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SHORT COMMUNICATION

Deep-water *Corallium rubrum* (L., 1758) from the Mediterranean Sea: preliminary genetic characterisation

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Abstract

The precious red coral *Corallium rubrum* (L., 1758) lives in the Mediterranean Sea and adjacent Eastern Atlantic Ocean on subtidal hard substrates. *Corallium rubrum* is a long-lived gorgonian coral that has been commercially harvested since ancient times for its red axial calcitic skeleton and which, at present, is thought to be in decline because of overexploitation. The depth distribution of *C. rubrum* is known to range from *c.* 15 to 300 m. Recently, live red coral colonies have been observed in the Strait of Sicily at depths of *c.* 600–800 m. This record sheds new light on the ecology, biology, biogeography and dispersal mechanism of this species and calls for an evaluation of the genetic divergence occurring among highly fragmented populations. A genetic characterization of the deep-sea red coral colonies has been done to investigate biological processes affecting dispersal and population resilience, as well as to define the level of isolation/differentiation between shallow- and deep-water populations of the Mediterranean Sea. Deep-water *C. rubrum* colonies were collected at two sites (south of Malta and off Linosa Island) during the cruise MARCOS of the R/V *Urania*. Collected colonies were genotyped using a set of molecular markers differing in their level of polymorphism. Microsatellites have been confirmed to be useful markers for individual genotyping of *C. rubrum* colonies. ITS-1 and mtMSH sequences of deep-water red coral colonies were found to be different from those found in shallow water colonies, suggesting the possible occurrence of genetic isolation among shallow- and deep-water populations. These findings suggest that genetic diversity of red coral over its actual range of depth distribution is shaped by complex interactions among geological, historical, biological and ecological processes.

Problem

Conservation of marine habitats and the sustainable management of marine resources are a binding priority in the modern world and this also applies to the deep-sea ecosystems, far too long considered basically unaffected by human activities. In recent years, evidence about human impact on deep-sea habitats has been documented in the scientific literature (*e.g.* Rogers 1999; Davies *et al.* 2007; for a review). Furthermore, the relative inaccessibility of such remote habitats to routine ocean exploration had somewhat justified an inattentive policy until recent times. In the last decade, international cooperative scientific programmes adopting sophisticated technology proved to be a turning point for the appreciation of the breath of biodiversity in the deep ocean, calling for the

implementation of proper actions. One example is the awareness of deep-water coral ecosystems as one of the most important natural heritages in the ocean and the necessity to protect them (Roberts *et al.* 2006; Davies *et al.* 2007). There is critical trade-off between exploitation and conservation of marine resources. Science should contribute to direct policies aimed at keeping the balance positive by assisting in supplying useful and basic information on threatened habitats and species. Therefore, an increasing number of studies have recently focused on marine deep-sea ecosystems (see Levin *et al.* 2007). However, studies on genetic diversity and population structuring of deep-sea organisms are scant (*e.g.* France 1994; Hensley *et al.* 1995; Le Goff-Vitry *et al.* 2004; Baco & Shank 2005; Shank & Halanych 2007), in particular those dealing with Mediterranean species (*e.g.* Maggio *et al.* 2009).

The precious red coral *Corallium rubrum* (Linnaeus, 1758) inhabits subtidal rocky habitats in the Mediterranean Sea and Eastern Atlantic Ocean (Zibrowius *et al.* 1984; Chintiroglou *et al.* 1989; Cattaneo-Vietti & Cicogna 1993; Rossi *et al.* 2008) and it has been commercially harvested since ancient times due to the high economic value of its red axial calcitic skeleton (Tescione 1973; Taviani 1997; Tsounis *et al.* 2007). *Corallium rubrum* is a gonochoric slow-growing gorgonian coral (Anthozoa, Gorgonacea) which undergoes internal fertilisation and produces brooded planula larvae that, upon release, search actively for a suitable substrate for settlement and metamorphosis (Vighi 1972). The genus *Corallium* includes 19 species (Bayer & Cairns 2003) and all of them, except *C. rubrum*, are widely distributed in tropical, subtropical and temperate oceans, including the Atlantic, the Indian, the Eastern Pacific, and the Western Pacific. *Corallium rubrum* is the only shallow-water species in the genus, differing from all other *Corallium* species, which occur at a depth greater than 500 m (Bayer 1964, 1996; Grigg & Bayer 1976; Bayer & Cairns 2003).

Three typologies of red coral populations have been described up to now: (i) Shallow-water populations in a depth range between 15 and about 60 m, dwelling on vertical cliffs and in caves; these populations have been commercially exploited for centuries and, at present, are made by small, short-lived colonies (Santangelo & Abbiati 2001); (ii) intermediate-water populations, at a depth range of about 60–300 m, made by larger, sparse, long-lived colonies (Santangelo *et al.* 1999; Tsounis *et al.* 2006); (iii) deep-water populations below 300 m depth that are poorly known.

Nowadays, the intermediate-water populations of *C. rubrum* are mainly commercially exploited, whereas the main bulk of available biological knowledge refers to the more accessible shallow-water populations. These studies mainly refer to reproductive patterns (Vighi 1972; Santangelo *et al.* 2003; Torrents *et al.* 2005; Tsounis *et al.* 2006), population dynamics and growth (Abbiati *et al.* 1992; Garrabou & Harmelin 2002; Marschal *et al.* 2004; Bramanti *et al.* 2005, 2007), and genetic structure (Abbiati *et al.* 1993; Costantini *et al.* 2007a,b). Conversely, only limited knowledge is available on the intermediate-water commercially valuable populations (Garrabou *et al.* 2001; Bramanti *et al.* 2005; Tsounis *et al.* 2006; Rossi *et al.* 2008; Torrents *et al.* 2008).

Live deep-water red coral colonies were observed for the first time in 2006, during the Marum-Quest ROV exploration of the Strait of Sicily by the R/V *Meteor* (Freiwald *et al.* 2009). Colonies were dwelling in scarcely accessible habitats (overhangs, scarps) and in association with deep-water stony corals (primarily *Madrepora oculata* and *Lophelia pertusa*).

In 2007, during the scientific cruise MARCOS by the R/V *Urania*, live colonies were collected for the first time in a depth range between 585 and 819 m, and these were used in the present study for genetic analysis. The existence of previously uncharted deep-water populations is opening new perspectives in the evaluation of the distribution and on the role that this habitat-forming species plays in ecosystem processes of the deep Mediterranean Sea.

Molecular tools provide a powerful approach to elucidate taxonomic position (including the potential presence of cryptic species), but also to investigate ecological processes by analysing genetic structuring and patterns of connectivity among populations. Size and interconnectivity of evolutionary significant units (ESU) are important parameters for conservation strategies, as small and isolated populations are vulnerable to inbreeding depression, which might reduce their evolutionary potential and increase their risk of extinction (Saccheri *et al.* 1998; Palumbi 2004).

The aims of this study are to provide a genetic characterization of the first collection of live red coral colonies from deep waters, to contrast observed patterns against those found in shallow-water populations, and to infer the ecological implications of the actual range of distribution of the species.

Material and Methods

Collections

Records of live red coral colonies, occasionally associated with dead colonies, were visually documented in 2006 at water depths of *c.* 600 m at three sites during Marum-Quest ROV 4000 m dives (Linosa Island, dive 673; Urania

bank, dive 677; South of Malta, dive 657; see Freiwald *et al.* 2009 for details on ROV dive stations). The first two sites refer to submarine volcanic edifices. The last site is located on sedimentary bedrock forming an escarpment (Freiwald *et al.* 2009) within the deep-water coral assemblages described by Schembri *et al.* (2007). *Corallium rubrum* have been observed in close association with living colonial and solitary scleractinians (*e.g. Lophelia pertusa*, *Madrepora oculata* and *Desmophyllum dianthus*), and mixed with large fossil corallites of *D. dianthus* and fossil shells of *Acesta excavata* (Freiwald *et al.* 2009).

In 2007, during the course of the R/V *Urania* MARCOS cruise (Fig. 1, Table 1), live colonies of red coral were collected for the first time at sites 673 and 657. At both sites, fragments of colonies were sampled in different stations. One to five live fragments of red coral were collected per station (Table 1). Onboard, each fragment was photographed and preserved in 80% ethanol at 4 °C.

Molecular analysis

Total genomic DNA was extracted from two to four polyps per fragment using the CTAB extraction procedure as described in Costantini *et al.* (2007a,b). Twelve fragments were genotyped using three types of molecular markers: six microsatellite loci; sequence polymorphism of a portion of the mitochondrial *mutS* homolog gene (mtMSH, Pont-Kingdon *et al.* 1995) and sequence polymorphism of the internal transcribed spacer one region (ITS-1) of the nuclear ribosomal DNA. Four microsatellite loci (COR9, COR15, COR48 and COR58) specifically developed for *Corallium rubrum* (Costantini & Abbiati

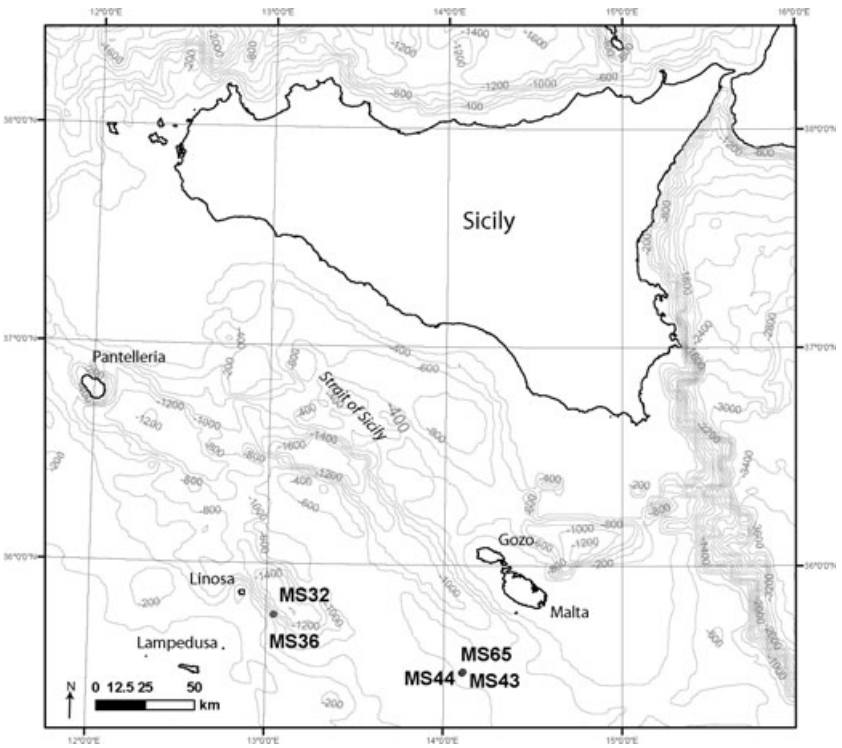

Fig. 1. Geographic positions of the two sampling locations (Malta and Linosa Island) in the Strait of Sicily and relative positions of sampling areas within locations at which *Corallium rubrum* colonies were collected.

Table 1. Collection locations [name of the MARCOS station (MS) with geographical coordinates and depth], and name of collected *Corallium rubrum* fragments at each station.

Site	Station	Lat. N	Long. E	Depth (m)	Name of fragments
657 Malta	MS43	35°30.720′	14°06.561′	607	MS43, MS43A, MS43B, MS43C, MS43-951
657 Malta	MS44	35°30.506′	14°06.230′	632	MS44
657 Malta	MS65	35°30.76′	14°06.42′	585	MS65A, MS65B, MS65C
673 Linosa	MS32	35°45.945′	13°02.625′	623	MS32
673 Linosa	MS36	35°46.010′	13°02.609′	819	MS36G, MS36D

2006), and two (CR3AL7 and CR3102A2) developed for *Corallium lauuense* (Baco *et al.* 2006) were analysed. Microsatellite loci were amplified using PCR conditions described in Costantini & Abbiati (2006) and Baco *et al.* (2006). Genotyping was carried out on an ABI 310 Genetic Analyser, using forward primers labelled with 6-FAM, HEX or TAMRA (MWG Biotech) and ROX HD400 (Applied Biosystems) as internal size standards. Allele sizing was performed using GENESCAN ANALYSIS software version 2.02 (Applied Biosystems).

Amplifications were done following the protocol of France & Hoover (2001) and Lepard (2003) for the mtMSH and Costantini *et al.* (2007a) for the ITS-1 region. PCR products were purified with an ExoSAP-IT kit (Amersham Pharmacia) and cycle sequenced in both directions using an ABI PRISM Big Dye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences were run on an ABI 310 Genetic Analyser (Applied Biosystems). mtMSH and ITS-1 sequences were edited and aligned manually using BIOEDIT version 7.0.4 (Hall 1999).

Microsatellite genetic diversity within sites was estimated as the mean number of alleles per locus (*N*a) and allelic range. Sequence genetic diversity within sites was estimated using sequence type diversity (*h*, Nei 1987) and nucleotidic diversity (π, Nei 1987) using ARLEQUIN version 3.1 (Excoffier *et al.* 2005). Both mtMSH and ITS-1 sequences were then aligned with those from shallow-water red coral samples collected at 11 locations along the Mediterranean coasts from Spain to Croatia (N = 280, depth range 20–40 m, Costantini *et al.* 2007a; and unpublished data). Analysis of the genetic divergence among sites (*e.g.* F_{ST} estimates of Weir 1996) was not done due to the small sample size of the deep-water collections.

Results

In the two sites, all six microsatellite loci analysed were polymorphic (Table 2). Overall, the number of alleles per locus ranged from 4 (in COR15) to 12 (in CR102A2), observed heterozygosity from 0.20 (in COR9) to 1 (in CR102A2 and COR58), and expected heterozygosity from 0.66 (in COR9) to 0.85 (in CR102A2) (Table 2). A significantly higher number of alleles were found in fragments from Malta sites compared to those from Linosa sites (Student's *t*-test: P = 0.001). This difference could be related to the lower number of fragments collected in Linosa.

Two fragments collected at Malta sites (MS 43-951 and MS43B) as well as two fragments collected at Linosa sites (MS36G and MS36D) showed identical genotypes at all microsatellite loci.

The mtMSH fragment was 567 bp in length (GenBank accession number GQ304902) corresponding to positions 2131–2695 of the mtMSH *Corallium ducale* sequence (GenBank accession number EU293805, Brugler & France 2008). All the analysed fragments showed identical sequences of the mtMSH gene. One synonymous transition (A/G) in the nucleotide 156 discriminates the mtMSH sequence of the deep-water colonies from the sequence found in shallow-water colonies collected along the Mediterranean coast from Spain to Croatia (N = 280, depth range 20–40 m, Costantini *et al.* unpublished data, GenBank accession number GQ304903).

The alignment of all the branch fragments ITS-1 sequences consisted of 257 positions. Four variable positions (nucleotide substitutions) were found. Overall, five different sequence types were identified (Table 3). Of the five different sequence types revealed, three were found in more than one location (sequence types 1–3–5, Table 3). Similarly to the results of microsatellites, fragments MS 43-951 and MS43B collected at Malta sites and fragments MS36G and MS36D collected at Linosa sites showed identical ITS-1 sequence types. The five sequence types found were aligned with the sequence types found by Costantini *et al.* (2007a) in shallow-water Mediterranean populations of red coral. Sequence types 1, 3 and 5 were identical to sequence types 42, 5 and 47 (GenBank accession numbers FJ876122, FJ876085, FJ876127, respectively) in Costantini *et al.* (2007a); sequence types 2 and 4 have never been found in shallow-water samples (named in GenBank ST52 and ST53 and with GenBank accession numbers GQ304900 and GQ304901, respectively).

Discussion

The bathymetric distribution of live colonies of red coral has long been considered to range between 15 and 200 m depth (Lacaze-Duthiers 1864; Carpine & Grasshof 1975; Weinberg 1978). This range has been extended down to 300 m by Zibrowius *et al.* (1984) and Rossi *et al.* (2008). In 2006 and 2007 (Freiwald *et al.* 2009 and this paper) live red coral colonies have been observed and subsequently collected down to 800 m depth, representing a major extension of the habitat of this species. These findings also suggest a greater similarity between *Corallium rubrum* and the other species belonging to the genus *Corallium* in terms of ecology and depth distribution. Genetic features of deep-sea red coral colonies have been analysed using molecular markers with different levels of polymorphism and resolution. Moreover, for the first time, cross-species amplification of microsatellite loci in the genus *Corallium* has been attempted. Microsatellites developed for *Corallium lauuense* have been used in

Table 2. Summary of genetic diversity at six microsatellite loci from *Corallium rubrum* samples: n, number of sampled individuals, Na, number of alleles per locus; H_e, expected heterozygosity; H_o, observed heterozygosity.

	Malta			Linosa		Overall
Station	MS43	MS44	MS65	MS32	MS36	
n	5	1	3	1	2	12
COR15						
Na	4	2	3	1	1	4
Allelic range	222–228	222–224	222–228	226	226	
H_e	0.70	0.50	0.61	0.00	0.00	0.72
H_o	0.80	1.00	1.00	0.00	0.00	0.67
COR48						
Na	4	2	5	2	2	7
Allelic range	169–184	160–175	172–208	178–184	178–184	
H_e	0.70	0.50	0.78	0.50	0.50	0.76
H_o	0.80	1.00	0.67	1.00	1.00	0.83
COR58						
Na	4	2	4	2	2	7
Allelic range	168–210	174–177	168–192	168–177	168–183	
H_e	0.69	0.50	0.67	0.50	0.50	0.76
H_o	1.00	1.00	1.00	1.00	1.00	1.00
COR9						
Na	4	1	2	0	1	5
Allelic range	179–185	183	179–193	-	185	
H_e	0.66	0.00	0.28	-	0.00	0.66
H_o	0.20	0.00	0.33	0.00	0.00	0.20
C3AL7						
Na	3	1	5	2	2	7
Allelic range	162–166	166	156–188	162–166	160–166	
H_e	0.58	0.00	0.78	0.50	0.50	0.74
H_o	1.00	0.00	1.00	1.00	1.00	0.92
CR102a2						
Na	4	2	6	2	2	12
Allelic range	270–318	266–276	272–320	270–320	270–318	
H_e	0.69	0.50	0.83	0.50	0.50	0.85
H_o	1.00	1.00	1.00	1.00	1.00	1.00
Multilocus						
H_e	0.67	0.33	0.66	0.40	0.33	
H_o	0.80	0.67	0.83	0.80	0.67	

C. rubrum, providing excellent results (no null amplifications have been observed), showing the possibility to extend the use of these markers to closely related taxa of Anthozoa, *e.g.* in species belonging to the same genus or in closely related genera (Jamison & Lasker 2008). Furthermore, microsatellites have been confirmed to be useful markers for individual genotyping of *C. rubrum* colonies, as suggested by Baco & Shank (2005) and Costantini *et al.* (2007a,b). Conversely, mitochondrial DNA, which is highly conserved in Anthozoa, provides little information on the genetic structure of the species even at large depth/geographic ranges.

Of the 12 fragments of colonies analysed, two fragments collected off Malta Island as well as two fragments collected off Linosa Island showed the same multilocus genotype at the six microsatellite loci, and identical mtMSH and ITS-1 sequences; these results strongly suggest that each pair of fragments may have come from a single colony.

Mitochondrial DNA of anthozoans is known to have a slow evolutionary rate, as observed by Shearer *et al.* (2002) and Hellberg (2006). Costantini *et al.* (2003) also found no intraspecific variation in shallow-water red coral populations using 16S and COI. Consistently, all the analysed red coral fragments showed identical sequences of the mtMSH gene. However, the deep-water red coral sequences differ by one synonymous substitution from those of shallow-water samples (Costantini *et al.* unpublished data, N = 280; depth range 20–40 m; sampling area: Mediterranean coasts from Spain to Croatia). Similar results have been obtained by France & Hoover (2002), who found a single nucleotide substitution in

Table 3. Sequence differences, distribution and genetic diversity of the five ITS-1 sequence types in *Corallium rubrum* samples. 33, 44, 259 and 264 represent nucleotide positions.

		Nucleotide position				Malta			Linosa		
GenBank accession no.		33	44	259	264	MS43	MS44	MS65	MS32	MS36	Total
FJ876122	1	A	G	A	T	1		2			3
GQ304900	2	G	A	.	.	3					3
FJ876085	3	.	A	.	.			1		2	3
GQ304901	4	.	A	R	.	1					1
FJ876127	5	.	A	.	C		1		1		2
	n					5	1	3	1	2	12
	H					3	1	2	1	1	
	h					0.7	1	0.67	1	0	
	π					0.004	0	0.002	0	0	

Dots indicate identical bases. Number of individuals per sample (n), total number of sequence type (H), sequence type diversity (*h*, Nei 1987), nucleotide diversity (π, Nei 1987).

COI sequences between shallow- and deep- water specimens of *Corallium ducale*. Such a fixed substitution of one nucleotide might suggest lack of connectivity between shallow- and deep-water populations in both *Corallium* species, but the small sample size of the deep-water collections does not allow further consideration on the evolutionary implications of this genetic variation. Further efforts have to be spent in investigating deep-water red coral populations to be able to infer the relative importance of geological and ecological processes in shaping red coral genetic diversity. Comparison of the genetic structure of populations along a depth gradient, from shallow to deep waters, and at a similar range of horizontal (geographical) distances would be useful. Incidentally, no shallow-water *C. rubrum* has been reported so far from the Malta archipelago (John Camilleri, personal communication).

In Anthozoan, ITS-1 sequence markers may suffer from intra-individual rDNA variability overlapping with intraspecific rDNA variation (Márquez *et al.* 2003; Vollmer & Palumbi 2004; Wei *et al.* 2006). Problems related to the ITS-1 level of intragenomic variability in red coral have been previously addressed (Costantini *et al.* 2007a). In the present study, unreadable sequences (due to the presence of one to three superimposed chromatogram peaks or to a frame shift in the sequences relative to indels), which may suggest intra-individual polymorphism, were not found. Patterns of the ITS-1 sequences in deep-water colonies slightly differed from those previously described in shallow-water red coral populations by Costantini *et al.* (2007a). Fragments from Linosa sites show the same sequence types previously found in shallow-water red coral samples (sequence types 5, 42, and 47, Costantini *et al.* 2007a), whereas fragments from Malta sites are characterized by two private sequence types. The sequence type 5 found in three deep-water coral fragments, was the most common found in shallow-water populations. Sequence types 42 and 47 were found in four colonies collected in Palinuro (Tyrrhenian Sea) and in most colonies collected in Vis (Adriatic Sea), respectively. The analyses of the ITS-1 sequence types (Costantini *et al.* 2007a) did not provide a clear geographic pattern of genetic structuring in shallow-water red coral samples at the Mediterranean scale. Despite the limited sample size, ITS-1 results from deep-water colonies may point to the occurrence of genetic isolation between the two sampling sites (Malta and Linosa shelves). Further sampling of red coral colonies in this area is needed to test hypotheses on depth or geographic gradients.

Not much is known about the evolution of the Mediterranean deep-sea fauna, mainly due to the technological/economical difficulties associated with the exploration of deep environments. Therefore, these preliminary results obtained from the first collection of deep-water live colonies of red coral are particularly relevant and contribute to the understanding of the red coral biology. Larger sample size together with collections from a broader geographical range (Atlantic and Mediterranean Sea) are needed to be able to identify biogeographic discontinuities and refugia populations as well as to delineate the evolutionary models of this species. Moreover, collection of samples in the same region in both shallow and deep waters would be useful to test hypotheses on the role of depth in determining patterns of genetic structure in *C. rubrum* and to provide a picture on the biology and ecology of the species, useful for the implementation of conservation and management strategies.

We are still only beginning to understand the principal ecological aspects of Mediterranean deep-water red coral populations, including the environmental factors (*e.g.* temperature, salinity, *etc.*), and biological processes (*e.g.* reproductive biology, recruitment, connectivity) which

regulate their life and distribution. Nevertheless, due to the vulnerability of red coral populations a precautionary approach is recommended by the implementation of conservation measures, such as creation of deep-sea protected areas, and implementation of deep-water monitoring programs.

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