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Integration of DNA barcoding for the initial recordings of Lessepsian fishes: a case study of the Indo-Pacific slender ponyfish *Equulites elongatus*

S. Sakinan*†, A. Karahan* and M. Ok*‡

**Middle East Technical University, Institute of Marine Science, Department of Marine Biology and Fisheries, Mersin, Turkey and ‡British Antarctic Survey, High Cross, Madingley Road, Cambridge, CB3 0ET, U.K.*

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In this study, the DNA barcode of a regional Lessepsian sighting of the slender ponyfish *Equulites elongatus* is integrated with morphometric and meristic descriptors as a case study to address further identification problems in the Mediterranean Sea. The study also aims to contribute to the regional mitochondrial cytochrome oxidase I information pool, to support other potential uses. The initial sighting of *E. elongatus* from the north-eastern Mediterranean coast of Turkey is provided from a trawl survey on 3 June 2015, where 76 specimens were captured during a 15 min tow.

Key words: *coI*; cytochrome oxidase; Mediterranean; meristics; morphology; Turkey.

The Mediterranean Sea is one of the most ecologically stressed regions in the world and its condition is deteriorating with intensive bio-invasion (Lejeusne *et al.*, 2010). The majority of the alien invaders in the Mediterranean Sea are those entering through the Suez Canal, termed as Lessepsians (Por, 1978). It has been shown that the intensity in the Lessepsian migration has increased in recent decades along with a rapid expansion in their distribution, which poses an increasing concern for the region (Lasram & Mouillot, 2009). In order to understand the dynamic of this invasion in the Mediterranean Sea and its effect on the fisheries, monthly trawl surveys have been carried out for 8 years in Mersin, Turkey. The factors driving the expansion of the distribution range of the invasive species are complex (Golani, 1998) and require substantial information, particularly, tracking of regional first sightings (Belmaker *et al.*, 2009). During the past decade, there has been significant attention to the Lessepsian sightings on the Mediterranean coast of Turkey owing to their increasing ecological importance. Considering also the heavy fishing pressure in the area and particularly trawling with poor selectivity (Ozbilgin *et al.*, 2015), it should be expected that a newly introduced

†Author to whom correspondence should be addressed. Tel.: +90 536 2490247; email: serdar.sakinan@gmail.com

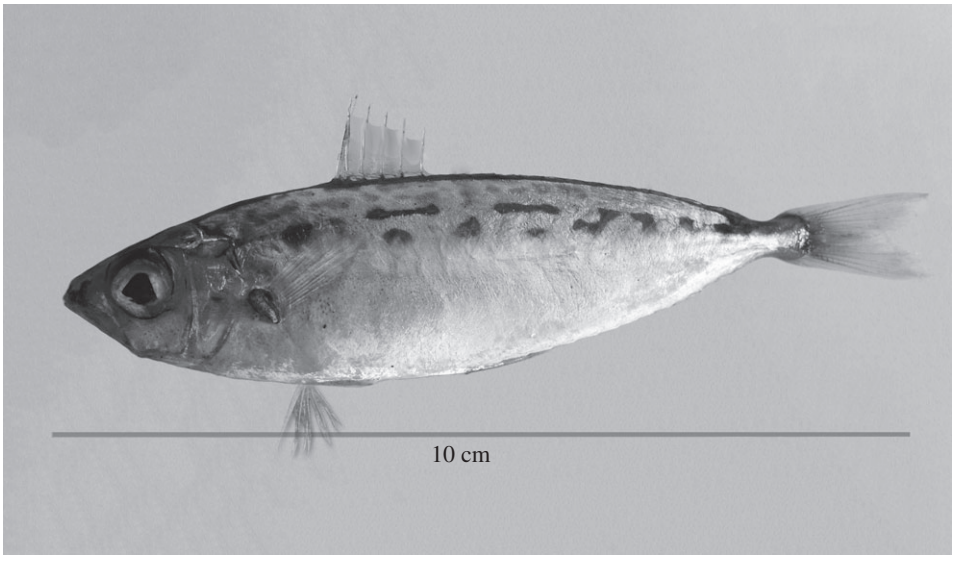

FIG. 1. The lateral appearance of a specimen of *Equulites elongatus* from the north-eastern Mediterranean Sea.

species would be immediately recognized and reported as soon as its introduction. Nevertheless, the time lag varies considerably from species to species between records from Israel and Turkey, which may indicate differences in dispersal times. Therefore, chronological information is important in sightings for understanding dispersal properties. Species identification based on conventional taxonomic characteristics alone can be difficult as a result of the potential morphological variations or ontogenetic changes (Kluge & Strauss, 1985). In particular, in the case of earlier developmental stages (eggs and larvae) or damaged samples, this could be even more problematic. This challenge can be addressed by DNA barcoding (Hebert & Gregory, 2005). DNA barcoding is a molecular technique that enables identification of a species based on short gene sequences obtained from mitochondrial gene cytochrome oxidase 1 (*coI*) (Hebert *et al.*, 2003). Documenting the DNA barcode of a newly introduced species in its new environment is important, particularly if there is no region-specific record in the international database such as the Barcode of Life Data (BOLD) system or GenBank (www.ncbi.nlm.nih.gov/genbank). The barcode data may help in tackling the identification problems as a complementary identification aid. Furthermore, it has been suggested that the barcode data may also potentially help understand the gene-flow conditions for an alien species (*e.g.* its haplotype diversity) (Hajibabaei *et al.*, 2007).

This study reports the DNA barcode of *Equulites elongatus* (Günther 1874) along with its morphometric and meristic descriptors. The species was recorded for the first time in the Mediterranean Sea on the Israeli coast by Golani *et al.* (2011). It is the second Lessepsian species from the Leiognathidae family in the area after *Equulites klunzingeri* (Steindachner 1898). Samples ($n = 76$; Fig. 1) were caught in a single haul during one of the monthly trawl surveys conducted on 3 June 2015 using a locally designed bottom trawl with a 14 mm mesh size at the codend. The tow duration is typically 30 min in this sampling programme. Because of the potentially higher vulnerability of this shallow sampling site (water depth = 15 m; Fig. 2), however, the haul

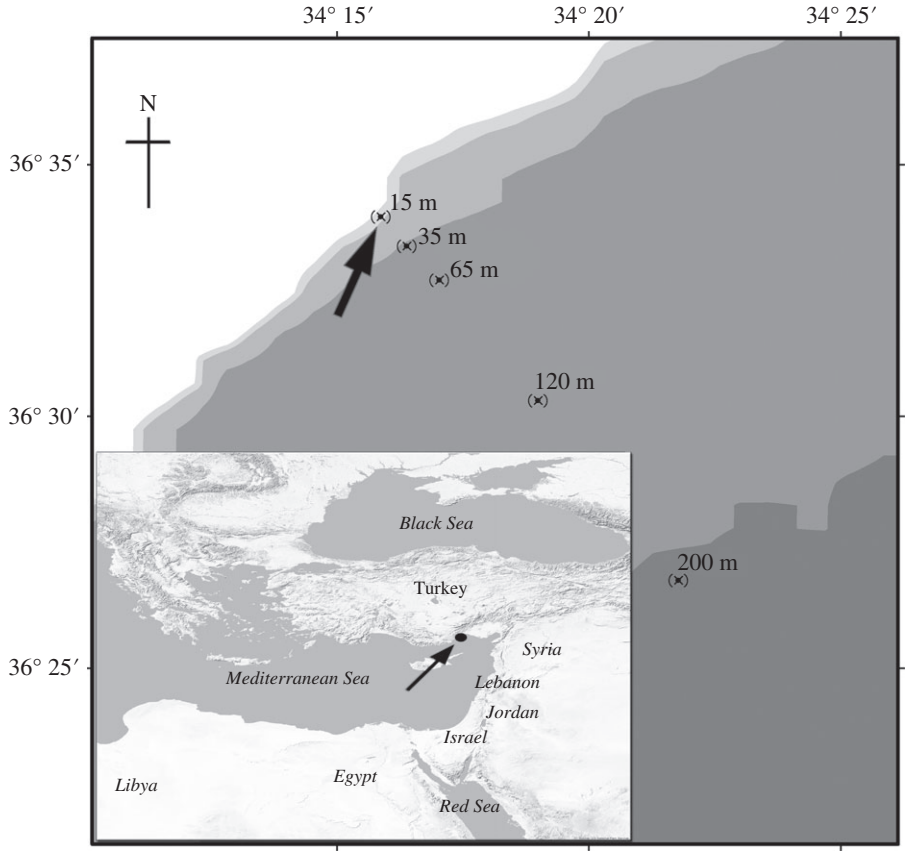

FIG. 2. Map of the study area with sampling sites (X) and the station where the specimens were caught (↑).

duration is limited to 15 min. Meristic counts and measurements follow Golani *et al.* (2011) and the references cited therein. Three of the specimens used for DNA sequencing were preserved in 70% ethanol and transferred to the fish collection deposited in: FFR (Rize University Zoology Museum of the Faculty of Fisheries; catalogue numbers = FFR09990.1–3). Genomic DNA was extracted from 100 mg of muscle tissue using the cetyl trimethyl ammonium bromide (CTAB) protocol (Stewart & Via, 1993). DNA samples were diluted 2:100 in sterile double distilled water (DDW) and kept at 4° C. The *coI* (*c.* 650 bp) was amplified following Ward *et al.* (2009) primers (*Fish-F1*, *Fish-R1*). Sequencing processes were performed by Macrogen Inc. (www.macrogen.com) for both directions. The *coI* sequences in both directions were edited and aligned using BioEdit 7.0.9.0 (Hall, 1999) software and checked for stop codons, insertions or deletions. Sequence data, chromatogram and primer details for specimens were submitted to BOLD (Ratnasingham & Hebert, 2007), which are available within the project file *Equulites elongatus* (FEE). Specimen and collection data, sequences, specimen images and trace files are listed in the same project folder as collection data on BOLD. The data were also submitted to GenBank *via* the BOLD system. *coI*-5P average pair-wise divergences within-species were determined using the

TABLE I. Morphometric measurements of specimens of *Equulites elongatus* specimens ($n = 40$) as proportions of standard (L_S) and head lengths (L_H)

	Dimension (mm)	Mean (mm)	Relative proportions
$\%L_S$			
Total length (L_T)	875–1044	948	110–131
Fork length (L_F)	795–951	877	100–120
L_S	721–845	795	–
Maximum body depth	195–248	221	25–31
Head length	192–220	206	24–28
Pre-dorsal length	287–391	307	36–49
Pre-anal length	385–441	417	48–55
Pre-pectoral length	195–240	214	25–30
Pre-pelvic length	236–273	254	30–34
Caudal height	92–110	104	12–14
$\%\ L_H$			
Eye diameter	64–81	72	32–38
Pre-orbital length	58–69	63	29–33

Kimura two-parameter (K2-P) distance model (Kimura, 1980) by MEGA 3.1 software (Tamura *et al.*, 2007). The phylogeographic relationships between haplotypes were estimated using the median-joining algorithm with default settings for constructing the network (weight = 10, $\varepsilon = 0$) in the programme NETWORK 4.6.1.2. (Bandelt *et al.*, 1999). The sequences of three previously barcoded specimens from Taiwan, Japan and China (ANGBF1833-12, FSCS030-06 and GBGC0932-06, respectively) were taken from the BOLD system database. *Equulites klunzingeri* (KM538355.1) was used as an outgroup during the NETWORK analysis.

The body of the *E. elongatus* specimens is elongate, slender and moderately compressed; snout sharp and pointed. The small protractile mouth points downward when protracted. Large eyes [32–38% of head length (L_H)], with bony ridges above eye located on top of the head. Nostrils a little in advance of the eyes, close together; anterior is small and round, posterior is slightly larger. Body covered with scales extending anteriorly ventral to the operculum and chest. Single dorsal fin contains eight spines, origin located slightly posterior to pelvic-fin origin. Dorsal spines are composed of a small spine, followed by three large and then four shorter spines, followed by 16 almost equally sized rays. The anal fin has three spines: a very small primary spine followed by

TABLE II. Meristic characteristics of *Equulites elongatus* specimens

Features	Number
Dorsal-fin rays	VIII + 16
Anal-fin rays	III + 14
Pectoral-fin rays	15
Pelvic-fin rays	I + 5
Caudal-fin rays	32

TABLE III. Nucleotide (A, adenine; C, cytosine; G, guanine; T, thymine; GC, guanine-cytosine) frequency distribution of three specimens of *Equulites elongatus*

	Minimum	Mean	Maximum	S.E.
G (%)	16·01	16·01	16·01	0
C (%)	31·15	31·15	31·15	0
A (%)	24·61	24·61	24·61	0
T (%)	28·23	28·23	28·23	0
GC (%)	47·16	47·16	47·16	0

the largest second spine and 14 rays. The short pectoral fin comprises 15 rays; the very short pelvic fin has a single spine and five rays. The silvery coloured body is covered with spots, upper part characterized by dark patches with lateral line conspicuous at the beginning, diffuse on posterior. Morphometric measurements are given in Table I. Meristic characteristics are given in Table II. A more thorough description was given by Golani *et al.* (2011).

coI sequences were obtained from three specimens of *E. elongatus*; each of the remaining sequences was 581 bp in length after trimming. GenBank accession numbers of these three individuals are KT381899, KT381900 and KT381901. There is no intraspecific distance between the individuals. The mean guanine-cytosine content (GC%) within the amplified region was counted as 47·16% (Table III). According to the BOLD system database, the barcode of *E. elongatus* has been previously recorded in Japan, Taiwan and China-Guangdong. The genetic K2P distance between the current observation and Japanese samples is only 2% (Table IV). The genetic K2P distance between the Taiwanese and Chinese specimens, however, is very high (11 and 17%, respectively). Such a discrepancy suggests that there has been previous misidentification of the specimens used for DNA barcoding in Chinese and Taiwanese samples; therefore, these samples were not included in the network analysis. All of the samples analysed here were clustered into a single haplotype which is different from that of the Japanese sample (Fig. 3). The outgroup species (*E. klunzingeri*) is 73 mutation steps away from the present study samples.

TABLE IV. Kimura two-parameter distance (below diagonal) and S.E. (above diagonal) values of present and previous study samples of *Equulites elongatus*

	Taiwan	China	Japan	Eq-1	Eq-2	Eq-3
Taiwan	–	0·02	0·02	0·02	0·02	0·02
China	0·15	–	0·02	0·02	0·02	0·02
Japan	0·11	0·18	–	0·01	0·01	0·01
Eq-1	0·11	0·17	0·02	–	0	0
Eq-2	0·11	0·17	0·02	0	–	0
Eq-3	0·11	0·17	0·02	0	0	–

Barcode of Life Data (BOLD) accession numbers from previous studies are: Taiwan, ANGBF1833-12; China-Guangdong, FSCS030-06; Japan, GBGC0932-06. The numbered abbreviations (Eq) indicate specimen barcode label.

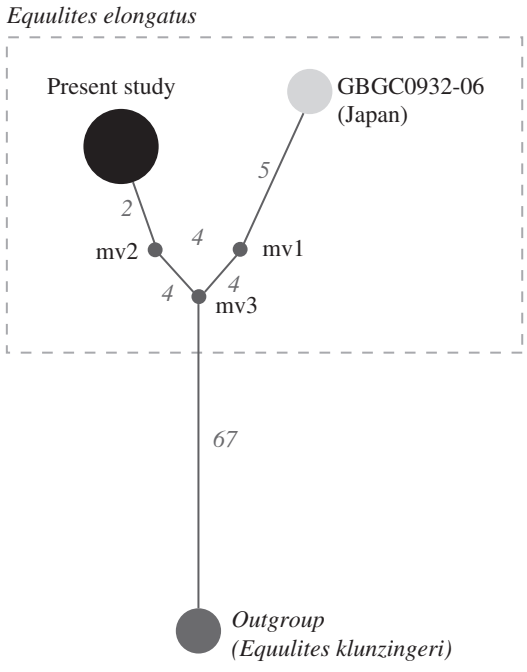

FIG. 3. The median-joining network analyses of haplotypes of *Equulites elongatus* with the outgroup *Equulites klunzingeri*. The pie size is proportional to the number of samples and colours indicate different region samples. The italic numbers represent mutation number between haplotypes. Mv, median vectors.

In terms of classical taxonomical measurement, the current observations were compared with those detailed by Golani *et al.* (2011). Almost all morphometric and meristic characteristics matched precisely. The only major difference is in the description of the anterior nostril. This was characterized as 'very small' compared with the posterior nostril hole by Golani *et al.* (2011); in the current study the anterior nostril is recognized as small, but not very small, in that its size is no less than half of the posterior. Recently, two more sightings of this species were reported from independent studies by Irmak *et al.* (2015) and Yokeş (2015), at 50 and 80 km, respectively, to the west of the survey area discussed here. Those reported by Yokeş (2015) were caught in December 2014 and April 2015, while Irmak *et al.* (2015) caught the specimens only 2 days later than the present study. In the survey area discussed here, however, no specimen of this species was caught during following surveys from July to December 2015 at the same location with the same equipment and for the same duration. In contrast, *E. klunzingeri* has been caught in almost every haul. Assuming that the spatial structure in habitat use is similar in these two species, it is perhaps too early to suggest that *E. elongatus* has established in the area successfully. *Equulites klunzingeri* is known to be highly vulnerable to trawling (Gücü & Bingel, 1994) owing to its high girth-to-length ratio. With this regard, *E. elongatus* may possibly be less affected by the fisheries owing to its more slender shape, which might be advantageous compared with *E. klunzingeri*. Assuming that *E. elongatus* possesses the same colonization capabilities, it will be interesting to observe its progression towards the west.

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