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Ecological Parallelism and Cryptic Species in the Genus *Ophiothrix* Derived from Mitochondrial DNA Sequences

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We addressed the long-standing problem of species assignment of two nominal species of the genus *Ophiothrix* (Echinodermata, Ophiuroidea) by phylogenetic analysis of a segment of the mitochondrial 16S rDNA. Our phylogeny identified two distinct mitochondrial lineages that do not correspond to the present species assignments. Individuals of the endemic Mediterranean species *O. quinquemaculata* were clustered with individuals of *O. fragilis* in both mitochondrial lineages. We thus suggest that these taxa are not biological species but ecotypes. Differences between the two ecotypes in morphological and physiological characteristics may be explained by adaptation to environmental conditions at different water depths. Despite the observed ecomorphological variability within each of the two major mitochondrial lineages, the large genetic distance (9.0–12.0%) between them does suggest the existence of two distinct biological species. Their reproductive isolation could result from differences in reproductive strategy rather than by ecological and/or morphological differentiation.

Key Words: **Ophiuroidea; Echinodermata; *Ophiothrix quinquemaculata; Ophiothrix fragilis;* polymerase chain reaction; 16S rDNA.**

INTRODUCTION

The Mediterranean Sea is a relatively young subtropical sea. Its eastern basin is a residue of the Thethys, while the western section originated from geological rotation events (Ott, 1988). After a first filling, one dramatic desiccation event was inferred from the presence of extended areas of evaporite in deep basins about 5 to 6 million years ago, separating two layers of deep-water sediments (Hsü *et al.*, 1973). Its present-day fauna originated from the Atlantic region since the Mediterranean Sea was refilled via the Strait of Gibraltar (Field and Gardner, 1991). Probably due to its intermittent connection to the Atlantic Ocean, many endemic species have evolved. There are 147 species of echinoderms described, 25 of which are endemic to the Mediterranean (Pérez-Ruzafa and López-Ibor, 1988), and representatives of eight families of ophiuroids colonize a wide variety of benthic habitats (Riedl, 1983). The genus *Ophiothrix* is particularly interesting since 1 of the 2 described species, *O. fragilis*, has a wide range of distribution, living in both the eastern Atlantic Ocean and the whole Mediterranean Sea, while the second species, *O. quinquemaculata*, is endemic to the Mediterranean. The 2 species are similar in their morphology but seem to have colonized different habitats. *O. fragilis* occurs in a wide variety of habitats, most of which are situated in the littoral zone. It has successfully colonized all types of substrate. In contrast, *O. quinquemaculata* is restricted to deeper zones with detritical bottom at depths between 20 and 60 m and sometimes even at a depth of 250 m (Tortonese, 1965; Riedl, 1983). It prefers soft substrate, but was also found on secondary hard substrate (coralligeneous rock). They inhabit regions of moderate current exposure and are suspension feeders with a characteristic feeding technique. They "sit" on the substrate using the anterior part of three arms, elevating the distal sections and the two remaining arms into the current to collect food particles with their tube feet (Warner, 1971; Warner and Woodley, 1975).

The identification of the two species is problematic since both display a high degree of morphological variation. They are currently distinguished by several diagnostic characters. Of these, the number, shape, and relative lengths of dorsal arm spines and the cross-sectional geometry of arm segments are most decisive. Since these major diagnostic characters can have intermediate states, additional characters must be examined. These are arm proportions, shape of radial shields, and shape of dorsal arm shields (see Table 1 for a character analysis of the surveyed individuals). The ecological separation of the two species is also problematic since *O. fragilis* was found to aggregate in the same manner and to display the same type of feeding in the

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TABLE 1

Morphological and Genetic Characterization of Five Specimens of *O. quinquemaculata* and Seven Specimens of *O. fragilis*

Ind. No.	Genotype	Lin.	Dorsal arm spines: **No.**	**Shape**	**Relative length**[a]	**Cross-sectional geometry of arm segments**	Shape of central disc	Color of: Central disc	Arms	Spines
					O. quinquemacula					
1	G3	I	**6**	**Long narrow**	**5**	**O.q-type**	Pentagonal	Gray with yellow dot	Alternating (2 white, 2 brown segments)	White
2	G3	I	**6**	**Long narrow**	**4**	**O.q.-type**	Round	Dark gray	White	White
3	G3	I	**6**	**Long narrow**	**5**	**O.q.-type**	Pentagonal	Light gray	White	White
4	G4	II	**6**	**Long narrow**	**5**	**O.q.-type**	Pentagonal	Dark gray	Brown	White
5	G4	II	**7**	**Intermediate**	**4**	**Intermediate**	Pentagonal	Brown	Alternating (3 brown, 1 red segment)	White
					O. fragilis					
6	G4	II	**6**	**Stocky**	**3**	**O.f.-type**	Round	Red marbled	Red	Red
7	G4	II	**7**	**Stocky**	**3**	**O.f.-type**	Round	Gray	Alternating (2 red, 2 brown segments)	Beige
8	G5	II	**7**	**Stocky**	**4**	**O.f.-type**	Pentagonal	Dark brown with white dot	Brown	Green
9	G7	II	**7**	**Stocky**	**3**	**O.f.-type**	Round	Dark brown	Brown	Beige
10	G4	II	**7**	**Stocky**	**2**	**O.f.-type**	Round	Dark brown with black dots	Brown	Green
11	G6	II	**7**	**Stocky**	**4**	**O.f.-type**	Pentagonal	Dark brown	Alternating (3 light, 1 dark brown segment)	Beige
12	G1	I	**7**	**Stocky**	**2**	**O.f.-type**	Pentagonal	Light brown	Alternating (2 light, 2 dark brown segments)	White
13	G2	I	**7**	**Stocky**	**2**	**O.f.-type**	Round	Purple	Light purple	White
14	G2	I	**7**	**Stocky**	**2**	**O.f.-type**	Round	Purple	Alternating (2 light, 2 dark brown segments)	White

Note. The diagnostic morphological characters most commonly used to differentiate the two *Ophiothrix* species (columns with boldface type) are the number, shape, and relative length of dorsal arm spines, as well as the cross-sectional geometry of the arm segments. *O. quinquemaculata* has six long narrow dorsal arm spines, with a drastic length increase to the fourth or fifth spine (referred to as O.q.-type in the table), while the seven dorsal arm spines of *O. fragilis* are stocky and their length increases continuously to the fifth spine (referred to as O.f.-type in the table). Two characters (shape of central disc and coloration) are listed to show their variability among the surveyed individuals. Ind. No., individual number; Lin., lineage.

[a] The measure for the relative length is the number of arm segments covered by the longest dorsal spine of each group.

Atlantic as *O. quinquemaculata* in the Mediterranean Sea (Broom, 1975). Additional taxonomic problems within this genus are caused by the distinction of several morphotypes of *O. fragilis*, with a degree of morphological differentiation similar to that found between *O. fragilis* and *O. quinquemaculata*. The taxonomy of *Ophiothrix* is still unsettled and the assignment of *O. fragilis* and *O. quinquemaculata* to one or two separate species is still disputed (Guille, 1964; Tortonese, 1965).

Molecular data addressing taxonomic problems among closely related taxa are scarce for echinoderms (Feral *et al.*, 1995, 1998; Laurin *et al.*, 1994; Poulin and Feral, 1994; Lafay *et al.*, 1995; Hensley *et al.*, 1995; Smith *et al.*, 1995). This is due to the fact that many mitochondrial "universal" primers did not amplify in some groups of echinoderms, such as ophiuroids. This fact is likely to be caused by the great evolutionary age of echinoderm classes, resulting from their early divergence. We have designed a specific primer for the 16S rDNA region and have attempted to clarify the taxonomy of the two *Ophiothrix* species.

MATERIALS AND METHODS

To test the degree of phylogenetic differentiation, we sequenced a 321-bp segment of the mitochondrial 16S rDNA of nine individuals of *O. fragilis* from the northern Adriatic Sea and the Irish Sea and five individuals of *O. quinquemaculata* from the northern Adriatic Sea. Individuals 1, 2, 3, 4, and 7 (see Table 1) were dredged 1.5 nautical miles south of Island "Sv. Ivan na Pucini"

(Rovinj, Croatia) at 36 m depth, while individuals 5 and 6 were dredged 1 nautical mile southeast of Island Sv. Ivan na Pucini at 32 m depth. Individuals 8, 9, 10, and 11 were collected at the Beach of Fazana (Pula, Croatia) at a depth of about 1 m, and Individuals 12, 13, and 14 were collected at Bay Stacka (Isle of Man, Great Britain) at a depth of 30 m. The specimens were classified twice independently, once by us, and a second time by D. Zavodnik (Center of Marine Research, Ruder Boskovic Institute, Rovinj, Croatia), by analyzing the morphological characters listed in Table 1.

DNA was extracted from gonad tissue of ethanol-preserved specimens. Total DNA was extracted using Chelex 100 (Bio-Rad). The orthologous segment of the 16S rDNA of three sea urchin species was obtained from GenBank to be used as outgroups: *Paracentrotus lividus* (Cantatore *et al.*, 1989; Accession No. J04815), *Strongylocentrotus purpuratus* (X12631), and *Arbacia lixula* (X80396).

Tissue was placed in 500 µl of 5% Chelex 100 (see also Walsh *et al.*, 1991) in sterile H_2O and incubated at 56°C for 3 h. After a brief vortexing, extracts were incubated at 95°C for 15 min and then stored at 4°C. Extracts were centrifuged at 13,000 rpm for 5 min before use. Aliquots (1 µl) of the supernatants were directly used for PCR.

Of the published 16S rDNA primers, one primer (16-sar; Kessing *et al.*, 1989) did not amplify in *Ophiothrix,* while the second did (16-sbr; Kessing *et al.*, 1989). We designed a new primer (5′-GAGTCCTGCCTGCCCAGTGA-3′, named 16-sar2) on the basis of seven published sequences covering a large phylogenetic spectrum. These species were *Asterina pectinifera* (Asakawa *et al.*, 1995; Accession No. D16387), *Bos taurus* (J01394), *Gallus gallus* (X52392), *Mus musculus* (J01420), *P. lividus* (J04815), *S. purpuratus* (X12631), and *Xenopus laevis* (M10217, X01600, X01601, and XD2890). Both primers were biotinylated at the 5′-end for nonradioactive sequencing.

Two amplifications (one double-stranded and one single-stranded) were conducted as described elsewhere (Sturmbauer and Meyer, 1993). Single-stranded amplification products were ultrafiltrated three times with 300 µl H_2O in spin colums (Millipore 30,000) before direct sequencing (Sequenase Images Non-Isotopic DNA Sequencing System; US Biochemical; Sanger *et al.*, 1977). Both strands were sequenced in six specimens of both species, while only one strand (using the 16 sbr primer) was sequenced of the remaining individuals. Individual 4 was processed twice independently, since its assignment turned out to be crucial for our conclusions. The DNA sequences were electrophoresed on 6% acrylamide–urea gels in Tris–borate–ethylenediamine–tetraacetate buffer (27 mM, pH 8.0).

Phylogenetic Analyses

The sequences were entered and aligned by eye in ESEE (version 1.09; Cabot and Beckenbach, 1989). A single insertion–deletion event had to be inferred in order to align the ingroup sequences. First, a "sliding window" analysis was performed to evaluate the degree of variation within the sequence data set (see also Sturmbauer *et al.*, 1996). The percentage of variation within windows of 9 bp was determined with a 3-bp overlap. The genetic variation was expressed as a percentage of the 27 possible base substitutions in a window of 9 bp. Two classes of variation were defined: $<10\%$ as regions of low variation and $>10\%$ as regions of high variation. Transition–transversion ratios were analyzed for these two regions separately and translated into weights to be used in parsimony analysis.

Phylogenetic analysis was performed by applying the parsimony and neighbor-joining methods (Saitou and Nei, 1987) in parallel, using PAUP (version 3.1.1; Swofford 1993) and NJBOOT2 (Tamura, 1993). For parsimony analysis the PAUP options "exhaustive search" and ACCTRAN were chosen.

The phylogenetic analysis was performed in two steps. In the first analysis we used three published sequences of sea urchins as an outgroup using the PAUP option "monophyletic sister group." Since the high degree of variation caused ambiguities in alignment, we restricted our analysis to a highly conserved region of 128 bp, 41 positions of which were variable and 36 of which were also phylogenetically informative. In a second step we analyzed all 321 bp of the genus *Ophiothrix,* polarizing our phylogenetic inferences by midpoint rooting. This analysis is based on 38 variable positions, 30 of which were also phylogenetically informative. Transversions were weighted two times over transitions in regions of low variation and with equal weights (but a base weight of 2) in regions of high variation, based on the observed relative frequencies. Indels were weighted like transversions due to their low frequency. For neighbor-joining, *p* distances including indels were used. Genetic distances are given as modified Kimura distances (Kimura, 1980), which were calculated by DNADIST in PHYLIP (Felsenstein, 1993). Differential weighting resulted in the same topology as using all equal weights. Thus, only the phylogenetic trees based on equal weights are shown here. Phylogenies were subjected to bootstrap analysis (Felsenstein, 1985) in both parsimony and neighbor joining. We also calculated decay indices of the branches (Bremer, 1994) for all branches using the computer program AutoDecay (Version 2.7.; Eriksson, 1996). The DNA sequences of this study are available from EMBL (Accession Nos. AJ002789–AJ002795).

RESULTS

Seven different mt genotypes were found among 14 sequenced individuals of the two species of *Ophiothrix.* These were grouped into two major mt lineages that corresponded neither to the present taxonomic assignments nor to geographic regions. Genotypes G1, G2,

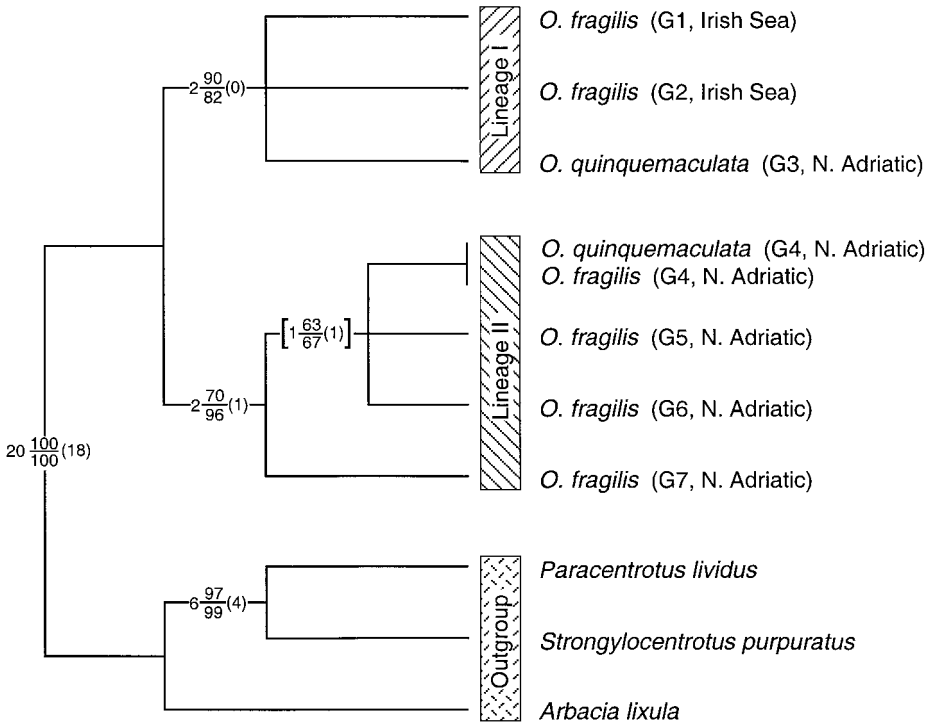

FIG. 1. Phylogenetic relationships of the seven identified mt genotypes of *Ophiothrix fragilis* and *O. quinquemaculata*. The phylogeny combines the results of two analytical steps, depicted as a strict consensus tree of all obtained most parsimonious trees and the neighbor-joining tree. The first step with *Paracentrotus, Strongylocentrotus,* and *Arbacia* as outgroups yielded two most parsimonious trees (tree length, 55 mutations; consistency index excluding uninformative sites, 0.94), and the second step focusing on *Ophiothrix* only resulted in four most parsimonious trees (tree length, 39 mutations; consistency index excluding uninformative sites, 0.97). Numbers above the branches are bootstrap values obtained by parsimony, and those below the branches are from neighbor joining. The numbers on the right-hand side of the bootstrap values are decay indices (Bremer, 1994); those to the left are the minimum number of synapomorphs assigned to the branch. Values in brackets are from the second step of analysis. The phylogeny is based on all equal weights, the outcome of which was identical to weighted parsimony according to the observed relative frequencies of transitions.

and G3 (lineage I) differed by between 1 and 6 mutations (0.3–1.9%) and genotypes G4, G5, G6, and G7 (lineage II) by between 1 and 3 mutations (0.3–0.9%), while between 28 and 35 mutations (9.0–12.0%) were found among individuals of different lineages. Each of the two major mt lineages contained individuals assigned to both *O. fragilis* and *O. quinquemaculata*. Two individuals of *O. quinquemaculata* shared the same genotype with three individuals of *O. fragilis* (genotype 4 in Table 1; see also Fig. 1). One mt lineage was exclusively found in the northern Adriatic Sea, and the second was found in both the Atlantic (Irish Sea) and the northern Adriatic Sea.

The phylogenetic analysis of the evolutionarily conserved segment of the 16S rDNA using *Paracentrotus, Strongylocentrotus,* and *Arbacia* as outgroups resulted in two most parsimonious trees of a length of 55 mutations [consistency index excluding uninformative sites, 0.94 (Kluge and Farris, 1969); total support index, 0.42 (Bremer, 1994)]. The topologies corroborated the split into two lineages according to the analysis of distances. The second analysis including only the genus *Ophiothrix* resulted in four most parsimonious trees of a length of 39 mutations (consistency index excluding uninformative sites, 0.97; total support index, 0.72), also resulting in the same major split as suggested by genetic distances (see Fig. 1).

DISCUSSION

The taxonomy of the genus *Ophiothrix* poses a long-standing problem to marine systematists that has been in dispute for over 130 years. Lyman stated in 1876 that "*Ophiothrix* is the *Salmo* of the echinoderms! Well defined and peculiar as a genus, it has a crowd of species, many of which are the dispair of the specific zoologist!" Owing to their extreme level of variation in morphology, color, and ecology, to an extent that even populations can be differentiated from each other, up to 12 species were described from the Mediterranean Sea (reviewed in Guille, 1964). Falling into the opposite extreme, Marktanner-Turneretscher (1887) and Von Marenzeller (1895) fused them all into a single species, *O. allopecurus*. Koehler (1924) established *O. quinquemaculata* as a valid species postulating it to be easily discernable from the second, more variable spe-

cies. He further described four variants (subspecies) of *O. fragilis,* each with distinct but overlapping features. Finally, Guille (1964) and Tortonese (1965) again questioned the species status of *O. quinquemaculata,* bringing the state of discussion back to the turn of the century.

Our phylogenetic analysis of mitochondrial DNA sheds light on two aspects of this conflict. The five individuals of *O. quinquemaculata* did not cluster into a single clade but were split into two distinct lineages. Each of these lineages contained individuals assigned to either *O. fragilis* or *O. quinquemaculata.* Moreover, some individuals assigned to different species turned out to have identical genotypes. Since *O. quinquemaculata* and *O. fragilis* possess distinct ecological and physiological characteristics (Baric, 1997; authors' unpublished results) we suggest that they are not biological species but ecotypes. Our finding of ecomorphological plasticity in *Ophiothrix* is similar to the results of Southward *et al.* (1995), who demonstrated high morphological variability of the vestimentiferan tube worm *Ridgeia piscesae.* The authors also related the observed morphological variability to habitat effects since no genetic differentiation among different populations was found.

The placement of individuals of *O. quinquemaculata* in both mitochondrial lineages indicates that the morphology of both lineages must be equally plastic, so that corresponding ecotypes were independently formed. That the presence of *O. quinquemaculata* in both mt lineages is a result of hybridization seems unlikely when genetic distances are considered: while individuals of the same lineage differ by a small number of mutations, the two identified mt lineages are highly distinct, being separated by Kimura distances of between 9 and 12%. Even if species status cannot be inferred from genetic distances alone, the large genetic distances among the two lineages point to the existence of two distinct species. For example, two species of sea urchins, *Strongylocentrotus purpuratus* and *S. droebachiensis,* differed by 6% in the mitochondrial genome (Palumbi and Wilson, 1990). Among individuals of a species of fiddler crabs from the Atlantic and Pacific coasts of the Isthmus of Panama a rate of 0.9% per million years was estimated for the orthologous segment of the 16S rDNA (Sturmbauer *et al.,* 1996). That the existence of cryptic species, especially in aquatic benthic habitats, seems to be a far more widespread phenomenon as previously thought is demonstrated by a quickly growing body of evidence from published genetic works (Grassle and Grassle, 1976; Vrijenhoek *et al.,* 1994; Southward *et al.,* 1995; Bastrop *et al.,* 1997, 1998).

Reproductive isolation among the two lineages could result from differences in the reproductive strategy rather than by ecological and/or morphological differentiation, since the delineation of *Ophiothrix* does not agree with morphological forms or species described so far nor to zoogeography. Similar phylogeographic surveys yielded a different result for other echinoderms. Analysis of Mediterranean and Atlantic populations of *Echinocardium cordatum,* based on rDNA and allozymes, showed a clear genetic separation between a Mediterranean and an Atlantic geographic cluster (Feral *et al.,* 1998). In *Ophiothrix,* lineage I has been found in both the Atlantic Ocean and the Mediterranean Sea, while the second lineage has so far been found in the Mediterranean Sea only. Whether lineage II is endemic to the Mediterranean can be answered only after larger sample sizes have been analyzed and additional samples from the Atlantic are included. One might speculate that lineage II represents the primary colonizers after creation of the Mediterranean Sea about 5 million years ago and has since evolved to a distinct species, while the individuals of lineage I represent a second invasion, caused by natural means or caused by ballast water of ships (Carlton, 1985, 1987; Carlton and Geller, 1993). That their invasion must have happened rather recently is suggested by the small observed Kimura distances of 1.6 to 1.9% among individuals of this lineage. A more exact timing of this event will be possible after more population samples of the Atlantic and the Mediterranean Sea are analyzed. Moreover, characterization of populations within the two identified mitochondrial lineages will profit from the use of more variable genetic markers such as polymorphic microsatellite DNA.

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