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SHORT COMMUNICATION

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First record of the buccaneer anchovy *Encrasicholina punctifer* (Fowler, 1938) (Clupeiformes; Engraulidae) in the Mediterranean Sea, confirmed through DNA barcoding

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1 | INTRODUCTION

The Levantine basin in the eastern Mediterranean Sea is a water body highly affected by many invasive species (Zenetos, Gofas, Morri, Rosso, & Violanti, 2012). Large ships with high volumes of ballast water require ever-deeper and larger port facilities. Biological invasions will further increase with the deepening of the Suez Canal (Galil, Boero, Campbell, Carlton, & Cook, 2015; Galil, Boero, Fraschetti, Piraino, & Campbell, 2015). Studies indicate the presence of 570 to 1,000 Lessepsian species in the Levantine basin, including 96 alien fish species (Fricke, Golani, & Appelbaum-Golani, 2015; Golani, 2010; Zenetos et al., 2012), have vital ecological and financial consequences. Monitoring of the fish stocks is highly critical and can provide valuable information for management.

Species of the Engraulidae are widely distributed (Froese & Pauly, 2016). The *Encrasicholina* genus represents Indo-Pacific species (Nelson, 2006). In earlier studies, *Encrasicholina punctifer* (Fowler, 1938) was synonymized with various *Stolephorus* species: *Stolephorus buccaneeri*, *S. punctifer* and *S. zollingeri* (Froese & Pauly, 2016). *Encrasicholina punctifer* is the currently recognized name for all of these species (Froese & Pauly, 2016).

Genetic analyses have become a more common monitoring tool for Lessepsian fishes during the last decade, and the information on their frequency of introduction or the founder effect can be ascertained using DNA-based methods (Bariche, Torres, Smith, Sayar, & Azzurro, 2015; Landi, Dimech, Arculeo, Biondo, & Martins, 2014; Moftah, Aziz, El Ramah, & Favereaux, 2011). DNA barcoding, a standardized method for species identification through the comparative analysis of short DNA sequences (Hebert & Gregory, 2005), would circumvent species identification difficulties; taxonomists could further use DNA barcoding as an additional tool for tackling the taxonomy of difficult-to-identify specimens. Specifically, DNA barcoding is also used for early detection and/or for fast and proper identification of Lessepsian fish migrants, preventing problems from occurring caused by cryptic morphology (Azzurro, Goren, Diamant, Galil, & Bernardi, 2015; Bariche et al., 2015).

The buccaneer anchovy, *E. punctifer*, is an important species in the fisheries of the Philippines, Japan, Hong Kong and Thailand. None of the *Encrasicholina* genus species were previously recorded in the ichthyofauna of the Mediterranean Sea. The present study aims to confirm the first occurrence of *E. punctifer* in the Mediterranean Sea by using two independent analyses (i.e. morphologic and molecular) of two specimens caught during a trawl survey in August 2014 in Mersin Bay, Levantine Sea, Eastern Mediterranean.

2 | MATERIALS AND METHODS

2.1 | Sampling and location

Encrasicholina punctifer were sampled during trawl surveys made by the Middle East Technical University–Institute of Marine Sciences (METU-IMS) in Mersin Bay on 6 August 2014, with start and finish trawling coordinates of 36.56095°N, 34.27495°E″; 36.57233°N, 34.29616°E″, respectively. Two *E. punctifer* (Figure 1a-b) specimens were caught, photographed, and preserved in 70% ethanol. The classification by Wongratana, Munroe, and Nizinski (1995) was used for taxonomic identification.

2.2 | DNA isolation and PCR

The genomic DNA from each specimen was extracted (100 mg muscle tissue) using the CTAB protocol (Stewart & Via, 1993). DNA samples

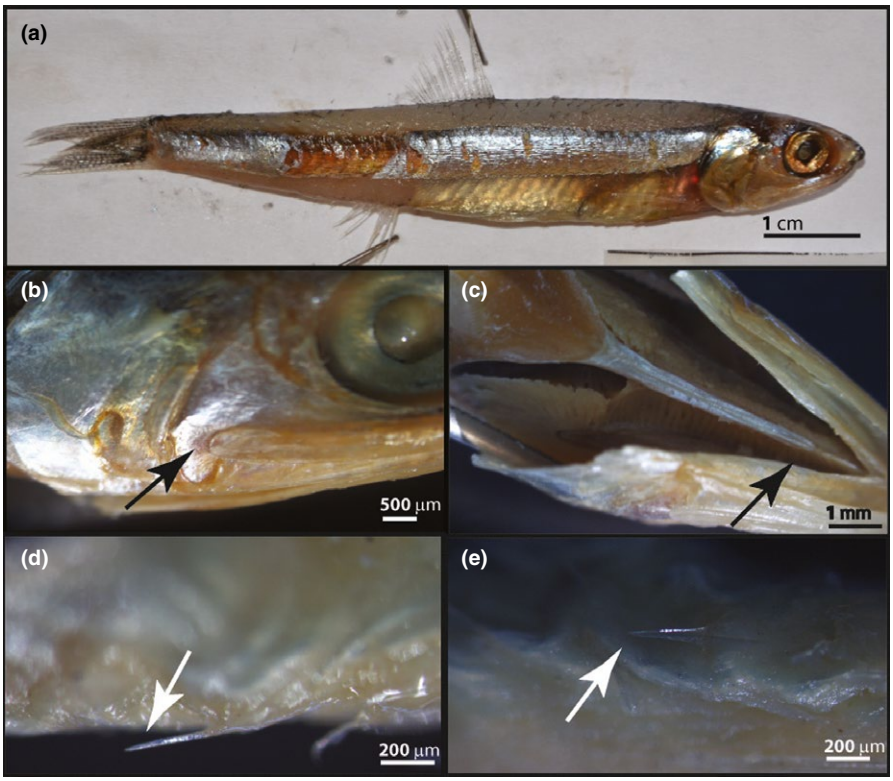

FIGURE 1 (a) Lateral appearance of *Encrasicholina punctifer* specimen, northeastern Mediterranean, (b) detail of maxilla tip, (c) appearances of isthmus muscle, (d, e) appearances of a small, sharp needle-like pre-pelvic scute

were diluted 2:100 in sterile double distilled water (DDW) and kept at 4°C. The cytochrome oxidase I (COI) gene (~650 bp) was amplified (Ward, Zemlak, Innes, Last, & Hebert, 2005) using primers (FishF1, FishR1). PCR results were screened on 1.3% agarose gel. Sequencing was performed in both directions by Macrogen Inc. (The Netherlands).

2.3 | Data analysis

COI sequences in both directions were edited and aligned using BioEdit v.7.0.9.0 (Hall, 1999) software and checked for stop codons, insertions or deletions. Sequence data, trace files, primer details, specimen collection data, voucher codes and specimen images were submitted to BOLD system (BOLD:AAF8837-IMS019-15 and BOLD:AAF8837-IMS020-15, http://www.boldsystems.org, see Ratnasingham & Hebert, 2007), which are available within the project file 'Lessepsian species'. The phylogeographic relationships between haplotypes and the species of currently and previously studied samples, which were downloaded from the NCBI and BOLD system databases, were estimated using the median joining algorithm with default settings for constructing the network (weight = 10 ε = 0) in the program NETWORK version 4.6.1.2. (Bandelt, Forster, & Röhl, 1999). The accession numbers of the NCBI mined and BOLD system samples barcode are provided in Table S1.

3 | RESULTS

3.1 | Morphologic description of the two specimens

The specimens had a slender, near cylindrical body, and rounded abdomen with four small, sharp needle-like pre-pelvic scutes (Figure 1a,d,e). There was no post-pelvic scute. A bright silver lateral stripe along the flanks was observed in both fresh and preserved samples (Figure 1a). The maxilla tip was blunt, scarcely projecting posteriorly beyond the second supra-maxilla and not reaching to the anterior border of the preopercle (Figure 1b). The isthmus muscle was short, not reaching anteriorly to the posterior border of the branchial membrane but preceeded by a small fleshy knob on the urohyal between the branchial membranes (Figure 1c). The lower gill rakers had a count between 28 and 30 for the first gill arch. Fourteen branched fin rays were observed for both dorsal and anal fins, 14–15 for the pectoral and 7–8 for the pelvic fin rays.

3.2 | Genetic analyses of the two specimens

Mitochondrial COI gene sequences were obtained from both *E. punctifer* specimens; each remaining sequence was 581 bp in length after trimming. There was no intraspecific distance between the two individuals. Mean GC and AT % values were counted as 45.44 and 54.56, respectively, with Molecular Weight = 176.67 Daltons for a single strand. During the NETWORK analysis only three base pair (bp) differences were observed between the samples in the present study and 22 previously studied *E. punctifer* samples from Taiwan, South Africa and Mozambique (Table S1 and Figure 2); 4 bp differences were observed for just one sample from South Africa (TZSAM005-05).

4 | DISCUSSION

Up to the present, two Indo-Pacific anchovy species (*Stolephorus insularis* and *Stolephorus indicus*) were recorded in the Mediterranean Sea

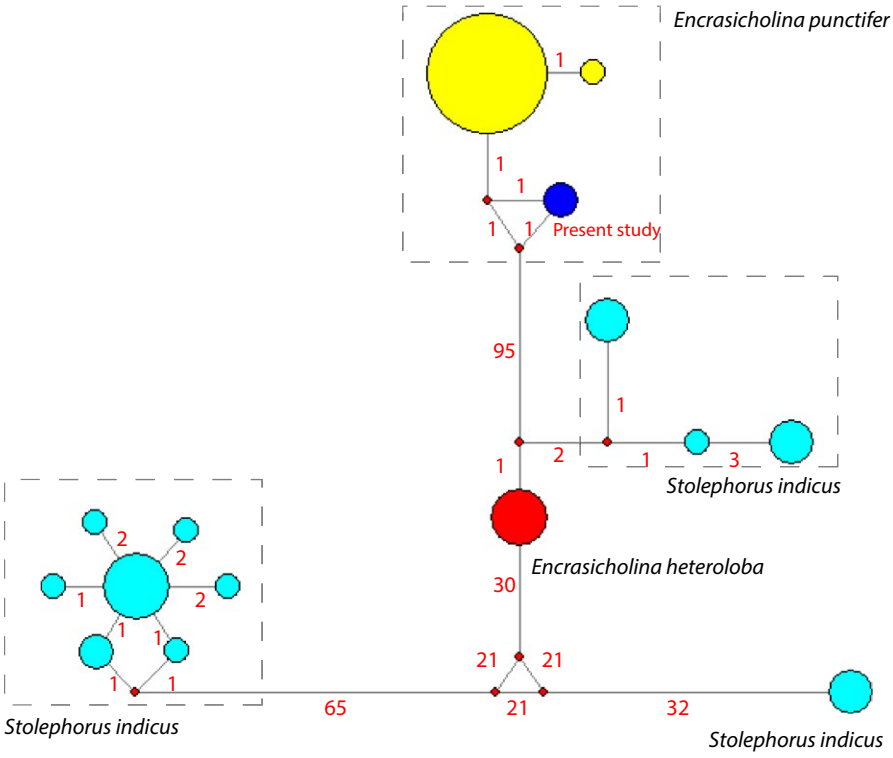

FIGURE 2 Median-joining network for COI haplotypes of present and previously studied samples. Pie sizes proportional to the number of samples. Colors = different species. Red numbers = mutation step between haplotypes/species. Red dot = median vectors. Blue cycle = two samples in present study

(Dalyan, Yemişken, Erguden, Turan, & Eryilmaz, 2014; Fricke, Golani, & Appelbaum-Golani, 2012; Fricke et al., 2015). *Encrasicholina punctifer* is easily distinguishable from the native *Engraulis encrasicolus* by the presence of pre-pelvic scutes, which are a morphological characteristic of both *Stolephorus* and *Encrasicholina* genera. On the other hand, *Encrasicholina* species are separated from *Stolephorus* by the short isthmus muscle (Wongratana et al., 1995); the isthmus muscles of the present study samples do not reach anteriorly to the posterior margin of the gill membrane (Figure 1c). The blunt maxilla tip is also one of the most important morphological characters for *E. punctifer* species (Figure 1b, Wongratana et al., 1995).

Results of molecular genetics (i.e. barcoding) have confirmed that the two specimens sampled were indeed *E. punctifer* in line with morphology. There are several records for *S. indicus* species in the NCBI database; *S. indicus* is a close relative of *E. punctifer* species. The COI gene analysis reveals at least 99 mutation steps between these two species (Table S1; Figure 2). There are also 97 mutation steps between the present study samples and the previously studied *E. heteroloba* (Lakra, Verma, Goswami, Lal, & Mohindra, 2011) species (Table S1 and Figure 2), clearly indicating that despite their similar morphology they are genetically distinct species. High mutation steps between *E. heteroloba*, *E. punctifer* and *S. indicus* species approve a well-resolved phylogeny for these anchovies.

One of the major obstacles for sustainable exploitation of fishing resources is the presence of wrong or ambiguous fish identification (Marko, Lee, Rice, Gramling, & Fitzhenry, 2004). The combined use of both molecular and morphometric tools can help to overcome this problem for native and alien species of the Mediterranean Sea. In this study, a species of *Encrasicholina* genus, *E. punctifer*, was recorded for the first time in the Mediterranean Sea in accord with both molecular and taxonomic tools. This study also provides the first DNA barcode record of this genus in the Mediterranean Sea.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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