in the IBD plots. Surprisingly, despite the small distance (0.6 km) between sampling localities at Alboran Island, the F_{ST} value suggests low gene flow between them. This could be explained by the location of one locality on the coast of the island, while the second is about 0.8 km offshore, and the strong currents that sweep through the area (Templado *et al.* 2006) may prevent planula exchange between these two closely situated areas.

The observed genetic structure of *A. calycularis* suggests that, as in other brooder scleractinian species, its larval biology (demersal planulae) is the main factor driving the low connectivity between closely situated localities and therefore the high genetic structure within them. In addition, local hydrodynamic processes within the three sampled areas may contribute to eventual dispersal events out of the place where larvae have been produced. Two other studies have been performed, with the use of allozyme loci, on related sympatric dendrophyllid species in the Mediterranean. In the case of the hermaphroditic brooder *Balanophyllia europaea* (Goffredo *et al.* 2004), gene flow measured as F_{ST} values was only found between short distances (patches 8–40 m apart), meanwhile genetic fragmentation was found at large distances (36–1941 km). A study on the gonochoric brooder species *Leptosammia pruvotti* (Goffredo *et al.* 2009) showed highest genetic differentiation at short distances (5–10 m apart) compared to large distances (2–872 km). Nevertheless, in both species, no statistical relationship between geographical and genetic distances was found, and it is suggested that this is attributed to insufficient time to reach equilibrium following historical changes in gene flow or colonization events (Slatkin 1993). The biology of the larvae could also affect the results found. While *B. europaea* and *L. pruvotti* are characterized by swimming larvae with neutral buoyancy and pelagic dispersal (Goffredo & Zaccanti 2004; Goffredo *et al.* 2009), *A. calycularis* releases larvae that acquire negative buoyancy and demersal behaviour. Such is also the case in the dendrophylliid brooder *Balanophyllia elegans*, from the NE Pacific coast, whose larvae have similar characteristics (Gerrodette 1981), and in which Hellberg (1995) found a strong correlation between genetic and geographic distances following the stepping-stone model.

Gene flow among populations of marine invertebrates is commonly characterized by occasional dispersal events over relatively long distances (Hellberg 2009; Nakajima *et al.* 2010). Those events include hydrographical conditions, attachment of larvae to small boulders or abalones and rafting by sea grass, among others (Hellberg 1995). It is widely recognized that, in general, marine species with limited larval dispersal show genetic structuring over a much finer spatial scale (Sherman *et al.* 2008). This local genetic structure has been seen as a general character of most shallow water corals (e.g. Ayre & Hughes 2000) and other brooder species, such as *Clavularia koellikeri* (Bastidas *et al.* 2002), *Corallium rubrum* (Costantini *et al.* 2007), or *Paramuricea clavata* (Mokhtar-Jamaï *et al.* 2011), among others. Those species are known to have low larval dispersal, consistent with the hypothesis that brooders are more likely to exhibit restricted gene flow (Carlon 1999).

Implications for management and conservation strategies of Astroides calycularis

Astroides calycularis is a threatened species included under Annex II of the Berne Convention and Annex II of the Barcelona Convention. It is also listed in the Spanish Catalogue of Threatened Species (Templado *et al.* 2004). The species is regressing from some areas as a result of different human-related impacts, such as destruction of rocky shores where the species dwells resulting in the fragmentation of the populations because of habitat loss, seawater pollution, recreational scuba diving, angling or illegal harvesting of the endolithic date mussels *Lithophaga lithophaga* (Moreno *et al.* 2008). Thus, any knowledge on the dispersal abilities of the species will help to know its potential capability to respond to these threats. Our genetic survey may also help to understand the genetic structure and connectivity patterns of the species as an argument for the design of Marine Protected Areas (MPAs) that cover its protection needs. Ideally, the effective design of reserves must account for the spatial scales of larval dispersal (Underwood *et al.* 2009), especially when MPA networks are planned (Jones *et al.* 2007). The genetic structure we found suggests that ecological connectivity is quite low between localities except over small scales. However, at a regional range, within each cluster, and even among clusters, sporadic gene flow may occur most probably depending on hydrodynamic conditions, configuration

and continuity of rocky substrates along the coastline and other external factors. Therefore, taking into account the important genetic structure found within localities, we recommend that conservation plans are based on local or regional approaches. We have initiated the survey of these parameters in three MPAs in the Alboran Sea, Chafarinas Islands, Alboran Island and Cabo de Gata. All of them were connected by some degree of gene flow with the nearby localities included in their corresponding cluster, even with other clusters, such as in the case of Alboran Island, which shows some degree of connectivity with the 'South Iberian Peninsula' cluster, with the localities of PC and GRA. Regardless, this evaluation is still in its beginning stages, and further approaches need to be assessed. Furthermore, studies on larval dispersal at smaller scales, or at other relevant scales, using other direct and indirect approaches might be evaluated to ensure the correct management and conservation of this and other threatened species. Further studies of interdisciplinary research on larval dispersal at smaller scales, or at other relevant scales, using other direct and indirect approaches might be evaluated to ensure the correct management and conservation of this and other threatened endemic species.

Acknowledgements

The authors want to acknowledge to P. Cabezas, I. Acevedo, J. Guallart, L. Sánchez-Tocino, O. Ocaña, J. Martínez-Ródenas, A. Frías, S. Revenga, M. Calvo, G. Gasparini, A. Addamo, M.C. Arroyo, E. Caroselli and F. Prada for their valuable help on the field with samples collection. Also, we would like to thank the directors and staff of the biological stations of Refugio Nacional de Caza de las Islas Chafarinas, Alboran Island and Cabo de Gata Marine Reserve, the Spanish General Secretary of Marine Fisheries and the Regional Governments for granting permits and for their valuable collaboration. We would like to thank R. García- Jiménez, T. Cuartero, E.G. Gonzalez, I.S. Magalhaes and M.H. Nielsen for the help done with the molecular laboratory and data analysis. We thank R. Barrientos, J. Pinzon and three anonymous reviewers for significantly improving this manuscript. S. Young significantly improve the English grammar and writing style. P. Casado-Amezúa was funded by a grant from the Spanish Research Council (CSIC). S. Goffredo was funded by the European Research Council (CoralWarm, ERC grant agreement n° 249930).This research was supported by BBVA Foundation and the Spanish Ministry of Science and Innovation (CTM2008-00496/MAR).

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This manuscript is part of P.C-A.'s PhD thesis which focuses on the application of molecular tools to assess population structure and connectivity in Mediterranean scleractinian corals for conservation purposes. S.G. is an academic researcher whose research focuses on the auto-ecology, reproductive patterns of sexual reproduction, population dynamics and connectivity of Mediterranean and Red Sea corals. J.T. is interested in evolutionary biology, phylogeography, reproductive strategies and conservation biology of marine invertebrates. A.M. is a staff scientist at the MNCN (CSIC), mainly interested in molecular systematics, in evolutionary processes originating biodiversity and in conservation.

Data accessibility

Individual-by-individual sampling locations and microsatellites scoring can be found in the Data File 'Microsatellite data set': DRYAD entry doi:10.5061/dryad.9rk8v438.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1. Summary statistics for thirteen microsatellite loci of *Astroides calycularis.*

Table S2. Pairwise F_{ST} (lower left) and D_{est} (upper right) values.

Table S3. Results of the assignment tests for first-generation migrants of *Astroides calycularis* as the percentage of individuals from the sampling site assigned to every location.

Table S4. Gene flow estimates among the clusters defined by STRUCTURE, based on a maximum likelihood approach of migration rates and population sizes ($\theta = 4\ N_e\mu$).

Fig. S1 Percentage of related pair-wise individuals.

Fig. S2 Plot of $\mathrm{Ln}P(X \mid K)$ as a function of the number of clusters (K) across the 20 runs.

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