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Molecular and morphological identification of an uncommon centrolophid fish

Communication

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Abstract: The use of both morphological and molecular methods has allowed a fast and reliable species assignment of a fish that local fishermen with over thirty years of experience had never seen before. The identified species, *Schedophilus medusophagus*, is rare along Italian coasts, and this is the first documented record in the Central Tyrrhenian Sea for over 35 years. Its abundance should be evaluated on a continuous basis, as it might reflect biological consequences of environmental and climatic change. The mitochondrial sequences obtained in this study constitute a useful molecular tag for future research and may contribute to the phylogenetic debate on the status of the genus *Schedophilus*, of which *S. medusophagus* is the type species. Based on the existing literature, these preliminary molecular data support the hypothesis that the genus is not monophyletic.

Keywords: *COI • Cornish blackfish • Mediterranean Sea • mtDNA • rare fish • Schedophilus medusophagus*

1. Introduction

Fish species are traditionally identified based on external morphological characters [1]. Nevertheless, classical identification can sometimes be rather difficult, for example when considering early-life stages and juvenile specimens; or in cases of species showing morphological interspecific similarity or high intraspecific variability. The development in the past two decades of molecular approaches based on PCR amplification, and of online sequence databases such as GenBank, EMBL-Bank and DDBJ, have considerably helped the identification of fish species. For example, 18 cryptic species have been recently identified [2] in a genus *Schindleria* which previously included only three species. As recently revised by Teletchea [3], the establishment of a DNA barcoding system, *i.e.* a molecular barcode inventory of known animal taxa, based on the mitochondrial gene cytochrome c oxidase I (COI) was proposed by Hebert *et al.* [4]. In the same year, Tautz *et al.* [5] emphasized the need of a DNA-based taxonomy system, which can act as a scaffold for taxonomic knowledge and as a convenient tool for species identification and description, still to be "firmly anchored within the knowledge, concepts, techniques and infrastructure of traditional taxonomy". Recently, Teletchea [6] drew this same conclusion "after 7 years and 1000 citations". Several studies [e.g., 7,8] document the benefit of complementing the traditional taxonomic data (morphology-based species identification) with molecular tools (DNA-based species identification) for fish species identification.

This study combined morphological and molecular approaches for the species assignment of a teleost collected in the Central Tyrrhenian Sea, that fishermen with over thirty years of experience in the area had never seen before. The molecular approach was carried out using several mitochondrial genes which, due to their characteristics (high copy number per cell, lack of introns, limited recombination, haploid mode of

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inheritance and appropriate rate of evolution), are easier and more straightforward to apply than nuclear DNA [3].

2. Experimental Procedures

On July 2008, a single specimen of an unknown fish was caught in the Central Tyrrhenian Sea, near Anzio (Italy), at a depth of between 80 to 100 meters. The specimen was measured, frozen 10 h after the catch, and kept at -20°C for 20 months. In March 2010, the specimen was defrosted and external morphological features and meristic characters were collected. No morphometric data were collected, as the front of the head of the specimen was damaged, probably by the trawl net.

Total genomic DNA was extracted from a pectoral fin clip of the specimen using the method of Aljanabi and Martinez [9]. Fragments of four mitochondrial regions – 16S rDNA (16S), cytochrome c oxidase I (COI), cytochrome b (Cyt *b*) and control region (CR) – were amplified using the following primers: L2510-16S and H3084-16S [10] for the 16S rDNA, GluFor [11] and 34Rev [12] for the Cyt *b*, FishF1 and FishR2 [13] for the COI, and Lpro2 and HdL1 [14] for the CR. PCR reactions were carried out in a total volume of 10 µl, containing 1 µl of 10X Buffer (BIOLINE, London, U.K.), 0.3 µl of $MgCl_2$ (50 mM), 0.2 µl of dNTP (2.5 mM), 0.1 µl of each primer (100 mM), 0.07 µl of 5 U μl^{-1} BIOTAQ (Gaithersburg, MD, USA), DNA polymerase and 10–100 ng of template. PCR was performed in a Biometra Thermocycler with an initial denaturation of 2 min at 95°C followed by 30 cycles of 30 s at 94°C, 30 s at 54°C, 1 min at 72°C and a final extension for 10 min at 72°C. PCR products were purified with SureClean Plus (Bioline) and sequenced on both strands with an automated DNA sequencer (BMR Genomics) using the same primers as those for the PCR. All sequences were visually verified on a chromatogram with Chromas Lite software version 2.01 (Technelysium Pty Ltd., Helensvale, Australia), aligned with the Clustal X program [15] for the complete reconstruction of the four mtDNA sequences. The four sequences were deposited in GenBank (accession numbers: HQ455052-HQ455055). Each sequence was BLASTed in GenBank, the NIH genetic sequence database; the COI sequence was also BLASTed in the Barcode of Life Data *Systems* (*BOLD*).

3. Results and Discussion

The specimen (Figure 1) was approximately 14.6 cm in length and 29.9 g in weight, and when freshly caught was dark red, dorsally purplish and ventrally light brown. The head was lighter than the body; and numerous pigment dots were present. The body was elongated, with a single, long, continuous dorsal fin. Meristic counts, collected using a stereomicroscope, were: 50 dorsal fin spines plus soft rays, 30 anal fin spines plus soft rays, 22 caudal fin rays, 19 pectoral fin rays, 4 ventral fin rays. There were 11 gill rakers on the first arch. Twelve spines were also visible on the margin of the preopercule. The collected morphological data confirmed that the specimen does not belong to any of the common Mediterranean families.

The molecular approach allowed us to circumscribe the number of possible teleost taxa to be considered for the morphological identification, up to the species level. The length of the studied sequences (some including the forward and the reverse primer sequences, or part of them) was: 646 bp for the 16S rDNA, 437 bp for the Cyt *b*, 629 bp for the COI, and 422 bp for the CR. The results of BLAST searches in GenBank and *BOLD* are given in Tables 1 and 2, respectively. The Cyt *b* and, mainly, the COI sequences provided the most

Figure 1. The specimen collected in the Tyrrhenian Sea (Photo: A. Fusari).

FAMILY	SPECIES	MAXIMUM IDENTITY (%)			
		16S	Cyt *b*	COI	CR
Centrolophidae	*Schedophilus ovalis*	98	90	90	n/a
	Schedophilus labyrinthicus	n/a	n/a	90	n/a
	Schedophilus velaini	n/a	87	n/a	n/a
	Seriolella caerulea	98	89	91	n/a
	Seriolella punctata	98	89	90	79
	Seriolella porosa	n/a	n/a	90	n/a
	Centrolophus niger	98	88	90	n/a
	Icichthys lockingtoni	98	88	91	n/a
	Hyperoglyphe japonica	98	89	90	n/a
	Hyperoglyphe antarctica	n/a	n/a	90	n/a
	Hyperoglyphe moselii	n/a	n/a	90	n/a
Nomeidae	*Cubiceps pauciradiatus*	92	85	n/a	87
	Psenes cyanophrys	91	80	n/a	78

Table 1. BLAST search results in the GenBank database for the four mtDNA genes of the unknown fish; n/a: not available.

discriminating information for the identification of the specimen. This is congruent with expectations. Indeed, according to Tautz *et al*. [5], sequences encoding ribosomal small subunit RNAs, whether of nuclear and mitochondrial origin, are highly conserved and thus not particularly useful for discriminating closely-related species. On the other hand, the mitochondrial CR is one of the fastest diverging regions of mtDNA. Therefore, it is more commonly used for the identification of genetically differentiated populations.

On the basis of genetic data, the specimen appears to belong to the family Centrolophidae, with a 100% probability of placement after a BLAST search in the BOLD data base (Table 2), which also places it in the genus *Schedophilus*, with a 98% probability (Table 2). As far as the species assignment is concerned (Table 3), the top match is with *Schedophilus huttoni*, which is, however, distributed in the South Pacific and Western Indian Oceans, and along the South African coast of the Atlantic Ocean. With the exception of *Schedophilus ovalis*, none of the remaining top-match species inhabits the Mediterranean Sea, or the Red Sea. This latter issue is particularly important given that one of our possible hypotheses was that we were dealing with an alien Lessepsian fish.

On the basis of the molecular data, we went back to morphological keys which are specific to the Centrolophidae [16-19] and we identified the specimen as a juvenile of the species *Schedophilus medusophagus* Cocco, 1839, which can be easily distinguished from the Mediterranean, congeneric, *S. ovalis* on the basis of meristic traits, and particularly due to the median fin

TAXONOMIC LEVEL	TAXON ASSIGNMENT	Pp (%)
Phylum	Chordata	100
Class	Actinopterygii	100
Order	Perciformes	100
Family	Centrolophidae	100
Genus	*Schedophilus*	98

Table 2. BLAST search results in the BOLD database for the COI sequence of the unknown fish. Pp (%), probability of placement.

SPECIES	Ss (%)
Schedophilus huttoni	98
Icichthys lockingtoni	92
Tubbia tasmanica	92
Schedophilus ovalis	91
Schedophilus maculatus	91
Schedophilus velaini	91
Schedophilus labyrinthicus	91
Seriolella caerulea	91
Seriolella porosa	91
Seriolella punctata	91
Hyperoglyphe japonica	91

Table 3. Top 11 matches for the COI sequence of the unknown fish within the Centrolophidae family detected with the BLAST search in the BOLD database. Ss (%), specimen similarity.

spines which, in the former, are weak and difficult to distinguish from rays, and, in the latter, strong and easily distinguishable.

The Cornish blackfish, *S. medusophagus*, is a subtropical mesopelagic species characterized by a vertical-age distribution. Juveniles and young adults live in the surface layers of the sea, and are commonly associated with pelagic medusae or floating objects, while adults are found at 500–600 m depth. *S. medusophagus* is distributed in the North-Eastern and North-Western Atlantic, and in the Western Mediterranean [16,17,20]. It is rare along the Italian coasts, although Bini [20] and Tortonese [16] reported its presence on the west coast of Italy from museum specimens coming from the Ligurian Sea, Naples and Sicily. Thus, this is the first documented record of the species in the Central Tyrrhenian Sea in over 35 years. As far as the Adriatic Sea is concerned, Dulčić [21] reported the first capture of larvae of *S. medusophagus* in the eastern central Adriatic and proposed that this record could represent an extension of the species range, compared to previous records for the species in the area [22,23]. Dulčić [21] also emphasized that, in the same period (mid 1990s), along with the Cornish blackfish, other mesopelagic species uncommon in the area had been recorded, and suggested that all these records could be connected to environmental factors, including increasing temperature. A similar explanation has also been hypothesized by Corsini-Foka and Frantzis [24] for the first record of the congeneric *S. ovalis* in the Aegean Sea, which was also interpreted as an expansion of the geographical distribution of the species. Therefore, though the present record of *S. medusophagus* along the Italian peninsular coast of the Central Tyrrhenian Sea is not an extension of the species range, its abundance should be evaluated on a continuous basis as it could be an indicator or example of the biological consequences of environmental and climatic change [21].

The sequences obtained in this study are the first mitochondrial sequences for the species, and thus constitute a useful identification tag for future samples. Meanwhile, they can help to clarify the phylogenetic status of the genus *Schedophilus* Cocco, 1839, of which *S. medusophagus* is the type species. This genus currently includes nine species (Froese and Pauly, www.fishbase.org, version 11/2010), with a diverse array of forms that show a morphological dichotomy. One form is hard-spined and firm-fleshed (e.g. *S. ovalis* and *S. velaini*) and the other is weak-spined and soft-fleshed (e.g. *S. medusophagus* and *S. huttoni*) [17,25]. Thus, the breadth and validity of the genus has been debated by various authors [e.g. 26-28]. In this context, an allozyme survey of 11 stromateoid species from Australian waters [25], including two *Schedophilus* species, clearly evidenced the separate clustering of the weak-spined *S. huttoni* and the hard-spined *S. labyrinthicus,* and the non monophyly of the genus. Bolch *et al*. [25] therefore proposed reconsidering the composition of the genus and suggested that the hard-spined, firm-fleshed group might be more correctly represented as a distinct centrolophid genus. They also emphasized that the taxonomic revision of the genus was hampered by the rarity of *Schedophilus* material, particularly of the type species *S. medusophagus* [25].

The BLAST search results obtained in the present study using the COI sequence and the BOLD database (Table 3) might also be considered congruent with the hypothesis of a non-monophyletic status of the genus *Schedophilus* and the existence of two groups within it. Indeed, the studied specimen of *S. medusophagus* shows the highest similarity value (98%) with the weak-spined *S. huttoni* species, lower values (91%) with the remaining hard-spined *Schedophilus* species, and values intermediate to these with two non-congeneric species of Centrolophidae, *Icichthys lockingtoni* and *Tubbia tasmanica*. As not all the COI sequences of the BOLD database are listed in Genbank, we were not able to perform any phylogenetic analyses within this study. However, in the phenetic BOLD TaxonId tree (not shown) *S. medusophagus* and *S. huttoni* are included in the same cluster, which is separate from the other *Schedophilus* species. These latter are instead grouped with other Centrolophidae species.

From a methodological point of view, the need for integration between the existing databases was apparent. In addition to the incomplete overlap between the BOLD and the GenBank sequences, the nomenclature reported in the BOLD and FishBase databases is not always congruent. For example, *S. labyrinthicus* and *S. velaini* are considered as synonyms in FishBase (Froese and Pauly, www.fishbase.org, version 11/2010) and as separate species in BOLD.

In conclusion, the benefits of the application of molecular tools to complement the traditional taxonomic data in the present study are unquestionable. However, though the application of the molecular approach is easier and faster than the morphological one, the resolving power of the molecular approach is also strictly correlated with taxon coverage, which is still limited, especially when rare species are considered.

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