Revista de Biología Marina y Oceanografía
Vol. 49, Nº2: 367-371, agosto 2014

RESEARCH NOTE

New contribution to the systematic status of various Mediterranean scorpionfish, as inferred from a mitochondrial DNA sequence

Nueva contribución al estado sistemático de diferentes peces escorpión del Mar Mediterráneo, inferida a partir de secuencias de ADN mitocondrial

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Abstract.- This study investigated the molecular phylogeny of 6 Mediterranean species of scorpionfish, belonging to the Scorpaenidae and Sebastidae family. Neighbor-Joining and Maximum Parsimony phylogenetic analyse, based on 424 base pairs of partial mitochondrial DNA sequences of the 12S-rRNA gene, revealed 2 main clades. One clade is represented by the *Scorpaena* genera (with the species *S. notata*, *S. porcus,* and *S. scrofa*) and another clade consists of the genera *Helicolenus*, *Pterois,* and *Scorpaenodes*. The molecular phylogeny showed that the *Scorpaenodes* genus (sub-family Scorpaeninae) is found within the clade of the species belonging to the other two sub-families (Pteroninae and Sebastinae). This pattern is in contrast with current classification and it, therefore, poses a number of problems if using only morphological characters when classifying these families.

Key words: Scorpionfish, mtDNA, 12S-rRNA, Mediterranean Sea

INTRODUCTION

The Scorpaeniformes order is represented in the Mediterranean Sea by two families: Scorpaenidae and Sebastidae, and both families include two sub-families Scorpaeninae and Pteroninae, and Sebastinae and Sebastolobinae, respectively. The Scorpeninae sub-family includes 3 genera (*Pontinus*, *Scorpaenodes* and *Scorpaena*) and 9 species, while the Pteroninae sub-family comprises a single genus and one species (*Pterois miles*). The Sebastinae and Sebastolobinae sub-families are both represented by one genus and one single species *Helicolenus dactylopterus* and *Trachyscopia cristulata echinata*, respectively (Froese & Pauly 2013, WoRMS[1]).

Some of these species have recently entered into the Mediterranean Sea through the Suez Channel or the Strait of Gibraltar. This is the case with *Pterois miles*, a lessepsian migrant, which has recently colonised the southeast of the Mediterranean Sea, and *Trachyscopia cristulata echinata* and *Scorpaena stephanica*, the latter two which have migrated from the Atlantic Ocean (Ciesm 2002). Many species of the Scorpaenidae and Sebastidae families are often very difficult to identify morphologically because the characters used in their identification are not easy to use. For this reason, there are occasionally many problems with correctly identifying the species, especially regarding those belonging to the *Scorpaena* genera.

Until now, many of the studies regarding the systematics and phylogeny of this order have been conducted on species outside the Mediterranean, thus highlighting the complexity of clarifying the position of the high taxonominal categories, *i.e.*, family and genera (Rocha-Olivares *et al.* 1999a, b; Kai *et al.* 2003, Kochzius *et al*. 2003, Shinohara *et al.* 2007). Only a few studies have been conducted on the Scorpaeniformes species in the Mediterranean Sea and these were addressed using cytogenetics (Caputo *et al*. 1998), meristic characters and a genetic analysis of the mitochondrial 16S rDNA gene (Turan *et al.* 2009). These studies have shown a discrepancy between morphological and genetic-cytogenetic results and highlighted the need for a taxonomical re-evaluation of the analysed species.

Hence, a sound knowledge of the systematics of Scorpaeniformes is important not only for taxonomic and evolutionary purposes but also regarding species delimitation in stock assessment studies. Furthermore, it is considered suitable to use molecular markers as a tool with which to discriminate each species and to understand the phylogenetic relationships within the Scorpaenidae family.

[1]WoRMS Editorial Board. 2014. World Register of Marine Species, VLIZ. <http://www.marinespecies.org/index.php>

The aim of this paper was to use mitochondrial DNA, and especially the 12S-rRNA portion, in order to genetically characterise the species analyzed in this paper and to study their phylogenetic relationships. 12S-rDNA is recognised as an important marker for improving species delimitation and for resolving taxonomic relationships in many groups of vertebrate and invertebrate families (Maggio *et al.* 2005, Sirna Terranova *et al.* 2007).

MATERIALS AND METHODS

A total of 30 specimens (5 individuals per species) were collected and analysed. A small piece of caudal fin from each specimen was preserved in ethanol (90%) and the total genomic DNA was extracted using a DNeasy Tissue Kit (Qiagen). The extracted DNA was suspended in distilled water and stored at -20°C until required. Amplification of the mitochondrial encoded 12S-rDNA gene was obtained using universal primers (Kocker *et al.* 1989). PCR was carried out in a Perkin Elmer Cetus Thermal cycler in a 100-µl solution containing 1 ng genomic DNA, 0.2 µM each dNTPs, 0.1 µM of each primer, 10 mM Tris–HCl (pH 9), 50 µM KCl, 1.5 mM MgCl2 and 2.5 U of Perkin Elmer Taq polymerase. The thermal cycling profile for the 12S-rDNA portion began at 94°C for 2 min as a hot start, followed by 5 cycles of 94°C (60 s), 48°C (45 s), and 72°C (60 s), 35 subsequent cycles of 94°C (120 s), 60°C (60 s), and 72°C (60 s), with a final step of 10 min at 72°C for the termination of PCR. PCR products were separated on 2% agarose gel, which had been stained with ethidium bromide. Subsequently, the bands were purified using a Qiaquick PCR purification Kit (Qiagen) and the PCR product sequenced in two directions in an ABI Prism 310 automated sequencer (Applied Biosystem).

Nucleotide sequences were aligned by the ClustalW Multiple Sequence Alignment program (Thompson *et al.* 1994) with default settings. In order to facilitate sequence comparison, we used homologous sequences of *Dicentrarchus labrax* (Perciformes, Moronidae) from GenBank (Accession Number X81566) as an outgroup in the analysis. The sequence analyses were performed using a 4.1 DNAsp version (Rozas & Rozas 1999) and MEGA version 5 (Tamura *et al.* 2011). Phylogenetic analysis was performed with the MEGA and PAUP computer programs (Swofford 2003), using Neighbor-Joining and Maximum Parsimony methods respectively.

RESULTS AND DISCUSSION

A total of 424 base pairs of 12S-rDNA were aligned, 307 were conserved and 112 were variable, of which 93 were parsimony informative (Table 1). The average nucleotide distance (Kimura 2 parameters) varied between a maximum value of 0.219 (*Scorpaena porcus vs. Scorpaenodes arenai*) and a minimum value of 0.088 (*Scorpaenodes arenai vs. Pterois miles*) (Table 2). As expected, most of the observed nucleotide variation was due to transitions.

Table 1. Variable sites of the 12S rDNA sequences of the 6 species of Scorpaeniformes analysed / Sitios variables de las secuencias de 12S rADN de las 6 especies de Scorpaeniformes analizadas

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                                                          111 1111111111
                11111 2222233333 3444566666 6777788889 9999999000 0223356666
           1367901456 3567901458 9018725678 9018901260 1234679012 5780492345

S.scrofa   aagtagaatc ggggactgcc cccgacatga gt-ttcacta tgctta-taa acccctttat
S.arenai   ggtcg.g.gt taaa.tgatt t.aat..c.c ..g.g.gt.t attcctgg.t g.t.ag..g.
S.notata   g.t.gaggg. ...a..... ..a..tg... t.-.....c. .....g-.g. ....t.cag.
S.porcus   .tt.gaggg. t..ag..... .ta..... ..-g...t.g ......-.g. .t.....ct.
H.dacty    cgtcg.ggat c.aa..gat. ..gat.g..c ..a.g....g .t.c..-.gt g...gg..t.
P.miles    cgtcg.g-gt ta.ag..att ..aat..cat .ca.atgt.t .t.c..c.tt g.tttg...a



           1111111111 2222222222 2223333333 3333333333 3333333344 44
           6677888888 0222333455 5691122244 4555555666 6667778811 12
           6708234567 0789079005 8814717801 4256789345 7890273978 90

S.scrofa   ataaattgaa tggtagagaa aggtacaaat gtgggcaagt ggtcatcatt ag
S.arenai   gcct..c.gc aaa..a..gg gc..gtgg.c .ct..tgg.g .taa..gg.. ..
S.notata   gc.....agt ..a.g.ga.. .aac..... a....gg... ..c..g.t.. .a
S.porcus   gc...c.a.t ...a..ga.. .a..... ac...gg.ta at..g...ag g.
H.dacty    gcgtt..agg a.a.....gg gc..gtg.tc .c...t.ga. atatgggg.a gn
P.miles    .cgt...a.. aaa..a..gg gc..gtgg.c ..aaatg..a .t.a..gg.. .a
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No difference was observed among individuals of the same analysed species. When comparing species, a greater number of transitions were observed between *Scorpaena porcus* and *Scorpaenodes arenai* (n. 49); this number was greater than that for transversions (n. 28). The average value of the transition/transversion ratio was 1.90, with a maximum value of 3.25 and a minimum value of 1.48. To estimate the phylogenetic relationships among species, phylogenetic trees were constructed, which were based on the methods of Neighbor-Joining and Maximum Parsimony. Both methods produced phylogenetic trees with the same topology and, for this reason we decided to show only the tree constructed by the Maximum Parsimony method (Fig. 1). The values allocated to the nodes were those calculated on 1,000 bootstrap replicates. All the analysed species were separated by significant bifurcations in which the values were always higher than 50%. The tree revealed two main clades, one represented by the *Scorpaena* genera (with the *S. notata*, *S. porcus* and *S. scrofa* species), and another consisting of the *Helicolenus*, *Pterois*, and *Scorpaenodes* genera.

Table 2. Nucleotide distance among the 6 species analysed according to Kimura-2 parameters / Distancia nucleotídica entre las 6 especies analizadas de acuerdo a los parámetros de Kimura-2

	S. porcus	*S. scrofa*	*S. notata*	*Sc. arenai*	*H. dactylopterus*
Scorpaena porcus					
Scorpaena scrofa	0.101				
Scorpaena notata	0.095	0.094			
Scorpaenodes arenai	0.218	0.197	0.209		
Helicolenus dactylopterus	0.168	0.170	0.176	0.108	
Pterois miles	0.195	0.179	0.207	0.088	0.126

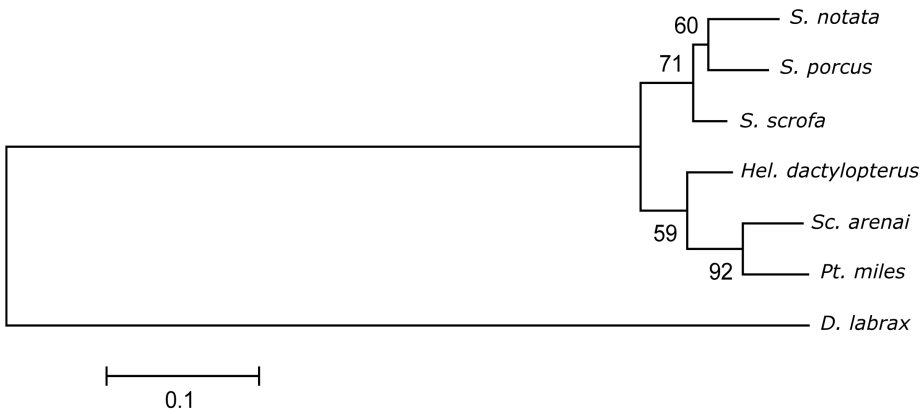

Figure 1. Maximum Parsimony tree based on 12S-rDNA nucleotide sequences of the 6 species of Scorpaeniformes analyzed. Numbers at nodes are bootstrap values based on 1000 Replicates. Scales bar represents an interval of Tamura-Nei genetic distance / Árbol de Parsimonia Máxima basado en las secuencias de nucleótidos del 12S-rADN de las 6 especies de Scorpaeniformes analizadas. Los números de los nodos son los valores de los bootstrap basados en 1000 réplicas. La barra de escala representa un intervalo de la distancia genética de Tamura-Nei

The data reported here place the *Scorpaenodes* genera of the Scorpaeninae subfamily in the same clade as the species belonging to the other 2 subfamilies (Pteroninae and Sebastinae). This conclusion contrasts with the current classification (Froese & Pauly 2014[2]) and reveals various problems with using only morphological characters. According to Turan *et al*. (2009), the use of only morphological characters to identify various species of Mediterranean Scorpaenidae is not sufficient. Indeed, morphology produces different results if compared with the sequences obtained from mitochondrial 16S-rDNA. Moreover, the data reported by Caputo *et al.* (1998) underline that differences in morphology in the *Scorpaena* species are limited, whereas the karyotype reorganisation is considerable. The analysis of our data with those reported in the literature revealed a weakness regarding the exclusive use of morphological characters or colour in some genera of Mediterranean scorpionfish. This has also been shown in other commercially important fish species in the Mediterranean, like groupers, in which the exclusive use of morphological traits is not satisfactory for identifying the species and establishing their phylogenetic relationships (Maggio *et al*. 2009). The use of the mitochondrial gene 12S-rRNA proved to be a valuable tool in analysing phylogenetic relationships among the examined species. Moreover, the use of mitochondrial gene 12S-rRNA highlighted that it should be used to analyse all the species belonging to different genera in order to obtain more satisfactory phylogenetic relationships within this family in the Mediterranean Sea. The marker used should be an important tool for the precise identification of the species and it should also be used for tracing individuals, thereby satisfying the provisions of the laws already in force in the European Union. These require (since 2011) that any fish available on the market is labelled with the species and region of origin. Finally, and considering the economical importance of scorpionfish, we would like to suggest that the 12S-rRNA mitochondrial marker should also be used as a tool for formulating management programmer for stock assessment.

ACKNOWLEDGMENTS

This study was supported by Fondi di Ateneo (ex 60%)

[2]Froese R & D Pauly. 2014. FishBase.<www.fishbase.org>

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Received 28 October 2013 and accepted 5 May 2014

Editor: Claudia Bustos