Journal of FISH BIOLOGY
Published for the Fisheries Society of the British Isles

Journal of Fish Biology (2011) **78**, 373–377
doi:10.1111/j.1095-8649.2010.02858.x, available online at wileyonlinelibrary.com

Discrimination of two picarel species *Spicara flexuosa* and *Spicara maena* (Pisces: Centracanthidae) based on mitochondrial DNA sequences

A. Imsiridou*, G. Minos*†, A. Gakopoulou*, V. Katsares*, T. Karidas* and G. Katselis‡

*Alexander Technological Educational Institute of Thessaloniki, Department of Aquaculture & Fisheries Technology, P. O. Box 157, GR-63200, Nea Moudania, Greece and ‡Technological Educational Institute of Messolonghi, Department of Aquaculture & Fisheries Management, GR-30200, Messolonghi, Greece

(Received 15 July 2010, Accepted 26 October 2010)

A DNA methodology based on PCR and sequencing analysis of the mtDNA 16S rRNA gene was developed for the discrimination of picarel *Spicara flexuosa* and blotched picarel *Spicara maena* (Pisces: Centracanthidae). The molecular results indicated that there is a clear discrimination between the two species, as all the individuals of *S. flexuosa* revealed the same 16S rDNA haplotype while the *S. maena* haplotype differs in 15 distinct nucleotides.

Key words: blotched picarel; genetic identification; picarel; sequencing; 16S rRNA.

Picarels or menolas are small-to-medium size inshore-pelagic schooling species. Picarel *Spicara flexuosa* Rafinesque is a non-migratory, gregarious, necto-benthonic fish occurring down to 130 m depth, but specimens regularly move in the water column (Lythgoe & Lythgoe, 1992). Blotched picarel *Spicara maena* (L.) is a gregarious to schooling species usually found on seagrass meadows, rocks and mud, at depths of 20–170 m (Miller & Loates, 1997). Both species belong to the Centracanthidae family, which contributes to the fisheries in the inshore areas of Greece (Mytilineou & Papaconstantinou, 1991).

As *S. flexuosa* and *S. maena* are protogynous hermaphrodites (Breder & Rosen, 1966) and due to the fact that *S. flexuosa* shows sexual dimorphism during the reproductive period, a systematic confusion appeared in the Centracanthidae family in the Mediterranean region. Numerous attempts to classify them have resulted in a confused synonymy (Pollard & Pichot, 1971) where Pollard & Pichot (1971), Tortonese (1975), Heemstra (1981, 1990), Arculeo *et al*. (1996), Quéro *et al*. (2003), Eschmeyer (2010) and Froese & Pauly (2010) proposed that *S. flexuosa* and *S. maena* are

†Author to whom correspondence should be addressed. Tel.: +30 23730 65313; email: gminos@otenet.gr

conspecific. On the other hand, many researchers (but Vasiliev (1980), Vasil'eva & Salekhova (1983), Tortonese (1986), Fischer *et al*. (1987), Papakonstantinou (1988), Costa (1991), Lythgoe & Lythgoe (1992), Debelius (1997), Miller & Loates (1997), Louisy (2002), Parin (2003), Golani *et al*. (2006) and Vasil'eva (2007)) consider the two species as different.

Some mitochondrial DNA (mtDNA) segments, notably the ribosomal DNA (rDNA) genes, are useful markers for determining relationships at different taxonomic levels (Arnason *et al*., 1991). 16S rDNA sequences have been used for phylogenetic analysis in fish taxa (Infante *et al*., 2004; Pardo *et al*., 2005). Molecular data provide a complementary approach to discriminate species distinguished by subtle morphological characters (Goetze, 2003). The aim of the present study was to evaluate the similarity and dissimilarity of *S. flexuosa* and *S. maena* species, as an attempt to solve the identification problem of the two Centracanthidae species, using the sequencing analysis of the 16S rDNA gene of the mtDNA.

Specimens were collected by professional fishermen in the Thermaikos Gulf (40° 23′ N; 22° 50′ E), in the northern Aegean Sea. Thirty-nine individuals of *S. maena* and 39 individuals of *S. flexuosa* were randomly collected from March to August 2008, a period which coincides with their spawning season. Total DNA was extracted from the muscle according to Hillis *et al*. (1996). A universal primer set (Palumbi, 1996) was used for the amplification of the 16S rDNA gene in both *S. flexuosa* and *S. maena*. The reaction mixture contained template DNA (*c*. 100 ng), 1× polymerase chain reaction (PCR) buffer, 2·2 mM $MgCl_2$, 20 pmol of each primer, 0·25 mM of each deoxynucleotide triphosphate (dNTP) and 0·5 U of Promega polymerase (www.promega.com). Amplification was started at 94° C for 3 min, followed by 31 cycles with 94° C for 50 s, 50° C for 50 s, 72° C for 50 s and a final extension at 72° C for 5 min. Electrophoresis of 3 µl of the PCR product was performed in 1× Tris–borate–EDTA (TBE) buffer for 1 h at 150 V, in 1·5% agarose gel containing 0·5 µg ml^{-1} ethidium bromide. The size of the PCR products was checked against a 100 base pairs (bp) DNA ladder and was *c*. 600 bp for both species. The resulting DNA fragments were visualized by UV transillumination and photographed. A sequencing analysis on a 3730 xl DNA Analyzer (Applied Bio systems, Inc.; www.appliedbiosystems.com) followed using both forward and reverse primers for cross-checking. The DNA sequences were deposited in GenBank (accession numbers FJ625835 and FJ625836). The nucleotide sequences of all individuals were aligned using the Clustal X software (Thompson *et al*., 1997) and the BioEdit software (Hall, 1999), set to default parameters and corrected by eye. Molecular analyses were conducted using MEGA version 3.1 (Kumar *et al*., 2004) and the *p*-distance model. The species *Spicara alta* (Osório) (GenBank: AF 247435.1) was used as out-group. A neighbour-joining (NJ) phylogenetic tree was constructed based on the *p*-distance model.

In total, 566 bp at the 5′-end of the mtDNA 16S rDNA gene for both species were sequenced. All *S. flexuosa* individuals revealed the same haplotype whereas all *S. maena* individuals revealed another haplotype, which was different in 15 nucleotides compared with *S. flexuosa* as a reference sequence. *p*-distance between *S. flexuosa* and *S. maena* was estimated at a value of $p = 0{\cdot}027$. The average nucleotide compositions of A, C, G and T were 21·38, 24·03, 26·33 and 28·27% for *S. flexuosa* and 22·26, 23·85, 25·44 and 28·45% for *S. maena*, respectively. The NJ tree revealed the complete genetic differentiation of the two species,

which also denotes that a reproductive isolation has been established between them.

Previous phylogenetic studies support the provisional inclusion of *Spicara* within sparids (Orrell *et al*., 2002, 2004; Yamanoue *et al*., 2007). According to these studies, *S. maena* is clustered with the sparid species *Spondyliosoma cantharus* (L.) (Orrell *et al*., 2002, 2004) or with the sparid *Pagrus major* (Temminck & Schlegel) (Yamanoue *et al*., 2007). Similarly, Chiba *et al*. (2009) found that the family Centracanthidae [*S. maena*, *S. flexuosa* and *Spicara smaris* (L.)] is included in the Sparidae clade and proposed that additional phylogenetic analyses are needed in order to clarify the validity of the family Centracanthidae. Considering the above, it is obvious that there is a systematic problem with the genus *Spicara*. There was only a single attempt for the genetic identification of *S. flexuosa* and *S. maena*, using allozyme electrophoresis (Arculeo *et al*., 1996). According to this study, *S. flexuosa* and *S. maena* are conspecific despite morphological differences, as no discriminating monomorphic locus was identified between the two species and genetic distance between *S. flexuosa* and *S. maena* was only $D = 0{\cdot}006$. On the other hand, the value $p = 0{\cdot}027$ revealed in the present study is higher than the limit value of 0·018 reported for intraspecies genetic distances in the family Sparidae (Junhong, 2007). In conclusion, since the 16S rDNA gene is a very good species-specific marker (Perez *et al*., 2005), the present data indicate a clear discrimination of the two species.

References

Arculeo, M., Mauro, A., Scelsa, G., Lo Brutto, S., Cammarata, M. & Parrinello, N. (1996). Protein differences among the Mediterranean species of the genus *Spicara*. *Journal of Fish Biology* **49,** 1317–1322. doi: 10.1111/j.1095-8649.1996.tb01798.x

Arnason, U., Gullberg, A. & Widegren, B. (1991). The complete nucleotide sequence of the mitochondrial DNA of the fin whale, *Balaenoptera physalus*. *Journal of Molecular Evolution* **33,** 556–568. doi: 10.1007/BF02102808

Breder, C. M. & Rosen, D. E. (1966). *Modes of Reproduction in Fishes.* Neptune City, NJ: T.F.H. Publications.

Chiba, S. N., Iwatsuki, Y., Yoshino, T. & Hanzawa, N. (2009). Comprehensive phylogeny of the family Sparidae (Perciformes: Teleostei) inferred from mitochondrial gene analysis. *Genes & Genetic Systems* **84,** 153–170. doi: 10.1266/ggs.84.153

Costa, F. (1991). *Atlante dei pesci dei mari italiani.* Milano: Gruppo Ugo Mursia Editore S.p.A.

Debelius, H. (1997). *Mediterranean and Atlantic Fish Guide.* Frankfurt: IKAN–Underwasserarchiv.

Fischer, W., Bauchot, M.-L. & Schneider, M. (Eds) (1987). *FAO Species Identification Sheets for Fishery Purposes, Mediterranean North Sea, Area 37*, Vol. 2. Rome: FAO.

Goetze, E. (2003). Cryptic speciation on the high seas; global phylogenetics of the copepod family Eucalanidae. *Proceedings of the Royal Society A* **370,** 2321–2331. doi: 10.1098/rspb.2003.2505

Golani, D., Öztürk, B. & Başusta, N. (2006). Centracanthidae. In *Fishes of the Eastern Mediterranean* (Golani, D., Öztürk, B. & Başusta, N., eds), pp. 168–169. Istanbul: Turkish Marine Research Foundation.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41,** 95–98.

Heemstra, P. C. (1981). Centracanthidae. In *FAO Species Identification Sheets for Fishery Purposes. Eastern Central Atlantic; Fishing Area 34, 47 (in part)* (Fischer, W., Bianchi, G. & Scott, W. B., eds). Ottawa: Department of Fisheries and Oceans.

Heemstra, P. C. (1990). Centracanthidae. In *Check List of the Fishes of the Eastern Tropical Atlantic (CLOFETA, II)* (Quero, J. C., Hureau, J. C., Karrer, C., Post, A. & Saldanha, L., eds), pp. 768–772. Paris: UNESCO.

Hillis, D. M., Moritz, C. & Mable, B. K. (1996). *Molecular Systematics.* Sunderland, MA: Sinauer & Associates Inc.

Infante, C., Catanese, G. & Manchado, M. (2004). Phylogenetic relationships among ten sole species (Soleidae, Pleuronectiformes) from the Gulf of Cadiz (Spain) based on mitochondrial DNA sequences. *Marine Biotechnology* **6,** 612–624. doi: 10.1007/s10126-004-3081-6

Junhong, X. (2007). Identification standard of Sparid species based on cytochrome b sequences. *South China Fisheries Science* **1,** 65–74. doi: CNKI:ISSN:1673-2227.0.2007-01-008

Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Briefings in Bioinformatics* **5,** 150–163. doi: 10.1093/bib/5.2.150

Louisy, P. (2002). *Guide d'identifications des Poissons Marins. Europe de l'ouest et Méditerranée.* Paris: ULMER.

Lythgoe, J. & Lythgoe, G. (1992). *Fishes of the Sea. The North Atlantic and Mediterranean.* Cambridge, MA: The MIT Press.

Miller, P. J. & Loates, M. J. (1997). *Fish of Britain and Europe.* London: Harper Collins Publishers.

Mytilineou, C. & Papaconstantinou, C. (1991). Age and growth of *Spicara flexuosa* (Rafinesque, 1810) (Pisces, Centracanthidae) in the Patraikos gulf (Greece). *Scientia Marina* **55,** 483–490.

Orrell, T. M. & Carpenter, K. E. (2004). A phylogeny of the fish family Sparidae (porgies) inferred from mitochondrial sequence data. *Molecular Phylogenetics and Evolution* **32,** 425–434. doi: 10.1016/j.ympev.2004.01.012

Orrell, T. M., Carpenter, K. E., Musick, J. A. & Graves, J. E. (2002). Phylogenetic and biogeographic analysis of the Sparidae (Perciformes: Percoidei) from cytochrome b sequences. *Copeia* **2002,** 618–631. doi: 10.1016/j.ympev.2004.01.012

Palumbi, S. R. (1996). Nucleic acids II: the polymerase chain reaction. In *Molecular Systematics* (Hillis, D. M., Moritz, C. & Mable, B. K., eds), pp. 205–247. Sunderland, MA: Sinauer & Associates Inc.

Papakonstantinou, C. (1988). *Check-list of Marine Fishes of Greece.* Athens: National Centre for Marine Research, Hellenic Zoological Society.

Pardo, B. G., Machordom, A., Foresti, F., Porto-Foresti, F., Azevedo, M. F. C., Bañón, R., Sánchez, L. & Martínez, P. (2005). Phylogenetic analysis of flatfish (order Pleuronectiformes) based on mitochondrial 16S rDNA sequences. *Scientia Marina* **69,** 531–543.

Parin, N. V. (2003). An annotated catalogue of fish-like vertebrates and fishes of the seas of Russia and adjacent countries: Part 3. Orders Perciformes (excluding suborders Gobioidei, Zoarcoidei and Stichaeoidei) and Tetraodontiformes. *Journal of Ichthyology* **43,** 1–40.

Perez, J., Álvarez, P., Martinez, J. L. & Garcia-Vazquez, E. (2005). Genetic identification of hake and megrim eggs in formaldehyde-fixed plankton samples. *ICES Journal of Marine Science* **62,** 908–914. doi: 10.1016/j.icesjms.2005.04.001

Pollard, D. A. & Pichot, P. (1971). The systematic status of the Mediterranean Centracanthidae fishes of the genus *Spicara*, and in particular *S. chryselis* (Val.) as indicated by electrophoresis studies of their eye-lens proteins. *Journal of Fish Biology* **3,** 59–72. doi: 10.1111/j.1095-8649.1971.tb05905.x

Quéro, J.-C., Porché, P. & Vayne, J.-J. (2003). *Guide des poisons de l'Atlantique européen (les guides du naturaliste)*. Lonay (Switzerland) and Paris: Delachaux et Niestlé S.A.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL X windows interface: flexible strategies for multiple alignment aided by quality analysis tool. *Nucleic Acids Research* **25,** 4876–4882. doi: 10.1093/nar/25.24.4876

Tortonese, E. (1975). *Fauna d'Italia*, Vol. 11. Bologna: Edizioni Calderini.

Tortonese, E. (1986). Centracanthidae. In *Fishes of the North-eastern Atlantic and the Mediterranean* (Whitehead, P. J. P., Bauchot, M.-L., Hureau, J.-C., Nielsen, J. & Tortonese, E., eds), pp. 908–911. Paris: UNESCO.

Vasiliev, V. P. (1980). The selective mortality of different chromosome morphs in *Spicara flexuosa* Raf. *Genetica* **52/53,** 327–331. doi: 10.1007/BF00121842

Vasil'eva, E. D. & Salekhova, L. P. (1983). The precision of diagnoses of the *Spicara* (Perciformes, Centracanthidae) species, based on osteological features. *Zoological Journal* **62,** 1044–1056.

Vasil'eva, E. D. (2007). *Fish of the Black Sea. Key to Marine, Brackish-water, Euryhaline, and Anadromous Species With Color Illustrations, Collected by S. V. Bogorodsky*. Moscow: VNIRO Publishing (in Russian, with English abstract).

Yamanoue, Y., Miya, M., Matsuura, K., Yagishita, N., Mabuchi, K., Sakai, H., Katoh, M. & Nishida, M. (2007). Phylogenetic position of tetraodontiform fishes within the higher teleosts: Bayesian inferences based on 44 whole mitochondrial genome sequences. *Molecular Phylogenetics and Evolution* **45,** 89–101. doi: 10.1016/j.ympev.2007.03.008

Electronic References

Eschmeyer, W. N. (2010). *Catalog of Fishes*. Available at http://research.calacademy.org/ichthyology/catalog/fishcatmain.asp/ (accessed 26 August 2010).

Froese, R. & Pauly, D. (Eds) (2010). *FishBase*. Available at http://www.fishbase.org/ (accessed July 2010).