

Combined analysis of four mitochondrial regions allowed the detection of several matrilineal lineages of the lessepsian fish *Fistularia commersonii* in the Mediterranean Sea

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The bluespotted cornetfish (Fistularia commersonii) is an Indo-Pacific species that in the last ten years colonized a large part of the Mediterranean basin. The aim of this study was to sequence some portions of the mitochondrial DNA (D-loop II, 16S, 12S and Cyt b) of this fish from different localities of the Mediterranean Sea, in order to evaluate the level of its genetic variability in this area. The genetic analysis performed on specimens from seven localities of Sardinia, Tunisia and Libya revealed the presence of at least five mitochondrial lineages. The results obtained, compared with previous studies, indicate that the use of a sufficient number of mitochondrial regions may allow a more accurate estimate of genetic variability in lessepsian invasions.

Keywords: biological invasions, bluespotted cornetfish, mitochondrial DNA, Mediterranean Sea, matrilineal lineages

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INTRODUCTION

In the last decades, the invasion of non-indigenous species in the Mediterranean Sea from the Red Sea through the Suez Canal, the so-called 'lessepsian migration' (Por, 1971), has dramatically increased (Galil, 2000). The lessepsian migration has always animated the interest of scientists (mainly ichthyologists), generally because of interactions between immigrants and the Mediterranean autochthonous species (see e.g. Rilov & Galil, 2009 and references therein). Conversely, less attention has been paid to the genetic structure of these colonizers (Hassan *et al.*, 2003; Hassan & Bonhomme, 2005; Azzurro *et al.*, 2006; Bernardi *et al.*, 2010).

The bluespotted cornetfish *Fistularia commersonii* Rüppell, 1838 (Osteichthyes: Fistulariidae) is an Indo-Pacific species recently recorded in the Mediterranean Sea. After its first report from the Middle East coast (Golani, 2000), the geographical distribution of this fish has rapidly extended to the eastern Mediterranean (Bilecenoglu *et al.*, 2002; Corsini *et al.*, 2002; Karachle *et al.*, 2004), to some areas near the Strait of Sicily (Azzurro *et al.*, 2004; Ben Souissi *et al.*, 2004)

and, more recently, to the western Mediterranean (Pais *et al.*, 2007; Hemida & Capapé, 2009) and the Adriatic Sea (Dulčić *et al.*, 2008). Pais *et al.* (2007) and Merella *et al.* (2010) suggested that *F. commersonii* colonized the Mediterranean as adult, because they found several Mediterranean specimens infected by two specific Indo-Pacific digeneans that cannot be harboured by larval stages of this fish. Golani *et al.* (2007), studying a partial sequence of the mitochondrial DNA (mtDNA) control region (D-loop I, also called HVS-I), argued that a genetic bottleneck occurred in the lessepsian migration of this species, as a result of a single invasion event mediated by as few as two females. This last finding is consistent with the evidence of a decrease of genetic variability in invaders (Spencer *et al.*, 2000; Sax *et al.*, 2007). However, genetic analysis until now performed on lessepsian fish showed no significant reduction of genetic diversity (Hassan *et al.*, 2003; Hassan & Bonhomme, 2005). It is worth noting that some of these studies were performed using multiple molecular markers and/or different nuclear or mitochondrial regions, since this approach is advisable to get more complete and reliable results (see e.g. Pogson *et al.*, 1995; Neigel, 1997; Clements *et al.*, 2003).

In this context, this study is aimed to provide new insights on the number and variability of matrilineal lineages of *F. commersonii* in the Mediterranean Sea throughout the

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Table 1. Data on the individuals of *Fistularia commersonii* examined.

Sample	Location	Area	Sampling date
ORs1	Oristano	Western Sardinia	November 2007
TSs1	Torre delle Stelle	Southern Sardinia	November 2007
ARs1	Arbatax	Eastern Sardinia	October 2005
ARs2	Arbatax	Eastern Sardinia	October 2007
ARs3	Arbatax	Eastern Sardinia	January 2008
CCs1	Capo Comino	Eastern Sardinia	September 2007
TEa1	Teboulba	Tunisia	December 2006
TEa2	Teboulba	Tunisia	December 2006
TEa3	Teboulba	Tunisia	December 2006
SFa1	Sfax	Tunisia	December 2006
TRa1	Tripoli	Libya	December 2006
TRa2	Tripoli	Libya	December 2006

combined use of different mitochondrial regions. Four regions of mtDNA were chosen: the second segment of the control region, D-loop II (also called HVS-II) the 16S rDNA, 12S rDNA, and Cytochrome b (Cyt b) of the coding region.

MATERIALS AND METHODS

Twelve specimens of *Fistularia commersonii* from different western and eastern Mediterranean areas (*sensu* Spalding *et al.*, 2007) were analysed: six from Sardinia (Oristano, west coast; Torre delle Stelle, south coast; Capo Comino and Arbatax, east coast), and six from North Africa (Teboulba and Sfax, Tunisia; Tripoli, Libya) (Table 1; Figure 1).

DNA was extracted from small portions of the muscle (about 15 mg) by means of the QIAGEN[®] DNeasy Tissue kit. Mitochondrial regions were amplified using the primers HCal2 and HCAH2 (Teske *et al.*, 2003) which amplified 396 bp of the control region D-loop II; L2510 and H3058 (Wilson *et al.*, 2001) which amplified 564 bp of the 16S rDNA gene; L1091 and H1478 (Kocher *et al.*, 1989) which amplified 350 bp of the 12S rDNA gene, L14725 (Pääbo *et al.*, 1991) and H15240 (Wilson *et al.*, 2001) which amplified

284 bp of the Cytochrome b gene. Each 25 µl PCR mixture contained about 15 ng/µl of total genomic DNA on average, 0.2 µM of each primer and 2.5 U of EuroTaq DNA Polymerase (Euroclone) in a reaction mix prepared according to the manufacturer's instructions. The MgCl₂ concentration was 2.5 mM.

Polymerase chain reaction was performed in a MJ PTC-100 Thermal Cycler (MJ research), programmed for 1 cycle of 2 minutes at 94°C, 35 cycles of 1 minute at 94°C, 1 minute at 54°C and 1 minute and 30 seconds at 72°C each. At the end, a post-treatment for 5 minutes at 72°C and a final cooling at 4°C were applied. For all PCR reactions, negative controls and replicates were included. Electrophoresis runs were carried out on 2% agarose gels, made using 0.5 × TBE buffer and stained with ethidium bromide (10 mg/ml), at 4 V/cm for 20 min.

Polymerase chain reaction products were purified using ExoSAP-IT (USB Corporation), and sequenced using an external core service. Sequences were aligned using the software BioEdit 7.0.5.2 (Hall, 1999) and deposited in GenBank (Accession Nos: 12S, GQ901988 to GQ901999; 16S, GQ902000 to GQ902011; Cyt b, GQ902012 to GQ902023; D-loop II, GQ902024 to GQ902035).

The best probabilistic model of sequence evolution was determined after evaluation by Modeltest 3.7 (Posada & Crandall, 1998), using the corrected Akaike information criterion (AICc). Since cluster analysis involved a dataset including four mitochondrial genes, they were tested for heterogeneity (Farris *et al.*, 1995) by the partition-homogeneity test, implemented in PAUP*4.0b10 (Swofford, 2003) in order to measure the significance of incongruence among datasets (Bull *et al.*, 1993; Chippindale & Wiens, 1994; Sullivan, 1996; Cunningham, 1997). The 99% confidence interval was used to determine the level of significance, following Farias *et al.* (2000).

To characterize genetic variation among individuals, the number of polymorphic sites (*S*), the number of haplotypes (*nH*), estimates of haplotype diversity (*h*), and nucleotide diversity (π), were computed using the software DnaSP 4.10 (Rozas & Rozas, 1999).

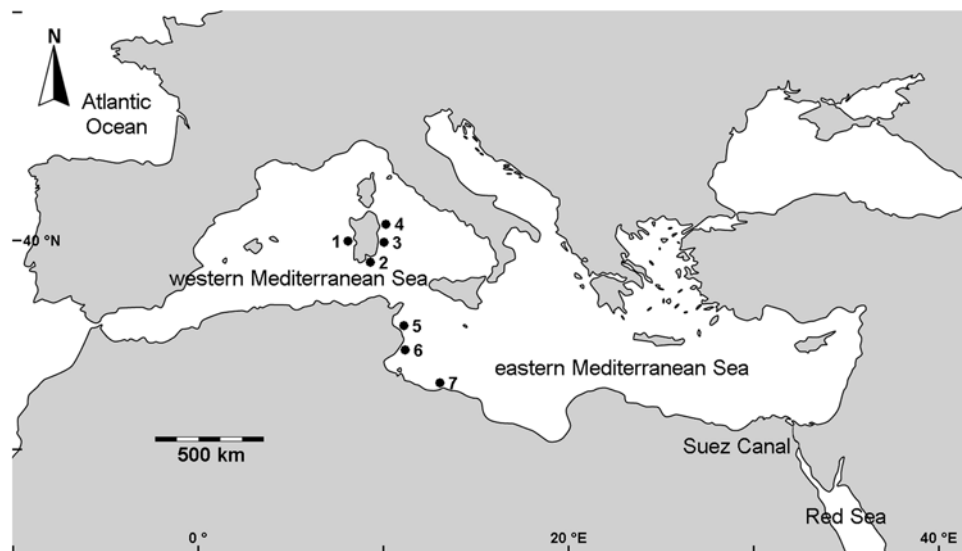


Fig. 1. Sites of sampling of *Fistularia commersonii*. (1) Oristano (ORs); (2) Torre delle Stelle (TSs); (3) Arbatax (ARs); (4) Capo Comino (CCs); (5) Teboulba (TEa); (6) Sfax (SFa); (7) Tripoli (TRa). The suffixes 's' and 'a' in the abbreviations indicate 'Sardinia' and 'North Africa', respectively.

Table 2. Summary of the genetic variability indices for each of the four mitochondrial regions analysed and for the pooled dataset.

Region	S	nH	h	π
D-loop II	9	2	0.409	0.009
16S	5	3	0.530	0.003
12S	3	3	0.318	0.002
Cyt b	0	1	0	0
D-loop II + 16S + 12S + Cyt b	17	5	0.667	0.004

Genetic relationships among individuals were inferred using the program Network 4.5.1.0 (www.fluxus-engineering.com) by means of the median-joining algorithm (Bandelt *et al.*, 1999), assigning the same weight to all the observed polymorphisms, and excluding from the analysis the insertion/deletions (indels) of presumably poor phylogenetic value (Redd & Stoneking, 1999).

RESULTS AND DISCUSSION

The level of genetic variability of the lessepsian migrant *Fistularia commersonii* was studied in different localities of the western and eastern Mediterranean Sea never sampled before. It is worth noting that, due to the rarity and irregular findings of this species in the western basin (with some records until autumn–winter 2007–2008, but no further reports in the following period), it was not possible to collect any additional samples beyond the 12 original ones.

The best model selected by AICc on D-loop II and 16S was K80, whereas 12S and Cyt b fitted the JC model. The partition–homogeneity test did not show significant heterogeneity ($P > 0.01$) among the four mitochondrial fragments. Thus, the four regions analysed (D-loop II, 16S, 12S and Cyt b) were combined in a resultant total length sequence of 1594 bp (including 1 indel), with 17 polymorphic sites 15 of which were parsimony-informative. The best model selected on the pooled dataset was K80.

The genetic diversity indices of the individuals of *F. commersonii* analysed for each mitochondrial region are reported in Table 2. The number of haplotypes ranged from one (Cyt b) to three (16S and 12S). The highest value of haplotype diversity ($h = 0.5$) was found in the 16S gene and the highest value of nucleotide diversity ($\pi = 0.009$) was evidenced in the D-loop II. However, the D-loop II— notwithstanding it provided good results in terms of genetic variability in some species of Syngnathiformes (Teske *et al.*, 2003; Sanna *et al.*, 2008)—did not give high levels of global differentiation for *F. commersonii*, as observed in an

analogous study on mitochondrial DNA of other fish (Tang *et al.*, 2006). Conversely, the 16S and 12S genes, although often highly conservative and generally used at higher systematic levels (Giribet *et al.*, 2001), showed to be the most variable and informative among the mtDNA regions considered. The Cyt b did not show any genetic variability, leading to consider this region of scarce importance to achieve useful information for further phylogeographic analysis on this species.

The network analysis (Figure 2) evidenced the occurrence of five haplotypes, grouped into two main haplogroups, both including individuals from North Africa and Sardinia with no geographical trend. Most of the specimens exhibited one of the two main haplotypes, whilst three individuals from Sardinia were differentiated for one to three mutations.

The main result obtained by the analysis of the combined dataset was the finding of five mitochondrial haplotypes, pointing out the presence of more than the two single matrilineal lineages previously described by Golani *et al.* (2007) in the Mediterranean Sea, after examining a single mtDNA region. Remarkably, on the basis of parasitological evidence, Pais *et al.* (2007) and Merella *et al.* (2010) suggested the possibility that *F. commersonii* invaded the Mediterranean with a large number of adult individuals, compatible with a not severe bottleneck event.

In this context, the present study highlights that, due to the heterogeneous levels of variability found for different mitochondrial regions of *F. commersonii*, the choice of a unique mtDNA segment could lead to a sub-estimation of this value even when high numbers of specimens are sampled. For this reason, the enlarged number of mitochondrial regions used in this work has allowed to detect a higher number of haplotypes in spite of the lower number of individuals analysed. Furthermore, although based on a narrow number of individuals, the present data suggest that the biogeographical barrier represented by the Strait of Sicily did not affect significantly either the dispersal or the genetic distribution of *F. commersonii* in the eastern and western parts of the Mediterranean Sea.

The results obtained provided new information on the levels of genetic variability for the analysed mitochondrial regions of *F. commersonii*. The knowledge of the polymorphism rates of D-loop II, 16S, 12S and Cyt b (Table 2) will allow to make a well-addressed choice of the most informative mtDNA regions depending on the specific purpose of the survey (such as population genetics, phylogeography and phylogeny). Indeed, different mutation and fixation rates shown by mitochondrial regions have been often used to depict multifaceted degrees of differentiation (see e.g. Saccone *et al.*, 1991).

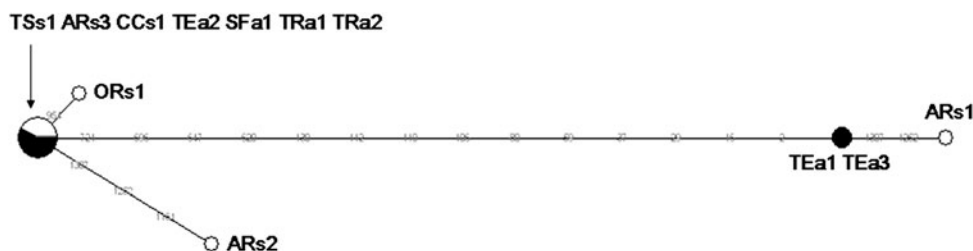


Fig. 2. Network plot obtained for the pooled dataset (D-loop II, 16S, 12S and Cyt b); in each spot, in white individuals from Sardinia and in black from North Africa. Numbers on network branches indicate the polymorphic sites.

In conclusion, the combined use of different mtDNA regions here proposed allowed a finer inference of the levels of genetic variability, and these results will be useful to shed some light on the debates arising from the supposed lack of reduction in genetic variability of Mediterranean populations of different lessepsian fish.

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