

Red Sea fishes in the Mediterranean Sea: a preliminary investigation of a biological invasion using DNA barcoding

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ABSTRACT

Aim More than 90 marine fish species in the Mediterranean have been determined to be alien species of Red Sea origin to date and therefore it is important to prioritize research into cataloguing their distribution and impacts. The aims of this study were to establish a barcode library for alien Mediterranean fishes of probable Red Sea origin and to initiate analyses of their invasion dynamics.

Location Mediterranean Sea.

Methods Specimens of exotic fishes were collected directly from the Mediterranean Sea off the coast of Lebanon or obtained from fish markets in Lebanon. Samples were first identified morphologically and later barcoded using the universal cytochrome *c* oxidase subunit I (*COI*) mitochondrial marker. Barcodes were compared with GenBank and BOLD database entries and analysed using genetic similarity indexes and neighbour-joining distance trees.

Results In total, 156 specimens were collected, corresponding to 43 species. The sequence similarity between these sequences and their closest GenBank and BOLD matches ranged between 100% and 83.5%. The 2% genetic distance criterion, often used as a threshold for assigning positive species identification, was met for 31 of 43 (72%) alien species. Sequences from the remaining species (28%) matched species in the databases that were either in the same genus (congeneric) or in the same family (confamilial). In two cases, namely *Plotosus lineatus* and *Sargocentron rubrum*, barcoding revealed a possible species complex (*P. lineatus*) and multiple unrecognized species existing in the Mediterranean Sea (*S. rubrum*).

Main conclusions Our study presents a preliminary DNA barcode library, useful for identifying correctly alien fish species of Red Sea origin in the Mediterranean Sea. The results show that most species could be identified, yet the data also uncovered some taxa with unresolved taxonomy and possible cases of unrecognized or cryptic species invasions.

Keywords

cytochrome oxidase I, DNA barcoding, Lessepsian migration, marine bioinvasions, Mediterranean, Red Sea

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INTRODUCTION

In 1869, the opening of the Suez Canal facilitated one of the greatest biological experiments of the contemporary world: the introduction of Red Sea species into the Mediterranean (Por, 1978, 2010). These species, commonly referred to as Lessepsian migrants, represent the largest source of alien

species in the Mediterranean, by far the most invaded marine system in the world (Edelist *et al.*, 2013). From an estimated 700 marine exotic species recorded to date in the Mediterranean, about half are of Indo-Pacific origin, and these include more than 90 Lessepsian fish species (Golani *et al.*, 2013; Galil *et al.*, 2015). The ecological and economic consequences of Lessepsian invasion are enormous (Sala *et al.*,

2011; Galil *et al.*, 2015) and huge efforts have been made to understand the mechanisms allowing species of Red Sea origin to be successful in their new environment (Belmaker *et al.*, 2013; Azzurro *et al.*, 2014).

Managing the risk posed by these species requires rapid, accurate and cost-effective species identification (Darling & Blum, 2007). Indeed, this is a critical aspect of monitoring biological invasions (Armstrong & Ball, 2005) and an essential requirement for early detection systems. Nevertheless, in some cases, this cannot be achieved using traditional (i.e. morphological) approaches. Hence, Lessepsian species in general, not just fishes, are often misidentified (McGlashan *et al.*, 2007; Galil, 2009; Golani *et al.*, 2013). The history of Lessepsian invasions broadly illustrates how species that are difficult to identify frequently remain unrecognized or misidentified (e.g. Azzurro *et al.*, 2015). DNA-based methods represent a foundation for species-level diagnosis, and molecular tools are increasingly being applied to monitor invasive species (Darling & Blum, 2007), including Lessepsian fishes (e.g. Bucciarelli *et al.*, 2002; Hassan *et al.*, 2003; Hassan & Bonhomme, 2005; Golani *et al.*, 2007; Bariche & Bernardi, 2009; Tenggardjaja *et al.*, 2014; Azzurro *et al.*, 2015). The case of the Lessepsian bioinvasion is unique in that the source and the route of invasion, i.e. the Red Sea via the Suez Canal, are known (Bernardi *et al.*, 2010). Yet, remarkably, the genetic information necessary for identification is known for fewer than 10% of the species (Bernardi *et al.*, 2010).

Among the variety of existing molecular approaches, DNA barcoding is the technique most often applied for the identification of unknown specimens (Hebert *et al.*, 2003a; Hebert & Gregory, 2005). This method involves sequencing a short DNA region from a specified 'barcode' region of the genome that is particularly suitable for distinguishing the species (Blaxter, 2004; Hebert & Gregory, 2005). The discriminatory power of DNA barcodes is predicated on the demonstration that divergence within species is smaller than between species (Kochzius *et al.*, 2010). This technique is particularly useful in studies aimed at identifying species transported from different geographical locations (Bergsten *et al.*, 2012). For this and other practical reasons, barcoding is the most widely used molecular technique for exotic species recognition (Armstrong & Ball, 2005; Darling & Blum, 2007; Cross *et al.*, 2011). It facilitates the identification of individuals of unconfirmed identity and also supports the monitoring of exotic species in many other ways, such as detection in environmental samples (Takahara *et al.*, 2013), assessing the invasion potential (Gaither *et al.*, 2013; Jackson *et al.*, 2015), localizing the sources of introduction (Tenggardjaja *et al.*, 2014), distinguishing between single and multiple introductions (Porco *et al.*, 2012), assessing propagule pressure (Darling & Blum, 2007) and recognizing multiple species in complex samples (e.g. for the identification of the invasive fish diet, as in Côté *et al.*, 2013).

Many potential barcode regions have been proposed and are in use; the mitochondrial cytochrome *c* oxidase subunit I

(*COI*), suggested as a standard system for cataloguing most forms of life, is the most widely used (Hebert *et al.*, 2003a; Ebach & Holdrege, 2005; Radulovici *et al.*, 2010), with some notable exceptions, such as, for example, cnidarians (McFadden *et al.*, 2011). *COI* sequences often show a high inter-specific and low intraspecific divergence, allowing efficient species identification (Hebert *et al.*, 2003b; Ward *et al.*, 2005, 2009).

The most frequent problem with DNA identifications is the absence of reference sequences (Darling & Blum, 2007). Recently, almost complete barcode coverage has been provided for fishes of the central (Landi *et al.*, 2014) and eastern Mediterranean (Keskin & Atar, 2013) but there is incomplete coverage for exotic fishes invading the Mediterranean.

How invading species cross the Suez Canal remains poorly understood and several alternatives have been proposed (Shefer *et al.*, 2004). However, before addressing this question, an introduced species, its route of invasion and its origin, must first be identified. Whereas most Indo-Pacific species found in the Mediterranean are assumed to be the result of Lessepsian migration (i.e. crossing the Suez Canal), some cases are very probably pet releases. For example, the Mediterranean presence of the clown triggerfish, *Balistoides conspicillum* and the yellow tang, *Zebrasoma flavescens*, both unknown in the Red Sea but common in the aquarium trade, are almost certainly the result of inadvertent releases (Weitzmann *et al.*, 2015). A comparison of the DNA sequence of an exotic fish in the Mediterranean with DNA sequences from various native biogeographical regions could potentially identify the method of introduction (e.g. Lessepsian migration, human transport or mariculture) of the studied species. DNA barcoding of exotic fish species would also provide the baseline information needed to study the frequency of entry into the Mediterranean, to distinguish between a single founding event, episodic entry or continuous entry of a species (e.g. Bucciarelli *et al.*, 2002; Hassan *et al.*, 2003; Hassan & Bonhomme, 2005; Golani *et al.*, 2007; Bariche & Bernardi, 2009). From a practical point of view, our ability to identify invaders is a vital step in mitigating the risk they pose (Bickford *et al.*, 2007), and DNA barcodes are an essential identification tool for when other methods prove unreliable. This is particularly important for the traceability and safety of food (Galimberti *et al.*, 2013), for example *Lagocephalus* spp. are highly toxic Lessepsian fishes that should not be consumed (Bentur *et al.*, 2008).

We sequenced a *c.* 63-base pair (bp) fragment of the *COI* for Lessepsian fish species in the eastern Mediterranean with the aim of generating a reference DNA barcode library. We set out to test the feasibility of using DNA barcodes for tracking Lessepsian species, exploring both the potential and limitations of this approach. We focused on fish species because they are relatively easy to identify and their invasion can be tracked over time. The number of alien fishes encountered in the eastern Mediterranean (*c.* 90) was also feasible for a reasonably comprehensive analysis.

MATERIALS AND METHODS

Specimen collections

The aim was to collect samples (fin clips) from five individuals per exotic species found in Lebanon, either directly by net or spear, or purchased from the local fish market. All specimens were identified based on morphometric and meristic characteristics, and fin clips were removed and fixed in 95% ethanol.

Molecular analyses

Fin clips were digested overnight at 55 °C with proteinase K in lysis buffer [10 mM Tris-HCl pH 8.0, 400 mM NaCl, 2 mM ethylene diamine tetraacetic acid (EDTA) and 1% sodium dodecyl sulfate (SDS)]. DNA was purified using chloroform extraction and isopropanol precipitation. The DNA extract was resuspended in 100 µL ultrapure water and stored at -20 °C. Four primers designed by Ward *et al.* (2005) were used together to amplify a 655-bp fragment of the mitochondrial *COI* gene: FishF1-5'-TCA ACC AAC CAC AAA GAC ATT GGC AC-3', FishF2-5'-TCG ACT AAT CAT AAA GAT ATC GGC AC-3', FishR1-5'-TAG ACT TCT GGG TGG CCA AAG AAT CA-3' and FishR2-5'-ACT TCA GGG TGA CCG AAG AAT CAG AA-3'. Each polymerase chain reaction (PCR) reaction of 25 µL contained 10–100 ng DNA template (0.5–2 µL), 100 nM each of the four primers, 200 µM each dNTP, 2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3 at 25 °C and 1 unit of *Taq* polymerase (prepared according to Engelke *et al.*, 1990). The reaction was amplified using the following programme: one cycle at 95 °C for 2 min; 35 cycles at 94 °C for 30 s, 54 °C for 30 s, 72 °C for 1 min; one cycle at 72 °C for 10 min. The PCR products were visualized on 1% agarose gels. PCR products were purified using commercial kits (illustra DNA and Gel Band Purification Kit, GE Healthcare, Sunnyvale, CA, USA). Each amplified DNA product was sequenced with a combination of FishF1 and FishF2 primers.

Assignments and identifications

Sequences were edited and aligned using GENEIOUS (Drummond *et al.*, 2010) and characterized by the absence of stop codons, insertions or deletions. Sequence data were deposited in the Barcode of Life Data system (BOLD) database, corresponding to accession numbers BLESF001-15–BLESF054-15. Species assignments and identifications were made both by estimating genetic distances with known sequences and by placing them in distance trees in order to gain a better understanding of their potential relationships with known sequences.

The barcode sequences obtained were compared with sequences present in GenBank using BLAST (Altschup *et al.*, 1990) and with the BOLD database (Ratnasingham & Hebert, 2007). Pairwise distances were estimated using the Kimura

2-parameter (K2P) distance model. GenBank and BOLD (hereafter called databases) sequences within a sequence divergence of 5% with our own sequences were selected and used to infer phylogenetic relationships, to avoid including too many sequences in the analyses. For those cases where there were no matches within 5% of our sequences, we expanded the search to 10% sequence identity. We then looked at potential relationships using a neighbour-joining (NJ) approach generated in R (R Core Team, 2013) using the *ape* package (Paradis *et al.*, 2004).

RESULTS

Species data set

We obtained a total of 156 samples (fin clips), representing 43 marine fish species (all fish nomenclature used in this study follows Froese & Pauly, 2015). Although the original aim was to collect five individuals per species, an average of only 3.73 sequences per species was achieved because of the availability of specimens and sequencing success. We initially distinguished the 43 species from the 156 sampled individuals based on morphological identifications (Table 1). This group of species represented our reference dataset. Although some Lessepsian fishes were common (e.g. *Siganus* spp.) others were very rare (e.g. *Heniochus intermedius*), and this lack of sample series challenged their use as representatives of the species. Morphological assignments of such species tend to cluster with previously described Lessepsian species, often with the assumption that previous identifications are correct, thus potentially perpetuating early mistakes. Consequently we assigned the 43 species to the known and described Lessepsian fish species, acknowledging that misidentifications were possible.

Species identification by DNA sequence matching

After reviewing the quality of the sequencing traces (electropherograms), a final consensus sequence was produced for each species. The reported sequences ranged from 522–598 bases for three species to 602–655 bases for 40 species. We found that the sequence similarity between our own sequences and their closest database match ranged between 83.5% and 100% (Table 2). As expected, we found an increase in genetic distance between species, genus and family levels (Fig. 1, Table 2), as found by other barcoding studies (Hubert *et al.*, 2008; Landi *et al.*, 2014). Overall, 24 out of 43 (55.8%) species assignments showed a perfect match between our identification and the databases (Table 2). The 2% genetic distance often used as a coarse threshold for positive identification assignment (e.g. Landi *et al.*, 2014) was met for 31 out of 43 (72.1%) Lessepsian species. Within those 31 species, 22 corresponded to GenBank and BOLD entries that matched our own morphological identifications, including *Ostorhinchus fasciatus*, which matched its synonym *Apogon quadrifasciatus* (Eschmeyer, 2013). For the remaining

Table 1 Fish samples of Mediterranean Lessepsian bioinvasers from Lebanon. The number of samples (*n*), number of haplotypes (*nH*), length of polymerase chain reaction (PCR) fragment (bp), location of natural range and date of first Mediterranean record are shown. Nomenclature of fish follows Froese & Pauly (2015).

Species	<i>n</i>	<i>nH</i>	bp	Natural range	Date first introduction
<i>Abudefduf vaigiensis</i>	1	1	545	Indo-Pacific	1959/1997
<i>Alepes djebaba</i>	5	1	657	Indo-Pacific	1927
<i>Atherinomorus forskalii</i>	4	2	641	Red Sea	1902
<i>Callionymus filamentosus</i>	2	1	598	Indo-Pacific	Before 1953
<i>Champsodon vorax</i>	1	1	615	Indo-Pacific	2010
<i>Crenidens crenidens</i>	1	1	631	West Indian Ocean	1970
<i>Decapterus russelli</i>	4	1	646	Indo-Pacific	2005
<i>Dussumieria elopsoidea</i>	1	1	639	Indo-Pacific	1949
<i>Equulites klunzingeri</i>	4	1	626	West Indian Ocean	1931
<i>Etrumeus golanii</i>	5	1	652	Red Sea	1961
<i>Fistularia commersonii</i>	5	1	652	Indo-Pacific/TEP	2000
<i>Hemiramphus far</i>	4	1	587	Indo-Pacific	1927
<i>Heniochus intermedius</i>	1	1	622	W. Indian Ocean	2002
<i>Herklotsichthys punctatus</i>	2	1	522	Red Sea	Before 1943
<i>Jaydia queketti</i>	1	1	617	West Indian Ocean	2004
<i>Jaydia smithi</i>	6	2	651	Indo-Pacific	2007
<i>Lagocephalus spadiceus</i>	10	2	649	Indo-Pacific	1950
<i>Lagocephalus sceleratus</i>	5	1	645	Indo-Pacific	2004
<i>Lagocephalus suezensis</i>	1	1	638	Red Sea	1977
<i>Liza carinata</i>	3	1	629	West Indian Ocean	1924
<i>Lutjanus argentimaculatus</i>	1	1	577	Indo-Pacific	1977
<i>Nemipterus randalli</i>	4	1	636	West Indian Ocean	2005
<i>Ostorhinchus fasciatus</i>	7	2	644	Indo-Pacific	2008
<i>Oxyurichthys petersi</i>	6	2	652	Red Sea	1982
<i>Parupeneus forskali</i>	1	1	642	Red Sea/Gulf of Aden	2012
<i>Pempheris rhomboidea</i>	5	1	652	Indo-Pacific	1978
<i>Platycephalus indicus</i>	2	1	641	Indo-Pacific	1953
<i>Plotosus lineatus</i>	4	1	652	Indo-Pacific	2001
<i>Pomadasys stridens</i>	2	1	633	West Indian Ocean	1969
<i>Pterois miles</i>	2	1	571	Indian Ocean	1991
<i>Sargocentron rubrum</i>	5	1	651	Indo-Pacific	1945
<i>Saurida undosquamis</i>	2	1	602	Indo-Pacific	1952
<i>Scarus ghobban</i>	2	1	652	Indo-Pacific/TEP	2001
<i>Scomberomorus commerson</i>	5	1	652	Indo-Pacific	1935
<i>Siganus luridus</i>	5	1	629	West Indian Ocean	1955
<i>Siganus rivulatus</i>	5	2	626	Red Sea/Gulf of Aden	1927
<i>Sillago suezensis</i>	5	1	634	Indo-Pacific	1977
<i>Sphyrnaena chrysotaenia</i>	1	1	568	Indo-Pacific	Before 1931
<i>Stephanolepis diaspros</i>	5	1	653	Red Sea/Arabian Gulf	1927
<i>Terapon puta</i>	4	1	654	Indo-Pacific	1973
<i>Torquigener flavimaculosus</i>	7	2	631	West Indian Ocean	1987
<i>Upeneus moluccensis</i>	5	1	651	Indo-Pacific	Before 1946
<i>Upeneus pori</i>	5	1	632	Red Sea/Gulf of Oman	1942

nine sequences that were within the 2% genetic distance threshold (*Decapterus*, *Equulites*, *Hemiramphus*, *Heniochus*, *Liza*, *Nemipterus*, *Siganus*, *Stephanolepis* and *Upeneus*), database assignments and our identifications showed matching genera but different species.

Our data also showed that for the remaining cases (12 out of 43; 30.2%), the Lessepsian fishes displayed sequence identities that matched database sequences by more than the 2% threshold (less than 98% similarity). Of those 12, three had matching identifications, meaning that the species we identified and the database species were identical, although their

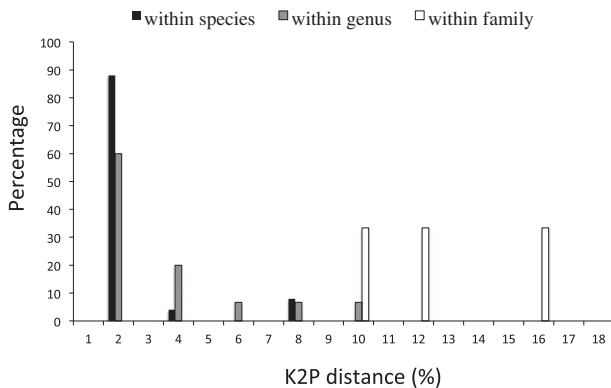
genetic similarity ranged from only 92.3% to 96.8% (Fig. 1, Table 2). The remaining nine species had matching genera (eight species, if *Jaydia* is considered to be the current nomenclature for *Apogon*) or did not match either genus or species (one species, *Herklotsichthys punctatus*). In this latter case, the closest database match belonged to the same family (Clupeidae). Finally, the closest database matches for three Lessepsian species were congeneric species: *Decapterus russelli* – *Decapterus maruadsi*, *Hemiramphus far* – *Hemiramphus archipelagus* and *Siganus luridus* – *Siganus sutor*. For those three species, databases did include the species we originally

Table 2 Barcoding sequence characteristics of Lessepsian invading fishes from Lebanon, Mediterranean Sea. The left column shows the species identified morphologically (nomenclature follows Froese & Pauly, 2015). The next four columns correspond to GenBank entries (Same indicates that they matched the left column), percentage similarity, the natural range of the species and the accession number. The four right-hand columns correspond to GenBank and BOLD entries that matched the left column but were not the closest match, or were individual specimens also collected in the Mediterranean.

Species	GenBank	%ident	Locality	Accession number	Species	%ident	Locality	Accession number
<i>Fistularia commersonii</i>	Same	100.0	Japan	AP005987				
<i>Pterois miles</i>	Same	100.0	Madagascar	JQ350295				
<i>Scarus ghobban</i>	Same	100.0	Persian gulf	HQ149928				
<i>Scomberomorus commerson</i>	Same	100.0	South Africa	HM007791				
<i>Sphyaena chrysotaenia</i>	Same	99.1	South Africa	JF494556	Same	100.0	BOLD	Private
<i>Upeneus moluccensis</i>	Same	99.5	Philippines	KF009674	Same	100.0	BOLD	Private
<i>Abudefduf vaigiensis</i>	Same	99.8	India	FJ237570				
<i>Callionymus filamentosus</i>	Same	99.8	Red Sea	JQ796948				
<i>Equulites klunzingeri</i>	<i>Equulites leuciscus</i>	99.8	Madagascar	DQ028034	Same	100.0	BOLD	Private
<i>Ostorhinchus fasciatus</i>	Same	99.8	South Africa	GU804948				
<i>Pempheris rhomboidea</i>	Same	99.8	Red Sea	KJ020196				
<i>Hemiramphus far</i>	<i>Hemiramphus archipelagus</i>	99.7	Persian gulf	HQ149857	<i>Hemiramphus far</i>	95.2	Philippines	KF714951
<i>Siganus luridus</i>	<i>Siganus sutor</i>	99.7	Madagascar	JQ350368	<i>Siganus luridus</i>	92.5	Madagascar	JQ350366
<i>Siganus rivulatus</i>	Same	99.7	Red Sea	KF434772				
<i>Atherinomorus forsskali</i>	Same	99.6	Red Sea	AB849032	<i>Atherinomorus lacunosus</i>	99.5	BOLD	Private
<i>Decapterus russelli</i>	<i>Decapterus maruadsi</i>	99.5	Malaysia	JX261150	Same	99.1	South Africa	JF493352
<i>Heniochus intermedius</i>	<i>Heniochus acuminatus</i>	99.5	Sri Lanka	FJ583541	<i>Heniochus diphreustes</i>	99.4	Madagascar	JF435030
<i>Nemipterus randalli</i>	<i>Nemipterus mesoprion</i>	99.5	India	EF609557	Same	100.0	Israel-Med.	KF564308
<i>Platycephalus indicus</i>	Same	99.5	Madagascar	JX488177				
<i>Terapon puta</i>	Same	99.5	India	KC774675	Same	100.0	BOLD	Private
<i>Lagocephalus guentheri</i>	Same	99.4	South Africa	JF493720	<i>Lagocephalus spadiceus</i>	100.0	BOLD	Private
<i>Lagocephalus scleratus</i>	Same	99.4	Unknown	JQ681800	Same	100.0	BOLD	Private
<i>Lagocephalus suezensis</i>	Same	99.4	Unknown	JQ681801	Same	100.0	BOLD	Private
<i>Stephanolepis diaspros</i>	<i>Stephanolepis auratus</i>	99.4	South Africa	KF025727	Same	100.0	BOLD	Private
<i>Alepes djebaba</i>	Same	99.1	Malaysia	HQ560999				
<i>Upeneus pori</i>	<i>Upeneus guttatus</i>	99.1	South Africa	KF489799	Same	100.0	BOLD	Private
<i>Pomadasystridens</i>	Same	99.0	Persian gulf	HQ149908	Same	100.0	Israel-Med	JQ741326
<i>Torquigener flavimaculosus</i>	Same	99.0	Unknown	JQ681843				
<i>Liza carinata</i>	<i>Liza klunzingeri</i>	98.8	India	JX983355				
<i>Crenidens crenidens</i>	Same	98.7	South Africa	JF493279				
<i>Dussumieria elopsoides</i>	Same	98.6	India	EU014224				
<i>Etrumeus golanii</i>	<i>Etrumeus teres</i>	97.9	California	GU440512	<i>Etrumeus golanii</i>	100.0	Israel-Med.	KF564305
<i>Sargocentron rubrum</i>	<i>Sargocentron seychellense</i>	97.9	Unknown	JX390736	<i>Sargocentron rubrum</i>	95.2	Turkey-Med	JQ623980
<i>Plotosus lineatus</i>	Same	96.8	India	EU148553	Same	100.0	BOLD	Private
<i>Parupeneus forsskali</i>	<i>Parupeneus heptacanthus</i>	96.3	Persian gulf	HQ149894				
<i>Champsodon vorax</i>	<i>Champsodon capensis</i>	94.5	South Africa	JF493109				
<i>Saurida undosquamis</i>	Same	92.7	India	FJ347931	<i>Saurida macrolepis</i>	100.0	Israel-Med.	KF564314
<i>Lutjanus argentimaculatus</i>	Same	92.3	Philippines	KF970482				
<i>Sillago suezensis</i>	<i>Sillago indica</i>	92.0	China	KM186884	<i>Sillago sihama</i>	100.0	Israel-Med.	FJ155363
<i>Jaydia smithi</i>	<i>Apogon ellioti</i>	90.9	South China Sea	JQ681488	<i>Apogon smithi</i>	100.0	BOLD	Private

Table 2 Continued

Species	GenBank	%ident	Locality	Accession number	Species	%ident	Locality	Accession number
<i>Oxyurichthys petersi</i>	<i>Oxyurichthys ophthalmonema</i>	90.7	South Africa	JF494033	<i>Oxyurichthys petersi</i>	99.5	Israel-Med.	KF564310
<i>Jaydia queketti</i>	<i>Apogon carinatus</i>	89.3	South China Sea	JQ681489	<i>Apogon queketti</i>	99.2	Israel-Med.	KF564297
<i>Herklotsichthys punctatus</i>	<i>Ethmidium maculatum</i>	83.5	Japan	AP011602	Same	100.0	BOLD	Private

**Figure 1** Distribution of Kimura 2-parameter (K2P) genetic distances within different taxonomic categories of fish collected from Lebanon, Mediterranean Sea.

identified as Lessepsians (*D. russelli*, *H. far*, *S. luridus*). Genetic similarity between our own Lessepsian species and those database entries varied between 92.5% and 99.1%, as shown in Table 2. This probably means that either our sequences or the database entries had been misidentified.

High intraspecific genetic distances

As expected, intraspecific divergence for the *COI* marker was generally very low, with few species (seven out of 43) showing more than one haplotype (Table 1) and, when present, haplotype differences involved a single base pair. We also found that six species that were sequenced from neighbouring countries perfectly matched our own sequences (Table 2), indicating that little variation within the *COI* marker was present.

Two species showed distances with presumed conspecifics (as determined by GenBank entries) greater than 2%, namely *Plotosus lineatus* (3.2%) and *Sargocentron rubrum* (4.8%). Distance trees of *P. lineatus* showed that genetic distances within this group were very large and probably indicated the presence of more than one species (Fig. 2c). This was supported by the presence, within this species complex, of a newly described species, *Plotosus japonicus* (Yoshino & Kishimoto, 2008). The situation for the other species, *Sargocentron 'rubrum'*, is more intriguing. The closest match to our own sequence was an entry identified as *Sargocentron seychellense* (Table 2, Fig. 2d). Surprisingly, a Mediterranean *S.*

rubrum sequence from Turkey, which was present in GenBank (Keskın & Atar, 2013), perfectly matched other database *S. rubrum* sequences (suggesting that this was probably a bona fide *S. rubrum*) but was not the closest match to our sequence (Table 2, Fig. 2d).

Distance trees

Distance trees allowed the discrepancies between our identifications and the GenBank data set to be placed within a broader context (see Appendix S1 in the Supporting Information for more information). As mentioned above, the majority of the sequences had genetic similarities with database entries greater than 98%, as shown, for example, by *Ostorhinchus fasciatus* and *Scarus ghobban* (Fig. 2a,b). We also found cases where no close matches were found, for example *Jaydia queketti* and *Jaydia smithi* (Fig. 2a). In these cases, phylogenetic reconstructions helped visualize the closest relatives of the focal species (*Apogon carinatus* and *Apogon ellioti* respectively). Out of 42 species, 35 identified Lessepsian species matched database entry identifications. Phylogenetic reconstructions showed that the remaining seven species were closely related to species belonging to the same genus or family (Appendix S1).

Origin of the Lessepsian species

Fishes of Red Sea origin collected in the Mediterranean are assumed to be the result of Lessepsian migration from the Red Sea via the Suez Canal. We therefore expected matching database species to originate from that region but also from the Indian Ocean, because many of these species are widely distributed across the Indo-Pacific. That was usually the case, bearing in mind that barcoding efforts are not geographically evenly distributed (Table 1). One interesting case was for the species we identified as *Abudefduf vaigiensis*, which perfectly matched *A. vaigiensis* individuals from India (Andamans in the eastern Indian Ocean) and the Philippines (Pacific Ocean) and other BOLD individuals presumably sampled in the Indian and Pacific Oceans. Although *A. vaigiensis* is an Indo-Pacific species and was identified in the Mediterranean relatively early (Tardent, 1959; Goren & Galil, 1998; Vacchi & Chiantore, 2000), later evidence, based on both morphological (Azzurro *et al.*, 2013; Deidun & Castriota, 2014) and molecular (Tsadok *et al.*, 2015) traits, showed that Atlantic *Abudefduf saxatilis* was also present in the western, central and eastern

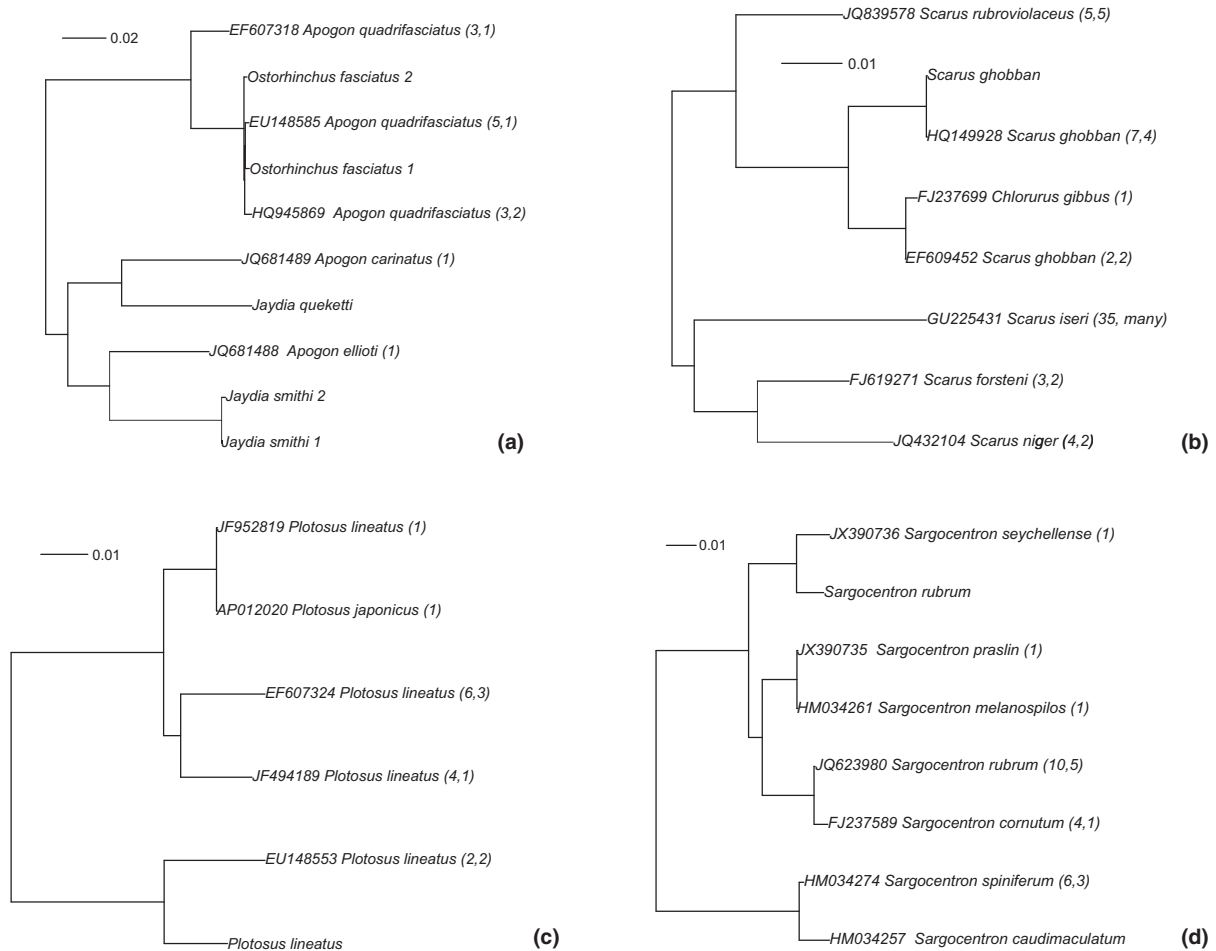


Figure 2 Phylogenetic relationships of example taxa collected from Lebanon, Mediterranean Sea. Neighbour-joining relationships of Apogonidae (a), *Scarus ghubban* (b), *Plotosus lineatus* (c) and *Sargocentron rubrum* (d). GenBank sequences are preceded by their accession numbers. Numbers in parentheses indicate the number of sequences available in GenBank (first number), and the number of independent entries (second number).

Mediterranean. Our results suggest that both species might be present in the Mediterranean. The case of the lionfish, *Pterois miles*, was also carefully scrutinized because of its potential to be a successful and devastating invader (Albins & Hixon, 2008; Albins, 2012). The closest match to our sequence was indeed *P. miles* and not *Pterois volitans*, confirming previous observations (Bariche *et al.*, 2013). In addition, we found that three GenBank entries perfectly matched our own sequences for this species. One individual was collected in the Mediterranean, in the Strait of Messina (GenBank KJ709588), another was a BOLD entry of unknown locality (GenBank FJ584026, BOLD TZAIB378-06), and the third was from Sri Lanka (GenBank FJ584029), thus consistent with an Indian Ocean origin for this Mediterranean invader.

DISCUSSION

DNA barcode-based assignments

The majority of Lessepsian migrant fishes are probably derived from populations present in the northern Red Sea.

Species deposited in gene databases may have been sampled from anywhere in the Indo-Pacific region (and are unlikely to have been sampled in the northern Red Sea), so it is important to recognize that even for the same species there might not be a 100% sequence match between Mediterranean and gene database sequences because of intraspecific variation and population structure across the Indo-Pacific (Hubert *et al.*, 2011). With this caveat in mind, we present a test case for the DNA barcode-based identification of 43 putative marine fish species collected in one of the most invaded regions of the world. Approximately 56% of the query sequences were identical to reference barcodes. Unambiguous species assignments were obtained in the majority of cases (72%), where matching sequences of the same species were available in the reference data set.

However, because there was only partial overlap between the reference and query species, a number of sequences could not be identified at the species level, resulting in 23% of the species with a sequence match of less than 97.9%. Nevertheless, for those species, a match at genus or family level was possible.

Mismatches and ambiguities in species assignments

The relatively high percentage (23%) of barcodes discussed above that showed a matching sequence identity of less than 97.9% should not necessarily be regarded as a failure of the DNA barcodes to discriminate among species. Notably, we did not find any case in our marine fish species data set in which COI did not allow us to recognize taxa among closely related species. For example, congeneric species such as *Lagocephalus scleratus*, *L. guentheri* and *Lagocephalus suzensis* were easily distinguished. For potential species complexes, such as *Plotosus*, barcodes indicate which taxa require particular attention.

In fact, most mismatches probably resulted from morphology-based misidentifications (either by ourselves or the authors of the sequences already deposited in gene databases) or from the lack of extensive barcoding efforts in the source region. For example, for *Etrumeus golanii*, a newly described species (DiBattista *et al.*, 2012), no COI sequences were found in the reference database. Interestingly, this Lessepsian species has long been misidentified with its closest GenBank match, *Etrumeus teres*. Taxonomical difficulties in species identification are a serious challenge for research of bioinvasion and management, given the sharp decline in the number of taxonomists (Wheeler *et al.*, 2004). Indeed, some fish species that have invaded the Mediterranean Sea belong to morphologically homogeneous groups that make their proper identification very difficult (e.g. Azzurro *et al.*, 2015). One practical reason for these oversights is that when an exotic species is first detected and incorrectly identified, subsequent studies can perpetuate the taxonomic error.

Several studies examining DNA barcodes of the fish fauna from other oceanic regions have found similar mismatches and ambiguities between DNA barcode data and current taxonomic knowledge (Landi *et al.*, 2014). In many of these cases, we cannot ascertain the identification of species with barcoding but we can indicate where taxonomic research efforts should be directed in the future, an example being the case of Lessepsian fishes with unresolved taxonomy, such as *Herklosichthys punctatus*, *Saurida undosquamis* and *Champsodon vorax*.

Intraspecific variability

Deep divergences within traditionally recognized species are quite common in barcoding studies aimed at building up a reference library (e.g. Puckridge *et al.*, 2013). They have been documented largely in marine ecosystems (Geller *et al.*, 2010) but also in most types of habitats, for species ranging from plants to microbes to mammals (Bickford *et al.*, 2007). We found high levels of intraspecific genetic diversity (above the 2% threshold) for *Plotosus lineatus* and *Sargocentron rubrum*, probably indicating the presence of species complexes for these two invaders or the possibility of taxonomic errors. The different lineages should be sought and identified in both the invading populations and in the Red Sea. The only plotosid in the Red Sea is currently identified as

P. lineatus (Golani & Bogorodsky, 2010). Future molecular studies could ascertain the source of this genetic variability and clarify whether a cryptic invasion has occurred or not. This would not be surprising given that the prevalence of cryptic species complexes in the tropics has probably been underestimated (Bickford *et al.*, 2007). The situation of *Sargocentron rubrum* is different, because eight species of squirrelfish belonging to the genus *Sargocentron* (including *S. rubrum*) are reported from the Red Sea (Golani & Bogorodsky, 2010). The Mediterranean sequence from Turkey is a good match with several other GenBank sequences identified as *S. rubrum*, so there is no reason to suspect a misidentification for that record. Our own sequence clusters with a sequence from the morphologically similar *S. seychellense*, but both species belong to the same *Sargocentron* clade (Dornburg *et al.*, 2012). Considering the high morphological resemblance of these fishes, clearly more than one species of *Sargocentron* have entered the Mediterranean from the Red Sea, but hitherto have remained unrecognized.

Origin of Lessepsian migrants

When geographical variability is present, DNA barcode data may highlight information below the species level, making it possible to identify the source of the introduction and the pathways followed by the exotic species to enter a new region. These two related questions have very important applied implications (Mack *et al.*, 2000) because they are of prime importance for preventing and managing invasions (Mack *et al.*, 2000; Gozlan *et al.*, 2010). The source of introduction of Lessepsian species is by definition the Red Sea, but the existence of alternative or additional routes of introduction remain possible for many of these species. Deep intraspecific divergences are common in invasive populations originating from more than one native source (admixed populations). For example, molecular studies have revealed distinct lineages of haplotypes in *Brachidontes pharaonis* (a bivalve), an acclaimed Lessepsian migrant that thrives along the eastern Mediterranean coast (Shefer *et al.*, 2004). These distinct lineages probably reflect different source populations for repeated invasions from the Red Sea. Similarly, for some putative Lessepsian fish species such as *A. vaigiensis* and *P. miles*, it is possible that alternative routes, or anthropogenic transport, have contributed to the introduction.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Appendix S1 A distance tree of the alien Mediterranean fishes based on neighbour-joining methods.

DATA ACCESSIBILITY

All sequences used in this work have been submitted to GenBank and BOLD.

BIOSKETCH

The authors' interests are focused on understanding the ecology and evolution of alien species in the Mediterranean. By combining taxonomic, ecological and genetic expertise, they work together on Lessepsian bioinvasion dynamics.

Author contributions: M.B. conceived the idea, acquired funds and collected the samples; M.T., C.S. and N.S. produced the DNA sequences; R.B. and G.B. analysed the data; M.B., E.A. and G.B. interpreted the results and carried out the writing.

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