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Molecular Systematic Analyses of Mediterranean Skates (Rajiformes)

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Abstract: Mitochondrial 16S ribosomal RNA was used to elucidate the pattern of relationships and systematic status of 4 genera, including 9 species of skates (*Rostroraja alba, Leucoraja fullonica, Dipturus oxyrinchus, D. batis, Raja clavata, R. montagui, R. asterias, R. miraletus,* and *R. radula*), living in the Mediterranean and Black Seas. The 16S rDNA dataset contained 39 variable and 29 parsimony informative sites. The mean nucleotide diversity (*Pi*) was 0.018. The genetic distances between pairs of species showed genetic homogeneity between *R. clavata* and *R. montagui*. The highest value of sequence divergence was detected between *D. oxyrinchus* and *R. asterias* (0.040). Low genetic distances were shown by the comparisons of *R. montagui* with *R. miraletus* and of *R. clavata* with *R. miraletus*. The phylogenetic tree revealed 4 main evolutionary lineages; the first lineage included *R. clavata, R. montagui*, and *R. miraletus. L. fullonica* was clustered with *R. asterias* in the second lineage. In the third lineage, *D. batis* and *D. oxyrinchus* showed a close relationship, which was the sister group to *R. radula*. *R. alba* was clustered separately from all others.

Key Words: 16S rRNA, mtDNA, sequence, phylogenetic relationships, Rajidae

Mitokondrial 16S rDNA Sekans Analizi ile Akdeniz Vatozlarının (Rajiformes) Moleküler Sistematik Analizi

Özet: Bu çalışmada Akdeniz ve Karadeniz'de yaşayan dört cins ve dokuz türü içine alan vatozlar (*Rostroraja alba, Leucoraja fullonica, Dipturus oxyrinchus, D. batis, Raja clavata, R. montagui, R. asterias, R. miraletus, R. radula*) arasındaki filogenetik ilişkinin şekli ve sistematik durumları mitokondrial 16S ribosomal RNA geni kullanarak ortaya çıkartılması planlanmıştır. 16S rDNA geni verilerinden 39 değişken ve 29 parsimoni informatif bölge saptanmıştır. Ortalama nükleotid çeşitliliği 0,018 olarak bulunmuştur ve türler arasındaki genetik uzaklık değerlerine bakıldığında *R. clavata* ve *R. montagui* arasında genetik homojenlik olduğu gözlenmiştir. En yüksek genetik farklılık değeri (0,040) *D. oxyrinchus* ve *R. asterias* türleri arasında gözlenmiştir. En düşük genetik farklılık değerleri ise *R. montagui* ve. *R. miraletus*, ve *R. clavata* ve *R. miraletus* türleri arasında gözlenmiştir. Filogenetik ağaç dört evrimsel soy ortaya çıkarmıştır; birinci soy *R. clavata*, *R. montagui* ve *R. miraletus* türlerini içermektedir. İkinci soyda *L. fullonica* ile *R. asterias* türleri birlikte gruplandırılmıştır. Üçüncü soyda *D. batis* ve *D. oxyrinchus* türleri birlikte gruplandırılmış olup *R. radula* ile yakın ilişki göstermiştir. *R. alba* ise soyağacında en farklı şekilde gruplandırılmıştır.

Anahtar Sözcükler: 16S rRNA, mtDNA, sekans, filogenetik ilişki, Rajidae

Introduction

The skates are found worldwide in marine waters, from the Atlantic to Pacific Oceans, and they are distributed from shallow coastal shelves to abyssal regions and are typically long-lived and feed upon invertebrates such as mollusks and crustaceans (McEachran and Miyake, 1990; Last and Stevens, 1994; Nelson, 1994). Skates have high species diversity (Last and Stevens, 1994; Nelson, 1994), but show high levels of morphological and ecological conservatism (Nelson, 1994; McEachran and Dunn, 1998), which usually lead to misclassification of species within the family (McEachran and Dunn, 1998). In the Mediterranean, the family Rajidae is represented by a complex of 33 species within 9 genera, *Raja*, *Rostroraja*, *Rajella*, *Malacoraja*, *Leucoraja*, *Dipturus*, *Breviraja*, *Amblyraja*, and *Bathyraja* (Stehmann and Burkel, 1984; Nelson 1994; McEachran and Dunn, 1998). The longnosed skate, *Dipturus oxyrinchus* (Linnaeus 1758), distributed in the Eastern Atlantic, central Norway to Senegal, and the Mediterranean Sea, is

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found on sand and sand-rock bottoms, and feeds on all kinds of bottom animals. *D. oxyrinchus* was formerly recognized as *Raja oxyrinchus* (Stehmann and Burkel, 1984). The blue skate, *Dipturus batis* (Linnaeus 1758), is a critically endangered species distributed in the Eastern Atlantic, from Norway and Iceland waters to Senegal, including the Mediterranean, and feeds on all kinds of bottom animals; large individuals prefer fish. *D. batis* was formerly classified in the genus *Raja* as *Raja batis*. The Shagreen ray, *Leucoraja fullonica* (Linnaeus 1758), feeds on all kinds of bottom animals, probably preferring fish, and is distributed throughout the Mediterranean, as well as in the Eastern Atlantic, from southern Iceland in the north to northern Morocco in the south (Stehmann and Burkel, 1984). *L. fullonica* was formerly classified in the genus *Raja* as *Raja fullonica* (Stehmann and Burkel, 1984). The bottlenosed skate, *Rostroraja alba* (Lacepède 1803), is an endangered species found in the Eastern Atlantic, from Ireland and England southward to central Mozambique, including the Mediterranean, and feeds on other elasmobranchs, crabs, and cuttlefish. *Rostroraja alba* was formerly classified in the genus *Raja* as *Raja alba*. The thornback ray, *Raja clavata* Linnaeus 1758, is a threatened species widely distributed in the Eastern Atlantic, ranging from Norway and Iceland to Northwest Africa, including the Mediterranean and Black Seas, and feeds on all kinds of bottom animals, preferably crustaceans. The spotted ray, *Raja montagui* Fowler 1910, is distributed in the Eastern Atlantic from the Shetlands to Mauritania, including the Mediterranean; it inhabits shelf waters and feeds mainly on crustaceans. The starry ray, *Raja asterias* Delaroche 1809, is endemic to the Mediterranean but may spread to the Strait of Gibraltar, northern Morocco and possibly south to Mauritania and inhabits inshore waters and it feeds on all kinds of benthic animals. The brown ray, *Raja miraletus* Linnaeus 1758, is distributed in the Eastern Atlantic, northern Portugal, and throughout the Mediterranean, is found over soft bottoms of the shelf and the uppermost slope, and feeds on all kinds of benthic animals. The rough ray, *Raja radula* Delaroche 1809, is endemic to the Mediterranean and feeds on all kinds of bottom animals.

In spite of the wide scientific interest given to skates, systematic relationships of the family Rajidae are still controversial. Molecular studies focusing on systematics of skates are still rare (Rocco et al., 1996; Tinti et al., 2003; Valsecchi et al., 2005). Mitochondrial DNA analysis is a useful molecular marker for systematics because of its special features (Meyer et al., 1990, Normark et al., 1991; Meyer, 1992). The pattern of maternal inheritance and rapid rate of evolutionary change of mtDNA compared to nuclear DNA make it a suitable tool for genetic studies among taxa of several fish groups at multiple taxonomic levels (Kocher and Stepien, 1997; Zardoya et al., 1999; Durand et al., 2002). The 16S rDNA sequence as well as other mtDNA gene markers was widely adopted to discriminate among phylogenetically related species of marine organisms (Kocher and Stepien, 1997; Heist and Gold, 1999; Perez et al., 2005).

In the present study, the pattern of relationships and systematic status of 4 genera, including 9 species of skates (*Rostroraja alba, Leucoraja fullonica, Dipturus oxyrinchus, D. batis*, *Raja clavata*, *R. montagui*, *R. asterias, R. miraletus*, and *R. radula*), living in the Mediterranean Sea were investigated with mitochondrial 16S rDNA gene sequence data.

Materials and Methods

Skates were collected in İskenderun Bay, northeast Mediterranean Sea and Black Sea during trawl surveys, and specifically recognized according to the available referenced guidelines and identification keys for Mediterranean species (Stehmann and Burkel, 1984; McEachran and Dunn, 1998). The number, location, and other details of the samples used in the sequence analyses are given in Table 1.

After mechanical removal of the skin, white muscle tissue (0.2-0.4 g) was sampled and stored in 98% ethanol. The amplification of mitochondrial 16S rDNA by PCR was performed with a profile of 94 °C for 4 min, followed by 35 cycles of 94 °C/30 s strand denaturation, 52 °C/20 s annealing, 72 °C/1 min 30 s primer extension, and a final 7 min elongation at 72 °C. The 16S rDNA amplification conditions were as follows: 1.5 µl 10 × polymerase buffer, 0.5 µl dNTP (10 mM), 0.3 µl *Taq* DNA polymerase (3 U/µl) equivalent to *Taq* DNA polymerase, 0.05 µl 16Fi140 primer (100 µM) (5´-CG(CT)AAGGGAA(ACT)GCTGAAA-3´), 0.05 µl 16Fi1524 primer (100 µM) (5´-CCGGTCTGAACTCAGATCACGTAG-3´), 3-5 µl DNA from AGOWA purification, and water for a total reaction volume of 15 µl. Amplified DNA was purified with Exo/Sap enzymes according to supplier's protocol (Cleveland, Ohio, USA). Finally, all the samples

Table 1. Sampling details of skate species and GenBank accession no for 16S rDNA gene. n, sample size used in the analyses.

Species	Sampling location	Latitude	n	GenBank Accession No.
Rostroraja alba	Northeastern Mediterranean Sea	36°05'N 36°65'E	2	EU476882 EU476884
Leucoraja fullonica	Northeastern Mediterranean Sea	36°05'N 36°65'E	2	EU476883 EU476889
Dipturus batis	Northeastern Mediterranean Sea	36°05'N 36°65'E	2	EU476891 EU476892
D. oxyrinchus	Northeastern Mediterranean Sea	36°05'N 36°65'E	1	EU476893
Raja montagui	Black Sea	41°10'N 39°36'E	1	EU476889
R. clavata	Black Sea	41°10'N 39°36'E	3	EU476886 EU476887 EU476888
R. miraletus	Northeastern Mediterranean Sea	36°05'N 36°65'E	1	EU476885
R. radula	Northeastern Mediterranean Sea	36°05'N 36°65'E	3	EU476894 EU476895 EU476896
R. asterias	Northeastern Mediterranean Sea	26°85'N 38°35'E	2	EU476897 EU476898

were sequenced in the forward (16Fiseq1463: 5´-TGCACCATTAGGATGTCCRGATCCAAC-3´) and reverse (16sarL: 5´-CGCCTGTTTAACAAAAACAT-3´) directions with an automated sequencing machine (Model ABI3730, Applied Biosystems).

Individual haplotypes were aligned using the CLUSTAL X Multiple Sequence Alignment program (Thompson et al., 1997), and final alignment was completed manually with BioEdit (Hall, 1999). mtDNA sequence data were analyzed to assess levels of pairwise nucleotide variation and to determine nucleotide composition for each taxon using MEGA 3.1 (Kumar et al., 2004). Modeltest (Posada and Crandall, 1998) was used to determine the best-fit model of DNA evolution. The TrN (Tamura and Nei, 1993) model was determined to be the appropriate model for our dataset. Phylogenetic analyses were performed using neighbor-joining (NJ), maximum parsimony (MP), and minimum evolution (ME) methods implemented in the MEGA 3.1 program. The robustness of the internal branches of trees was assessed by bootstrapping (Felsenstein, 1985) with 1000 replicates. The values above, below, and inside branches of the tree indicate the bootstrap percentages for the neighbor joining (NJ), maximum parsimony (MP), and minimum evolution (ME) analyses, respectively.

One species from the family Squatinidae (Elasmobranchii) was included in the molecular phylogenetic trees and rooted as an outgroup species from published sequences in GenBank under accession number *Squatina squatina*, DQ922643.

Results

After alignment, the partial 16S rDNA gene sequences consisted of 718 bp fragments. Examination of the gene reveals a lack of cytosine (C; 18.8%) and abundance of thymine (T; 32.5%). The 16S rDNA dataset contained 39 variable and 29 parsimony informative sites. The mean nucleotide diversity (*Pi*) was 0.018. The sequence analysis of 16s rDNA revealed 8 different haplotypes. Haplotype diversity was 0.90. The minimum spanning tree illustrating the phylogenetic relationships between 16S haplotypes is given in Figure 1. The *L. fullonica* haplotypes were located between haplotypes of the species of the genus Raja.

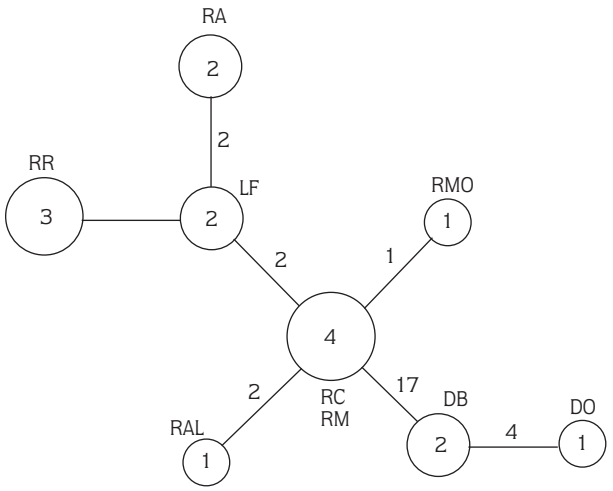

Figure 1. Minimum spanning tree that shows the relationships among the haplotypes. Each circle represents a different sequence. The size of the circle represents the sequence's relative frequency; connecting line is also labeled with the number of positional differences between the 2 sequences. DO, *D. oxyrinchus;* DB, *D. batis*; RM, *R. miraletus*; RC, *R. clavata*; RAL, *R. alba*; LF, *L. fullonica*; RR, *R. radula*; RMO, *R. montagui*; RA, *R. asterias*.

The Tamura-Nei genetic distances between pairs of species (Table 2) showed that there was no sequence divergence between *R. clavata* and *R. montagui*, and the highest value of sequence divergence was between *D. oxyrinchus* and *R. asterias* (0.040). Low genetic distances were also shown by the comparisons of *R. montagui* with *R. miraletus* and of *R. clavata* with *R. miraletus*. Phylogenetic trees constructed with NJ, ME, and MP showed identical topologies (Figure 2), and 4 main evolutionary lineages were recognized. The first lineage included *R. clavata*, *R. montagui*, and *R. miraletus*. The close relationship between these 3 species was supported by good bootstrap values. *L. fullonica* was clustered with *R. asterias* in the second lineage and well supported by bootstrap analysis. In the third lineage, *D. batis* and *D. oxyrinchus* showed a close relationship, from which *R. radula* was more separated. *R. alba* was clustered separately from all others.

Discussion

The nucleotide variation among 16S rDNA haplotypes showed sequence divergences that clearly separated most rajid species including *R. miraletus, R. radula*, and *R. alba.* On the other hand, a low level of genetic divergence was observed between some of the rajid species, which is not within the range frequently observed between species. 16S rDNA gene sequence data used in this study clustered *D. batis* and *D. oxyrinchus* together but under the genus *Raja*. *R. radula* was more divergent than the 2 *Dipturus* species. This finding throws doubt on the present taxonomic status of the genus *Dipturus*, which used to be classified as the genus *Raja* (*R. batis* and *R. oxyrinchus*; Stehmann and Burkel, 1984). A similar observation was also reported by Tinti et al. (2003), who found a closer 16S rDNA relationship of *R. asterias*, *R. clavata*, and *R. montagui* to *D. oxyrinchus* than to *R. miraletus*. Moreover, Valsecchi et al. (2005) reported that the position of *R. miraletus* and *R. undulata* with respect to *D. oxyrinchus* was phylogenetically uncertain, even using different data sets and methods of analysis. On the other hand, the alternative hypothesis can also be given that *R. radula* belongs to the genus *Dipturus*. The close classification of *L. fullonica* with *R. asterias* in the *Raja* clade also calls into question the present taxonomic status of *L. fullonica,* since the past classification of this species was *Raja fullonica* (Stehmann and Burkel, 1984). The haplotype relationships also support the past classification (Figure 1). Based on the present data set, the 16S rDNA

Table 2. Pairwise genetic distance between the skate species.

	Species	1	2	3	4	5	6	7	8	9
1	*R. alba*	-								
2	*R. montagui*	0.003	-							
3	*R. clavata*	0.003	0.000	-						
4	*L. fullonica*	0.006	0.003	0.003	-					
5	*R. miraletus*	0.005	0.002	0.002	0.005	-				
6	*D. batis*	0.030	0.028	0.028	0.030	0.030	-			
7	*D. oxyrinchus*	0.037	0.035	0.035	0.037	0.037	0.007	-		
8	*R. radula*	0.026	0.023	0.023	0.021	0.025	0.032	0.039	-	
9	*R. asterias*	0.010	0.007	0.007	0.003	0.008	0.033	0.040	0.025	-

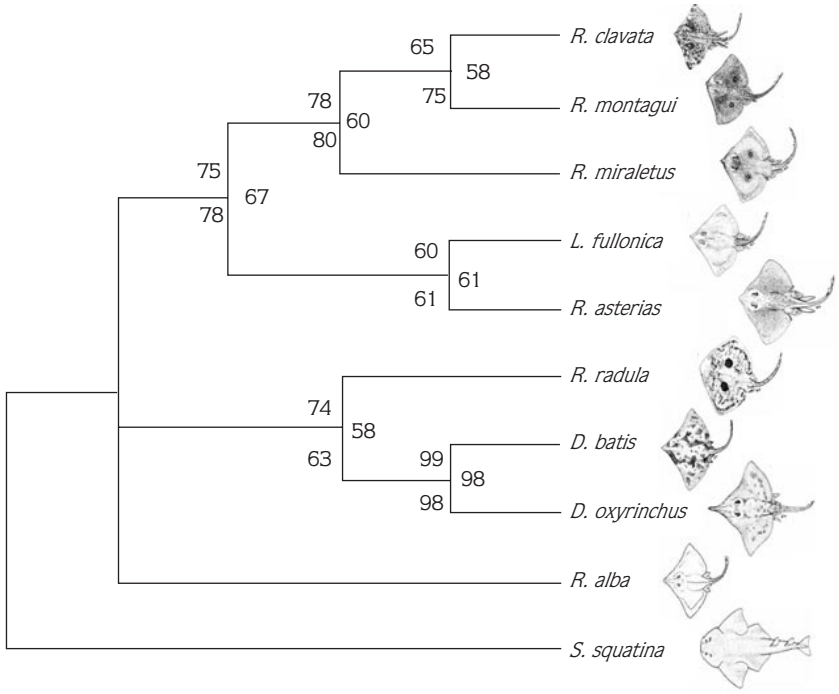

Figure 2. Neighbor-joining phylogenetic tree based on 16S rDNA rajid sequences. The values above, below, and inside branches indicate the bootstrap percentages for the neighbor joining (NJ), maximum parsimony (MP), and minimum evolution (ME) analyses, respectively. All trees were rooted using the homologous 16S rDNA sequence of *S. squatina* as out-group. Skate drawings are taken from fishbase.org.

gene marker may not be an appropriate marker for the identification of these rajid species, especially for *R. clavata* and *R. montagui*. Tinti et al. (2003) also made this suggestion since they investigated phylogenetic relationships of 4 *Raja* species (*R. clavata*, *R. montagui*, *R. asterias*, and *R. miraletus*) and 1 *Dipturus* species (*D. oxyrinchus*) and found low level of sequence divergence between *R. clavata* and *R. asterias* using 16S rDNA. Similarly, Valsecchi et al. (2005) reported that 16S and control region sequence data sets are not good markers for inferring evolutionary relationships in Rajidae.

A second alternative hypothesis concerns a recent hybridization between some species of skates (Avise, 1994; Tinti et al., 2003). The very similar biological and ecological features of the species together with their low evolutionary divergence might favor the production of natural F_1 hybrids from inter-specific mating events in the areas where they are sympatric. However, due to the maternal inheritance of mtDNA in vertebrates, mitochondrial markers can identify only the maternal genome contribution. These markers cannot visualize inter-specific hybridization phenomena that may occur between species with overlapping reproductive, biological, and ecological characters.

On the other hand, as an alternative explanation for this low divergence, skates show high morphologic plasticity. The high plasticity of the characters of these species might affect the correct identification of specimens and lead to misclassification. Therefore, in that circumstance, we sequenced 2 or more same species as different. Thus, further analyses on a larger data set and additional molecular markers (i.e. nuclear genes) will be needed to address an explanation among those proposed.

All these findings lead us to make more extensive comparative analyses including more rajid species distributed in the biogeographic region and using other sequence and morphological markers to resolve this systematic incongruity.

In conclusion, the 16S rDNA data revealed that the inferred species-level topology of *D. alba*, *D. oxyrinchus*, and *L. fullonica* is not congruent with the existing

taxonomic classification since they appear to belong to the genus *Raja*. Moreover, the species *R. clavata* and *R. montagui* are genetically contiguous. Additional molecular genetic analyses based on different parts of the mtDNA and nuclear genome could improve the findings presented here.

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