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ORIGINAL ARTICLE

Genetic evidence for an undescribed species previously considered as *Sillago sihama* from the northern Red Sea

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Abstract

The augmentation of traditional taxonomy by the addition of genetic methods, particularly DNA analysis, has revealed that many species that appeared identical morphologically throughout their distribution range are actually divided into two or more genetically distinct species. The whiting, *Sillago sihama*, is a fish that was considered to be of a wide Indo-Pacific distribution. Not long ago, what was considered to be *S. sihama* invaded the Mediterranean from the Red Sea via the Suez Canal. In our study we sequenced a 655-bp region of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) from Hong Kong, southern Red Sea and Mediterranean populations of *Sillago*. We discovered that the first two (HK and southern Red Sea) are genetically similar while the sequencing of the Mediterranean population shows a significant divergence, confirming the existence of two distinct species. We further sequenced a segment of 178 bp (of the 655 bp) from a formaldehyde-fixed specimen from the northern Red Sea (Gulf of Suez) and found it to be identical to that of the Mediterranean population. We were thus able to confirm that the northern Red Sea population is the source for the invading population. The mitochondrial control region (D-loop) analysis of the Mediterranean population revealed that all 38 specimens have a single mitochondrial haplotype.

Key words: *Mitochondrial DNA, D-loop, Lessepsian migration,* Sillago

Introduction

The family of Sillaginidae consists of small to moderately sized benthic fishes that inhabit the shallow waters of the Indian and Pacific Oceans (McKay 1985, 1992). The family consists of approximately 30 species divided into 3 genera, 2 of which, *Sillaginopsis* and *Sillaginotes*, consist of a single species each, and the remainder belonging to the genus *Sillago*. Most of the *Sillago* species have a restricted distribution, although *Sillago sihama* (Forsskal, 1755) is known to have the widest distribution of all species in this family. Its original distribution was considered to extend in the western Pacific Ocean from Korea in the north to northern Australia and in the Indian Ocean from east Africa to the Red Sea (McKay 1985, 1992).

Accumulated studies investigating the genetic structure of fish species with a wide geographic distribution that were considered to be different populations of the same species revealed that despite similar or indistinguishable morphology they constitute a distinct taxon and are therefore termed cryptic species (Knowlton 2000). In many cases they were consequently elevated to a specific level (Bernardi & Goswami 1997; Knowlton 2000; Bucciarelli et al. 2002; Kimura et al. 2007).

The opening of the Suez Canal in 1869 connected the Red Sea and its tropically originated fauna with the Mediterranean Sea and its primarily temperate-originating fauna. With its man-made opening, the Mediterranean is exposed to a massive colonization of Red Sea organisms, termed Lessepsian migration (Golani 2010). Very few organisms have traversed

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in the opposite direction from the Mediterranean southward to the Red Sea.

At least 84 Lessepsian fish species have been recorded to date in the Mediterranean (Golani 2010; Golani et al. 2011a; Goren et al. 2011), among them the whiting *Sillago sihama*. It was first recorded in the Mediterranean in 1977 from the coast of Lebanon (Mouneimne 1977) and immediately developed a large population in its new habitat (Golani et al. 2002). However, Golani et al. (2011b) have shown that the Mediterranean and the northern Red Sea populations consist of another species distinct from *S. sihama* and resurrected the name *Sillago erythraea* Cuvier, 1829.

The use of DNA in taxonomic research (also known as DNA barcoding) has been shown to be very useful in fish studies (Ward et al. 2005) as well as other taxa (Hebert et al. 2004; Hajibabaei et al. 2006 and many more) in the last decade. DNA barcoding helps researchers to understand evolutionary and genetic relationships, and study species identification, discovery and biodiversity (Hebert et al. 2003; Lim et al. 2009). A DNA sequence from a 650-base fragment of the 5′ end of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) is the standard for species-level identification through DNA barcoding with success rates ranging from 98 to 100% (Dasmahapatra & Mallet 2006). It has proven to be a reliable tool for identifying invasive species (Smith & Fisher 2009), sister species (Tavares & Baker 2008) and cryptic species (Burns et al. 2008; Locke et al. 2010).

The COI sequencing does not reveal enough polymorphism for population studies, and therefore the mitochondrial DNA non-coding control region (D-loop) is a common tool for analysis (Munwes et al. 2010; Palencia et al. 2010; Zgonjanin et al. 2010).

The main objective of this study was to genetically barcode different fish identified as *Sillago sihama* that originated from areas separated by a large geographic distance, in order to determine their taxonomic status.

Materials and methods

Sample collection and DNA extraction

Fifty specimens of *Sillago* spp. were collected from three different locations (Table I): 8 from Hong Kong, 4 from the southern Red Sea (Eritrea) and 38 from the Mediterranean Sea (22 from Haifa bay, 8 from Michmoret, 5 from Herzliya and 3 from Jaffa). Four specimens of *Sillago ingenuua* (McKay, 1985) were also collected from Taipei. All specimens were deposited in the Fish Collection of the Hebrew University of Jerusalem (HUJ). Adult fish muscles (about 50 mg) were used for DNA sample preparation using the AccuPrep® genomic DNA extraction kit (Bioneer, Daejeon, Korea). Two formalin-fixed *S. sihama* individuals, one from the northern Red Sea and one from the southern part, were also analysed (Table I). The formalin-fixed DNA was extracted using the QIAamp DNA mini kit (Qiagen, Gaithersburg, MD, USA).

DNA sequencing

Approximately 650 bp were amplified from the 5′ region of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) using the following primers (Ward et al. 2005):

FishF1: 5′ TCAACCAACCACAAAGACATTG GCAC 3′
FishR2: 5′ ACTTCAGGGTGACCGAAGAAT CAGAA 3′

Table I. Details of specimens used in this study and relevant GenBank numbers.

Species	Location	Date	Haplotype	No. of samples	GenBank numbers
Sillago sihama	Hong Kong	May 2005	Hong Kong 1	5	FJ155369[b]
			Hong Kong 2	3	FJ155370[b]
	Eritrea, Southern Red Sea	Feb. 2005	Eritrea 1	3	FJ155365[b]
			Eritrea 4	1	FJ155367[b]
	Zula Bay, Eritrea, Southern Red Sea	Mar. 1962	Southern Red Sea	1[a]	GQ338320[c]
	Abu Zenima, Gulf of Suez Northern Red Sea	Apr. 1970	Northern Red Sea	1[a]	GQ338319[c]
	Mediterranean	May 2007	Mediterranean 1	38[e]	FJ155362[b]; GQ306205[d]
	Michmoret	Oct. 2007		8	
	Herzliya	Nov. 2007		5	
	Jaffa	Apr. 2008		3	
	Haifa Bay			22	
Sillago ingenuua	Taipei, Taiwan	May 2005	Taipei 1	4	FJ155368[b]

[a]Formalin-fixed samples. [b] 654-bp COI fragment sequences. [c] 178-bp COI fragment sequences. [d] 1102-bp D-loop sequences. [e] All 38 Mediterranean samples shared the same haplotype.

PCR reactions were carried out in 25-µl reaction volumes containing 1X PCR buffer (including 1.5 mM $MgCl_2$), 0.2 mM of each dNTPs, 1 µM of each primer, 1 unit of Super-Term Taq polymerase (Hoffmann-La Roche), and about 100 ng template DNA. PCR reactions were processed in an MJ Research thermal cycler with the following thermal regime: an initial step of 2 min at 95°C followed by 35 cycles of 0.5 min at 94°C, 0.5 min at 57°C, and 1 min at 72°C, followed by 3 min at 72°C and then held at 15°C. PCR products were visualized on 1.5% agarose gels and sequenced bidirectionally using the PCR primers on an ABI 377 DNA Sequencer (Applied Biosystems, Foster City, CA) following manufacturer's instructions.

A segment of 178 bp from the COI gene from low-quality DNA (extracted from formalin-fixed fish samples) was amplified, using the following newly designed primers:

Form 197F: 5′ GCCCCTGATATGGCATT CCC 3′
Form 382R: 5′ GTGATGAAGTTAATTGCTC CTAAAATTGA 3′

PCR reactions were carried out as described above with the following thermal regime: an initial step of 2 min at 95°C followed by 40 cycles of 0.5 min at 94°C, 0.5 min at 57°C, and 1 min at 72°C, followed by 3 min at 72°C and then held at 15°C. PCR products were visualized and sequenced as described above.

D-Loop specific mtDNA sequences of Mediterranean *Sillago* sequences were obtained by PCR amplification using four newly designed primers from the flanking cytochrome *b* and 12S genes:

CytbF1: 5′ TCGCATCCTTCTTATACTTCTT CCT 3′
CytbF2: 5′ GATGTTGTCATTCTCACCTGAA TTGG 3′
12SR1: 5′ GTGCGGATACTTGCATGTGTA A 3′
12SR2: 5′ TAGGGCTAAGCATAATAGGGTAT CTAATC 3′

Two inner primers were designed in order to get the full bidirectional sequences of the D-loop:

CytbF3: 5′ TCTAGCAGGCTGACTAGAGAA-TAAA 3′
12SR3: 5′ CAGTGTTATGCTTTAATTAAGC-TACACTA 3′

PCR reactions were carried out as described above with the following thermal regime: an initial step of 2 min at 95°C followed by 30 cycles of 45 s at 94°C, 45 s at 57°C, and 1 min at 72°C, followed by 3 min at 72°C and then held at 15°C. PCR products were visualized and sequenced as described above.

Data analysis

BioEdit Sequence Alignment Editor Ver. 7.0.9.0 (Hall 1999) was used to align the different haplotypes from this study and from GenBank (Table II). Phylogenetic trees were constructed using the neighbour-joining approach, and the significance of the branches was computed by the likelihood ratio method. Phylogenetic analysis was carried out using PHYLIP© (version 3.69), written by Felsenstein (2009). All sequences were sent to GenBank (GQ338319-20; GQ306205-07; FJ155362-70).

Table II. The 18 *Sillago* spp. haplotypes that were used for constructing Figure 1, their GenBank numbers, description, specimen voucher numbers and sample location.

Name in Figure 1	GenBank #	Description	Specimen voucher #	Location
robusta	EF609470	*S. robusta*	BW-A1551	Australia
flindersi	EF609468	*S. flindersi*	BW-A1560	Australia
ingenuua A	EF609469	*S. ingenuua*	BW-A1540	Australia
ingenuua T	FJ155368	*S. ingenuua*	HUJ19498	Taipei
japonica	HM131485	*S. japonica* haplotype 1	JXM1	China
maculata	FJ223187	*S. maculata*	MBCSC:Z711002	China
ciliata	FJ223183	*S. ciliata*	BW-A1513	Australia
analis	FJ223177	*S. analis* haplotype 1	(Not available)	Australia
bassensis	EF609466	*S. bassensis*	BW-A1546	Australia
aeolus	HM131473	*S. aeolus* haplotype 1	MDY1	China
sihama HK1	FJ155369	*S. sihama* isolate Hong Kong 1	HUJ19497-1	Hong Kong
sihama HK2	FJ155370	*S. sihama* isolate Hong Kong 2	HUJ19497-2	Hong Kong
sihama C	EU600155	*S. sihama*	SIL-SZ1	China
sihama A	EF609471	*S. sihama*	BW-A1541	Australia
sihama Ind	EF609617	*S. sihama*	WL-M186	India
sihama Er1	FJ155365	*S. sihama* isolate Eritrea 1	HUJ19716-1	Eritrea
sihama Er4	FJ155367	*S. sihama* isolate Eritrea 4	HUJ19716-4	Eritrea
sihama Med	FJ155362	*S. sihama* isolate Mediterranean Jaffa 1	HUJ19696	Mediterranean

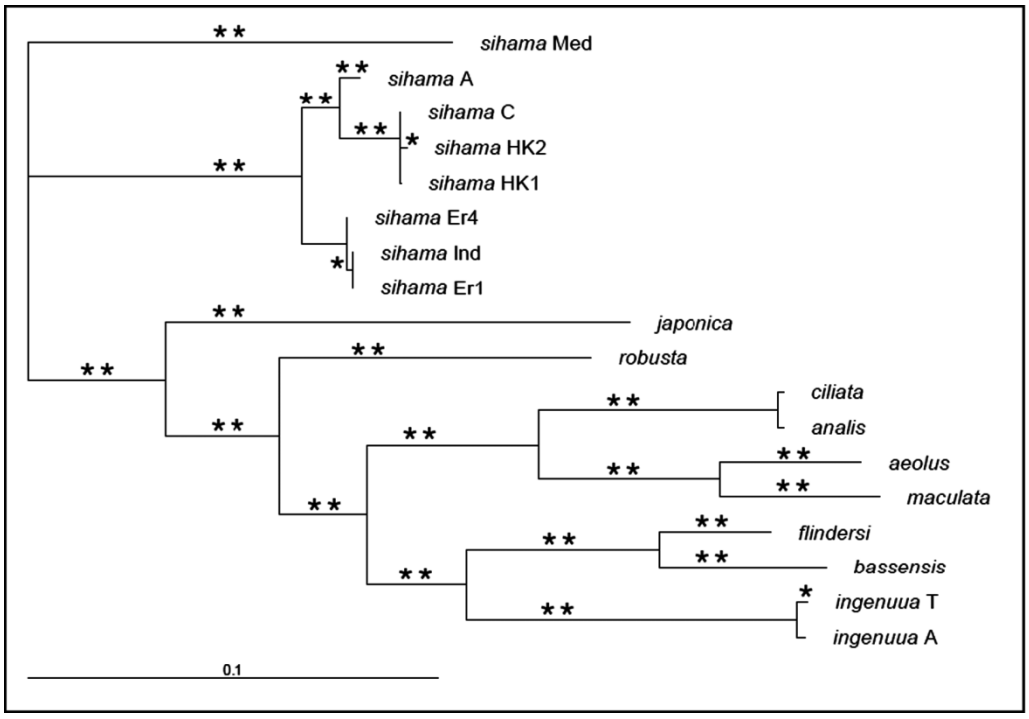

Figure 1. A neighbour-joining phylogenetic tree for 18 *Sillago* spp. haplotypes, based on differences in the cytochrome *c* oxidase subunit I gene. A single asterisk (*) next to a branch denotes a significant ($0.01 \leq P_{value} < 0.05$) likelihood ratio for that branch, and a double asterisk (**) corresponds to $P_{value} < 0.01$.

Results

All 38 Mediterranean samples had the same sequence (haplotype '*sihama* Med'). Three of the Eritrean samples shared one haplotype ('*sihama* Er1') and the fourth had a different one ('*sihama* Er4'). Five of the eight Hong Kong samples shared one haplotype ('*sihama* HK1') and the other three had the '*sihama* HK2' haplotype. All of the Taipei *Sillago ingenuua* samples shared the same haplotype ('*ingenuua* T'). Comparison with published *Sillago* cytochrome *c* oxidase subunit I gene haplotypes was performed by looking into 3 haplotypes of *S. sihama* ('*sihama* Ind' from India, '*sihama* C' from China and '*sihama* A' from Australia). Another haplotype of *S. ingenuua* ('*ingenuua* A' from Australia) and 8 haplotypes of other *Sillago* species: *S. analis* ('*analis*'), *S. ciliate* ('*ciliate*'), *S. maculata* ('*maculata*'), *S. bassensis* ('*bassensis*'), *S. japonica* ('*japonica*'), *S. aeolus* ('*aeolus*'), *S. robusta* ('*robusta*') and *S. flindersi* ('*flindersi*') from the NCBI GenBank database (Table II) were used. The resulting phylogenetic tree is shown in Figure 1. The tree clearly demonstrates a highly significant division of the sample into three main branches: one consisting only of the *S. sihama* Mediterranean haplotype, one comprises all the other *S. sihama* haplotypes, and a more diverse third branch including haplotypes of the non-*sihama Sillago* species only.

DNA from the southern Red Sea, as well as the northern Red Sea, specimens were successfully amplified and sequenced. The amplified segment contained 178 bp of the 654 bp of the COI gene. The sequences were compared to the corresponding segments of the *S. sihama* haplotypes from the previous section (Figure 2). As this smaller segment did not include some of the nucleotide differences obtained in the larger COI segment, a consensus sequences for the different geographic origins was received and the haplotypes were named accordingly, as presented in Figure 2 (Australia, Hong Kong, China, Eritrea and India). The phylogenetic tree clearly shows that the northern Red Sea sample is identical to the Mediterranean *Sillago*, while the southern Red Sea sample is very close to the other *sihama* samples.

The mitochondrial encoded control region (D-loop) of 38 individuals from the Mediterranean (see Table I for the different locations and dates) was PCR-amplified and sequenced. Out of the 1102 aligned nucleotides used for the subsequent phylogenetic analysis, none were variable. No point mutations, insertions or deletions were observed

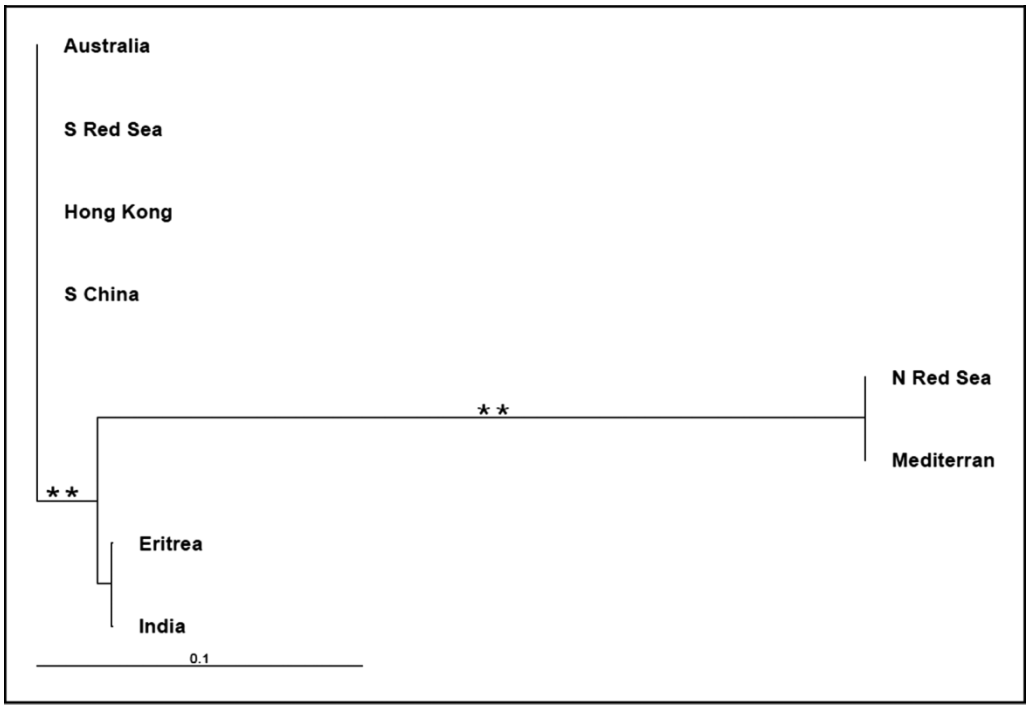

Figure 2. A neighbour-joining phylogenetic tree for 8 *Sillago sihama* haplotypes, based on differences in a 178-bp segment of the 654-bp of the COI gene. The double asterisk (**) next to a branch denotes a highly significant ($P_{value} < 0.01$) likelihood ratio for that branch.

within the Mediterranean *Sillago* population and all of the individuals share one haplotype.

Discussion

Sillago sihama was considered as having a wide Indo-Pacific distribution and it was suggested that it consists of more than one taxon. For instance, McKay (1992) shows significant differences in the shape of the swimbladder between the Australian and Red Sea specimens.

The COI barcoding method has proved to be a reliable tool for taxonomic analysis of many species (Hebert et al. 2004; Waugh 2007) including fish (Helfman et al. 2009). The COI-based phylogenetic tree constructed and based on our findings (Figure 1) clearly demonstrates the differences between various *Sillago* species and is consistent with the morphologic-based taxonomy (McKay 1992).

Therefore, it can be concluded that the immigrating Mediterranean population did not originate from the southern Red Sea *Sillago sihama*, and the two populations previously considered to be the same species are genetically distinct species. Due to the lack of fresh material from the northern Red Sea and especially from the Gulf of Suez, considered to be the 'launching pad' for Lessepsian migrants (Golani 1993), a formalin-fixed specimen was used, taken from Abu Zenima, Gulf of Suez, which is deposited in the Hebrew University Fish Collection. The reason that fresh material from the northern Red Sea could not be obtained is that it is apparently rare in the Gulf of Aqaba, probably due to the topography of the gulf. However, in the Gulf of Suez, where it is abundant, it was, unfortunately, impossible to collect for political reasons. The technical challenge of extracting DNA from formalin-fixed samples limited the analysis to a short sequence of only 178 bp. Comparing this short sequence to the other samples revealed a perfect match between the northern Red Sea specimen and the Mediterranean specimens, while the southern Red Sea specimen belongs to the *S. sihama* branch (Figure 2). Therefore, it can be concluded that there are two genetically distinct species in the Red Sea, and the Mediterranean population originated from the northern Red Sea. This result agrees with Golani et al. (2011b), who showed that, based on the morphology of the swimbladder, the number of vertebrae and the squamation of the operculum, the northern Red Sea and the Mediterranean populations together constitute a distinct species that differs from *Sillago sihama*. Golani et al. (2011b) have resurrected the name *S. erythraea* Cuvier for this species.

A similar case of two distinct lineages between the southern and northern Red Sea was found in the hardyhead silverside, *Atherinomorus lacunosus*, which is also a Lessepsian migrant (Bucciarelli et al. 2002).

A further morphological study (Kimura et al. 2007) elevated this two lineage taxa to the specific level, *Atherinomorus lacunosus* (Forster, 1801) and *Atherinomorus forskalii* (Ruppell, 1838).

There are numerous cases of fish species that are confined to the northern Red Sea or even to the Gulf of Suez. Some examples are: *Hippocampus suezensis* Dunker 1940 (Syngnathidae), *Diplogrammoides gruveli* Smith, 1963 (Callionymidae), *Lagocephalus suezensis* Clark and Golhar, 1953 (Tetraodontidae) and *Bathygobius fishelsoni* Goren, 1978 (Gobiidae).

Endemism in the northern Red Sea, and in particular in the Gulf of Suez, was discussed by Fricke (1983), Golani (1999), Por (2010) and others. According to these authors, the Gulf of Suez has had many instances of species isolation as a result of vast fluctuations of temperature and sea levels during glacial periods, repeatedly becoming hypersaline, and as a consequence resulting in high levels of endemism.

The Mediterranean population seems to have very limited variation. Although sampled at different locations and times (Table I), all individuals had the very same sequence. Even a detailed look at the most variable region, the non-coding D-loop of the mitochondrial DNA, revealed no polymorphism. Many population studies use the D-loop (AKA variable region) as a tool to study their variation (Li et al. 2008; Khan et al. 2008 and many others). The mtDNA has a high mutation rate due to the limited DNA repair mechanisms (Brown et al. 1979; Larsen et al. 2005; Neiman & Taylor 2009). The D-loop is the only non-coding region of the mtDNA, and therefore has a weaker selection pressure. As a result, the D-loop is usually a very polymorphic region reflecting the accumulation of DNA changes over short periods of time. In fish, the coding genes are used for barcoding at the species, genus and family levels, while the D-loop is used for population studies. In *S. sihama* as well as other *Sillago* species like *S. ciliata*, *S. analis* and *S. maculata*, sequence comparisons of the D-loop revealed a high degree of polymorphism (GenBank accession no.: JN626248-248; FJ223190-202).

Several studies of fish migrants, including Lessepsian migrant fish, have shown a reduction of genetic variability in the colonizing populations as compared to the source population (see Golani et al. 2007). The single haplotype of all 38 *S. sihama* specimens suggests that the Mediterranean population did in fact undergo such a loss of genetic variability. However, due to contingencies that prevented sampling from the Northern Red Sea and the Suez Canal, we refrain at this stage from categorically stating that a genetic bottleneck occurred. Future molecular studies of the northern Red Sea populations are necessary to resolve this quandary.

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