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Genetic cryptic species as biological invaders: the case of a Lessepsian fish migrant, the hardyhead silverside *Atherinomorus lacunosus*

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Abstract

Marine cryptic species, taxa that are morphologically identical but genetically distinct, may be important and underestimated components of the ecosystem. The understanding of several ecological interactions, such as marine bioinvasions, could be altered by the correct description of the bioinvaders. Here, we have focused our study on the hardyhead silverside, *Atherinomorus lacunosus*, a Lessepsian migrant. Lessepsian migrants are those species that are invading the Mediterranean from the Red Sea via the Suez Canal. We PCR amplified and sequenced the mitochondrial control region from individuals collected from the Mediterranean and the northern and southern Red Sea. We found that the two Red Sea populations are likely to correspond to two previously undescribed cryptic species. We also found that the Mediterranean individuals group with the northern Red Sea species. The Mediterranean population showed high levels of genetic diversity and did not share haplotypes with the northern Red Sea population. Lessepsian invasion by *A. lacunosus* probably occurred repeatedly and is likely to still be occurring.

Keywords: Lessepsian migrants; *Atherinomorus lacunosus*; Cryptic species; Biological invasions

1. Introduction

The number of cryptic species, taxa that cannot be distinguished morphologically but are genetically distinct, may be vastly underestimated in the marine environment

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(Knowlton, 2000). This could have important repercussions in our understanding of ecological processes, including the dynamics of marine bioinvasions. Marine biological invasions have recently become an important issue both in conservation as well as in theoretical ecology (Carlton and Geller, 1993; Holland, 2000). One essential aspect in understanding and managing bioinvasions is the correct taxonomic assignment of the invading species. Thus, undetected or misidentified marine cryptic species could be a complicating factor in our understanding of bioinvasions.

The opening of the Suez Canal in 1869, connecting the tropical Red Sea to the subtropical waters of the eastern Mediterranean Sea, provided the local faunas with an invasion route and the scientists with a means to study invasion mechanisms. The preferred invasion direction, from the Red Sea into the Mediterranean, has defined a group of organisms termed Lessepsian Migrants (Por, 1978). Among fishes alone, 56 species are known to have used this route of invasion, causing ecological impact to the native Mediterranean fauna (Por, 1978; Golani, 1993, 1999, 2000). The subject of our study, the hardyhead silverside, *Atherinomorus lacunosus*, was the first fish of Red Sea origin to be recorded in the Mediterranean following the opening of the Suez Canal (Tillier, 1902). It is a small inshore pelagic species, with a very wide geographic distribution from Australia and Japan, through the Indian Ocean to East Africa and the Red Sea, and now in the Mediterranean Sea (Ivantsoff and Crowley, 1991). The taxonomy of this species has been tumultuous. From a single widespread species, Ivantsoff and Crowley (1991) created a complex of several species, with more restricted geographic ranges. Nevertheless, the range of *A. lacunosus* itself remained very large.

While successful invasions have been studied for a long time (e.g. Safriel and Ritte, 1980), it is still difficult to determine which populations (if not all) are the actual source of successful migrants. It is likely that ecological characteristics of the species such as habitat preferences (Golani, 1993) and life cycle strategies (Swearer, personal communication) are important components. Among species with strongly structured populations, it is also possible that not all populations contribute equally to the successful migrating population. Considering the drastically different habitat conditions between the Red Sea and the Mediterranean, not all species and likewise probably not all populations are capable of migrating successfully through the Suez Canal.

This study had therefore two distinct goals. Considering the taxonomic issues involving *A. lacunosus* and its very wide distribution our first goal was to ensure that the invading species was not part of a complex of cryptic species. Secondly, we wanted to determine if a molecular approach could be used to detect population structure between Red Sea and newly established Mediterranean populations.

2. Materials and methods

2.1. Collections and DNA extractions

Individuals of the hardyhead silverside, *A. lacunosus*, were collected by experimental beach seine, over sandy shore substrate, at a depth of up to 1.5 m. Collections were done from two sites in the Red Sea: Eilat, Israel, in the north ($n = 12$), and the Dahlak

Islands, Eritrea, in the south ($n = 10$). In the Mediterranean, captures were performed along the Israeli coast at Jaffa ($n = 1$, MED1), and Hadera ($n = 11$, MED2-12) (Fig. 1). A sequence from a closely related species from the same genus, Ogilby's silverside *Atherinomorus ogilbyi*, was obtained from GenBank (Chaplin et al., unpublished), and used to compare intra-generic relationships. A sequence from the same family (Atherinidae), *Leptatherina wallacei*, obtained from Genbank (Chaplin et al., unpublished), was used as an outgroup.

Muscle tissue was dissected and preserved at ambient temperature in 95% ethanol. Tissues were digested overnight at 55 °C in 500 ml of extraction buffer (400 mM NaCl, 10 mM Tris, 2 mM EDTA, 1% SDS, Proteinase K). Then, DNA was purified by standard chloroform extraction and isopropanol precipitation (Sambrook et al., 1989).

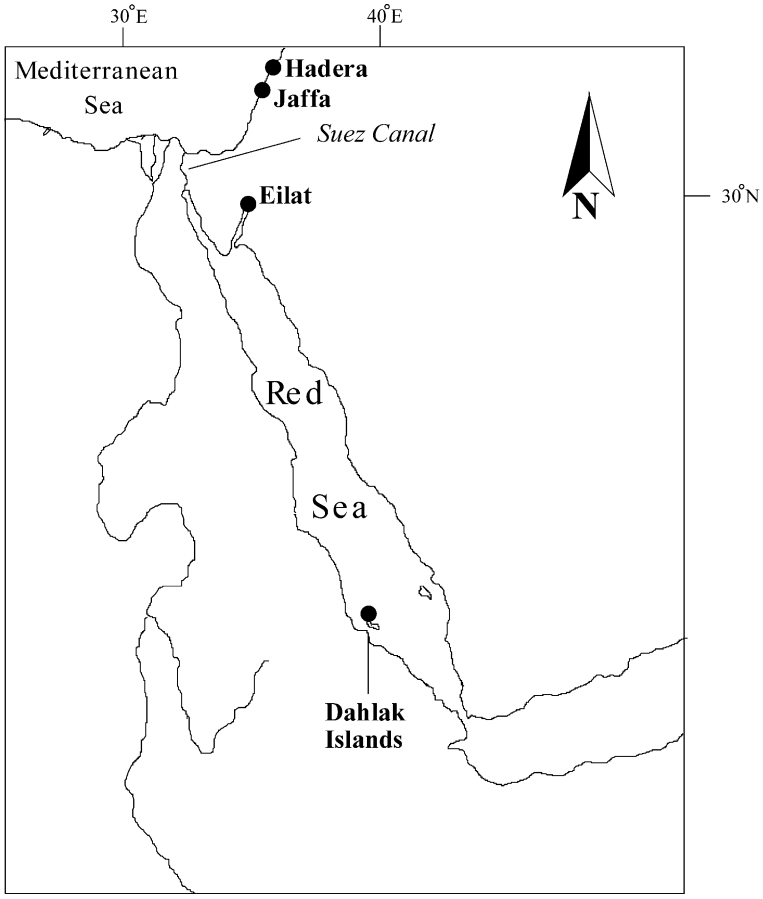

Fig. 1. *A. lacunosus* sampling locations. Samples were collected at the following localities: Jaffa, and Hadera, Israel, Mediterranean Sea; Dahlak Islands, Eritrea, southern Red Sea; Eilat, Israel, northern Red Sea.

2.2. Polymerase chain reaction (PCR) amplification

Amplification of the mitochondrial control region (also called D-loop) was accomplished with universal primers CR-A and CR-E described in Lee et al. (1995). Each 100-ml reaction contained 10 to 100 ng of DNA, 10 mM Tris HCL (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 2.5 units of Taq DNA Polymerase (Perkin-Elmer, Norwalk, CT), 150 mM of each dNTP, and 0.3 mM of each primer, and was amplified with a cycling profile of 45 s at 94 °C, 45 s at 48 °C, 1 min at 72 °C, for 35 cycles. After purification following the manufacturer's protocol (ABI, Perkin-Elmer), sequencing was performed with the primers used in the PCR amplification on an ABI 373 automated sequencer (Applied Biosystems, Foster City, CA).

2.3. Sequence analysis

We used the computer program Clustal V implemented by Sequence Navigator (Applied Biosystems) to align the mitochondrial control region sequences. Phylogenetic relationships were assessed using the Neighbor-Joining and the Maximum Parsimony methods implemented by the Software package PAUP (Phylogenetic Analyses Using Parsimony, version 4.0, Swofford, 1998). Topological confidence was evaluated with 2000 bootstrap replicates (Felsenstein, 1985). Alternative topologies were estimated using a Kishino and Hasegawa test implemented by PAUP. Genetic distances were calculated using a Kimura 2 parameter method. Fst and haplotype diversity were calculated using the software package DNAsp (Rozas and Rozas, 1997) following Hudson et al. (1992).

3. Results and discussion

3.1. Sequences

A portion of the mitochondrially encoded control region (also called D-loop) of 34 individuals (10 from the Dahlak islands, 12 from Eilat, and 12 from the Mediterranean) was PCR amplified and sequenced. Out of the 413 aligned nucleotides, which were used for the subsequent phylogenetic analysis, 68 were variable and 58 were phylogenetically informative. As expected, more transitions were observed than transversions (ratio was 2.0); however, no evidence of saturation was found (not shown). No insertions or deletions were observed.

3.2. Phylogenetic reconstructions

A phylogenetic reconstruction based on the Neighbor-Joining method is shown in Fig. 2. Data partitioned in three distinct lineages. Two sister lineages, one comprising the species *A. ogilbyi*, and the other comprising all the *A. lacunosus* individuals collected at the Dahlak Islands, were found. This last group was well-supported (100% of the bootstrap replicates). The third lineage included all the *A. lacunosus* individuals from the Mediterranean and from Eilat. This group was also very well supported (100% of the

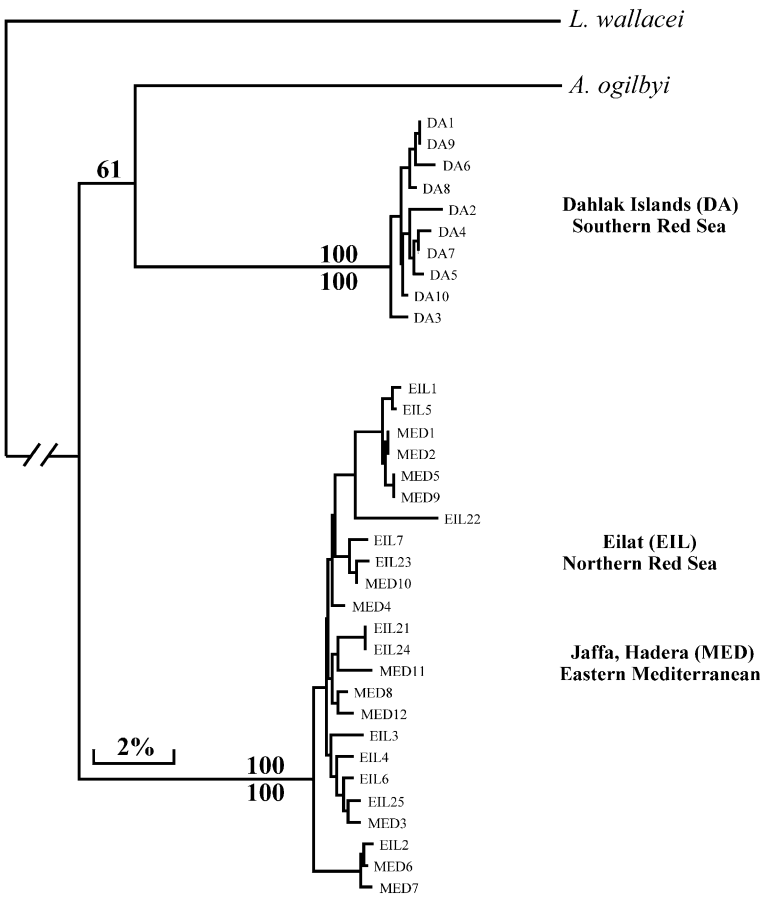

Fig. 2. Phylogenetic relationships between *A. lacunosus*. The phylogenetic tree, based on mitochondrial control region (D-loop) sequences, was obtained using the Neighbor-Joining Method implemented by the software package PAUP (version 4.0). Labels correspond to sampling localities as follows Mediterranean Sea (MED); Dahlak Islands (DA) southern Red Sea; Eilat (E) northern Red Sea. See Fig. 1 for geographic location. The length of each branch is proportional to the number of nucleotide substitutions. Scale bar represents 2% sequence divergence. The tree is rooted using *L. wallacei* as an outgroup. Bootstrap support of the major branches, when above 60%, is shown above the nodes (for the Neighbor-Joining Method) and below the nodes (for the maximum parsimony method).

bootstrap replicates). Haplotype diversity was found to be very high: 0.98 (S.D. 0.05) for Dahlak Islands samples, 0.98 (S.D. 0.04) for Eilat samples, and 0.97 (S.D. 0.04) for Mediterranean samples. When using the Maximum Parsimony method, many (>100) parsimonious trees were found (length = 698 steps, Consistency Index = 0.88). Although a large number of parsimonious trees were found, in all cases these trees partitioned the samples in the same three lineages. The sister relationship between *A. ogilbyi* and *A. lacunosus* from the Dahlak Islands was poorly supported ($<50\%$ bootstrap support). A Kishino and Hasegawa statistical test did not find a significant difference between this

topology and the two other possible topologies: a sister relationship between *A. ogilbyi* and *A. lacunosus* from the Mediterranean + Eilat, or a sister relationship between the two *A. lacunosus* clades ($p>0.4$). These three lineages are therefore to be viewed as a trichotomy. A Kishino and Hasegawa test always rejected the grouping of the Mediterranean samples with the Dahlak population rather than the Eilat population ($p<0.001$).

When considering genetic distances, *A. ogilbyi* was separated from the *A. lacunosus* Dahlak population and Mediterranean + Eilat group by 14.5% and 15.7% sequence divergence (average pairwise sequence divergences), respectively, while these two populations were separated by a sequence divergence of 12.8%. These values are well above the expected upper values for intra-specific variation (5.8%, McCune and Lovejoy, 1998).

As mentioned above, a cladistic analysis did not place *A. lacunosus* in a monophyletic group, but rather found a trichotomy that included a different species (*A. ogilbyi*) and two other clades. Furthermore, the sequence divergence between the two cryptic *A. lacunosus* is similar to the distance of either a congeneric species, *A. ogilbyi*, and this distance is within the expected range of divergence between species and well above the expected within-species range. In contrast, the Mediterranean population and the population from the northern Red Sea site (Eilat) formed a single lineage. Thus, these results suggest that the two Red Sea samples are likely to represent genetic cryptic species.

3.3. Origin of Lessepsian migrants

Our phylogenetic analysis revealed that while two pairs of Mediterranean individuals shared the same haplotype and one pair of Eilat individuals shared the same haplotype, no haplotypes were shared between Eilat and Mediterranean individuals. The difference between these two populations was further shown by high pairwise Fst values ($Fst = 0.045$). Average pairwise genetic divergences within the Eilat population ($1.88 \pm 0.76\%$), and within the Mediterranean population ($1.53 \pm 0.73\%$) were statistically identical and were also identical to the average pairwise genetic divergence of the combined samples ($1.79 \pm 0.74\%$). Unlike what would be expected from a single (or few) invasion events that would result in strong founding effects and low genetic diversity, these results indicate that the Mediterranean population is likely to be the product of a constant or repeated influx of *Atherinomorus* from the Red sea to the Mediterranean. Further analyses, with larger sample sizes, will be necessary to fully describe the nature of this invasion.

4. Conclusion

Marine biological invasions are becoming a reality with sometimes devastating effects (Verlaque and Fritayre, 1994). In order to implement efficient responses, the correct identification of the invaders is essential. Here we have shown that biological invaders do not escape the predicted high level of cryptic genetic species in the marine realm. Indeed, silversides (Atherinidae) are a taxonomically difficult group. Many workers (see Ivantsoff and Crowley, 1991) have shown that within the wide distribution of *A. lacunosus*, there

may be several distinct populations or even several distinct species. Our results support this claim. The first Lessepsian migrant, *A. lacunosus* is likely to be a member of a group of several cryptic species, as we found that within the Red Sea (a very small part of its geographic range), at least two cryptic species are found. The invading species seems to have originated from a cryptic genetic species, possibly restricted to the northern Red Sea. Further investigations will shed more light on this unique invading species. **[SS]**

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