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Employing DNA barcoding as taxonomy and conservation tools for fish species censuses at the southeastern Mediterranean, a hot-spot area for biological invasion

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ABSTRACT

This study evaluates the utility of DNA barcoding (mitochondrial cytochrome oxidase subunit I; COI) as a biodiversity and conservation applied tool for identifying fish fauna from the southeastern Mediterranean (the continental coast of Israel), a hot-spot area for biological invasion, also with an eye to establish the foundation for follow-up studies that will use environmental DNA (eDNA) tracks of native and invasive species, and for the application of eDNA concepts and methodologies in nature conservation. We established a dataset of 280 DNA barcodes, representing 110 marine fish species (all identified by a taxonomist), belonging to 75 native and 35 Lessepsian migratory species that were tested within and against the BOLD system database. Most of the query sequences showed 98% matches with reference DNA barcodes in the BOLD system excluding two *Trachurus* species, three *Dasyatis pastinaca* and two *Rhinobatos rhinobatos* individuals. Relatively high intraspecific genetic distances were observed in two Elasmobranchii species (8.83%–18%), suggesting possible cryptic species. In contrast, relatively low interspecific genetic distances were found between two Actinopterygii species (1.54%). Gobiidae family members were observed as being the most scattered on the Bayesian tree. Out of the 110 fish species, 48 were taxonomically discordant with the BOLD BINs (4 at the family level, 15 at the genus level and 29 at the species level), 53 were concordant and 9 were singletons. Discordancy was noted for some *Diplodus* and *Fistularia* species during the BOLD BIN analysis. *Apogon queketti, Champsodon nudivittis* and *Cheilodipterus novemstriatus* were uploaded to the BOLD system for the first time. We elucidated 177 haplotypes, 122 for the native fish species and 55 for the Lessepsian species. The results will allow the evaluation of invasive species success and will help developing improved policies for the conservation of Mediterranean biodiversity.

1. Introduction

Multiple drivers, cumulatively assigned as climate change and accelerated anthropogenic impacts (Jackson, 2010), affect the world's oceans, the largest biome on the planet. Oceans are also impacted by over-harvesting and destructive fishing practices (FAO, 2006), altogether reducing ecological resilience, degrading marine and oceanic habitats, including all ocean biodiversity hotspots (Nellemann, Hain, & Alder, 2008). The aforementioned factors have led to species and biodiversity changes in marine ecosystems, including plants, invertebrates and fish in all oceanic waters, and miniature oceans like the Mediterranean Sea (Lejeusne, Chevaldonné, Pergent-Martini, Boudouresque, & Pérez, 2010). Approximately 716 fish species inhabit the Mediterranean Sea (Froese & Pauly, 2014) and nearly 600 of them are indigenous, mostly of Atlantic origin that decrease in numbers gradually as one move eastward (Golani, Öztürk, & Başusta, 2006). This restricted number of fish species in the eastern Mediterranean basin has been supplemented by tropical species, as over 80 species in the list are non-indigenous species of Indo-Pacific and Red Sea origin (Bariche, 2010; Golani, 2010; http://www.faoeastmed.org/pdf/publications/EastMed_TD04.pdf). As biological invasions are now

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occurring at increasingly alarming rates (Galil, 2009; Hellmann, Byers, Bierwagen, & Dukes, 2008; Lodge et al., 2012; Ruiz, Carlton, Grosholz, & Hines, 2014), the augmented introduction of alien and invasive species becomes an important driver for biodiversity changes that occur in all oceans, including the Mediterranean Sea (Coll et al., 2010; Galil, Boero, Fraschetti, et al., 2015; Galil, Boero, Campbell, et al., 2015), as the genetic characteristics of invasive species that may be classified into two categories: 1) (pre)adapted but relatively reduced genetic diversity resulting from population bottlenecks; 2) rapid evolution associated with enhanced levels of diversity due to recurrent introduction events (Lee, 2002; Voisin et al., 2005). While exotic species do not necessarily competitively exclude native marine species (Briggs, 2007), there are increasing examples of dramatic changes in species distributions following the emergence of invasive species (Daskalov, Grishin, Rodionov, & Mihneva, 2007). This is even more apparent in the Mediterranean Sea, primarily within the Levantine basin, which is a hotspot for biological invasion (Galil, 2009).

Biodiversity is the term given to the current variety of life on earth and natural patterns it forms that has been shaped by ca. 3.5 billion years of evolution. It is commonly referred to the combination of species present in an ecosystem, as to the diversity of genes at various levels of biological organizations (Ardura, Planes, & Garcia-Vazquez, 2011; Frankham, 1995; Hedrick, 2001; Wang, Hard, & Utter, 2002). Each species is an integral part of its ecosystem; a valid organism list and accurate and quick species identifications are fundamental to understanding the health states of biological assemblages and the changes in species distributions (Keppel et al., 2012). Traditionally, morphology was the key factor in describing, delineating and naming species whose physiological, behavioral and anatomical features became irrefutable. Yet, the exact number of the species present in the marine arena will probably never be determined due to, among other causes, the looming extinction of marine species, which drives the quest for finding an efficient way to document biodiversity. It is universally acknowledged that the classical taxonomic tools are able to identify only a fraction of all species (Carew, Pettigrove, & Hoffmann, 2005) and that species extinctions/biological introductions may outpace their discovery. Similarly, cryptic species, sub-species and closely related taxa reduce the capacity to monitor the full species diversity repertoires presented by marine organisms. To solve these difficulties, the topic of DNA barcoding has emerged, and within approximately one decade since its inception (Hebert, Cywinska, Ball, & DeWaard, 2003; Hebert & Gregory, 2005), it is offering new insights not only into species delineation but also to species conservation (Ardura, Linde, Moreira, & Garcia-Vazquez, 2010; Francis et al., 2010). It is in this context that DNA barcoding is recommended for conservation efforts, though raising debates regarding its potential to aid the development of strategies that minimize biodiversity loss (Moritz & Cicero, 2004; Hebert & Gregory, 2005; Smith, Fisher, & Hebert, 2005; Witt, Threloff, & Hebert, 2006). Furthermore, the accumulation of large databases of aligned referenced sequences offers new ways of cataloguing biodiversity and novel conservation approaches. DNA barcodes thus may help identifying cryptic and polymorphic species, being used as a tool for evaluating biological invasion and as a supplementary tool for the life history stages of unknown species, and thus they are particularly helpful when morphology is ambiguous or uninformative (Schander & Willassen, 2005; Kress, García-Robledo, Uriarte, & Erickson, 2014).

The Levant, encompassing the south-eastern Mediterranean Sea, is a hot spot area for species invasion (Katsanevakis et al., 2014). The opening of the Suez Canal about 146 years ago had instigated the substantial movement of invasive Red Sea species (Lessepsian migration; Galil, Boero, Fraschetti, et al., 2015; Galil, Boero, Campbell et al., 2015) into the Mediterranean Sea, a massive biological invasion that continues by a northward path along the Levant shores. The initial sightings of the northward invasive migrators are generally reported near the Israeli coasts, and they are then followed by further northward observations at longer distances such as the Lebanese, Syrian and Turkish coast, towards the western Mediterranean (Bariche, Letourneur, & Harmelin-Vivien, 2004; Galil, 2007; Galil, Boero, Fraschetti et al., 2015; Galil, Boero, Campbell et al., 2015; Johnston & Purkis, 2014). In addition to the species that arrive via the Lessepsian migration, approximately 5000 vessels reach the Israeli Mediterranean ports annually, and the ballast water on-board 20% of them comes from the Indo-Pacific Ocean (Safriel, 2014). Thus, following the introduction of non-native fishes (as many invertebrates), the fish biota in the Levantine basin of the Mediterranean Sea have been subjected to significant changes in the last century (Goren & Galil, 2005), primarily along the Israeli Mediterranean coasts, raising challenges to all environmental conservation measures. Here, we evaluate for the first time the use of the COI gene in identifying native and invasive fish species biodiversity and intra-species population divergence near the Israeli Mediterranean coasts. Considering the vulnerability of the region (Galil, 2000), a molecular census of the area biota is severely needed in order to register the current biodiversity, understand the shifts in marine biodiversity and conserve fish diversity. Following the above, the present study tests the utility of DNA barcoding as a biodiversity and conservation applied tool for identifying fish fauna and invasion potentials of some Lessepsian fish species from the southeastern Mediterranean, a hot-spot area for biological invasion.

2. Materials and methods

2.1. Sampling

Fish were collected twice a year for a duration of two years (2011–2012) along the continental coast of Israel (SE Mediterranean Sea; Locations: Haifa, Nitzanim and Ashdod; Fig. 1), at depths of 20–40 m, on board the commercial vessels Moti and Or-David. Each transect was repeated 4–6 times (2–3 times during the day and 2–3 times during the night). The sampling was performed using an otter trawl. The net was 12 m wide and 2 m high, with a codend mesh of 42 mm. The duration of each tow was ca 90 min at a speed of ca 2.5 knots. Transects were planned according to the geographical seascape of the substrate, since the trawl can collect material only on a soft bottom, and so the transects' routes were designed to avoid natural and artificial obstacles (e.g., rocks, shipwrecks). Sampling dates, regions and coordinates are given in Table S1 (Appendix A).

On the ship's deck, the collected material was sorted according to the lowest taxon level possible, counted and labelled with the sample number. Then 1–5 specimens from each taxon (designated as the voucher specimens) were photographed and measured, the detailed geographical information was recorded and tissue samples were taken for DNA extraction, after which the sampled specimens were stored in 96% alcohol. The voucher specimens were brought to the Steinhardt National Natural History Museum and Research Center at Tel Aviv University, where each specimen was examined by a taxonomist for its final identification, after which the specimens were registered and archived in the national museum collection.

2.2. DNA isolation and PCR

Muscle tissue collected from each sampled specimen was placed separately in a 1.5 ml vial and the DNA extractions were carried out according to Paz et al. (2003). The DNA samples were diluted 1:100 in sterile double distilled water and kept at 4 °C. The cytochrome oxidase I (COI) gene (~650 bp) was amplified according to the

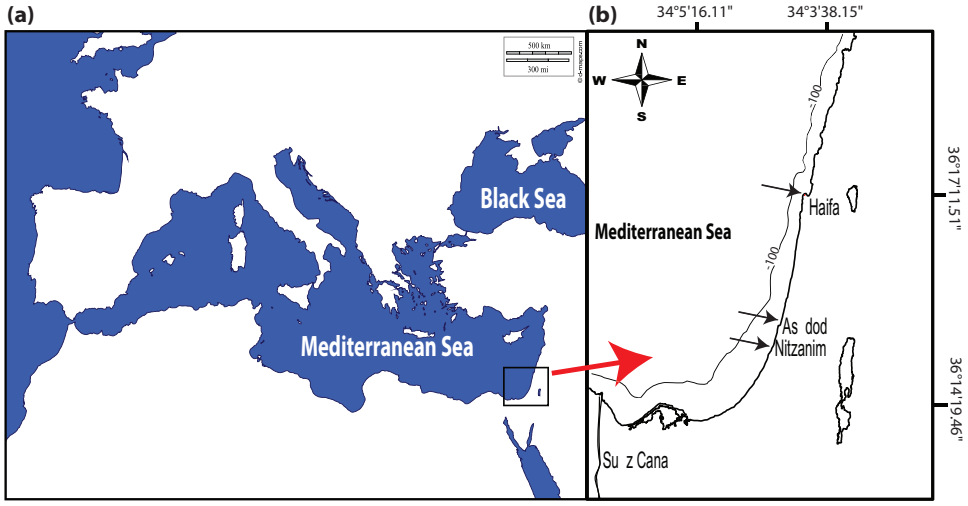

Fig. 1. a) A map representing the entire Mediterranean basin (from d-maps.com). b) The sampling sites for the 110 species collected along the Israeli Mediterranean coasts: Haifa, Ashdod and Nitzanim.

Folmer et al. (1994 and Ward, Zemlak, Innes, Last, and Hebert (2005) primers (FishF1, FishR1; FishF2, FishR2). The PCR outcomes were screened for the existence of PCR products on 1.3% agarose gel. The sequencing processes were performed by Macrogen Inc. (Seoul, South Korea) for both directions.

2.3. Data analysis

Data submission and alignment – The sequence data, trace files and primer details for specimens were submitted to the Barcode of Life Data System [BOLD, http://www.boldsystems.org, (see Ratnasingham & Herbert, 2007)], which is available within the project file 'Marine Biota of Israel Mediterranean-BIM)' (Table S1, Appendix A). The collection data and specimen images are listed in the same project folder. Sequence alignment was performed using both the Multiple Sequence Comparison by Log-Expectation (MUSCLE vs. 3.8.31, Edgar, 2004) implemented on the BOLD system and BioEdit v.7.0.9.0 (Hall, 1999) software.

Distance, gap and haplotype analysis – Distance and barcode gap analyses were carried out using the tools implemented on the BOLD system. The divergences within and between species and also between genus and family were calculated using both, the Kimura's two-parameter (K2P, Kimura, 1980) and the p-distance models, as possible with the "Distance Summary" tool available in BOLD. K2P model is now widely used to assign an unknown specimen to a known species to identify novel sequences, and to determine whether an unknown specimen is a distinct new species (Hebert, Cywinska, et al., 2003; Hebert, Ratnasingham, & DeWaard, 2003; Hsu, Ning, Gwo, & Zeng, 2013; Pereira, Pazian, Hanner, Foresti, & Oliveira, 2011). On the other hand the use of K2P distance in barcode analyses has been challenged and the p-distance has been proposed to be a better model (Collins, Boykin, Cruickshank, & Armstrong, 2012; Shen, Guan, Wang, & Gan, 2016; Srivathsan & Meier, 2012). Therefore, both, the K2P and p-distance models were used to test for the presence of the "Barcode Gap" and for the "Distance Analysis". The Barcode Gap analysis provides the distribution of the distances within each species and the distance to the nearest neighbor of each species. Haplotypes analyses were performed using the DNAsp version 5.0 software (Rozas & Rozas, 1999; Rozas, Sánchez-DelBarrio, Messeguer, & Rozas, 2003).

Discordance analysis- We applied the Barcode Index Number (BIN) system clusters (Ratnasingham & Hebert, 2013) as well, allotting to COI sequence data into OTUs (Operational Taxonomy Units; termed BINs in BOLD), independent of any prior taxonomic assignment. As such, it provides means of confirming the concordance between barcode sequence clusters and species designations. This was performed by comparing the taxonomy on the input records against all other records in the same BINs, including those submitted and managed by other users. The BIN Discordance report helps compare the taxon (species, genus, family and order level) of selected records to all the other samples in the BINs they are associated with, revealing less than 2% intraspecific differences in the sequence data.

Bayesian tree – Bayesian inferences were conducted with MrBayes [version 3.1.2 (80), Ronquist et al., 2012] using a single GTR + G model for nucleotide datasets. The model parameters, including the branch lengths, were unlinked between partitions. Bayesian analyses were computed using two incrementally heated Metropolis-coupled Markov Chain Monte Carlo (mcmc) runs for 10 million generations, with trees and associated model parameters sampled every 1000 generations. The minimal and average ESSs (effective sample size) of the runs were used to calculate the diagnostic values. The convergence of the analyses was validated by the standard deviation of split frequencies reaching 0.01 and by graphical monitoring of the likelihood values over time, using Tracer v1.4 (Rambaut & Drummond, 2013) (Fig. S1, Appendix B). The final models were visualized with FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree). Root trees and midpoint parameters were used for the Bayesian analysis tree.

3. Results

The COI sequences were obtained from 282 fish specimens (266 Actinopterygii and 16 Elasmobranchii) belonging to 110 species (75 native and 35 Lessepsian migratory species), 92 genera, 57 families and 18 orders. Of the 282 COI sequences, a stop codon was detected in two samples (*Callionymus filamentosus*: BIM-C1 and *Dasyatis pastinaca*: BIM-D12) that were excluded from the analyses. Whereas there was no error code for a *Torpedo torpode* (BIM184-13) specimen, it was not included in some BIN analyses on the BOLD system but the sequence was used for other analyses. Each of the remaining sequences was between 508 and 679 bp in length, suggesting that NUMTs (Zhang & Hewitt, 1996; nuclear DNA sequences originating from mitochondrial DNA sequences) were not sequenced (Table S1, Appendix A).

After the alignment and trimming of both direction sequences for each species, 177 haplotypes were observed, 122 for the native fish species and 55 for the Lessepsian species (Table S2, Appendix

Table 1
The Distance Summary reports for sequence divergence between barcode sequences at the species, genus and family level.

Label	n	T	C	Min Dist(%)		Mean Dist(%)		Max Dist(%)		SE Dist(%)	
				K	P	K	P	K	P	K	P
Within Species	246	76	318	0.00	0.00	0.35	0.3	18.0	15.6	0.01	0.01
Within Genus	97	15	175	1.54	1.52	15.8	13.8	28.2	23.3	0.03	0.03
Within Family	162	14	1149	7.46	7.00	17.0	14.9	26.6	21.9	0.01	0.01

T: taxa, C: comparisons, P: Pairwise distance, K: Kimura's two-parameter (K2P) distance model.

A). Usually, 3 or 4 specimens from each species were sampled. The numbers of variants (haplotypes) observed for the COI marker in each Lessepsian migrant fish species (Table 2) revealed a single haplotype for 19 species, two haplotypes for 13 species, three haplotypes for two species and four haplotypes for one species.

3.1. The compatibility of the taxonomic and the molecular methods

The 98% compatibility was observed between the taxonomic identifications and DNA barcode results for the most of the species, excluding two *Trachurus* species, three *Dasyatis pastinaca* and two *Rhinobatos rhinobatos* individuals. Two *Trachurus mediterraneus* and one *Trachurus trachurus* specimen were clustered into one OTU. Three *D. pastinaca* individuals were clustered into two OTUs (as BIMD06-BIME81 and BIME79) and two *R. rhinobatos* individuals (BIM H38 and BIMS47) into two OTUs.

3.2. Distances and gaps analyses from the BOLD system

According to the results of the "Barcode gap analysis", the minimum inter-specific distance for the Nearest Neighbor was detected between *Trachurus mediterraneus* and *T. trachurus* (K2P = 1.54%, *p*-distance = 1.52%) and the maximum between *Bregmaceros atlanticus* and *Etrumeus golanii* (K2P = 25.18%, *p*-distance = 21.21); the maximum intra-specific distance was observed in *Dasyatis patinaca* (K2P = 8.83, *p*-distance = 8.12%) and *Rhinobatos rhinobatos* (K2P = 18.00%, *p*-distance = 15.61%) (Fig. S2, Appendix B). The distance summary results are given in Table 1. Intraspecific distances, distance to nearest neighbor and "gap" within species, within genus are shown in Fig. S2-S5 (Appendix B). Whereas, P-distance model records were observed lower than the K2P distance model records for all data, no conflict recorded between the results of these two models for either min/max intraspecific distance or nearest neighbor model.

3.3. Singleton and discordance reports

Out of the 110 fish species, 48 were assigned as taxonomically discordant with the BOLD BINs (4 at the family level, 15 at the genus level and 29 at the species level), 53 were concordant and 9 were singletons (Table S3, S4-Appendix A). The BOLD BIN IDs of the singleton species are listed in Table S4 (Appendix A). One of the most noticeable cases of discordant species was the genus *Diplodus,* where the species *Diplodus sargus*, *Diplodus capensis*, *Diplodus noct*, *Diplodus sargus helenae*, *Diplodus sargus ascensionis*, *Diplodus sargus sargus* and *Diplodus sargus kotschyi* were clustered together with a distance of approximately 0.2% (BOLD:ACE3794). In a same way, the distances between the samples from *Diplodus vulgaris*, *Diplodus prayensis*, *Diplodus sargus* and *Diplodus fasciatus* were only about 1%. *Fistularia commersonii*, *Fistularia corneta* and *Fistularia petimba* were clustered together with less than 2% differences (BOLD: AAB5992). The distances between the species *Sardinella aurita, Sardinella lemuru, Sardinella longiceps, Sardinella brasiliensis* and *Sardinella sindensis* were less than 2% (BOLD: AAB7268). Out of 308 sample records (BOLD: AAA8614), less than 1% differences were observed between *Trachurus trachurus, Trachurus mediterraneus*, *Trachurus novaezelandiae*, *Trachurus picturatus*, *Trachurus novaezelandiae*, *Trachurus declivis*, *Trachurus murphyi*, *Trachurus japonicas*, *Trachurus capensis*, *Trachurus lathami*, *Trachurus symmetricus*, *Trachurus trecae*, *Trachurus picturatus murphyi* and *Trachurus delagoa*.

3.4. The Bayesian trees

The average standard deviation of the split frequencies for the Bayesian analysis was 0.01, the potential scale reduction factor (PSRF) was 1.00 and the effective sample size (ESS) was 470 for run 1 and 756 for run 2. A credible set of trees is <15002 and a 95% credible set contains 7501 trees (25% discarded as burn-in). The Bayesian analysis of 110 fish species (Fig. 2) reveals that the most distant clade is the Elasmobranchii subclass, consisting of a fifteen-sample sequences, clustered on a separate branch from the Actinoptergii subclass. The longest mean branch is depicted for the Torpenidae family members of the Elasmobranchii subclass and for a single species from three families: from the Actinoptergii subclass: Apogonidae (*Cheilodipterus novemstriatus),* Soleidae *(Solea solea)* and Bregmaceratidae *(Bregmaceros atlanticus)*. The location of the families on the tree revealed that members of the Gobiidae and the Serranidae families are the most scattered on the tree. Sparidae, Mullidae and Carangidae revealed the most regular family pattern, as almost all the family members clustered together. Also, with the exception of one sample, all the Apogonidae family members were clustered together. The tree further shows two Centracanthidae species (*Spicara maena* and *Spicara smaris)* located in one of the Sparidae family clusters and one Champsodontidae species (*Champsodon nudivittis*) included in the Mullidae family cluster (Fig. 2).

4. Discussion

Considering the wide range of conservation aspects and rationales, DNA barcoding, while not sufficient enough to rigorously address population-level questions (Moritz & Cicero, 2004), is a highly adequate tool for early detection of genetic depletion of exploited and cryptic species (Ardura et al., 2011; Rubinoff, 2006). The seemingly less important lists of species/OTUs (operational taxonomic units)/cryptic species/subspecies, may develop into indispensable tools in ecological conservation. This also reflects one of the challenges posed on DNA barcoding as an integrated part in various scientific, social, economic and political fields associated with the effective conservation of biodiversity (Krishnamurthy & Francis, 2012).

4.1. Invasion

The Mediterranean's increasing role as an international commercial shipping hub and the Suez Canal's enlargement steps (Galil, Boero, Fraschetti, et al., 2015; Galil, Boero, Campbell et al., 2015) have all contributed to a resurgence of introductions since the 1950s (Coll et al., 2010). The Levantine basin, particularly, is considered as the most invaded water body out of all the European

Table 2
The haplotype numbers for the 35 Lessepsian fish species sampled from the Israeli Mediterranean coasts.

Species numbers	Species name	n	h	Origin
1	*Alepes djedaba*	3	1	Indo-Pacific
2	*Apogon queketti*	1	1	Western Indian Ocean
3	*Apogon smithi*	5	1	Indo-Pacific
4	*Callionymus filamentosus*	3	1	Indo-Pacific
5	*Champsodon nudivittis*	1	1	Indo-West Pacific
6	*Cheilodipterus novemstriatus*	1	1	Western Indian Ocean
7	*Cynoglossus sinusarabici*	2	2	Western Indian Ocean
8	*Cynoglossus* sp.	1	1	Western Indian Ocean
9	*Decapterus russelli*	5	2	Indo-West Pacific
10	*Equulites klunzingeri*	5	2	West Indian Ocean
11	*Etrumeus golanii*	4	3	Western Indian Ocean
12	*Fistularia commersonii*	4	2	Indo-Pacific
13	*Herklotsichthys punctatus*	1	1	Western Indian Ocean
14	*Lagocephalus sceleratus*	3	1	Indo-West Pacific
15	*Lagocephalus guentheri*	3	1	Indo-West Pacific
16	*Lagocephalus suezensis*	3	2	Western Indian Ocean
17	*Nemipterus randalli*	4	1	West Indian Ocean
18	*Oxyurichthys petersii*	1	1	Western Indian Ocean
19	*Parexocoetus mento*	2	2	Indo-Pacific
20	*Petroscirtes ancylodon*	2	2	Western Indian Ocean
21	*Plotosus lineatus*	5	1	Indo-Pacific
22	*Pomadasys stridens*	5	2	West Indian Ocean
23	*Sargocentron rubrum*	4	2	Indo-West Pacific
24	*Saurida macrolepis*	4	2	Indo-West Pacific
25	*Scomberomorus commerson*	1	1	Indo-West Pacific
26	*Siganus luridus*	1	1	West Indian Ocean
27	*Siganus rivulatus*	3	2	West Indian Ocean
28	*Sillago sihama*	4	1	Indo-West Pacific
29	*Sphyraena chrysotaenia*	5	4	Indo-Pacific
30	*Stephanolepis diaspros*	2	2	Western Indian Ocean
31	*Terapon puta*	3	1	Indo-West Pacific
32	*Torquigener flavimaculosus*	3	1	West Indian Ocean
33	*Trypauchen vagina*	1	1	Indo-Pacific
34	*Upeneus moluccensis*	3	2	Indo-West Pacific
35	*Upeneus pori*	5	3	Western Indian Ocean
Total		103	55	

n: individual numbers, h: haplotype numbers.

seas, with censuses pointing to 570–1000 alien species (Coll et al., 2010; Galil, 2009; Zenetos et al., 2012), including many introduced species already establishing permanent populations while extending their distribution ranges to other Mediterranean sites. In the 2009 census (Galil, 2009), 125 alien species were recorded as having spread to at least four Mediterranean countries. Whereas some small fish and juveniles were probably not sampled because of the mesh size, this study well represents most of the adult fish species in the region.

The rapid increase in ship sizes that pass through the Suez Canal, from the "Post-Suezmax" (> 12,000 TEU) to the latest container vessels (> 19,000 TEU), which in turn required the enlargement of ports' facilities and the deepening of the canal, is envisaged to enhance biological invasion to the Mediterranean Sea, raising more conservation awareness (Galil, Boero, Fraschetti, et al., 2015; Galil, Boero, Campbell et al., 2015). This upsurge in non-native species is clearly apparent in our fish censuses, since 35 of the 110 barcoded species were alien species, of which three, one sample/haplotype from each, *Apogon queketti, Champsodon nudivittis* and *Cheilodipterus novemstriatus,* were uploaded to the BOLD system for the first time.

4.2. The first barcodes

Apogon queketti is a bethopelagic fish species, with the first Mediterranean Sea record (Iskenderun Bay, Turkey) in 2004–2005 (Eryilmaz & Dalyan, 2006). *Champsodon nudivittis* has also been recorded for the first time in Iskenderun Bay in 2008 (Çiçek & Bilecenoglu, 2009). *Cheilodipterus novemstriatus* has been recorded for the first time by Goren, Lipsky, Brokovich, & Abelson (2010) around the Tel-Aviv coastline (Israel). The present study provides the first barcode records of these three species. Whereas *A. queketti* clustered with its own family members (Apogonidae) in the Bayesian tree, *C. novemstriatus* was clustered far from the other members of the Apogonidae. In a similar way, *C. nudivittis* (Champsodontidae) samples were clustered within the Mullidae family. The nearest neighbor for *A. queketti* was *A. simithi* (K2P = 15.1, p-distance = 13.4) and the closest neighbor of *C. novemstriatus* was *Ostorhinchus fasciatus* (Apogonidae) (K2P = 19.2, p-distance = 16.5) and for *C. nudivittis M. barbatus* (K2P = 5.5, p-distance = 5.3).

4.3. Species boundaries

The use of DNA barcoding for conservation purposes is in its emerging stages, as detailed biodiversity accounts are not yet available for a wide range of biogeography zones and various taxonomically discordant groups may develop. When comparing the obtained sequences in the present research with the BOLD BINs records we revealed 48 taxonomically discordant groups (4 at the family level, 15 at the genus level and 29 at the species level; a 2% divergent threshold). According to Ratnasingham & Hebert (2007), discordance between the BIN assignments and the current taxonomy may reflect taxonomic errors, sequence contaminations, deficits in RESLs (refined single linkage algorithm), or the inability of sequence variation at COI to diagnose species due to introgression or their young age. In the present study all of the samples passed the contamination test and all of the samples were identified by an expert taxonomist. Still, when we consider the suggestion that "climate change will decrease worldwide biodiversity through cross-breeding between invasive and native species hybridization"

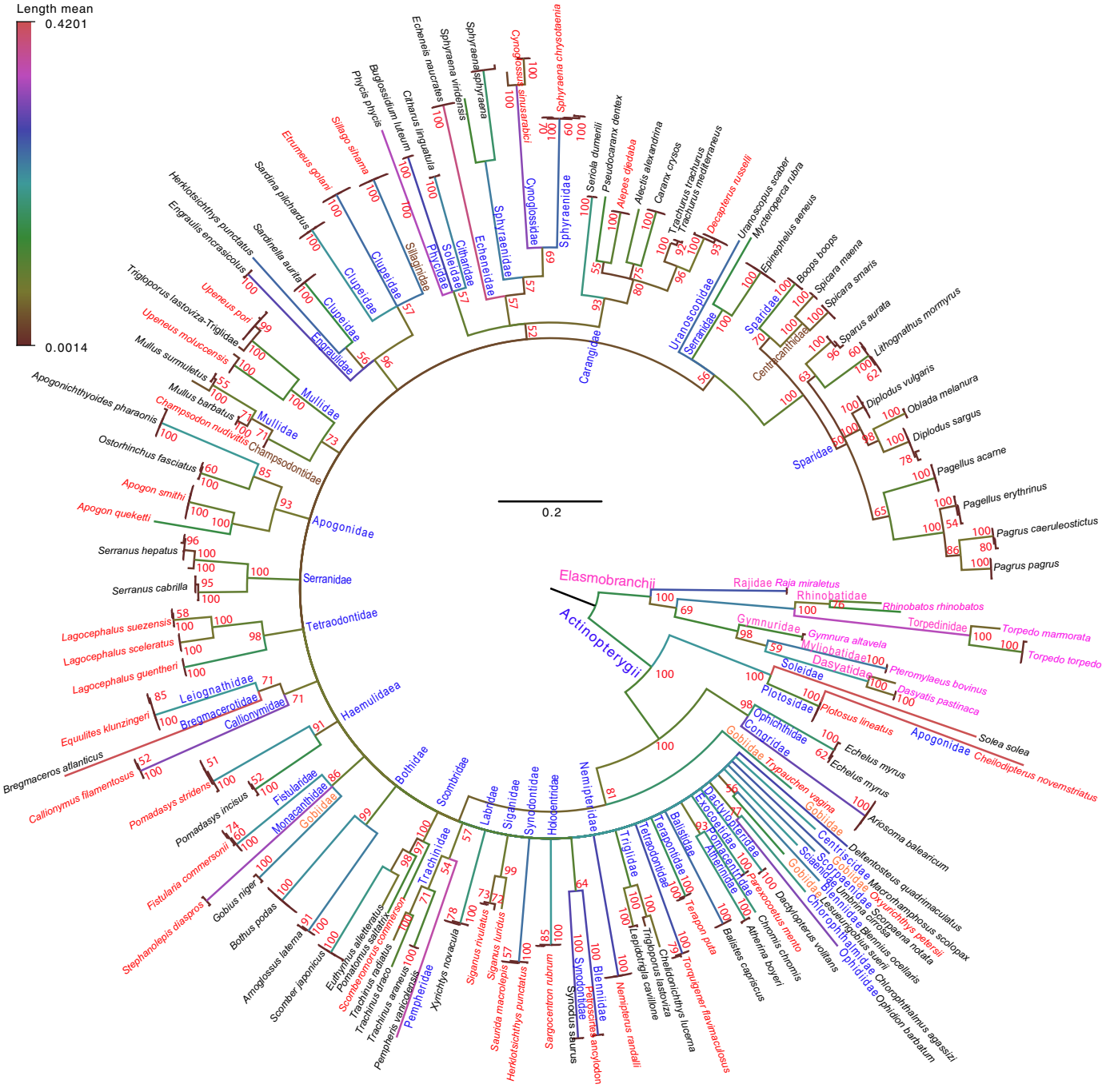

Fig. 2. The Bayesian phylogenetic tree for the 110 species collected from FigTree (midpoint, root tree). Branch colors represent the branch's mean length and node label represent bootstrap value. Elasmobanchii members are pink, Actinopterygii species are in blue and invasive species in red, with the most dispersed family coloured orange (Gobiidae). Brown marked species represent a family branch, which is in another family cluster. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(Muhlfeld et al., 2014) there is a possibility of introgression for some species.

Further consideration should be given to the emerging topic of species boundaries, a crucial biodiversity point. For instance, discordancy between *Trachurus mediterraneus* and *T. trachurus* were seen both in the present study data and previously submitted data to the BOLD system. Studying two mtDNA regions, Cárdenas et al. (2005) elucidated five distinct groups for the genus *Trachurus* with *T. trachurus* and *T. mediterraneus* clustering into two different groups. We, however, observed less than a 2% (1.52-1.54%) distance between them at the barcode region, and our result is supported by the BOLD BINs discordance report as well. This might indicate that a revision of the genus COI gene threshold value is necessary (Meyer & Paulay 2005; Virgilio, Jordaens, Breman, Backeljau, & de Meyer, 2012; Collins and Cruickshank (2013).

Other examples are the genera *Diplodus* and *Fistularia*. Domingues, Santos, Brito, Alexandrou, and Almada (2007) pointed to the possibility that *D. sargus* might have disappeared from the Atlantic coast of Europe during glacial peaks, suffering from population bottlenecks in the Canaries and Mauritania, while surviving in Madeira, the Azores and the Mediterranean. On the other hand Bargelloni et al. (2005) reported little intraspecific differentiation at

the base of both the mtDNA and allozyme markers for Atlantic and the Mediterranean populations of *D. sargus*. Based on two-mtDNA gene regions analyses *D. sargus* and *D. vulgaris* were clustered into different clusters by Summerer, Hanel, & Sturmbauer (2001). Possible misidentification and/or low COI resolution might be the reason of the discordancy for this genus species.

With regards to *Fistularia* species, Golani et al. (2007) and Tenggardjaja et al. (2014) compared the Red Sea and the Mediterranean Sea *Fistularia commersonii* populations using two mtDNA gene regions (D-loop and COI) and a nuclear gene, showing a highly significant genetic diversity between samples. In contrast, the *F. commersonii* species in the present study (considered as Red Sea origin) was clustered together with the Eastern Pacific Ocean species *F. corneta* and the Indo-Pacific species *F. petimba*. Whereas *F. commersonii* is often misidentified as *F. petimba* (a reddish or brownish-orange deep-water species with bony plates along the dorsal midline; Froese & Pauly, 2014), this is not the case for *F. corneta*. This can be explained by the complex history of intersea relationships. Compared to the Mediterranean Sea and Indian Ocean, the Red Sea is relatively young, having developed early in the Tertiary period (early Miocene; Sherman, 1998; Steininger & Wessely, 1999; Rohling et al., 1998, 2014). At the beginning of the Pliocene, the Mediterranean and Red Sea basins were parts of a single hydrographic system, and it is believe that the Red Sea was not connected to the Indian Ocean during the Mio-Pliocene. The Strait of Bab el Mandeb, which connects the Red Sea to the Indian Ocean, was opened during a more recent episode of seafloor spreading (Hsü, Stoffers, & Ross, 1978). It is possible that a low evolutionary rate and a recent entrance to the Red Sea made these different water system species similar.

4.4. Hidden diversity

Observing cases of high intra-specific distances within two Elasmobranhii species is another conservation related outcome of the present study. High intra-specific genetic distances were reported in the present study between three *Dasyatis pastinaca* (5,89-8.83%) and two *Rhinobatos* specimens (18%). Ward, Zemlak, Innes, Last, & Hebert (2005); Ward, Holmes, White, & Last (2008); Ward, Hanner, & Hebert, (2009) also revealed high intra-specific genetic divergences (c. 3–4%) for some Dasyatidae species, illustrating how barcoding can be used for conservation, by elucidating hidden diversity and highlighting taxa of potential revisionary interest. Above outcomes may also be explained by the presence of cryptic species, similarly to results obtained for other taxa, such as insects (Resch et al., 2014). Beside the Elasmobranchii, our barcoding study also revealed high hidden diversity in the family Gobiidae necessitates detail taxonomic examinations as new conservation considerations.

4.5. Genetic diversity and invasive success

Genetic diversity is a crucial component of biodiversity and fundamental to species survival. It is the basis of reproductive performance, resistance to diseases and capacity of adaptation to environmental changes (Frankham, 1995; Hedrick, 2001; Wang et al., 2002). A high genetic diversity is considered as one of the valuable causes of invasive success, allowing species to escape the harmful effects of inbreeding (Keller & Waller, 2002; Spielman, Brook, & Frankham, 2004) and to adapt to new environments (Allendorf & Lundquist, 2003; Sakai et al., 2001). Recording the haplotypes of 35 Lessepsian fish species, all sampled from an area very close to the Suez Canal, not only may revise conservation considerations but may also help in elucidating invasion success of different haplotypes, another important conservation consideration. Bariche et al. (2015) worked on 43 marine fish species from the Lebanon, recording maximum of 2 haplotypes per species as compared to up to 4 haplotypes for each of the 35-lessepsian fish species in the Israeli Mediterranean coasts (this study); while only a single haplotype was recorded for the species *Etrumeus golanii, Upeneus pori* and *Sphyraena chrysotaenia* (Bariche et al., 2015), we recorded 3 or 4 haplotypes. It is possible say that some of the *Etrumeus golanii* and *Upeneus pori* species haplotypes did not reach yet the Lebanese coasts. Such an analysis adds information for invasive species success and accordingly enhances conservation strategies when considering native and invasive species in a specific biogeographic region.

5. Conclusions

With the significant decline in biodiversity, species extinction has been enhanced (Jenkins, 2003), many of which are species with taxonomic statuses that have not been established, an outcome relevant to fisheries, and conservation of marine biodiversity (Kress et al., 2014). One of the major obstacles for the sustainable use of fishing resources and the efficient management of fisheries is the existence of wrong or ambiguous fish identification (Marko et al., 2004), a hindrance that can be overcome by using the barcoding tool. To maximize the value of the DNA barcode data for conservation, clear hypotheses should be set, and appropriate methods should be employed for testing each distinct hypothesis, necessitating collaborative work between molecular biologists, ecologists, taxonomists and bio-informaticians (Ardura et al., 2010; Collins and Cruickshank (2013); Joly et al., 2014). Here we present DNA barcodes for 110 fish species that inhabit the Israeli Mediterranean coasts, and compare the present study outcomes to the BOLD system database. The species assignments of the present study were generally straightforward, with species barcodes forming unambiguous monophyletic clusters but with tree-branches that are not always compatible with the classical taxonomy, as high intra- and low interspecific distances within and between some species. We believe that our results will pave the way for future eDNA studies to further track the distributions of native and invasive fish species.

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References

Çiçek, E., & Bilecenoglu, M. (2009). A new alien fish in the Mediterranean sea: champsodon nudivittis (Actinopterygii: perciformes: Champsodontidae). *Acta Ichthyologica Et Piscatoria*, *39*(1), 67–69. http://dx.doi.org/10.3750/AIP2009.39.1.14

Allendorf, F. W., & Lundquist, L. L. (2003). Introduction: population biology, evolution, and control of invasive species. *Conservation Biology*, *17*(1), 24–30. http://dx.doi.org/10.1046/j.1523-1739.2003.02365.x

Ardura, A., Linde, A. R., Moreira, J. C., & Garcia-Vazquez, E. (2010). DNA barcoding for conservation and management of Amazonian commercial fish. *Biological Conservation*, *143*(6), 1438–1443. http://dx.doi.org/10.1016/j.biocon.2010.03.019

Ardura, A., Planes, S., & Garcia-Vazquez, E. (2011). Beyond biodiversity: fish metagenomes. *PUBLIC LIBRARY OF SCIENCE*, *6*(8), e22592. http://dx.doi.org/10.1371/journal.pone.0022592

Bargelloni, L., Alarcon, J. A., Alvarez, M. C., Penzo, E., Magoulas, A., Palma, J., et al. (2005). The Atlantic-Mediterranean transition: discordant genetic patterns in two seabream species, *Diplodus puntazzo* (Cetti) and *Diplodus sargus* (L.). *Molecular Phylogenetics and Evolution*, *36*(3), 523–535. http://dx.doi.org/10.1016/j.ympev.2005.04.017

Bariche, M., Letourneur, Y., & Harmelin-Vivien, M. (2004). Temporal fluctuations and settlement patterns of native and Lessepsian herbivorous fishes on the Lebanese coast (eastern Mediterranean). *Environmental Biology of Fishes*, *70*(1), 81–90. http://dx.doi.org/10.1023/b:ebfi.0000022928.15148.75

Bariche, M., Torres, M., Smith, C., Sayar, N., Azzurro, E., Baker, R., et al. (2015). Red Sea fishes in the Mediterranean Sea: a preliminary investigation of a biological invasion using DNA barcoding. *Journal of Biogeography*, *42*(12), 2363–2373. http://dx.doi.org/10.1111/jbi.12595

Bariche, M. (2010). *Champsodon vorax* (Teleostei: champsodontidae), a new alien fish in the Mediterranean. *Aqua International Journal of Ichthyology*, *16*(4), 197–200.

Briggs, J. C. (2007). Marine biogeography and ecology: invasions and introductions. *Journal of Biogeography*, *34*(2), 193–198. http://dx.doi.org/10.1111/j.1365-2699.2006.01632.x

Cárdenas, L., Hernández, C. E., Poulin, E., Magoulas, A., Kornfield, I., & Ojeda, F. P. (2005). Origin, diversification, and historical biogeography of the genus Trachurus (Perciformes: carangidae). *Molecular Phylogenetics and Evolution*, *35*(2), 496–507. http://dx.doi.org/10.1016/j.ympev.2005.01.011

Carew, M. E., Pettigrove, V., & Hoffmann, A. A. (2005). The Utility of DNA markers in classical taxonomy: using cytochrome oxidase I markers to differentiate Australian Cladopelma (Diptera: chironomidae) midges. *Annals of the Entomological Society of America*, *98*(4), 587–594. http://dx.doi.org/10.1603/0013-8746(2005)098[0587:TUODMI]2.0.CO;2

Coll, M., Piroddi, C., Steenbeek, J., Kaschner, K., Ben Rais Lasram, F., Aguzzi, J., et al. (2010). The biodiversity of the Mediterranean Sea: estimates, patterns, and threats. *PUBLIC LIBRARY OF SCIENCE*, *5*(8), e11842. http://dx.doi.org/10.1371/journal.pone.0011842

Collins, R. A., & Cruickshank, R. H. (2013). The seven deadly sins of DNA barcoding. *Molecular Ecology Resources*, *13*(6), 969–975. http://dx.doi.org/10.1111/1755-0998.12046

Collins, R. A., Boykin, L. M., Cruickshank, R. H., & Armstrong, K. F. (2012). Barcoding's next top model: an evaluation of nucleotide substitution models for specimen identification. *Methods in Ecology and Evolution*, *3*(3), 457–465. http://dx.doi.org/10.1111/j.2041-210x.2011.00176.x

Daskalov, G. M., Grishin, A. N., Rodionov, S., & Mihneva, V. (2007). Trophic cascades triggered by overfishing reveal possible mechanisms of ecosystem regime shifts. *Proceedings of the National Academy of Sciences of the United States of America*, *104*(25), 10518–10523. http://dx.doi.org/10.1073/pnas.0701100104

Domingues, V. S., Santos, R. S., Brito, A., Alexandrou, M., & Almada, V. C. (2007). Mitochondrial and nuclear markers reveal isolation by distance and effects of Pleistocene glaciations in the northeastern Atlantic and Mediterranean populations of the white seabream (Diplodus sargus, L.). *Journal of Experimental Marine Biology and Ecology*, *346*(1–2), 102–113. http://dx.doi.org/10.1016/j.jembe.2007.03.002

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*(5), 1792–1797. http://dx.doi.org/10.1093/nar/gkh340

Eryilmaz, L., & Dalyan, C. (2006). First record of apogon queketti gilchrist (Osteichthyes: apogonidae) in the mediterranean sea. *Journal of Fish Biology*, *69*(4), 1251–1254. http://dx.doi.org/10.1111/j.1095-8649.2006.01185.x

FAO (2006). Global Forest Resources Assessment 2005. Progress towards sustainable forest management. FAO Forestry Paper 147. Forestry Paper, 147, 350 pp. ISBN 978-92-5-106654-6.

Folmer, O., Black, M., Hoeh, W., Lutz, R., & Vrijenhoek, R. (1994). DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, *3*(5), 294–299. http://dx.doi.org/10.1371/journal.pone.0013102

Francis, C. M., Borisenko, A. V., Ivanova, N. V., Eger, J. L., Lim, B. K., Guillén-Servent, A., et al. (2010). The role of DNA barcodes in understanding and conservation of mammal diversity in Southeast Asia. *PUBLIC LIBRARY OF SCIENCE*, *5*(9), e12575. http://dx.doi.org/10.1371/journal.pone.0012575

Frankham, R. (1995). Conservation genetics. *Annual Review of Genetics*, *29*, 305–327. http://dx.doi.org/10.1146/annurev.genet.29.1.305

Froese, R., & Pauly. D. (2014). Fishbase. FishBase. Retrieved from www.fishbase.org.

Galil, B. S. (2000). A sea under siege –Alien species in the Mediterranean. *Biological Invasions*, *2*, 177–186. http://dx.doi.org/10.1023/A:1010057010476

Galil, B. S. (2007). Seeing red: alien species along the mediterranean coast of Israel. *Aquatic Invasions*, *2*(4), 281–312. http://dx.doi.org/10.3391/ai.2007.2.4.2

Galil, B. S. (2009). Taking stock: inventory of alien species in the Mediterranean Sea. *Biological Invasions*, *11*(2), 359–372. http://dx.doi.org/10.1007/s10530-008-9253-y

Galil, B. S., Boero, F., Campbell, M. L., Carlton, J. T., Cook, E., Fraschetti, S., et al. (2015). Double trouble: the expansion of the Suez Canal and marine bioinvasions in the Mediterranean Sea. *Biological Invasions*, *17*(4), 973–976. http://dx.doi.org/10.1007/s10530-014-0778-y

Galil, B., Boero, F., Fraschetti, S., Piraino, S., Campbell, M., Hewitt, C., et al. (2015). The enlargement of the Suez Canal and introduction of non-Indigenous species to the Mediterranean Sea. *The Limnology and Oceanography Bulletin*, *24*(2), 41–43.

Golani, D., Öztürk, B., & Başusta, N. (2006). *The fishes of the eastern mediterranean sea*. Turkish Marine Research Foundation [Pub no: 24, pp. 256].

Golani, D., Azzurro, E., Corsini-Foka, M., Falautano, M., Andaloro, F., & Bernardi, G. (2007). Genetic bottlenecks and successful biological invasions: the case of a recent Lessepsian migrant. *Biology Letters*, *3*(5), 541–545. http://dx.doi.org/10.1098/rsbl.2007.0308

Golani, D. (2010). Colonization of the Mediterranean by Red Sea fishes via the Suez Canal-Lessepsian migration. In D. A.-G. B. Golani (Ed.), *Fish invasions of the Mediterranean Sea: change and renewal* (pp. 145–188). Sofia-Moscow: Pensoft Publishers.

Goren, M., & Galil, B. S. (2005). A review of changes in the fish assemblages of Levantine inland and marine ecosystems following the introduction of non-native fishes. *Journal of Applied Ichthyology*, *21*(4), 364–370. http://dx.doi.org/10.1111/j.1439-0426.2005.00674.x

Goren, M., Lipsky, G., Brokovich, E., & Abelson, A. (2010). A flood of alien cardinal fishes in the eastern Mediterranean – First record of the Indo-Pacific Cheilodipterus novemstriatus (Ruppell, 1838) in the Mediterranean Sea. *Aquatic Invasions*, *5*(suppl. 1), 49–51. http://dx.doi.org/10.3391/ai.2010.5.S1.012

Hall, T. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* [citeulike-article-id:691774].

Hebert, P. D. N., & Gregory, T. R. (2005). The promise of DNA barcoding for taxonomy. *Systematic Biology*, *54*(5), 852–859. http://dx.doi.org/10.1080/10635150500354886

Hebert, P. D. N., Cywinska, A., Ball, S. L., & DeWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings. Biological Sciences/The Royal Society*, *270*(1512), 313–321. http://dx.doi.org/10.1098/rspb.2002.2218

Hebert, P. D., Ratnasingham, S., & DeWaard, J. R. (2003). Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1), S96. http://dx.doi.org/10.1098/rsbl.2003.0025

Hedrick, P. W. (2001). Conservation genetics: where are we now? *Trends in Ecology and Evolution*, *16*(11), 629–636. http://dx.doi.org/10.1016/S0169-5347(01)02282-0

Hellmann, J. J., Byers, J. E., Bierwagen, B. G., & Dukes, J. S. (2008). Five potential consequences of climate change for invasive species. *Conservation Biology*, *22*(3), 534–543. http://dx.doi.org/10.1111/j.1523-1739.2008.00951.x

Hsü, K. J., Stoffers, P., & Ross, D. A. (1978). Messinian evaporites from the mediterranean and red seas. *Elsevier Oceanography Series*, *21*, 71–72. http://dx.doi.org/10.1016/S0422-9894(08)71076-6

Hsu, T. H., Ning, Y., Gwo, J. C., & Zeng, Z. N. (2013). DNA barcoding reveals cryptic diversity in the peanut worm Sipunculus nudus. *Molecular Ecology Resources*, *13*(4), 596–606. http://dx.doi.org/10.1111/1755-0998.12097

Jackson, J. B. C. (2010). The future of the oceans past. *Philosophical Transactions of the Royal Society of London*, *365*(1558), 3765–3778. http://dx.doi.org/10.1098/rstb.2010.0278

Jenkins, M. (2003). Prospects for biodiversity. *Science (New York, N.Y.)*, *302*(5648), 1175–1177. http://dx.doi.org/10.1126/science.1088666

Johnston, M. W., & Purkis, S. J. (2014). Are lionfish set for a Mediterranean invasion? Modelling explains why this is unlikely to occur. *Marine Pollution Bulletin*, *88*(1-2), 138–147. http://dx.doi.org/10.1016/j.marpolbul.2014.09.013

Joly, S., Davies, T. J., Archambault, A., Bruneau, A., Derry, A., Kembel, S. W., et al. (2014). Ecology in the age of DNA barcoding: the resource, the promise and the challenges ahead. *Molecular Ecology Resources*, *14*(2), 221–232. http://dx.doi.org/10.1111/1755-0998.12173

Katsanevakis, S., Coll, M., Piroddi, C., Steenbeek, J., Ben Rais Lasram, F., Zenetos, A., et al. (2014). Invading the Mediterranean Sea: biodiversity patterns shaped by human activities. *Frontiers in Marine Science*, *1*(32), 1–11. http://dx.doi.org/10.3389/fmars.2014.00032

Keller, L. F., & Waller, D. M. (2002). Inbreeding effects in wild populations. *Trends in Ecology and Evolution*, http://dx.doi.org/10.1016/s0169-5347(02)02489-8

Keppel, G., Van Niel, K. P., Wardell-Johnson, G. W., Yates, C. J., Byrne, M., Mucina, L., et al. (2012). Refugia: identifying and understanding safe havens for biodiversity under climate change. *Global Ecology and Biogeography*, *21*(4), 393–404. http://dx.doi.org/10.1111/j.1466-8238.2011.00686.x

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, *16*(2), 111–120. http://dx.doi.org/10.1007/bf01731581

Kress, W. J., García-Robledo, C., Uriarte, M., & Erickson, D. L. (2014). DNA barcodes for ecology, evolution, and conservation. *Trends in Ecology & Evolution*, *30*(1), 25–35. http://dx.doi.org/10.1016/j.tree.2014.10.008

Krishnamurthy, P. K., & Francis, R. A. (2012). A critical review on the utility of DNA barcoding in biodiversity conservation. *Biodiversity and Conservation*, *21*(8), 1901–1919. http://dx.doi.org/10.1007/s10531-012-0306-2

Lee, C. E. E. (2002). Evolutionary genetics of invasive species. *Trends in Ecology & Evolution*, *17*(8), 386–391. http://dx.doi.org/10.1016/S0169-5347(02)02554-5

Lejeusne, C., Chevaldonné, P., Pergent-Martini, C., Boudouresque, C. F., & Pérez, T. (2010). Climate change effects on a miniature ocean: the highly diverse, highly impacted Mediterranean Sea. *Trends in Ecology and Evolution*, *25*(4), 250–260. http://dx.doi.org/10.1016/j.tree.2009.10.009

Lodge, D. M., Turner, C. R., Jerde, C. L., Barnes, M. A., Chadderton, L., Egan, S. P., et al. (2012). Conservation in a cup of water: estimating biodiversity and population abundance from environmental DNA. *Molecular Ecology*, *21*, 2555–2558. http://dx.doi.org/10.1111/j.1365-294X.2012.05600.x

Marko, P. B., Lee, S. C., Rice, A. M., Gramling, J. M., Fitzhenry, T. M., McAlister, J. S., et al. (2004). Fisheries: mislabelling of a depleted reef fish. *Nature*, *430*(July), 309–310. http://dx.doi.org/10.1038/430309b

Meyer, C. P., & Paulay, G. (2005). DNA barcoding: error rates based on comprehensive sampling. *PLoS Biology*, *3*(12), 1–10. http://dx.doi.org/10.1371/journal.pbio.0030422

Moritz, C., & Cicero, C. (2004). DNA barcoding: promise and pitfalls. *PLoS Biology*, *2*(10), e354. http://dx.doi.org/10.1371/journal.pbio.0020354
Muhlfeld, C. C., Kovach, R. P., Jones, L. A., Al-Chokhachy, R., Boyer, M. C., Leary, R. F., et al. (2014). Invasive hybridization in a threatened species is accelerated by climate change. *Nature Climate Change*, *4*(7), 1–5. http://dx.doi.org/10.1038/NCLIMATE2252
Nellemann, C., Hain, S., & Alder, J. (2008). In Dead Water—Merging of climate change with pollution, over-harvest, and infestations in the world's fishing grounds. Environment. ISBN: 978-82-7701-048-9.
Paz, G., Douek, J., Mo, C., Goren, M., & Rinkevich, B. (2003). Genetic structure of *Botryllus schlosseri* (Tunicata) populations from the Mediterranean coast of Israel. *Marine Ecology Progress Series*, *250*, 153–162. http://dx.doi.org/10.3354/meps250153
Pereira, L. H. G., Pazian, M. F., Hanner, R., Foresti, F., & Oliveira, C. (2011). DNA barcoding reveals hidden diversity in the Neotropical freshwater fish *Piabina argentea* (Characiformes: characidae) from the Upper Paraná Basin of Brazil. *Mitochondrial DNA*, *22*(Suppl 1(October)), 87–96. http://dx.doi.org/10.3109/19401736.2011.588213
Rambaut, A., & Drummond, A. J. (2013). Tracer V1.6. Available from http://beast.bio.ed.ac.uk/Tracer. Retrieved from http://beast.bio.ed.ac.uk/software/tracer/.
Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: the barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes*, *7*, 355–364. http://dx.doi.org/10.1111/j.1471-8286.2006.01678.x
Ratnasingham, S., & Hebert, P. D. N. (2013). A DNA-Based registry for all animal species: the barcode index number (BIN) system. *PUBLIC LIBRARY OF SCIENCE*, *8*(7) http://dx.doi.org/10.1371/journal.pone.0066213
Resch, M. C., Shrubovych, J., Bartel, D., Szucsich, N. U., Timelthaler, G., Bu, Y., et al. (2014). Where taxonomy based on subtle morphological differences is perfectly mirrored by huge genetic distances: DNA barcoding in Protura (Hexapoda). *PUBLIC LIBRARY OF SCIENCE*, *9*(3), e90653. http://dx.doi.org/10.1371/journal.pone.0090653
Rohling, E. J., Fenton, M., Jorissen, F. J., Bertrand, P., Ganssen, G., & Caulet, J. P. (1998). Magnitudes of sea-level lowstands of the past 500,000 years. *Nature*, *394*(July), 162–165. http://dx.doi.org/10.1038/28134
Rohling, E. J., Foster, G. L., Grant, K. M., Marino, G., Roberts, A. P., Tamisiea, M. E., et al. (2014). Sea-level and deep-sea-temperature variability over the past 5.3 million years. *Nature*, *508*(7497), 477–482. http://dx.doi.org/10.1038/nature13230
Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). Mrbayes 3.2: Efficient bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–542. http://dx.doi.org/10.1093/sysbio/sys029
Rozas, J., & Rozas, R. (1999). DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics (Oxford, England)*, *15*(2), 174–175. http://dx.doi.org/10.1093/bioinformatics/15.2.174
Rozas, J., Sánchez-DelBarrio, J. C., Messeguer, X., & Rozas, R. (2003). DnaSP, DNA polymorphism analyses by the coalescent and other methods. *Bioinformatics*, *19*(18), 2496–2497. http://dx.doi.org/10.1093/bioinformatics/btg359
Rubinoff, D. (2006). Utility of mitochondrial DNA barcodes in species conservation. *Conservation Biology*, *20*(4), 1026–1033. http://dx.doi.org/10.1111/j.1523–1739.2006.00372.x
Ruiz, G. M., Carlton, J. T., Grosholz, E. D., & Hines, A. H. (2014). Global invasions of marine and estuarine habitats by non-indigenous species: mechanisms, extent, and consequences. *American Zoologist*, *37*(6), 621–632. http://dx.doi.org/10.1093/icb/37.6.621
Safriel, U. N. (2014). The Lessepsian invasion—a case study revisited. *Israel Journal of Ecology & Evolution*, *59*(4), 214–238. http://dx.doi.org/10.1080/15659801.2013.930994
Sakai, A. K., Allendorf, F. W., Holt, J. S., Lodge, M., Molofsky, J., With, K. A., et al. (2001). The population biology of invasive species. *Annual Review of Ecology and Systematics*, *32*, 305–332. http://dx.doi.org/10.1146/annurev.ecolsys.32.081501.114037
Schander, C., & Willassen, E. (2005). What can biological barcoding do for marine biology? *Marine Biology Research*, *1*(1), 79–83. http://dx.doi.org/10.1080/17451000510018962
Shen, Y., Guan, L., Wang, D., & Gan, X. (2016). DNA barcoding and evaluation of genetic diversity in Cyprinidae fish in the midstream of the Yangtze River. *Ecology and Evolution*, http://dx.doi.org/10.1002/ece3.2060
Sherman, K. (1998). Assessment, sustainability, and monitoring of coastal ecosystems: an ecological perspective. *Large marine ecosystems of the indian ocean. assessment, sustainability and management*, 3–22.
Smith, M. A., Fisher, B. L., & Hebert, P. D. N. (2005). DNA barcoding for effective biodiversity assessment of a hyperdiverse arthropod group: the ants of Madagascar. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *360*(1462), 1825–1834. http://dx.doi.org/10.1098/rstb.2005.1714
Spielman, D., Brook, B. W., & Frankham, R. (2004). Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(42), 15261–15264. http://dx.doi.org/10.1073/pnas.0403809101
Srivathsan, A., & Meier, R. (2012). On the inappropriate use of Kimura-2-parameter (K2P) divergences in the DNA-barcoding literature. *Cladistics*, *28*(2), 190–194. http://dx.doi.org/10.1111/j.1096-0031.2011.00370.x
Steininger, F. F., & Wessely, G. (1999). From the tethyan ocean to the paratethys sea: oligocene to neogene stratigraphy: paleogeography and paleobiogeography of the circum-Mediterranean region and the oligocene to neogene basin evolution in Austria. *Mitteilungen Des Österreichische Geologische Gesellschaft*, *92*, 95–116.
Summerer, M., Hanel, R., & Sturmbauer, C. (2001). Mitochondrial phylogeny and biogeographic affinities of sea breams of the genus Diplodus (Sparidae). *Journal of Fish Biology*, *59*(6), 1638–1652. http://dx.doi.org/10.1006/jfbi.2001.1796
Tenggardjaja, K., Jackson, A. M., Leon, F., Azzurro, E., Golani, D., & Bernardi, G. (2014). Genetics of a Lessepsian sprinter the bluespotted cornetfish. *Fistularia Commersonii. Israel Journal of Ecology and Evolution*, *59*(4), 181–185. http://dx.doi.org/10.1080/15659801.2013.898402
Virgilio, M., Jordaens, K., Breman, F. C., Backeljau, T., & de Meyer, M. (2012). Identifying insects with incomplete DNA barcode libraries, African fruit flies (Diptera: tephritidae) as a test case. *PUBLIC LIBRARY OF SCIENCE*, *7*(2) http://dx.doi.org/10.1371/journal.pone.0031581
Voisin, M., Engel, C. R., & Viard, F. (2005). Differential shuffling of native genetic diversity across introduced regions in a brown alga: aquaculture vs. maritime traffic effects. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(15), 5432–5437. http://dx.doi.org/10.1073/pnas.0501754102
Wang, S., Hard, J. J., & Utter, F. (2002). Genetic variation and fitness in salmonids. *Conservation Genetics*, *3*(3), 321–333. http://dx.doi.org/10.1023/a:1019925910992
Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., & Hebert, P. D. N. (2005). DNA barcoding Australia's fish species. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *360*(1462), 1847–1857. http://dx.doi.org/10.1098/rstb.2005.1716
Ward, R. D., Holmes, B. H., White, W. T., & Last, P. R. (2008). DNA barcoding Australasian chondrichthyans: results and potential uses in conservation. *Marine and Freshwater Research*, *59*(1), 57–71. http://dx.doi.org/10.1071/MF07148
Ward, R. D., Hanner, R., & Hebert, P. D. N. (2009). The campaign to DNA barcode all fishes, FISH-BOL. *Journal of Fish Biology*, *74*(2), 329–356. http://dx.doi.org/10.1111/j.1095-8649.2008.02080.x
Witt, J. D. S., Threloff, D. L., & Hebert, P. D. N. (2006). DNA barcoding reveals extraordinary cryptic diversity in an amphipod genus: implications for desert spring conservation. *Molecular Ecology*, *15*(10), 3073–3082. http://dx.doi.org/10.1111/j.1365-294x.2006.02999.x
Zenetos, A., Gofas, S., Morri, C., Rosso, A., Violanti, D., García Raso, J. E., et al. (2012). Alien species in the mediterranean sea by 2012. a contribution to the application of european unionís marine strategy framework directive (MSFD). part 2. introduction trends and pathways. *Mediterranean Marine Science*, *13*(2), 328–352. http://dx.doi.org/10.12681/mms.327
Zhang, D. X., & Hewitt, G. M. (1996). Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution*, *11*(6), 247–251. http://dx.doi.org/10.1016/0169-5347(96)10031-8