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Characterization of the zooxanthellate and azooxanthellate morphotypes of the Mediterranean gorgonian *Eunicella singularis*

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Abstract The gorgonian *Eunicella singularis* (Esper, 1794) is abundant on rocky bottoms at Cap de Creus (42°18′49″ N; 003°19′23″ E) in the western Mediterranean, and this study compared zooxanthellate colonies from relatively shallow depths with azooxanthellate colonies living at depths to 60 m. The goal was to determine the taxonomic status of a previously described subspecies, *E. singularis aphyta*. Sampling at 10-m intervals from 20 to 60 m using scuba or a remotely operated vehicle (ROV) in 2004 and 2010 allowed examination of colony shape, sclerite variability, genetic variability, and the presence/absence of zooxanthellae. Two morphotypes were identified: a shallow morphotype with candelabra-like colonies at 20–30 m has zooxanthellae, while a deep morphotype with more ramified colonies at 40–60 m lacks symbionts. Sclerite differences among colonies were also identified along the depth gradient. The mitochondrial marker *msh1* did not discriminate between the two morphotypes and indeed did not discriminate among several Mediterranean species of *Eunicella*. Other genetic markers will be needed to firmly establish the taxonomic status of the two depth-related morphotypes.

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Introduction

Morphological variation is ubiquitous within coral species, creating significant challenges for the advancement of taxonomic, evolutionary, and ecological studies (Vermeij et al. 2007). Though the morphology of some species is conserved even under varying environmental factors (Weinbauer and Velimirov 1998; Rodriguez-Lanetty et al. 2003), in other species intraspecific morphological variation is correlated with differences in environmental regimes like current speed and direction (Lewis and Von Wallis 1991). Morphological plasticity characterizes many gorgonian (Brazeau and Lasker 1988; West et al. 1993; Sánchez et al. 2007) and coral species (Muko et al. 2000; Todd et al. 2004; Einbinder et al. 2009), with changes along depth gradients reflecting differences in hydrodynamic and light regimes (Sebens 1987).

Among the approximately 20 gorgonian species in the Mediterranean Sea, four belong to the genus *Eunicella*: *Eunicella verrucosa*, *Eunicella filiformis*, *Eunicella cavolinii*, and *Eunicella singularis* (Esper 1794; Rossi 1959; Carpine and Grasshoff 1975; Weinberg 1976). Both *E. verrucosa* and *E. filiformis* have a primarily Atlantic distribution; *E. verrucosa* has been reported from the Mediterranean Sea as rare or in patchy populations, whereas

E. filiformis has only been recorded from the Straits of Gibraltar area and Alboran Sea (Rossi 1959; Carpine and Grasshoff 1975; Grasshoff 1992) where the hydrographic regime also permits the sporadic presence of *Eunicella labiata* and *Eunicella gazella* that are typically found along the African coast (Grasshoff 1992; López-González pers. obs.). Conversely, *E. cavolinii* and *E. singularis* are common in the western Mediterranean (Rossi 1959; Carpine and Grasshoff 1975) where they are among the main structural species in the coralligenous community (Gili and Ros 1985; Ballesteros 2006). *E. cavolinii* is common in the eastern part of the western Mediterranean basin and in the Adriatic Sea, but is absent or occasionally found along coasts west of Marseille (Carpine and Grasshoff 1975; Weinberg 1976). This species is found primarily on vertical rock walls in shallow coastal waters and as deep as 150 m on coralligenous formations (Rossi 1959; Carpine and Grasshoff 1975). Colonies of *E. cavolinii* typically have planar branching with many short primary branches and their color ranges from faint yellow to orange (Rossi 1959; Weinberg 1976).

Eunicella singularis is the only Mediterranean gorgonian species known to host symbiotic algae; it is abundant throughout the western Mediterranean and Adriatic Sea, and is occasionally present in the eastern Mediterranean. This species is common on rocky bottoms in shallow waters (Carpine and Grasshoff 1975; Weinberg 1976), as well as on coralligenous formations in deeper sublittoral waters (Rossi 1959; Gori et al. 2011a). Colonies of *E. singularis* have long primary branches arranged in parallel, with few ramifications, and their color ranges from bright to grayish-white (Rossi 1959; Weinberg 1976). A subspecies of *E. singularis* was described based on colonies lacking symbiotic algae (i.e., *E. singularis aphyta*, Théodor 1969). However, while some authors recognized this subspecies (Weinberg 1976), others treated the symbiotic and aposymbiotic colonies as intraspecific varieties (Carpine and Grasshoff 1975). As the aposymbiotic form of *E. singularis* is quite rare in the depth range where investigations of Mediterranean gorgonians were concentrated in the last 40 years (0–40 m), the controversy over the taxonomic status of this form waned.

Knowledge of the distribution and ecology of shallow populations of *E. singularis* has increased considerably over the last decades (e.g., Weinberg and Weinberg 1979; Ribes et al. 2007; Linares et al. 2008) but there were a few studies of populations from nearshore rocky bottoms at 40–150 m depth (Sink et al. 2006; Virgilio et al. 2006). Furthermore, there is no recent information on deep sublittoral colonies attributable to the aposymbiotic form of *E. singularis* (Théodor 1969). Recent underwater exploration techniques have facilitated scientific observation of benthic communities below 40 m depth. In the Mediterranean Sea, dense populations of gorgonians were recently observed at 60 and 100 m (Bo et al. 2009, 2011; Cerrano et al. 2010), including dense stands of *E. singularis* in waters down to 70 m (Gori et al. 2011a, b). Small colonies dominate the shallow water populations of *E. singularis* (Linares et al. 2008), whereas large colonies, with more continuous coverage of the substrate, dominate populations at 60 m depth (Gori et al. 2011b). Moreover, colony morphology of *E. singularis* varies with depth. Colonies in shallow water down to 30–35 m exhibited a candelabra-like colony morphology and an overall grayish-white color (Weinberg 1976), whereas colonies from 30–35 to 70 m displayed a variable colony morphology and bright white color (Gori et al. 2011a, b). The latter colonies were considered representatives of the aposymbiotic form of *E. singularis* of Théodor (1969), which raises a question about the taxonomic status of the aposymbiotic form.

To elucidate whether the bathymetric forms of *E. singularis* represent distinct taxonomic units, we examined differences between colonies of *E. singularis* collected from 20 to 60 m depth by analyzing the following gorgonian descriptors: (1) colony shape, (2) sclerite size and shape, (3) genetic variability in a mitochondrial marker (*msh1*), and (4) the presence of symbiotic algae in the tissue. Results of the analyses on *E. singularis* were compared with those on *E. cavolinii* and *E. verrucosa*, which are the other congeneric species in the western Mediterranean exclusive of the Alboran Sea.

Materials and methods

Colony shape

Pictures of colonies of *E. singularis* used to analyze colony shape were obtained from the north and east side of Cap de Creus (42°18′49″ N; 003°19′23″ E) in the northwestern Mediterranean Sea (Online Resource 1). Colonies from 20, 30, 40, 50, and 60 m depth were photographed in November 2004 with the ROV Phanthom XTL equipped with a 700 horizontal lines camera. In each picture, colonies were perpendicular to the camera and two parallel laser beams, which provided a scale for the images, were in the same plane as the colony. A total of 100 pictures of *E. singularis* (20 from each 10-m increment in the depth range sampled) was selected for analysis. Pictures of *E. cavolinii* (20–25 m depth) were obtained in March 2009 from the north coast of the Isola d'Elba (42°49′18″ N; 010°09′52″ E) in the northern Tyrrhenian Sea (Online Resource 1) by scuba diving with a digital camera in an underwater housing, equipped with an underwater flash. A ruler placed near each colony provided scale for the images. A total of 20 pictures of *E. cavolinii* were selected for analysis. Finally, pictures of *E. verrucosa* (15–25 m depth)

were obtained in April 2010 from Tarragona (41°06′07″ N; 001°15′12″ E) (Online Resource 1) also by scuba diving with the same equipment used for photographing *E. cavolinii*. A total of 20 pictures of *E. verrucosa* were selected for analysis. The selected pictures were analyzed with Carnoy (Schols and Smets 2001) and ImageJ (Abramoff et al. 2004), and each image was calibrated using either the laser beams or the ruler as reference.

From each picture, the maximum height and maximum width of the gorgonian colony were measured; mean width was calculated as the mean of three measurements taken at equidistant positions and perpendicular to the height; the surface area of the fan was measured (Weinbauer and Velimirov 1995); the number of ramifications and the number of branches of each order were counted (Brazeau and Lasker 1988); the length of all the primary branches was measured. For each colony, the following shape features were calculated: height to width ratio, height to mean width ratio, ramification density (number of ramifications per fan surface area), primary branch density (number of primary branches per fan surface area), order of the colony's base, maximum length and mean length of the primary branches, bifurcation ratio (Strahler 1957; Brazeau and Lasker 1988), and tributary to source ratio of primary and secondary order branches (defining "source" as any branch that joins another branch of equal order, and "tributary" as any branch that joins a higher order branch; Brazeau and Lasker 1988). Distance-based permutational multivariate analysis of variance (PERMANOVA; Anderson 2001; McArdle and Anderson 2001) performed using the PERMANOVA.exe software (Anderson 2005) was used to test the null hypothesis of no significant differences in overall shape of the colony with respect to depth and species. Each term of the analysis was tested using 9,999 random permutations of appropriate units (Anderson and ter Braak 2003), and significant terms relevant to the hypothesis were investigated using a posteriori pairwise comparisons with the PERMANOVA t statistic and 9,999 permutations. Data were standardized with respect to their mean absolute deviation (MAD), and a Euclidean distance matrix was built based on the standardized data. Furthermore, an ordination of all the analyzed colonies ($n = 140$) based on the Euclidean distances was obtained with a principal component analysis (PCA) performed with the R-language function Princomp from the Vegan library (Oksanen et al. 2005) of the R software platform (R Development Core Team 2007). Finally, a clustering of all the analyzed colonies ($n = 140$) based on the Euclidean distances and using a Ward aggregation for the ordination performance was obtained with a cluster analysis performed with the R-language function As.phylo that is available in the Ape library (Paradis et al. 2004). The R-language function Simprof, from the Clustsig library (Whitaker and Christman 2010), was used to assess the number of significant clusters produced; the expected distribution of the data was created from 10,000 generated similarity profiles, and 9,999 similarity profiles were generated for use in comparing the observed test statistic with its null distribution.

Sclerite variability

Samples from colonies of *E. singularis* used to analyze the sclerite size and shape were collected from the east side of Cap de Creus (42°18′44″ N; 003°19′05″ E; Online Resource 1) at 20, 30, 40, 50, and 60 m in June 2010. At each depth, a portion of a primary branch was collected from 20 colonies by scuba diving and stored in ethanol. Additionally, samples from 20 colonies of *E. cavolinii* (20–25 m depth) were collected in March 2009 from the northern side of the Isola d'Elba, and samples from 20 colonies of *E. verrucosa* (15–25 m depth) were collected in January 2009 from Tarragona (Online Resource 1).

Samples from each colony were immersed in bleach until the organic matter was dissolved and the sclerites were disaggregated. Sclerites were cleaned with distilled water, deposited on a glass coverslip attached to an aluminum stub with colloidal silver, and then sputter-coated with Au-Pd. Sclerites were photomicrographed with a Scanning Electron Miscroscope (SEM) HITACHI S-3500 N at 5.0 kV. Measurements of 20 balloon clubs and 10 spindles from each colony were taken at 1000× and 400×, respectively. For each balloon club, the following measurements were recorded: length, width, spiny end and collar widths (Weinberg 1976); the head of each balloon club was classified into one of five categories according to the degree of roughness (Online Resouce 2). For each spindle, length and maximum width were recorded (Weinberg 1976). For each colony, the following sclerite features were calculated: mean length and mean width of the balloon clubs, mean width of the balloon club spiny ends, mean width of the balloon club collars, mean roughness of the balloon club heads, mean length of the spindles, and mean width of the spindles. Distance-based PERMANOVA (Anderson 2001; McArdle and Anderson 2001) was used to test the null hypothesis of no significant differences in the overall sclerite size and shape with respect to depth and species. Data were standardized with respect to their MAD, and the standardized data were used to build a Euclidean distance matrix. An ordination of all the analyzed colonies ($n = 70$) based on the Euclidean distances was obtained with PCA. Finally, the same cluster analysis used to analyze colony shape was also used to analyze sclerite variability.

Genetic marker

Colonies of *E. singularis* from 20 and 60 m depth, *E. cavolinii*, and *E. verrucosa* were analyzed using the octocoral-specific, mitochondrial, mismatch repair gene homolog (*msh1*) ($n = 16$; 2 individuals of *E. singularis* from 20-m depth, 4 individuals of *E. singularis* from 60-m depth, 3 individuals of *E. cavolinii*, and 7 individuals of *E. verrucosa*). As identical mitochondrial (COI) and nuclear (ITS2) sequences have already been obtained for *E. singularis* and *E. cavolinii* (Calderón et al. 2006), we chose *msh1* to be sequenced (Online Resource 3) as it exhibits a relatively high rate of substitution in Octocorallia (France and Hoover 2001; van der Ham et al. 2009; McFadden et al. 2011). Though variation in *msh1* may not distinguish among all congeners (Lepard 2003; McFadden et al. 2011), it is the most variable molecular marker available for the study of multiple octocorallian species (Thoma et al. 2009; McFadden et al. 2011).

Presence of symbiotic algae

Portions of the samples collected from colonies of *E. singularis*, used for sclerite analysis, were also analyzed for the presence of symbiotic algae. Ten colonies of *E. singularis* from each 10-m depth increment were preserved in 10 % formol, rinsed in distilled water, decalcified in a 10 % formic acid solution, and dehydrated in an ethanol series with increasing concentrations (i.e., 70, 96, and 100 % EtOH). After dehydration, samples were placed in a 1:1 mixture of 100 % EtOH and resin (Technovit 7100) for 2 h, then embedded in a biphasic resin (Technovit 7100) and stored in the dark at 4 °C for 48 h. Finally, samples were placed in resin (Technovit 7100) and left to harden for 3 d at room temperature. The resin blocks containing the samples were longitudinally cut into 3-μm thick sections and stained with hematoxylin and eosin. Stained sections were observed with a microscope to determine whether symbiotic algae were present in the tissue of each colony analyzed ($n = 50$; 5 depths × 10 individuals per depth).

Environmental parameters at Cap de Creus

Environmental parameters were recorded monthly from September 2009 to August 2010 at Cap de Creus (42°18′44″ N; 003°19′05″ E; Online Resource 1). Temperature, salinity, density, and photosynthetically active radiation (PAR, 400 to 700 nm) were measured at 1-m depth intervals from 5 to 60 m with a Seabird 19 and a Seabird 25 conductivity temperature and depth sensors (CTDs) equipped with Biospherical Instruments Inc QSP-2300 and a Li-Cor underwater spherical quantum sensor LI-193, respectively.

Results

Colony shape

Colony shape was significantly different among colonies of *E. singularis* collected at different depths, and colonies of *E. cavolinii* and *E. verrucosa* (PERMANOVA, Pseudo-$F = 8.75$, $p < 0.01$). Colonies of *E. cavolinii* and *E. verrucosa* have a similar colony shape that significantly differed from colonies of *E. singularis* collected at any depth (Fig. 1; Table 1). Colonies of *E. singularis* showed a gradient in colony shape going from shallow to deep (Fig. 1; Table 1): no significant differences were found between colonies of *E. singularis* from 20 and 30 m, whereas colonies from 20 m significantly differed from all others; colonies from 30 m were similar to colonies from 40 and 50 m, but statistically differed from colonies at 60 m; finally, colonies from 40 and 50 m were similar to those from 60 m (Fig. 1; Table 1). The first two principal components of the PCA explained 77.9 % of the data variance with the first axis explaining 55.9 %. The PCA indicated that these differences were mainly due to four groups of features, while the bifurcation ratio and the tributary to source ratios did not contribute to the explanation of variance (Fig. 2). A clear covariance existed between the mean and maximal length of the primary branches, the height to width and mean width ratios, and the ramification and primary branch densities (Fig. 2). Colonies of *E. singularis* from shallow waters (20 and 30 m) were characterized by large primary branches, high height to width ratios (colonies are taller than wide), low ramification and primary branch densities, and a low order of the colony base (Figs. 1, 2 and Online Resource 4). In contrast, colonies of *E. cavolinii* and *E. verrucosa* had a large number of short primary branches, height to width ratios close to one (colonies approximately as tall as wide), and a higher order of the colony base (Figs. 1, 2 and Online Resource 4). Colonies of *E. singularis* from 40, 50, and 60 m displayed colony shapes intermediate between the shallow water colonies of *E. singularis* and the colonies of *E. cavolinii* and *E. verrucosa* (Figs. 1, 2 and Online Resource 4). The cluster analysis of colony shapes recovered two main groups, which were comprised of: (1) all colonies of *E. cavolinii* (20 of 20) and *E. verrucosa* (19 of 20), and (2) colonies of *E. singularis* from 20 m (18 of 20) and 30 m (17 of 20) (Fig. 3). Colonies of *E. singularis* from 40 m generally grouped with conspecifics from shallow waters (13 of 20) as well as colonies from 50 m (12 of 20). However, colonies of *E. singularis* from 60 m generally grouped with colonies of *E. cavolinii* and *E. verrucosa* (13 of 20) (Fig. 3).

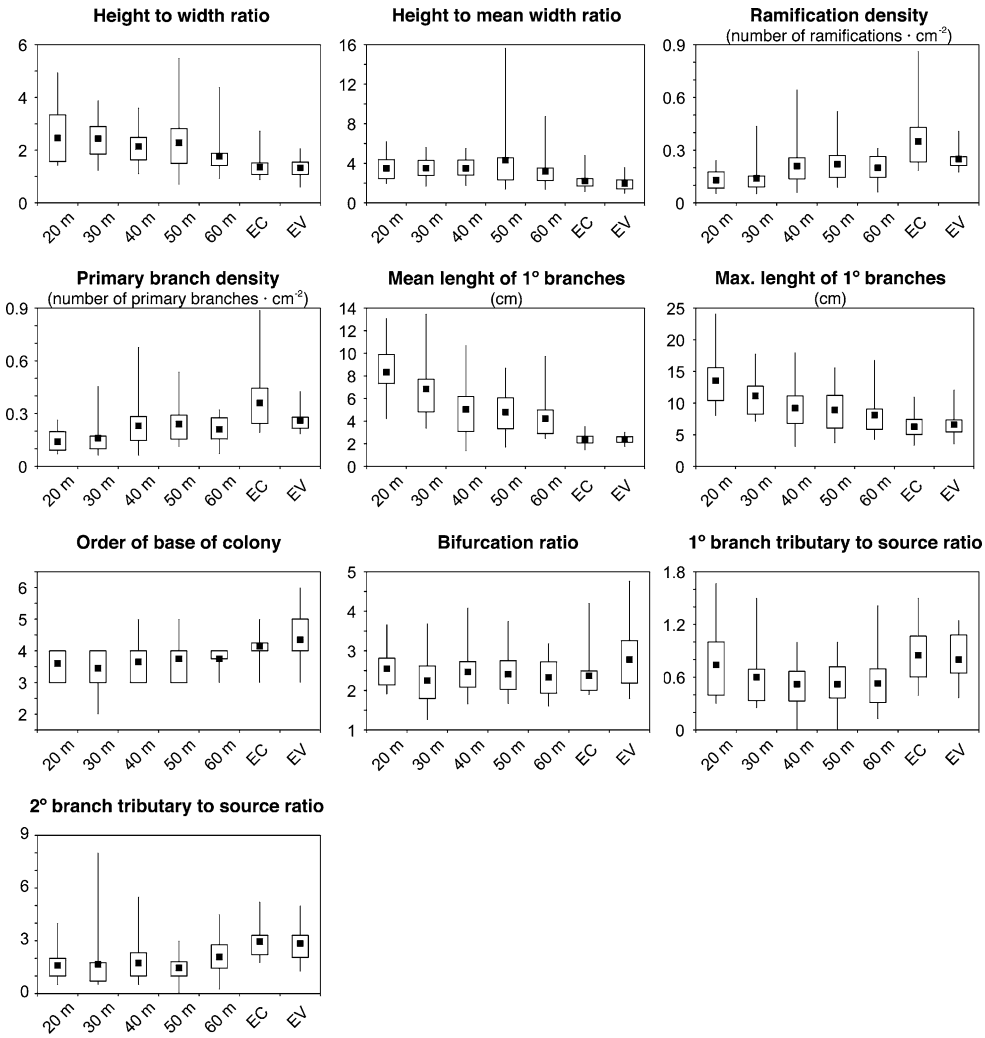

Fig. 1 Colony shape features of *E. singularis* at each of five depths sampled, and for *E. cavolinii* (EC) and *E. verrucosa* (EV) ($n = 140$); *black square* indicates mean value, *box* indicates first and third quartiles, and *line* indicates range between minimum and maximum values

Sclerite variability

Sclerite size and shape were significantly different among colonies of *E. singularis* from different depths, and colonies of *E. cavolinii* and *E. verrucosa* (PERMANOVA, Pseudo-$F = 14.65$, $p < 0.01$). The colonies of *E. singularis* from 20 and 30 m shared similar sclerites, while colonies from 40 m as well as colonies of *E. verrucosa* showed significantly different sclerites (Fig. 4; Table 1). Finally, colonies of *E. singularis* from 50 and 60 m had similar sclerites to colonies of *E. cavolinii* (Fig. 4, Table 1). The first two principal components of the PCA explained 76.1 % of the data variance with the first axis explaining 60.6 %. The PCA indicated that these differences were mainly due to three groups of features, with a clear covariance between balloon club and spindle lengths, and a slight covariance among the balloon club collar and spiny end widths, and spindle width (Fig. 5). Colonies of *E. singularis* from 20 and 30 m were mainly characterized by large and wide sclerites, with balloon clubs having large collars and spiny ends, and smooth heads (Figs. 4, 5 and Online Resources 5 and 6). Colonies from 40 m mainly had sclerites that were the same length as the sclerites in colonies from shallower waters, but were thinner and had rougher balloon club heads (Figs. 4, 5 and Online Resource 7). Colonies of *E. singularis* from 50 and 60 m and *E. cavolinii* had smaller sclerites with rough balloon club heads (Figs. 4, 5 and Online Resources 8, 9 and 10). Finally, sclerites from colonies of *E. verrucosa* were the thinnest and had the roughest balloon club heads (Figs. 4, 5 and Online Resource 11). Cluster analysis aggregated together colonies of *E. singularis* from 20 m (10 of 10) and 30 m (8 of 10), and several colonies from 40 m (4 of 10; Fig. 6). Additionally, the analysis grouped together all the colonies of *E. verrucosa* (10 of 10) that were related to a group composed of the remaining colonies of *E. singularis* from 40 (6 of 10), 50 (9 of 10), 60 m (10 of 10), and colonies of *E. cavolinii* (9 of 10) (Fig. 6).

Genetic marker

All 16 of the *msh1* sequences obtained, representing colonies of *E. singularis* from 20 m and 60 m depth or the

Table 1 Pairwise test results for the colony shape and the sclerite size and shape comparison among colonies of *E. singularis* sampled at each depth, and *E. cavolinii* and *E. verrucosa*

Depth/species	Colony shape		Sclerite size and shape	
	t	*p*	*t*	*p*
20 m vs. 30 m	1.161	0.2485	1.063	0.3365
20 m vs. 40 m	2.448	0.0011**	2.804	0.0001***
20 m vs. 50 m	2.356	0.0023**	3.698	0.0001***
20 m vs. 60 m	3.240	0.0001***	3.497	0.0001***
20 m vs. EC	5.539	0.0001***	3.559	0.0001***
20 m vs. EV	5.688	0.0001***	7.019	0.0002***
30 m vs. 40 m	1.582	0.0594	2.244	0.0059**
30 m vs. 50 m	1.661	0.0479	3.739	0.0002***
30 m vs. 60 m	2.287	0.0019**	3.430	0.0001***
30 m vs. EC	4.812	0.0001***	3.461	0.0002***
30 m vs. EV	4.865	0.0001***	6.940	0.0001***
40 m vs. 50 m	0.643	0.7605	2.508	0.0021**
40 m vs. 60 m	0.880	0.5071	2.040	0.0071**
40 m vs. EC	3.161	0.0001***	2.144	0.0064**
40 m vs. EV	3.106	0.0002***	5.731	0.0001***
50 m vs. 60 m	1.169	0.2418	1.183	0.2193
50 m vs. EC	2.870	0.0002***	1.604	0.0382
50 m vs. EV	2.863	0.0005***	5.786	0.0001***
60 m vs. EC	3.068	0.0001***	0.962	0.4278
60 m vs. EV	2.726	0.0001***	4.559	0.0001***
EC vs. EV	1.893	0.0148	3.981	0.0001***

Significant *p* values are indicated with two (*p* value <0.01), or three asterisks (*p* value <0.001)

other two species of *Eunicella*, were identical: only a single *msh1* haplotype was observed and it was shared among all the analyzed colonies (Online Resource 12). These results led us not to sequence the remaining samples that had been preserved for genetic analysis.

Presence of symbiotic algae

Analysis of histological preparations of *E. singularis* showed that all colonies from 20 and 30 m had zooxanthellae in their tissues, whereas all colonies from 40, 50, and 60 m lacked zooxanthellae (Online Resource 13).

Environmental parameters at Cap de Creus

Both temperature and salinity were nearly constant throughout the water column from October 2009 to February 2010. In March 2010, surface waters were less saline than deeper waters, probably due to heavy rains in the study area during that month. Water column stratification began to develop in April 2010 with a thermocline forming around 30 m but was partially disrupted at the end of June due to strong winds. The water column was fully stratified in July and August 2010 with a thermocline at 35 m. Stratification was stretched between 30 and 45 m in September (Online Resource 14). PAR decreased exponentially with depth; over an annual average, when compared to readings from 20 m, PAR was 37.8 ± 13.1 % at 30 m, 14.1 ± 7.1 % at 40 m, 4.6 ± 2.8 % at 50 m, and 1.4 ± 1.0 % at 60 m.

Discussion

The results of this study suggest the existence of two distinct morphotypes of *E. singularis* at Cap de Creus. A "shallow morphotype" in colonies at 20 and 30 m, corresponds to the commonly known *E. singularis*. These colonies have symbiotic algae in their tissues and an overall candelabra-like shape with few ramifications and large primary branches. Their balloon club sclerites are large, have smooth heads, wide collars and spiny ends. In contrast, a "deep morphotype" of *E. singularis* characterizes colonies at 40–60 m that lack symbiotic algae in their tissues. These colonies have more ramifications and shorter primary branches compared with the shallow morphotype. The balloon club sclerites of this deep morphotype vary with increasing depth showing intermediate dimensions at 40 m, while being smaller at 50 and 60 m (resembling sclerites of *E. cavolinii*).

Eunicella singularis is known as the Mediterranean gorgonian with the longest and thickest branches, the lowest ramification density, and the greatest height to width ratio (Rossi 1959; Carpine and Grasshoff 1975; Weinberg 1976; Weinbauer and Velimirov 1998). Colonies of *E. cavolinii* are characterized by low height to width ratio, short and ramified branches (Carpine and Grasshoff 1975; Weinberg 1976), and an overall colony shape resembling that of *Leptogorgia sarmentosa* or *E. verrucosa* rather than *E. singularis* (Rossi 1959; Weinbauer and Velimirov 1998). Sclerites of *E. singularis* are larger and the balloon clubs have wider collars and spiny ends compared with those of *E. cavolinii* (Rossi 1959; Weinberg 1976). Additionally, the heads of balloon clubs are smooth in *E. singularis* but are rough in *E. cavolinii* (Rossi 1959; Carpine and Grasshoff 1975). Furthermore, sclerites of *E. verrucosa* are distinct from any other species of *Eunicella* commonly encountered in the Mediterranean Sea (Rossi 1959; Carpine and Grasshoff 1975; Weinberg 1976).

A previous study has shown no differences in colony shape between colonies at 20-m and 30-m depth (Weinbauer and Velimirov 1998) but our studies over a wider depth range show significant gradual changes in shape with depth. Phenotypic plasticity confers adaptability to the range of environmental conditions encountered by

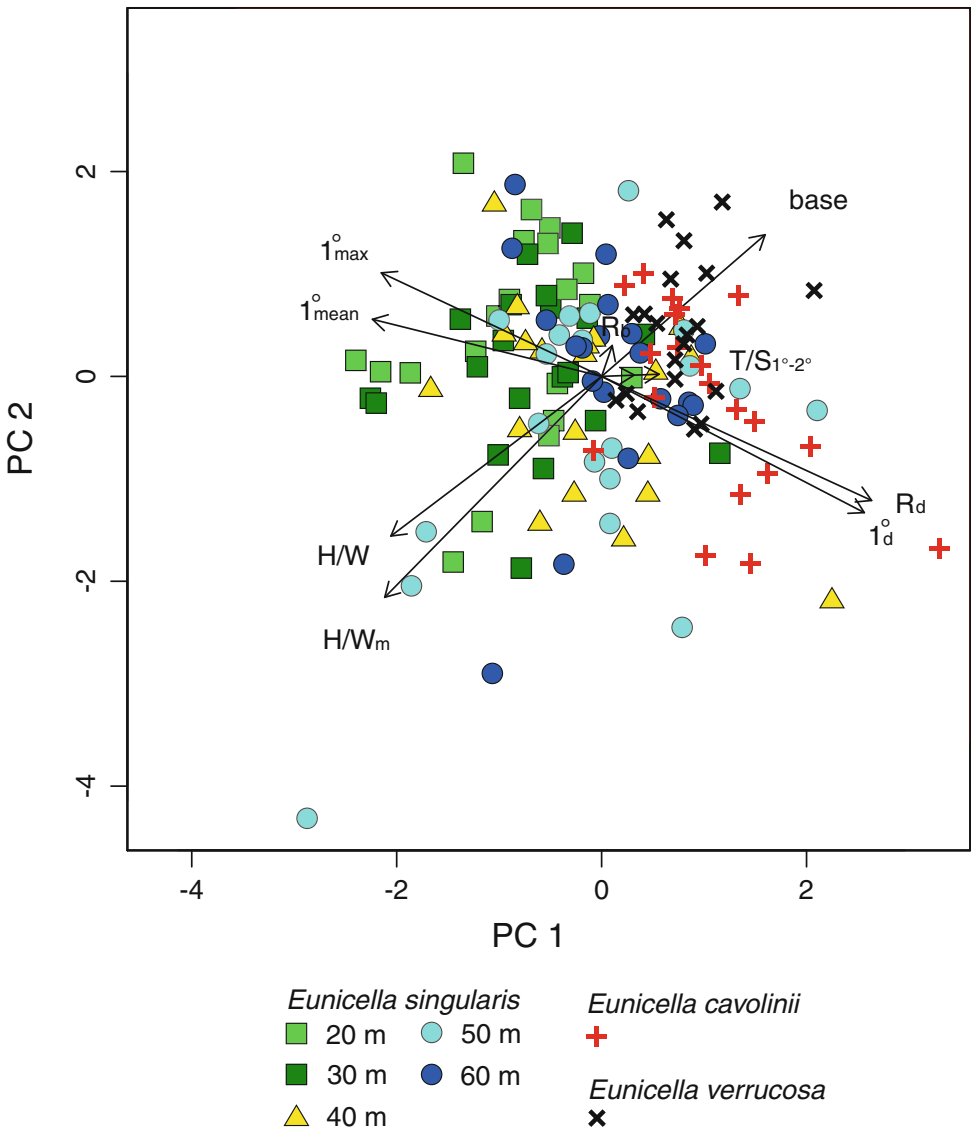

Fig. 2 Principal component analysis (PCA) biplot showing ordination of studied colonies ($n = 140$) with respect to colony shape, and roles of analyzed features; *H/W* height to width ratio, *H/Wm* height to mean width ratio, *Rd* ramification density, *1°d* primary branch density, *base* order of colony's base, *1°max* maximum length of primary branches, *1°mean* mean length of primary branches, *Rb* bifurcation ratio, *T/S1°* tributary to source ratio of primary branches, *T/S2°* tributary to source ratio of secondary branches

sessile organisms (Bradshaw 1965; Smith et al. 2007) and provides the capacity for the colony to adapt its form to the environment in which it lives (Marfenin 1997; Shaish et al. 2007). Depth-related differences in colony shape, and morphological adaptations to different light intensities are well known for stony corals and tropical gorgonians, where deeper colonies have longer branches, and lower tributary to source and width to length ratios than shallower ones (Brazeau and Lasker 1988; West et al. 1993; Sánchez et al. 2007). Differences in water turbulence were previously reported as the main cause of variability in colony shape of *E. singularis* from shallow waters (Théodor 1963; Skoufas et al. 2000). Additionally, branching networks were narrower and primary branches were significantly shorter in sheltered compared to exposed colonies of *E. cavolinii*, whereas the ramification density was significantly higher in sheltered colonies as well as in colonies from 70 and 80 m depths (Velimirov 1976; Weinbauer and Velimirov 1995). Colonies of the shallow morphotype of *E. singularis* mainly inhabit horizontal and subhorizontal rocky bottoms where a candelabra-like shape, with few ramifications and large primary branches that bend readily, reduces drag (Weinbauer and Velimirov 1998). This may allow the shallow colonies to withstand strong water movements caused by storm-induced waves (Hiscock 1983; Bongaerts et al. 2010). Additionally, strong waves often transport detached algae that could easily get entangled in colonies with many ramifications thus disproportionally increasing the perceived hydrodynamic forces, while colonies with long and vertical branches could make entanglement of detached algae less likely.

The elastic properties of the axial skeleton of gorgonians allow colonies to bend and return to an erect position (Jeyasuria and Lewis 1987), but sclerites limit the extent of

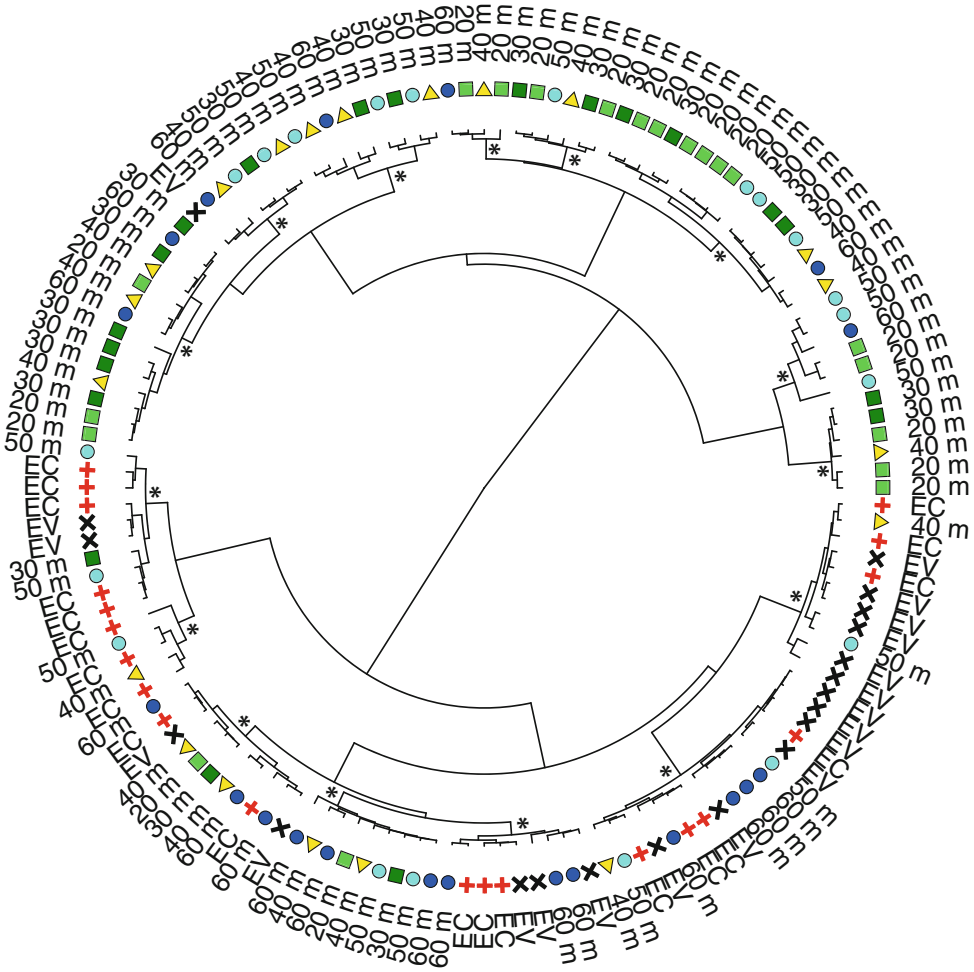

Fig. 3 Cluster analysis of studied colonies ($n = 140$) aggregated with respect to colony shape; *asterisks* indicate significant clusters determined by the Simprof test ($p < 0.05$)

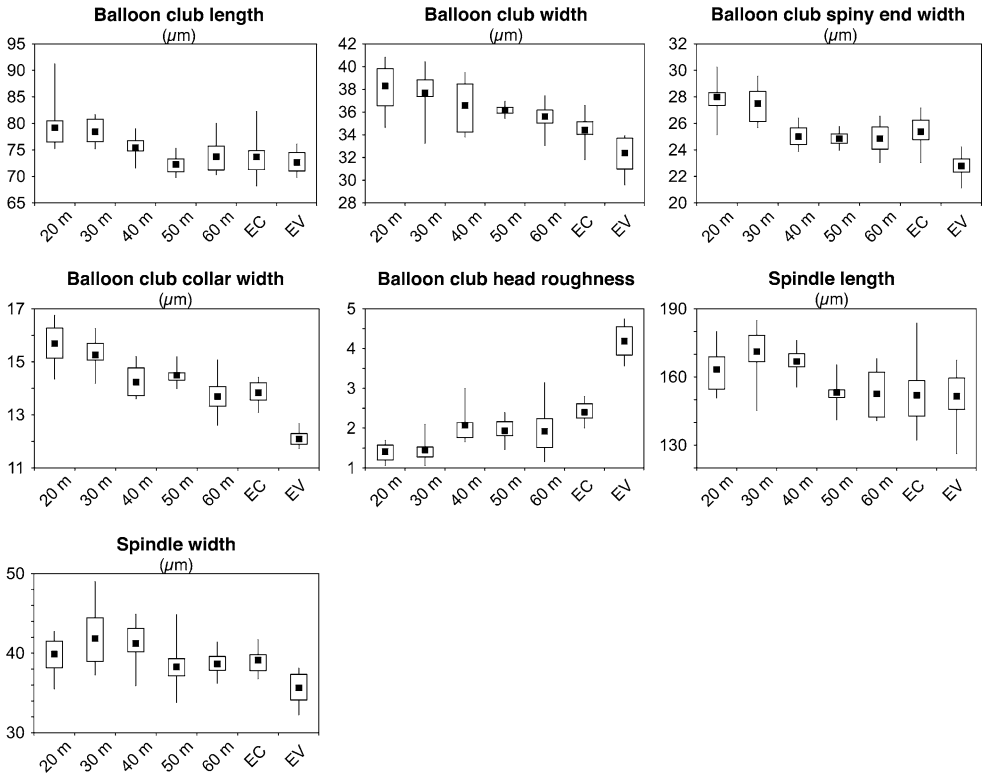

Fig. 4 Sclerite features of colonies of *E. singularis* at each of five depths sampled, and for *E. cavolinii* (EC) and *E. verrucosa* (EV) ($n = 70$); *black square* indicates mean value, *box* indicates first and third quartiles, and *line* indicates range between minimum and maximum values

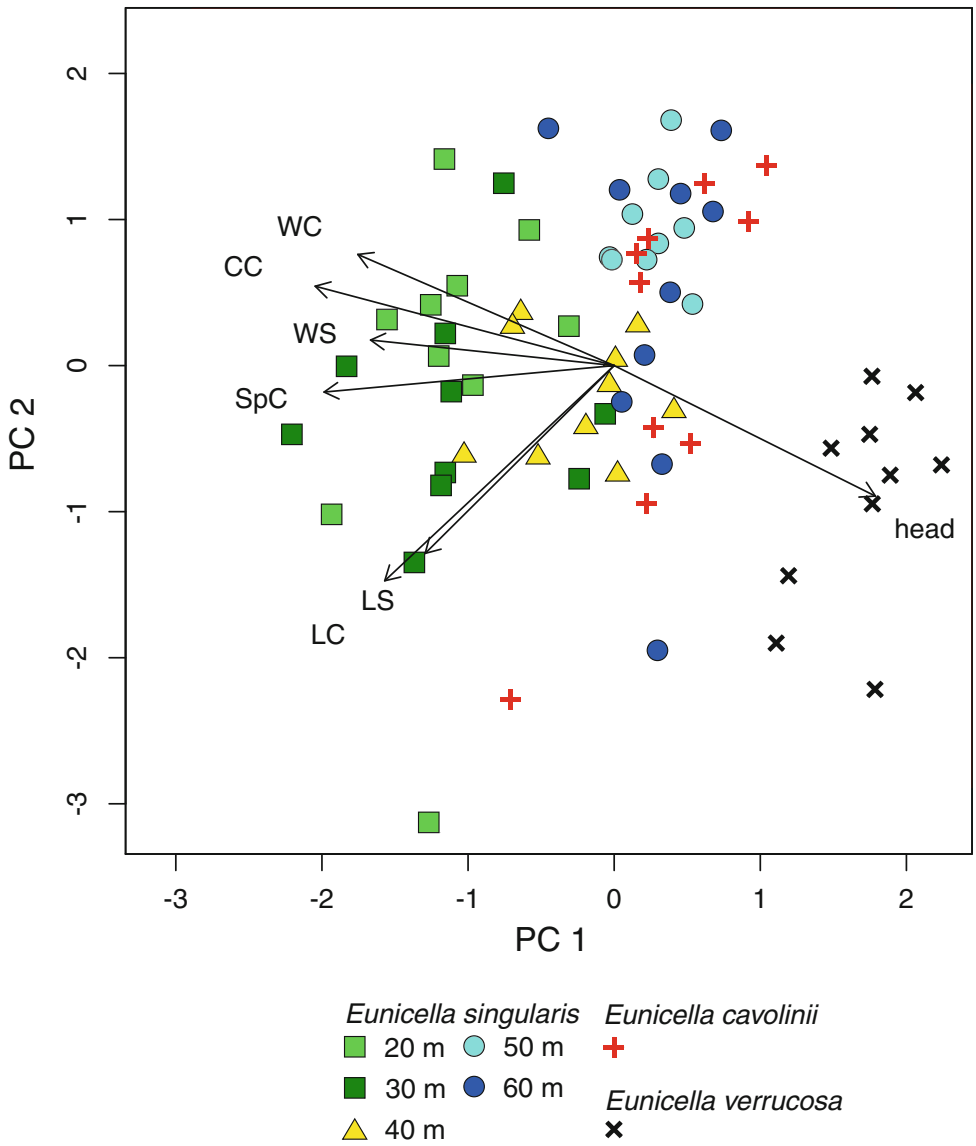

Fig. 5 Principal component analysis (PCA) biplot showing ordination of studied colonies ($n = 70$) with respect to sclerite size, and roles of analyzed features; *LC* mean balloon club length, *WC* mean balloon club width, *SpC* mean balloon club spiny end width, *CC* mean balloon club collar width, *head* mean balloon club head roughness, *LS* mean spindle length, *WS* mean spindle width

these movements as they are a major determinant of the colony's overall structure (Lewis and Von Wallis 1991). In species of *Eunicella*, balloon clubs form a single layer that comprises the outer cortex of the colony; clubs are perpendicular to the axis with the heads exposed on the colony surface. Immediately subjacent to the outer cortex is a layer of densely packed spindles. This arrangement of clubs and spindles limits compression effects (Lewis and Von Wallis 1991). Just as the shallow morphotype of *E. singularis* had larger sclerites than did the deep morphotype, colonies of *E. cavolinii* exposed to strong hydrodynamic conditions had larger balloon clubs than colonies in sheltered sites (Velimirov 1976). Additionally, shallow colonies of *E. singularis* have larger spindles in exposed compared with sheltered sites (Skoufas 2006). Colony shape and sclerite features in exposed colonies of *E. cavolinii* are similar to those in our study of shallow colonies of *E. singularis* from 20 and 30 m. This shows that environmental conditions can strongly influence the macro- and microarchitectural features of gorgonians.

In our study area, the reduction in the annual average of PAR values at and below 40-m depth to <15 % of the PAR values at 20-m depth correlates with the absence of symbiotic zooxanthellae in the gorgonian tissue. This restriction by depth between the zooxanthellate and azooxanthellate morphotypes also corresponds to the approximate depth of the summer thermocline in the study area. As *E. singularis* reproduces in late May and June when the water column is fully stratified (Ribes et al. 2007; Gori et al. 2007), it is possible that larvae of *E. singularis* from shallow colonies are mostly restricted in their recruitment to substrates above the thermocline, whereas larvae from the deep morphotype remain below the thermocline. However, depending on conditions during the summer

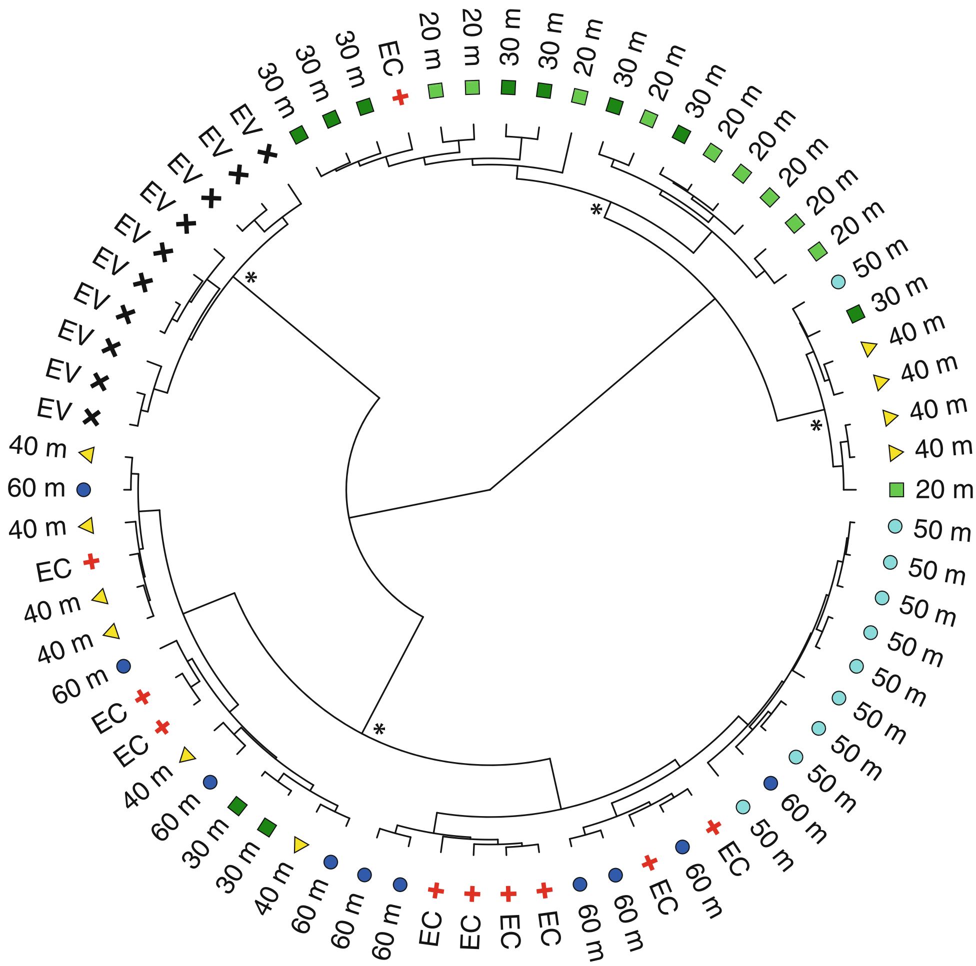

Fig. 6 Cluster analysis of studied colonies ($n = 70$) aggregated by sclerite size; *asterisks* indicate significant clusters determined by the Simprof test ($p < 0.05$)

(Rossi et al. 2011), there may be some years when larvae from shallow colonies are able to recruit to deeper depths, and/or larvae from deeper colonies could recruit to shallower bottoms. As direct transmission of symbiotic algae from the female parent to newly released larvae is suggested for *E. singularis* (Théodor 1969; Weinberg and Weinberg 1979), this variable year-to-year displacement of the thermocline could result in partial overlap in the distribution of shallow and deep morphotypes of *E. singularis* at 30–40 m and could explain the close proximity of colonies of *E. singularis* with and without zooxanthellae observed at 35–38 m (Théodor 1969). When described by Théodor, the deep morphotype of *E. singularis* was proposed as a subspecies (*E. singularis aphyta*; Théodor 1969; Weinberg 1976). Based on our results, we support the existence of two distinct morphotypes of *E. singularis*, with gradual changes in colony shape and sclerite features with depth. The discriminating characteristic of the two morphotypes is the presence/absence of symbiotic algae, indicating that *E. singularis* is not an obligatory symbiotic species.

The mitochondrial *msh1* gene has been used to test the phylogenetic utility of morphology-based classifications of cnidarians (France 2007; McFadden et al. 2009). However, *msh1* did not discriminate between the two morphotypes of *E. singularis* nor did it discriminate among different species of *Eunicella*. A similar result was previously obtained for other genetic markers in which the COI sequences of *E. singularis* and *E. cavolinii* were identical and the ITS2 sequence of a specimen of *E. singularis* was the same as one of the ITS2 haplotypes recovered from *E. cavolinii* (Calderón et al. 2006). This could be a consequence of phenotypic variation within a single species of *Eunicella* or a recent speciation event that is not yet apparent in the genetic markers examined. Thus, further research is necessary to find molecular markers for resolving evolutionary relationships in these species of *Eunicella*, and firmly establish the taxonomic status of the two depth-related morphotypes. Perhaps microsatellites (Molecular Ecology Resources Primer Development Consortium et al. 2010), or additional nuclear molecular markers, will prove useful as they have for other coral and gorgonian species (Gutiérrez-Rodríguez et al. 2004, 2005; Le Goff-Vitry et al. 2004).

The present study is the first to analyze the deep, azooxanthellate morphotype of *E. singularis*, since it was first described by Théodor (1969). It is now important to

explore the frequency and abundance of this aposymbiotic form in other parts of the Mediterranean Sea in order to quantify its ecological role.

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