Graellsia, 68(1): 207-218
enero-junio 2012
ISSN: 0367-5041
doi:10.3989/graellsia.2012.v68.057

SLIGHT GENETIC DIFFERENTIATION BETWEEN WESTERN AND EASTERN LIMITS OF *ASTROIDES CALYCULARIS* (PALLAS, 1776) (ANTHOZOA, SCLERACTINIA, DENDROPHYLLIIDAE) DISTRIBUTION INFERRED FROM COI AND ITS SEQUENCES

P. Merino-Serrais[1❖], P. Casado-Amezúa[1], Ó. Ocaña[2],
J. Templado[1] & A. Machordom[1*]

ABSTRACT

P. Merino-Serrais, P. Casado-Amezúa, Ó. Ocaña, J. Templado & A. Machordom. 2012. Slight genetic differentiation between western and eastern limits of *Astroides calycularis* (Pallas, 1776) (Anthozoa, Scleractinia, Dendrophylliidae) distribution inferred from COI and ITS sequences. *Graellsia*, 68(1): 207-218.

Understanding population genetic structure and differentiation among populations is useful for the elaboration of management and conservation plans of threatened species. In this study, we use nuclear and mitochondrial markers (internal transcribed spacers -ITS and cytochrome oxidase subunit one -COI) for phylogenetics and nested clade analyses (NCA), thus providing the first assessment of the genetic structure of the threatened Mediterranean coral *Astroides calycularis* (Pallas, 1766), based on samples from 12 localities along its geographic distribution range. Overall, we found no population differentiation in the westernmost region of the Mediterranean; however, a slight differentiation was observed when comparing this region with the Tyrrhenian and Algerian basins.

Keywords: Threatened coral; population genetic structure; Mediterranean Sea.

RESUMEN

P. Merino-Serrais, P. Casado-Amezúa, Ó. Ocaña, J. Templado & A. Machordom. 2012. Leve diferenciación genética entre los límites occidental y oriental de distribución de *Astroides calycularis* (Pallas, 1776) (Anthozoa, Scleractinia, Dendrophylliidae), inferida a partir de secuencias de COI e ITS. *Graellsia*, 68(1): 207-218 (in English).

El estudio de la estructura de las poblaciones y su diferenciación a nivel genético es de gran utilidad para la elaboración de planes de manejo y conservación de especies amenazadas. En este estudio, utilizamos marcadores nucleares y mitocondriales (espaciadores internos de genes ribosomales -ITS y citocromo oxidasa, subunidad I -COI) y métodos de análisis filogenéticos y de clados anidados (NCA), para realizar la primera valoración de la estructura genética del coral naranja *Astroides calycularis* (Pallas, 1766), una especie amenazada del Mediterráneo, a partir de muestras de 12 localidades a lo largo de su área de distribución. En las localidades situadas en la región más occidental del Mediterráneo se encontró cierta homogeneidad genética, mientras que al comparar estas localidades con las de las cuencas argelina y del mar Tirreno se observó una ligera diferenciación.

Palabras clave: Coral amenazado; estructura genética poblacional; mar Mediterráneo.

[1] Museo Nacional de Ciencias Naturales (MNCN-CSIC). c/ José Gutiérrez Abascal, 2. 28006 Madrid, Spain.
[2] Museo del Mar de Ceuta. Muelle Cañonero Dato s/n. 51001 Ceuta, Spain.
* Corresponding author A. Machordom (e-mail address: annie@mncn.csic.es) Telephone: +34 91 411 13 28 (ext. 1126) Fax: +34 91 564 50 78
❖ Current address: Laboratorio de Circuitos Corticales (CTB-CSIC). Universidad Politécnica de Madrid, Campus Montegancedo s/n. 28223 Pozuelo de Alarcón, Madrid, Spain.

Introduction

Polyspecific coral reefs disappeared from the Mediterranean Sea at the end of the Messinian, Late Miocene (Esteban, 1996). In spite of this, 33 species of scleractinian corals can currently be found in this sea (approximately half of them are colonial species). Among them, only one species, *Cladocora caespitosa* (Linnaeus, 1767), remains as a monospecific coral-reef builder. Although many of these species are affected by disruption or regression due to global climate change and human impact, none of them are included in the annexes of the Habitat Directive, and only one, *Astroides calycularis* (Pallas, 1766), has been included in Annex II of the Bern and Barcelona Conventions (list of endangered or threatened species).

Astroides calycularis is a colonial azooxanthellate coral commonly known as "Mediterranean orange coral" because of the deep orange colour of its coenosarc and polyps (Zibrowius, 1995). It belongs to the family Dendrophylliidae, which comprises nearly 170 species worldwide (Cairns, 2001) and represents approximately 23% of all azooxanthellate coral species (Picciani *et al.,* 2011). Zibrowius (1995) suggested that *A. calycularis* resembles some species of *Tubastrea*, a genus that occurs worldwide in tropical seas, and in particular a relatively similar species found in the Cape Verde islands. More recently, based on skeletal biomineralization patterns and 28S rRNA sequences, it has been found that the monotypic genus *Astroides* forms a monophyletic group with the genera *Balanophyllia* and *Tubastrea* (Cuif *et al*. 2003). But this only demonstrates that the dendrophylliid taxa analysed were in accordance with the current taxonomy and classification, contrary to other families as, for instance, Caryophylliidae or Pocilloporiidae (see Kerr, 2005 or Kitahara *et al*., 2010). However, a dendrophylliid phylogeny based only on morphological data, showed *Tubastrea* and *Turbinaria* as the sister group of *Astroides*, *Balanophyllia* not being directly related to *Astroides* (Cairns, 2001).

A detailed description of this coral has been provided by Zibrowius (1980), and more recently, Goffredo *et al.* (2011) studied the colony and polyp biometric relationships and intra-colony polyp population size structure. This coral lives in shaded habitats (e.g., vertical walls, overhangs and cave entrances), prefers areas with high hydrodynamics and can be found from the water surface to a depth of approximately 50 metres; however, it is mainly found in shallow waters. In some places, this coral is the dominant species covering up to 80-90% of the surface of the walls. In places of high hydrodynamics, it typically forms massive colonies with polygonal corallites. In sheltered or deeper places, colonies tend to have a bush-shaped morphology with nearly circular corallites. The ecological importance of *A. calycularis* as a biobuilder has been previously shown in a study based on North African populations (Ocaña, 2005).

This coral has been characterised as gonochoric, both at the polyp and colony level, and as planula-brooder (Goffredo *et al.,* 2010). Field observations performed by various authors have characterised the larvae as having negative buoyancy and a demersal behaviour, and thus crawling until finding a substrate to settle on (Lacaze-Duthiers, 1873; Goffredo *et al.,* 2009).

Some authors regard *A. calycularis* as an ancient Tethyan species (Ocaña *et al*., 2007, 2009). It is an indicator of Quaternary climate oscillations, since it is a warm-water species with a tolerance for a narrow temperature range (Bianchi & Morri, 1993). Based on fossil evidence, it was widely distributed throughout the Western Mediterranean Sea during certain periods of the Pleistocene (Zibrowius, 1995), but disappeared from the Northern Mediterranean areas during colder periods. This response is similar to that of other warm-water corals, such as the closely related genus *Tubastrea* (see Ocaña *et al*., 2007, 2009).

Currently, the range distribution of *A. calycularis* is restricted to the south-central part of the Western Mediterranean Sea. In particular, it is found in the following regions: the southeastern Iberian Peninsula, from the Strait of Gibraltar to Cape Palos (Murcia); the northern coasts of Africa, from the Strait of Gibraltar to Cape Bonn in Tunisia; around Sicily and nearby islands; and the Gulf of Naples in the Tyrrhenian Sea (Zibrowius, 1995; Bianchi, 2007; Goffredo *et al.,* 2010). In addition, some records report its existence in the Atlantic coast of southern Spain and northern Morocco (Zibrowius, 1980; Bianchi, 2007). Recently, *A. calycularis* has also been found in the Adriatic Sea, along the coast of Croatia (Grubelić *et al.,* 2004) up to the Gulf of Venice (Casellato *et al.,* 2007). The recent range expansion of this species into the Adriatic Sea seems to have been influenced by the warming of seawater, by the prevailing sea current system and by the rocky coastal configuration (Grubelić *et al*.,

2004). However, currently, this species is disappearing in some places because of destruction or loss of habitat caused by human activities, such as coastal development, pollution, diving, angling and illegal fishing of the endolithic date-mussel *Lithophaga lithophaga* (Templado *et al.,* 2004; Moreno *et al.,* 2008).

To better understand the processes impacting regression of this coral during its history, its current expansion to the northeast and to establish the proper conservation plans, it is important to determine the genetic structure and the extent of gene flow among different populations along its geographical range. It is difficult to disentangle the effects of contemporary gene flow with those of historic population extinctions, expansions and colonisations, all of which have led to the present-day species distribution and population structure. To date, population genetic studies of Mediterranean scleractinian corals are limited to the solitary species *Balanophyllia europaea* (Goffredo *et al.,* 2004) and *Leptopsammia pruvoti* (Goffredo *et al.,* 2009); in both cases, these studies were based on allozyme electrophoresis.

Therefore, the main aim of this study is to provide the first characterisation of the population genetic structure of *A. calycularis*, contributing with this knowledge to the management and conservation of this species.

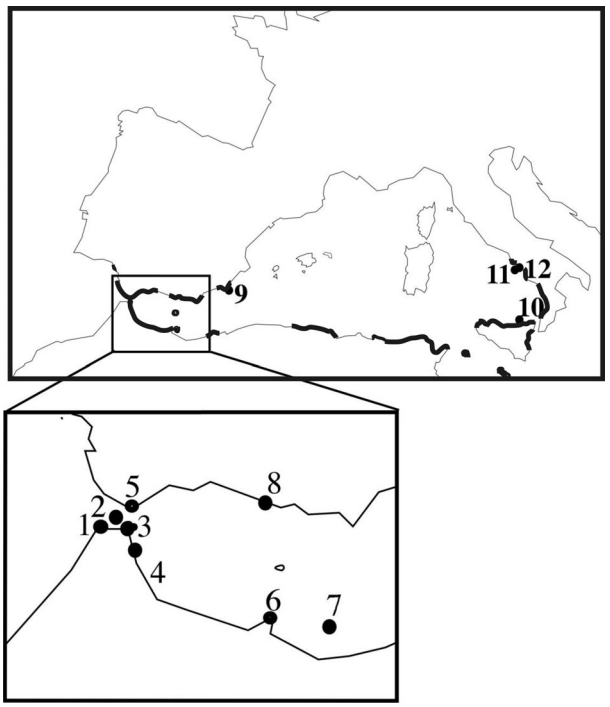

Fig. 1.– Map of sampling sites. The thick black line indicates the current species distribution range. Numbers are referred to in Table 1.

Fig. 1.– Localidades de muestreo. La línea gruesa indica la distribución actual de la especie. Los números corresponden con los de la Tabla 1.

Materials and methods

Sampling collection

Colonies of *A. calycularis* were collected from 12 sites along its distribution range (Fig. 1, Table 1). We selected superficial colonies (0-5 m) from vertical walls and cave entrances where the population density was higher. At each site, colonies were removed from the rocky substrate with a knife. In order to avoid sampling the same colony twice, colonies were removed from different patches.

DNA extraction and amplification

Total DNA was extracted from individual polyps that were preserved in absolute ethanol using ChargeSwitch gDNA Micro Tissue Kit (Invitrogen). Partial sequences of the mitochondrial cytochrome oxidase subunit I (COI) and the nuclear region of ribosomal internal transcribed spacers (ITS1+5.8S+ITS2=ITS) were amplified by polymerase chain reaction (PCR) using the following primers: LCO1490 5′-GGTCAACAAATCATAAAGATATTGG-3′ (Folmer *et al*., 1994) and COI-H 5′-TCAGGGTGACCAAAAAATCA-3′ (Machordom *et al*., 2003); ITS2.1 5′-CGTAGGTGAACCTGCGGAAGGATC-3′ and ITS2.2 5′-CCTGGTTAGTTTCTTTTCCTCCGC-3′ (Hugall *et al*., 1999). For both sets of primers, the PCR mix contained 1-3 µl of DNA, 5 µl of the corresponding buffer (with 10 x 2 mM $MgCl_2$), 1 µl of dNTPs mix (10 mM), 0.7 µl of both primers (10 µM), 1.5-2.5 U T*th* DNA polymerase (Biotools) and ddH_2O for a final reaction volume of 52 µl. The following PCR conditions were used: in the COI amplification, 94ºC (4 min), 40 cycles of 94ºC (1 min), 45ºC (1 min), 72ºC (1 min), and a final extension at 72ºC (10 min); and in the ITS amplification, 94ºC (5 min), 30 cycles of 94ºC (45 s), 57.5ºC (1 min), 72ºC (1 min), and a final extension at 72ºC (10 min). Amplified fragments (approximately 700 bp)

Table 1.– Populations analysed for each locus, number of specimens analysed per locality (N), and their GenBank accession numbers.

Tabla 1.– Poblaciones analizadas por locus, número de especímenes analizados por población (N) y sus números de acceso en GenBank.

MAP LOCALITY	POPULATION/ REGION/BASIN	COORDINATES	DATE OF SAMPLING	N		ACCESSION NUMBERS	
				COI	ITS	COI	ITS
1	Tangier/North Africa/ Alboran Sea	35°47'11.72"N; 5°55'49.84"W	September 2006	10	9	JQ343161-JQ343170	JQ343102-JQ343110
2	Perejil Island/North Africa/ Alboran Sea	35°54' 48.11"N; 5°25'03.34"W	October 2005	8	9	JQ343145-JQ343152	JQ343085-JQ343093
3	Hacho Mount-Ceuta/ North Africa/Alboran Sea	35°53'54.19"N; 5°15'50.29"W	October 2005	7	7	JQ343138-JQ343144	JQ343078-JQ343084
4	Negro Cape/North Africa/ Alboran Sea	35°41'16.81"N; 5°16'31.73"W	December 2005	8	8	JQ343153-JQ343160	JQ343094-JQ343101
5	Tarifa/Iberian Peninsula/ Alboran Sea	36°0'36.29"N; 5°35'37.75"W	July 2006	2	2	JQ343128-JQ343129	JQ343063-JQ343064
6	Tres Forcas Cape/ North Africa/Alboran Sea	35°25'56.50''N; 2°59'34.61''W	November 2005	10	10	JQ343171-JQ343180	JQ343068-JQ343077
7	Chafarinas Islands/ North Africa/Alboran Sea	35°10'40.78"N; 2°26'17.93"W	September 2005	4	3	JQ343181-JQ343184	JQ343065-JQ343067
8	Coast of Granada/Iberian Peninsula/Alboran Sea	36°43'40.50''N; 3°41'38.10''W	March 2006	8	9	JQ343185-JQ343192	JQ343111-JQ343119
9	Murcia/Iberian Peninsula/ Algerian	37°34'42.96"N; 0°50'31.77"W	August 2005	2	2	JQ343126-JQ343127	JQ343061-JQ343062
10	Eolie/Southern Italy/Tyrrhenian	38°31'34.45"N; 14°56'20.52"E	July 2005	2	2	JQ343130-JQ343131	JQ343120-JQ343121
11	Capri/Southern Italy/Tyrrhenian	40°32'58.92"N; 14°15'36.96"E	October 2005	4	2	JQ343134-JQ343137	JQ343122-JQ343123
12	Massa Lubrense/Southern Italy/Tyrrhenian	40°36'30.16"N; 14°19'59.16"E	October 2005	2	2	JQ343132-JQ343133	JQ343124-JQ343125

were purified by ethanol precipitation prior to sequencing both strands using "BigDye Terminator" (Applied Biosystems) sequencing reactions. Sequence gels were run on an ABI 3730 genetic Analyzer (Applied Biosystems).

Sequence analysis

Following removal of the primer regions, DNA sequences obtained for each specimen and marker were aligned and checked using Sequencher 4.6 (Gene Code Corporation). All alignments were validated by eye. A Blast search was performed on GenBank sequences in order to determine the most similar sequences to use as outgroups; *Tubastrea coccinea* for COI (GenBank Accession No. DQ445807) and *Tubastrea aurea* for ITS (GenBank Accession No. AY722796) were chosen as outgroups.

The analyses were first performed for each gene separately. The congruence among tree topologies of COI and ITS genes was assessed by the partition homogeneity test in PAUP* (Swofford, 2002). Nucleotide saturation was evaluated by plotting transition and transversion changes against uncorrected (`p´) divergence values. Sequence analysis was based on the principles of maximum parsimony (MP), neighbour-joining (NJ) and maximum likelihood (ML), as well as Bayesian principles. The evolutionary model that best fit our data was selected using Modeltest 3.06 (Posada & Crandall, 1998). Maximum likelihood analysis was performed by Quartet Puzzling (using 1000 replicates) or heuristic search. Support in the phenetic and parsimony analyses was estimated by bootstrapping (1000 repetitions) (Felsenstein, 1985), and by posterior probabilities in Bayesian analyses.

To test the genetic structure of the observed variation, we used nested clade phylogeographic analysis (NCPA) software (Panchal, 2006) to relate genetic structure to the geographic distribution of the samples. The haplotype network was manually converted into a series of nested clades, following the rules provided by Templeton (2004) and applied by the NCPA program (Panchal, 2006). A chi-squared test of geographical association of clades and biological inference from nested clades was applied as the basis for the biological interpretation, according to Templeton (2004).

Results

The obtained sequences (a total of 67 for COI and 65 for ITS) were submitted to GenBank with the accession numbers JQ343061 to JQ343125 for ITS and JQ343126 to JQ343192 for COI (Table 1). We counted on 56 specimens in which both regions could be sequenced. Of the 1334 characters included in the matrices (658 for the COI gene and 676 for the ITS region), 1201 characters were constant, 99 variable characters were uninformative, and only 34 characters were informative (when gaps were treated as fifth state of characters, and only 24 when they were treated as missing data). No nucleotide saturation was found. According to COI codon positions, the most informative position was the third, and all of the substitutions were synonymous. The ITS region showed more variability than the COI gene (25 of the global 34 substitutions and 15/24 were due to changes in ITS regions, considering gaps as fifth state of characters and missing data, respectively). Base composition was homogeneous in all of the taxa analysed. The empirical proportions of the different nucleotides were as follows: A=0.232, C=0.209, G= 0.251 and T= 0.306.

The divergence between the outgroup (*Tubastrea*) and ingroup ranged from 1.28% to 7.33% (for COI and ITS respectively, with respect to specimens from Italy). The percentage of divergence between the 8 different *A. calycularis* haplotypes, excluding the outgroup, ranged from 0% for specimens belonging to the same population to 1.39% (for COI) and 2.06% (for ITS) between the Italian Massa Lubrense samples and the Murcia samples. Within the western samples (i.e., those from North Africa and the Iberian Peninsula, Alboran Sea basin), the population of Murcia (Algerian basin) presented the highest distances with respect to the other populations.

Given that the partition homogeneity test showed no significant differences (p= 0.29) between individual data corresponding to COI and ITS, the data from those sequences were analysed and presented together. The best-fit model for this global matrix was the "transversional model with equal base frequencies" (TVM+I, where I= 0.84).

The phylogenetic tree showed the relationships among the 8 different haplotypes (considering gaps as missing data) found in this study (Fig. 2). The main cluster (Fig. 2, node 6) represented samples collected along the Iberian Peninsula coasts and in North Africa (Alboran Sea basin). At the base of this group, the population from Algerian basin (south-eastern Spain, Murcia) was found to be the most divergent with respect to the other populations. The Italian samples, Tyrrhenian basin, which clustered together based on their geographical origin, grouped a greater number of haplotypes in a smaller area, that is, both Eolie and Massa Lubrense samples were grouped together while the two samples from Capri appeared in different groups. Support values for the main nodes were strong for the terminal branches only (Fig. 2). The lack of support for other branches is likely due to the scarce variation of characters (only 34 informative characters including gaps or 24 characters excluding gaps).

The nested clade analysis (NCA) on the genetic haplotype network is shown in Fig. 3. Eleven different haplotypes were detected; 8 of these haplotypes corresponded to the ones previously defined in the phylogenetic trees. Three new haplotypes were detected when gaps were considered as a fifth character state, thus accounting for the discrepancy between analyses.

The null hypothesis of no association between the position of haplotypes in the network and geographical location was rejected ($P< 0.05$) for several clades.

The results of NCA showed a network of haplotypes and haplotypes/missing intermediate steps, which were organised in different levels ranging from level 1 (the most simple) to level 5 (the complete set of haplotypes). Based on the final phylogeographic network (Fig. 3), in level 1, we found that all of the haplotypes in the western group (Alboran Sea basin) were directly related, thus forming a homogeneous group with no interme-

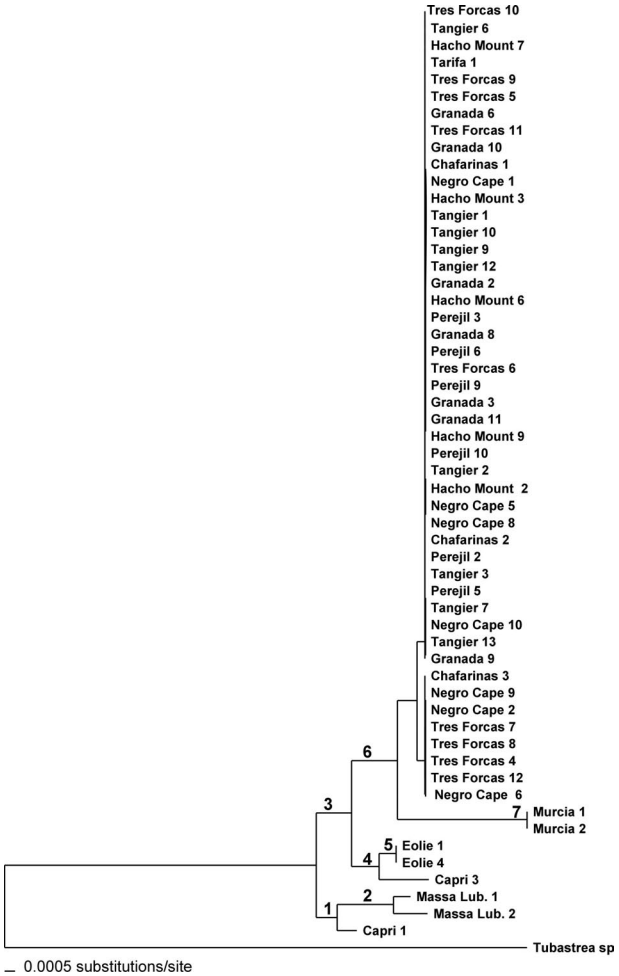

Fig. 2.– Phylogenetic hypothesis based on COI and ITS sequences. Numbers on branches indicate the posterior probability (in percentage) or bootstrap values. Those values were: **1**: –, 61, –, –; **2**: 100, 78, 87, 83; **3**: 92, –, –, 65; **4**: 77, 97, 65, –; **5**: 96, 94, 75, 67; **6**: 96, 57, 84, 82; **7**: 100, 100, 99, 100, for Bayesian inference, maximum parsimony, neighbour-joining and maximum likelihood, respectively (– represents values under 50 or not recovered by such method).

Fig. 2.– Reconstrucción filogenética basada en las secuencias de COI e ITS. Los números sobre las ramas indican la probabilidad posterior (en tanto por ciento) o los valores de bootstrap. Estos valores fueron **1**: –, 61, –, –; **2**: 100, 78, 87, 83; **3**: 92, –, –, 65; **4**: 77, 97, 65, –; **5**: 96, 94, 75, 67; **6**: 96, 57, 84, 82; **7**: 100, 100, 99, 100; para la inferencia bayesiana, máxima parsimonia, *neighbour joining* y máxima verosimilitud respectivamente (– representa valores por debajo de 50 o no recuperados por un determinado método).

diate steps, except for haplotypes K (Tangier 6) and F (Murcia, Algerian basin), which had 1 and 8 intermediate steps, respectively. The largest difference was observed in the eastern group (Tyrrhenian basin) haplotypes in which from 5 to 14 intermediate steps were found. The two haplotypes most closely related between the two geographical areas had 10 intermediate steps.

In level 2, we found association between the haplotypes from Eolie (D) and one of the haplotypes from Capri (C). Some clusters included haplotypes from both sides of the Gibraltar Strait, such as cluster 2-13, which included haplotypes G (a widely distributed haplotype in the western group) and H (from the Moroccan locality Negro Cape 4), and cluster 2-4, which included two minority haplotypes observed in samples taken from around the Strait of Gibraltar, J (Tres Forcas, Tarifa, Ceuta) and K (Tangiers).

In level 3, all of the haplotypes from the western group, except for haplotypes I and F, appeared in cluster 3-6. The eastern group was divided into three different sets: the 5 clades grouped into clusters 3-1, 3-2 and 3-4.

The association observed in level 4 interestingly showed a geographical split. Although nearly all of the western haplotypes appeared in cluster 4-3, haplotype I was found in cluster 4-1, even though only one step differentiates haplotype I from haplotypes H or J, both of which are found in the same area as haplotype I. In fact, haplotype I was in cluster 4-1 with haplotypes D (from Eolie) and C (from Capri). Therefore, two of the three clusters represented haplotypes found only in Italy or only in Morocco/Spain, while the third cluster included samples from both of these geographical areas.

The chi-squared test of geographical association of clades and biological inference from the NCA supported statistically significant differences only for clades 3-6 (haplotypes K, J, H and G from sites in northeast Morocco and Tarifa), 4-1 (haplotypes C, D and I from sites in the Tyrrhenian basin and Chafarinas Islands) and 4-3 (haplotypes K, J, H, G and F from sites in northeast Morocco, Tarifa and Murcia) (Table 2). The inferred pattern of biological interpretation for all of the clades was allopatric fragmentation, except in the case of clade 3-6, which remained inconclusive. Overall restricted gene flow or dispersal was found (Table 2).

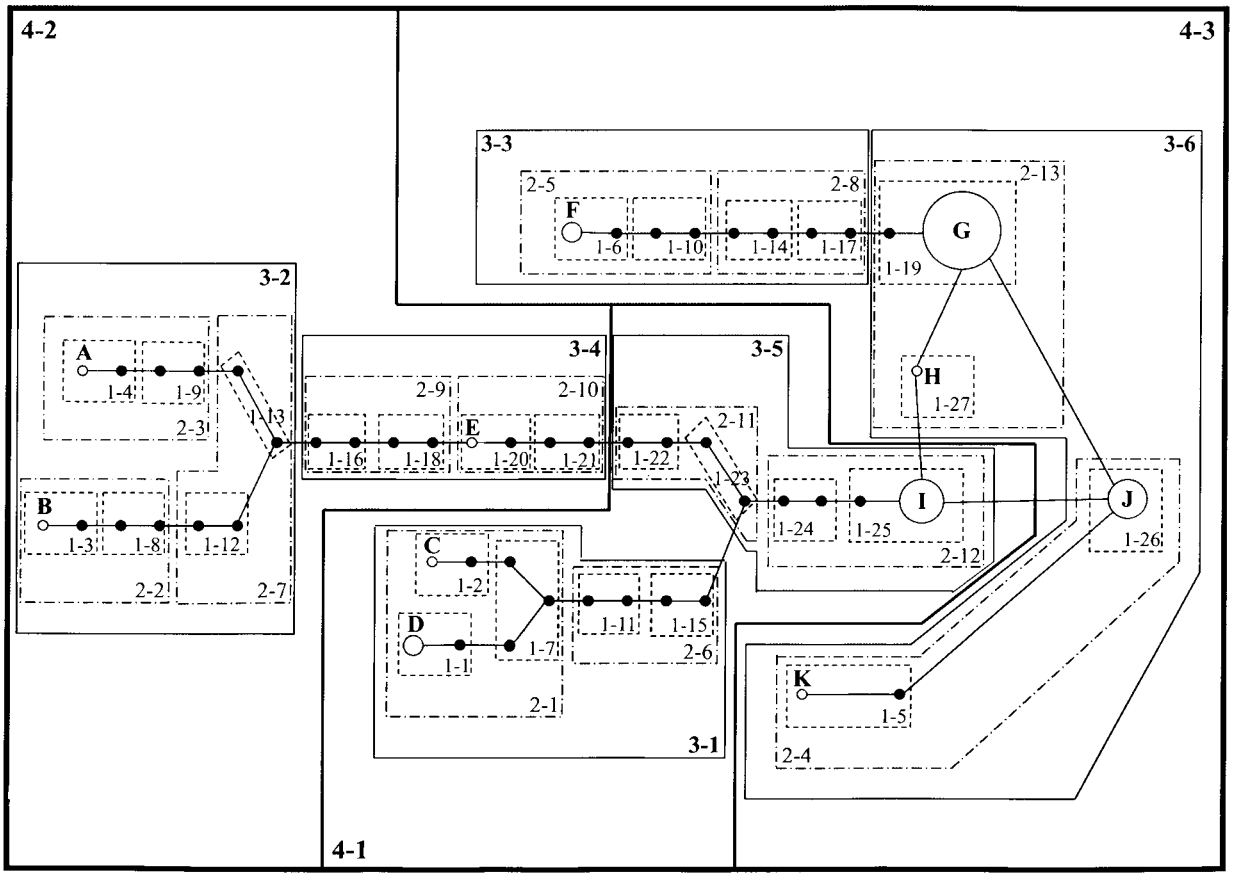

Fig. 3.– Nested clade analysis: white circles indicate sample haplotypes and black circles are missing intermediate haplotypes inferred by TCS program. Numbers of clades indicate their levels in the total network. The letter inside the boxes identifies the haplotypes. **A**: Massa Lubrense1; **B**: Massa Lubrense2; **C**: Capri3; **D**: Eolie1, Eolie4; **E**: Capri1; **F**: Murcia1, Murcia2; **G**: Chafarinas1, Chafarinas2, Tres Forcas6, Tres Forcas11, Hacho Mount2, Hacho Mount3, Hacho Mount6, Hacho Mount9, Perejil2, Perejil3, Perejil5, Perejil6, Perejil9, Perejil10, Negro Cape1, Negro Cape8, Negro Cape9, Negro Cape10, Granada2, Granada3, Granada6, Granada8, Granada9, Granada10, Granada11, Tangier1, Tangier2, Tangier3, Tangier7, Tangier9, Tangier10, Tangier12, Tangier13; **H**: Negro Cape6; **I**: Chafarinas3, Tres Forcas4, Tres Forcas7, Tres Forcas8, Tres Forcas12, Negro Cape2, Negro Cape9; **J**: Tarifa1, Tres Forcas5, Tres Forcas9, Tres Forcas10, Hacho Mount7; **K**: Tangier6.

Fig. 3.– Análisis de clados anidados: los círculos blancos indican haplotipos muestreados y los negros representan haplotipos intermedios no encontrados, inferidos por el programa TCS. Los números de los clados indican su nivel con respecto a la red completa. Las letras sobre las cajas identifican cada haplotipo. **A**: Massa Lubrense1; **B**: Massa Lubrense2; **C**: Capri3; **D**: Eolie1, Eolie4; **E**: Capri1; **F**: Murcia1, Murcia2; **G**: Chafarinas1, Chafarinas2, Tres Forcas6, Tres Forcas11, Hacho Mount2, Hacho Mount3, Hacho Mount6, Hacho Mount9, Perejil2, Perejil3, Perejil5, Perejil6, Perejil9, Perejil10, Negro Cape1, Negro Cape8, Negro Cape9, Negro Cape10, Granada2, Granada3, Granada6, Granada8, Granada9, Granada10, Granada11, Tangier1, Tangier2, Tangier3, Tangier7, Tangier9, Tangier10, Tangier12, Tangier13; **H**: Negro Cape6; **I**: Chafarinas3, Tres Forcas4, Tres Forcas7, Tres Forcas8, Tres Forcas12, Negro Cape2, Negro Cape9; **J**: Tarifa1, Tres Forcas5, Tres Forcas9, Tres Forcas10, Hacho Mount7; **K**: Tangier6.

Discussion

Overall, the results obtained in this study suggest a scarce genetic structure of *A. calycularis* among the samples analysed. Both the phylogenetic inferences and the networks generated from NCA showed a slight differentiation between the Italian samples and the samples from the westernmost Mediterranean, and within the westernmost Mediterranean samples, between those from

Table 2.– Geographical association of clades and biological inference from nested clade analysis. P is the probability of obtaining a χ^2 statistic larger than or equal to the observed statistic by randomly permuting the nested contingency 10,000 times. For each clade with significant geographical associations as detected by the permutation test, the inference chain and the biological interpretation, according to Templeton (2004), are indicated.

Tabla 2.– Asociación geográfica de clados e inferencia biológica deducida de los análisis de clados encajados. P es la probabilidad de obtener por azar un valor de χ^2 mayor o igual al observado en una permutación de 10.000 réplicas. Para cada test en el que se encontró una asociación significativa se indican la cadena de inferencia y la interpretación biológica según las propuestas de Templeton (2004).

Clade	Permutional χ^2 statistic	P	Chain of inference	Inferred pattern
2-1	3.00	0.332		Null hypothesis cannot be rejected
2-4	6.00	0.494		Null hypothesis cannot be rejected
2-13	5.97	0.387		Null hypothesis cannot be rejected
3-6	17.34	0.015	1-2 NO	Inconclusive outcome
4-1	9.00	0.034	1-19 NO	Allopatric fragmentation
4-2	3.00	0.333		Null hypothesis cannot be rejected
4-3	42.00	0.003	1-19 NO	Allopatric fragmentation
Total clade	**68.68**	**0.0001**	**1-2-3-5-6*-7-8 YES**	**Restricted gene flow/dispersal**

Murcia (southeastern Spain) and the rest of the samples from the Alboran Sea.

The population of Murcia is placed in the Algerian basin and northeast of the Almeria-Oran oceanographic Front (AOF), which constitutes the eastern boundary of the Alboran Sea. The AOF is a thermohaline density front generated by the convergence between the inflow of Atlantic water through the Strait of Gibraltar and the Mediterranean water (Tintore *et al.,* 1988). It has been regarded as an oceanographic barrier for dispersal of some species (Patarnello *et al.,* 2007; Mokhtar-Jamaï *et al*., 2011). This oceanographic front appears to be more efficient in population isolation than the strong current of the Gibraltar Strait, which purportedly should prevent gamete interchange between the northern and southern parts of the Gibraltar Strait (Zane *et al*., 2000). However, in this study, we found that several clusters contained haplotypes originated from both sides of the Gibraltar Strait, such as clusters 2-13 and 2-4, which had two minority haplotypes observed in samples taken near the Strait of Gibraltar (Tangier and Hacho Mount-Ceuta, on the African side, and Tarifa, on the European side).

Indeed, the westernmost examined populations shared haplotypes, including between those from the Gibraltar Strait and the rest of the Moroccan and Iberian populations, thus showing no significant barriers for gamete interchange. Thus, it is difficult to determine a genetic structure in this area for *A. calycularis*, despite its being a barrier or transition zone for numerous species (Lo Brutto *et al*., 2004; Baus *et al*., 2005; González-Wangüemert *et al*., 2006; Atarhouch *et al*., 2007). Recent life histories of such populations may explain the low variation in the species studied here. For instance, the reduction in the effective size or regressions/expansions of populations may be due to previous glaciations (Duran *et al*. 2004; Lemaire *et al*., 2005) and present-day human activities.

Another factor that may explain this low level of variation is the selection of markers for analysis. Our genetic studies were based on the analysis of two commonly used genes: one nuclear region (ITS) (Diekmann *et al*., 2001) and one mitochondrial gene (COI) (Medina *et al*., 1999; van Oppen *et al.,* 2002). Genetic data for scleractinian coral populations remains surprisingly limited, mainly due to the lack of adequate markers (Ridgway & Gates, 2006). Mitochondrial DNA of anthozoans is known to have a slow evolutionary rate (Hellberg, 2006; Costantini *et al.,* 2010). Studies in Octocorals (Alcyonacea) suggest that the slow evolution of the mitochondrial genome may be due to a mitochondrial DNA mismatch-repair system encoded by the gene mtMSH (France & Hoover, 2002). In addition, ITS sequence markers may display intra-individual rDNA variation (Wei *et al.,* 2006). Problems related to the ITS-

1 level of intraspecific variability have been previously addressed in the Mediterranean red coral *Corallium rubrum* (Costantini *et al.,* 2007). In our study, ITS sequences had three times more changes compared to the sequences of the COI fragment, despite having sequenced a similar length for both regions. Several taxonomical and phylogeographical questions have been addressed with the use of this nuclear marker (Wörheide *et al*., 2002; Lam & Morton, 2003), in some cases using its secondary structure as a character (Chen *et al*., 2004).

Even though the variation rate of mitochondrial genes can be as much as 10 or 20 times slower than single-copy nuclear genes (McFadden *et al*., 2004), these genes continue to be used for phylogenetic and phylogeographical analysis (Medina *et al*., 1999, van Oppen *et al*., 2001), mainly in combination with other markers (Romano & Palumbi, 1997; Cuif *et al*., 2003), due to their usefulness or possible comparison with other studies.

Regardless, the near lack of genetic structure found in this study should be re-examined using other variable markers, such as microsatellites isolated for this species (Molecular Ecology Resources Primer Development Consortium *et al.,* 2010), which will confirm if the observed lack of structure is real or derived from the genetic markers used.

Given the genetic data presented here (even with remarkably low variation), conservation strategy policies must focus on maintaining the continuity of the species habitat (threatened by human development) to ensure connectivity between adjacent populations. Some differentiated populations, such as in Murcia, in the western area, have to be especially protected due to their isolation and unique genetic characteristics.

Acknowledgements

We thank the managers and people who contributed to the sampling collections, directly or by the corresponding sampling permits. This study was supported by the BBVA Foundation, the Spanish Ministry of Science and Innovation (CTM2008-00496/MAR and CGL2011-23306) and the "Organismo Autónomo Parques Nacionales". PMS was funded by the project "Biología y divulgación para la conservación y mejor gestión de la especie *Astroides calycularis* y sus hábitats en los litorales de Ceuta y Melilla", granted by the Spanish Environment Ministry. PCA was funded by a grant from the Spanish Research Council (CSIC). We are grateful also to the two anonymous reviewers for improving the manuscript, and to Melinda Modrell for her conscientious revision of the English.

References

Atarhouch, T., Rami, M., Naciri, M. & Dakkak, A., 2007. Genetic population structure of sardine (*Sardina pilchardus*) off Morocco detected with intron polymorphism (EPIC-PCR). *Marine Biology*, 150: 521-528.

Baus, E., Darrock, D. J. & Bruford, M. W., 2005. Gene-flow patterns in Atlantic and Mediterranean populations of the Lusitanian sea star *Asterina gibbosa. Molecular Ecology*, 14: 3373-3382.

Bianchi, C. N., 2007. Biodiversity issues for the forthcoming tropical Mediterranean Sea. *Hydrobiologia*, 580: 7-21.

Bianchi, C. N. & Morri, C., 1993. Range extension of warm-water species in the northern Mediterranean: evidence for climatic fluctuations? *Porcupine Newsletter*, 5: 156-159.

Cairns, S. D., 2001. A generic revision and phylogenetic analysis of the Dendrophylliidae (Cnidaria: Scleractinia). *Smithsonian Contributions to Zoology*, 615: 1-75.

Casellato, S., Masiero, L., Sichirollo, E. & Soresi, S., 2007. Hidden secrets of the Northern Adriatic: "Tegnúe", peculiar reefs. *Central European Journal of Biology*, 2(1): 122-136.

Chen, C. A., Chang, C., Wei, N. V., Chen, C. H., Lein, Y. T., Lin, H. E., Dai, C. F. & Wallace, C. C., 2004. Secondary structure and phylogenetic utility of the ribosomal Internal transcribed spacer 2 (ITS) in Scleractinian corals. *Zoological Studies*, 43(4): 759-771.

Costantini, F., Fauvelot, C. & Abbiati, M., 2007. Genetic structuring of the temperate gorgonian coral (*Corallium rubrum*) across the western Mediterranean Sea revealed by microsatellite and nuclear sequences. *Molecular Ecology*, 16: 5168-5182.

Costantini, F., Taviani, M., Remia, A., Pintus, E., Schembri, P. J. & Abbiati, M., 2010. Deep-water *Corallium rubrum* (L., 1758) from the Mediterranean Sea: preliminary characterisation. *Marine Ecology*, 31: 261-269.

Cuif, J. P., Lecointre, G., Perrin, C., Tillier, A. & Tillier, S., 2003. Patterns of septal biomineralization in Scleractinia compared with their 28S rRNA phylogeny: a dual approach for a new taxonomic framework. *Zoologica Scripta*, 32(5): 459-473.

Diekmann, O. E., Bak, R. P. M., Stam, W. T. & Olsen, J. L., 2001. Molecular genetic evidence for probable reticulate speciation in the coral genus *Madracis* from a Caribbean fringing reef slope. *Marine Biology*, 139: 221-233.

Duran, S., Palacín, C., Becerro, M. A., Turon, X. & Giribet, G., 2004. Genetic diversity and population

structure of the commercially harvested sea urchin *Paracentrotus lividus* (Echinodermata, Echinoidea). *Molecular Ecology*, 13: 3317-3328.

Esteban, M., 1996. An overview of Miocene reefs from Mediterranean areas: General trends and facies models. In: E. K. Franseen, M. Esteban, W. C. Ward & J. M. Rouchy (eds.). *Models for carbonate stratigraphy from Miocene Reef Complexes of the Mediterranean regions*. SEPM (Society for Sedimentary Geology). Concepts in Sedimentology and Paleontology Series, no. 5: 3-53.

Felsenstein, J., 1985. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39: 783-791.

Folmer, O., Black, M., Hoeh, W., Lutz, R. & Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, 39: 294-299.

France, S. C. & Hoover, L. L., 2002. DNA sequences of the mitochondrial COI gene have low levels of divergence among deep-sea octocorals (Cnidaria: Anthozoa). *Hydrobiologia*, 471: 149-155.

Goffredo, S., Caroselli, E., Gasparini, G., Marconi, G., Putignano, M. T., Pazzini, C. & Zaccanti, F., 2011. Colony and polyp biometry and size structure in the orange coral *Astroides calycularis* (Scleractinia: Dendrophylliidae). *Marine Biology Research*, 7(3): 272-280.

Goffredo, S., Di Ceglie, S. & Zaccanti, F., 2009. Genetic differentiation of the temperate-subtropical coral *Leptopsammia pruvoti* in the Mediterranean Sea. *Israel Journal of Ecology & Evolution*, 55: 99-115.

Goffredo, S., Gasparini, G., Marconi, G., Putignano, M. T., Pazzini, C. & Zaccanti F., 2010. Gonochorism and planula brooding in the Mediterranean endemic orange coral *Astroides calycularis* (Scleractinia: Dendrophylliidae). Morphological aspects of gametogenesis and ontogenesis. *Marine Biology Research*, 6(5): 421-436.

Goffredo, S., Mezzomonaco, L. & Zaccanti, F., 2004. Genetic differentiation among populations of the Mediterranean hermaphroditic brooding coral *Balanophyllia europaea* (Scleractinia: Dendrophylliidae). *Marine Biology*, 145(6): 1075-1083.

González-Wangüemert, M., Perez-Ruzafa, A., García-Charton, J. A. & Marcos, C., 2006. Genetic differentiation and gene flow of two sparidae subspecies, *Diplodus sargus sargus* and *Diplodus sargus cadenati* in Atlantic and south-west Mediterranean populations. *Biological Journal of the Linnean Society*, 89: 705-717.

Grubelić, I., Antolic, B., Despalatovic, M., Grbec, B. & Beg Paklar, G., 2004. Effect of climatic fluctuations on the distribution of warm-water coral *Astroides calycularis* in the Adriatic Sea: new records and review. *Journal of the Marine Biological Association of the United Kingdom*, 84: 599-602.

Hellberg, M. E., 2006. No variation and low synonymous substitution rates in coral mtDNA despite high nuclear variation. *BMC Evolutionary Biology*, 6: 24-32.

Hugall, A., Stanton, J. & Moritz, C., 1999. Reticulate evolution and the origins of the ribosomal internal transcribed spacer diversity in apomictic *Meloidogyne*. *Molecular Biology and Evolution,* 16: 157-164.

Kerr, A. M., 2005. Molecular and morphological supertree of stony corals (Anthozoa: Scleractinia) using matrix representation parsimony. *Biological Reviews*, 80: 543-558.

Kitahara, M. V., Cairns, S. D., Storlaski, J., Blair, D. & Miller, D. J., 2010. A comprehensive phylogenetic analysis of the Scleractinia (Cnidaria, Anthozoa) based on mitochondrial CO1 sequence data. *PLoS ONE*, 5(7): e11490.

Lacaze-Duthiers, H., 1873. Développement des coralliaires. Actinaires à Polypiers. *Archives de Zoologie Expérimentale Générale*, 2: 269-348.

Lam, K. & Morton, B., 2003. Morphological and ITS1, 5.8S, and partial ITS2 ribosomal DNA sequences distinctions between two species *Platygyra* (Cnidaria: Scleractinia) from Hong Kong. *Marine Biotechnology,* 5: 555-567.

Lemaire, C., Versini, J. J. & Bonhomme, F., 2005. Maintenance of genetic differentiation across a transition zone in the sea: discordance between nuclear and cytoplasmic markers. *Journal of Evolutionary Biology,* 18(1): 70-80.

Lo Brutto, S., Arcuelo, M. & Parrinello, N., 2004. Congruence in genetic markers used to describe Mediterranean and Atlantic population of European hake (*Merluccius merluccius* L. 1758). *Journal of Applied Ichthyology*, 20: 81-86.

Machordom, A., Araujo, R., Erpenbeck, D. & Ramos, M. A., 2003. Phylogeography and conservation genetics of endangered European Margaritiferidae (Bivalvia: Unionidae). *Biological Journal of the Linnean Society*, 78: 235-252.

McFadden, C. S., Tullis, I. D., Hutchinson, M. B., Winner, K. & Sohm, J. A., 2004. Variation in coding (NADH Dehydrogenase Subunits 2, 3, and 6) and noncoding intergenic spacer regions of the mitochondrial genome in Octocarallia (Cnidaria: Anthozoa). *Marine Biotechnology*, 6: 516-526.

Medina, M., Weil, E. & Szmant, A. M., 1999. Examination of the *Montastrea annularis* species complex (Cnidaria: Scleractinia) using ITS and COI

sequences. *Marine Biotechnology*, 1(1): 89-97.

Mokhtar-Jamaï, K., Pascual, M., Ledoux, J. B., Coma, R., Féral, J. P., Garrabou, J. & Aurelle, D., 2011. From global to local genetic structuring in the red gorgonian *Paramunicea clavata*: the interplay between oceanographic conditions and limited larval dispersal. *Molecular Ecology*, 20(16): 3291-3305.

Molecular Ecology Resources Primer Development Consortium, Abdoullaye, D., Acevedo, I., Adebayo, A. A., Behrmann-Godel, J., Benjamin, R. C., Bock, D. G., Born, C., Brouat, C., Caccone, A., Cao, L. Z., Casado-Amezúa, P., Catanéo, J., Correa-Ramírez, M. M., Cristescu, M. E., Dobigny, G., Egbosimba, E. E., Etchberger, L. K., Fan, B., Fields, P. D., Forcioli, D., Furla, P., García de León, F. J., García-Jiménez, R., Gauthier, P., Gergs, R., González, C., Granjon, L., Gutiérrez-Rodríguez, C., Havill, N. P., Helsen, P., Hether, T. D., Hoffman, E. A., Hu, X., Ingvarsson, P. K., Ishizaki, S., Ji, H., Ji, X. S., Jimenez, M. L., Kapil, R., Karban, R., Keller, S. R., Kubota, S., Li, S., Li, W., Lim, D. D., Lin, H., Liu, X., Luo, Y., Machordom, A., Martin, A. P., Matthysen, E., Mazzella, M. N., McGeoch, M. A., Meng, Z., Nishizawa, M., O'Brien, P., Ohara, M., Ornelas, J. F., Ortu, M. F., Pedersen, A. B., Preston, L., Ren, Q., Rothhaupt, K. O., Sackett, L. C., Sang, Q., Sawyer, G. M., Shiojiri, K., Taylor, D. R., Van Dongen, S., Van Vuuren, B. J., Vandewoestijne, S., Wang, H., Wang, J. T., Wang, L., Xu, X. L., Yang, G., Yang, Y., Zeng, Y. Q., Zhang, Q. W., Zhang, Y., Zhao, Y. & Zhou, Y., 2010. Permanent Genetic Resources added to Molecular Ecology Resources Database 1 August 2009-30 September 2009. *Molecular Ecology Resources*, 10(1): 232-236.

Moreno D., de la Linde A., Arroyo M. C. & López-González P. J., 2008. *Astroides calycularis* (Pallas, 1766). In: J. M. Barea-Azcón, E. Ballesteros-Duperón & D. Moreno (coords.). *Libro rojo de los invertebrados de Andalucía*. Tomo 1. Consejería de Medio Ambiente, Junta de Andalucía. Sevilla: 281-287.

Ocaña, O., 2005. *Biología y divulgación para la conservación y mejor gestión de la especie* Astroides calycularis *y sus hábitats en los litorales de Ceuta y Melilla*. Informe científico inédito realizado para el Ministerio de Medioambiente. 71 pp.

Ocaña, O., Opresko, D. M. & Brito, A., 2007. First record of the black coral *Antipathella wollastoni* (Anthozoa: Antipatharia) outside of Macaronesian Waters. *Revista de la Academia Canaria de Ciencias,* [2006], 18(4): 125-138.

Ocaña, O., Ramos, M. A. & Templado, J., 2009. *Los paisajes sumergidos de la región de Ceuta y su biodiversidad.* Museo del Mar de Ceuta. Ceuta. 254 pp.

Panchal, M., 2006. The automation of Nested Clade Phylogeographic Analysis. Bioinformatics, 23: 509-510.

Patarnello, T., Volckaert, F. A. M. J. & Castilho, R., 2007. Pillars of Hercules: is the Atlantic-Mediterranean transition a phylogeographical break? *Molecular Ecology,* 16(21): 4426-4444.

Picciani, N., Pires, D. O. & Silva, H. R., 2011. Cnidocysts of Caryophylliidae and Dendrophylliidae (Cnidaria: Scleractinia): taxonomic distribution and phylogenetic implications. *Zootaxa*, 3135: 35-54.

Posada, D. & Crandall, K. A., 1998. MODELTEST: testing the model of DNA substitution. *Bioinformatics*, 14: 817-818.

Ridgway, T. & Gates, R. D., 2006. Why are there so few genetic markers available for coral population analysis? *Symbiosis*, 41: 1-7.

Romano, S. L. & Palumbi, S. R., 1997. Molecular evolution of a portion of the mitochondrial 16S ribosomal gene region in scleractinian corals. *Journal of Molecular Evolution*, 45: 397-411.

Swofford, D. L., 2002. PAUP*: Phylogenetic Analysis Using Parsimony (* and other Methods). Version 4.0. Sinauer Associates, Sunderland, Massachussetts.

Templado, J., Calvo, M., Luque, A. A., Garvía, A., Maldonado, M. & Moro, L., 2004. *Guía de los invertebrados y peces marinos españoles protegidos por la legislación nacional e internacional.* Ministerio de Medio Ambiente, Serie Técnica. Madrid. 214 pp.

Templeton, A. R., 2004. Statistical phylogeography: methods of evaluating and minimizing inference errors. *Molecular Ecology*, 13: 789-809.

Tintore, J., Violette, P. E., Blade, I. & Cruzado, A., 1988. A study of an intense density front in the eastern Alboran Sea: the Almeria-Oran Front. *Journal of Physical Oceanography*, 18: 1384-1397.

van Oppen, M. J. H., McDonald, B. J., Willis, B. & Miller D. J., 2001. The evolutionary history of the coral genus *Acropora* (Scleractinia, Cnidaria) based on a mitochondrial and nuclear marker: reticulation, incomplete lineage sorting, or morphological convergence? *Molecular Biology and Evolution*, 18(7): 1315-1329.

van Oppen, M. J. H., Wörheide, G. & Takabayashi, M., 2002. Nuclear markers in evolutionary and population genetic studies of scleractinian corals and sponges. In: K. Moosa (ed.). *Proceedings of the 9th International Coral Reef Symposium*, Bali: 339-346.

Wei, N. W. V., Wallace, C. C., Dai, C. F., Pillary, K. R. M. & Chen, C. A., 2006. Analyses of the ribosomal internal transcribed spacers (ITS) and the 5.8S gene indicate that extremely high rDNA heterogeneity is a unique feature in the scleractinian coral genus *Acropora* (Scleractinia; Acroporidae). *Zoological Studies*, 45: 404-418.

Wörheide, G., Hooper, J. N. A. & Degnan, B. M., 2002. Phylogeography of western Pacific *Leucetta `chagosensis´* (Porifera: Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). *Molecular Ecology*, 11(9): 1753-1768.

Zane, L., Ostellari, L., Maccatrozzo, L., Bargelloni, L., Cuzin-Roudy, J., Buchholz, F. & Patarnello, T., 2000. Genetic differentiation in a pelagic crustacean (*Meganyctiphanes norvegica*: Euphausiacea) from the North East Atlantic and the Mediterranean Sea. *Marine Biology*, 136: 191-199.

Zibrowius, H., 1980. Les scléractiniaires de la Méditerranée et de l´Atlantique nord-oriental. *Mémoires de l´Institut Océanographique (Monaco),* 11: 1-284.

Zibrowius, H., 1995. The "Southern" *Astroides calycularis* in the Pleistocene of the northern Mediterranean – an indicator of climatic changes (Cnidaria, Scleractinia). *Geobios*, 28(1): 9-16.

Recibido / *Received*, 2-02-2012
Aceptado / *Accepted*, 24-05-2012
Publicado impreso / *Published in print*, 30-06-2012