Journal of the Marine Biological Association of the United Kingdom, 2011, 91(6), 1289–1293.
doi:10.1017/S0025315410001451

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

DARIA SANNA[1], PAOLO MERELLA[2], TIZIANA LAI[1], SARRA FARJALLAH[3], PAOLO FRANCALACCI[1], MARCO CURINI-GALLETTI[1], ANTONIO PAIS[4] AND MARCO CASU[1]

[1]Dipartimento di Zoologia e Genetica Evoluzionistica, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy, [2]Sezione di Parassitologia e Malattie Parassitarie, Dipartimento di Biologia Animale, Università di Sassari, Via Vienna 2, 07100 Sassari, Italy, [3]Unité de Recherche: Génétique, Biodiversité et Valorisation des Bioressources UR/09-30, Institut Supérieur de Biotechnologie de Monastir, Monastir 5000, Tunisia, [4]Sezione di Acquacoltura e Gestione delle Risorse Acquatiche, Dipartimento di Scienze Zootecniche, Università di Sassari, Via E. De Nicola 9, 07100 Sassari, Italy

*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

Submitted 12 April 2010; accepted 12 July 2010; first published online 27 September 2010

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

Corresponding author:
M. Casu
Email: marcasu@uniss.it

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 $MgCl_2$ 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 GQ9023; 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).

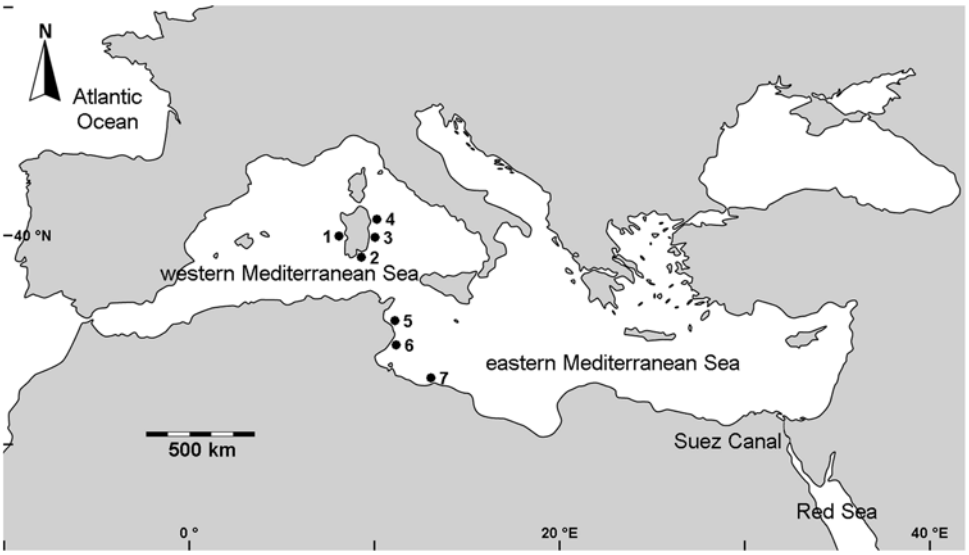

Fig. 1. Sites of sampling of *Fistularia commersonii*. (1) Oristano (ORs); (2) Torre delle Stelle (TSs); (3) Arbatax (ARs); (4) Capo Comino (CCs); (5) Teboulba (TEa); (6) Sfax (SFa); (7) Tripoli (TRa). The suffixes 's' and 'a' in the abbreviations indicate 'Sardinia' and 'North Africa', respectively.

https://doi.org/10.1017/S0025315410001451 Published online by Cambridge University Press

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).

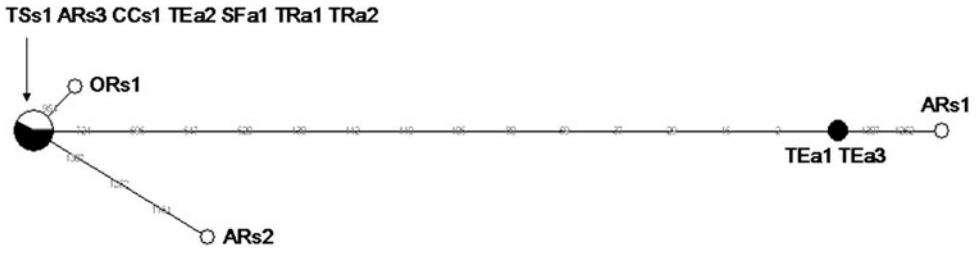

Fig. 2. Network plot obtained for the pooled dataset (D-loop II, 16S, 12S and Cyt b); in each spot, in white individuals from Sardinia and in black from North Africa. Numbers on network branches indicate the polymorphic sites.

https://doi.org/10.1017/S0025315410001451 Published online by Cambridge University Press

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.

ACKNOWLEDGEMENTS

We wish to thank two anonymous referees for their helpful comments that contributed to improve this paper. This research was supported by the 'Centro di Eccellenza' of the Università di Sassari.

REFERENCES

Azzurro E., Pizzicori P. and Andaloro F. (2004) First record of *Fistularia commersonii* (Fistularidae) from the central Mediterranean. *Cybium* 28, 72–74.

Azzurro E., Golani D., Bucciarelli G. and Bernardi G. (2006) Genetics of the early stage of invasion of the lessepsian rabbitfish *Siganus luridus*. *Journal of Experimental Marine Biology and Ecology* 333, 190–201.

Bandelt H.J., Forster P. and Rohl A. (1999) Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16, 37–48.

Ben Souissi J., Zaouali J., Bradai M.N. and Quignard J.P. (2004) Lessepsian migrant fishes off the coast of Tunisia. First record of *Fistularia commersonii* (Osteichthyes, Fistularidae) and *Parexocoetus mento* (Osteichthyes, Exocoetidae). *Vie et Milieu* 54, 247–248.

Bernardi G., Golani D. and Azzurro E. (2010) The genetics of lessepsian bioinvasions. In Golani D. and Appelbaum-Golani B. (eds) *Fish invasions of the Mediterranean Sea: change and renewal*. Sofia and Moscow: Pensoft Publishers, pp. 71–84.

Bilecenoglu M., Taskavak E. and Kunt K.B. (2002) Range extension of three lessepsian migrant fish (*Fistularia commersonii, Sphyraena flavicauda, Lagocephalus suezensis*) in the Mediterranean Sea. *Journal of the Marine Biological Association of the United Kingdom* 82, 525–526.

Bull J.J., Huelsenbeck J.P., Cunningham C.W., Swofford D.L. and Waddell P.J. (1993) Partitioning and combining data in phylogenetic analysis. *Systematic Biology* 42, 384–397.

Chippindale P.T. and Wiens J.J. (1994) Weighting, partitioning, and combining characters in phylogenetic analysis. *Systematic Biology* 43, 278–287.

Clements K.D., Gray R.D. and Choat J.H. (2003) Rapid evolutionary divergences in reef fishes of the family Acanthuridae (Perciformes: Teleostei). *Molecular Phylogenetics and Evolution* 26, 190–201.

Corsini M., Kondilatos G. and Economidis P.S. (2002) Lessepsian migrant *Fistularia commersonii* from the Rhodes marine area. *Journal of Fish Biology* 61, 1061–1062.

Cunningham C.W. (1997) Is congruence between data partitions a reliable predictor of phylogenetic accuracy? *Systematic Biology* 46, 464–478.

Dulčić J., Scordella G. and Guidetti P. (2008) On the record of the lessepsian migrant *Fistularia commersonii* (Rüppell, 1835) from the Adriatic Sea. *Journal of Applied Ichthyology* 24, 101–102.

Farias I.P., Ortí G. and Meyer A. (2000) Total evidence: molecules, morphology, and the phylogenetics of cichlid fishes. *Journal of Experimental Biology* 288, 76–92.

Farris J.S., Kallersjo M., Kluge A.G. and Bult C. (1995) Constructing a significance test for incongruence. *Systematic Biology* 44, 570–572.

Galil B.S. (2000) A sea under siege—alien species in the Mediterranean. *Biological Invasions* 2, 177–186.

Giribet G., Edgecombe G.D. and Wheeler W.C. (2001) Arthropod phylogeny based on eight molecular loci and morphology. *Nature* 413, 157–161.

Golani D. (2000) First record of the bluespotted cornetfish from the Mediterranean Sea. *Journal of Fish Biology* 56, 1545–1547.

Golani D., Azzurro E., Corsini-Foka M., Falautano M., Andaloro F. and Bernardi G. (2007) Genetic bottlenecks and successful biological invasions: the case of a recent lessepsian migrant. *Biology Letters* 3, 541–545.

Hall T.A. (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* 41, 95–98.

Hassan M. and Bonhomme F. (2005) No reduction in neutral variability of mitochondrial and nuclear genes for a Lessepsian migrant, *Upeneus moluccensis*. *Journal of Fish Biology* 66, 865–870.

Hassan M., Harmelin-Vivien M. and Bonhomme F. (2003) Lessepsian invasion without bottleneck: example of two rabbitfish species (*Siganus rivulatus* and *Siganus luridus*). *Journal of Experimental Marine Biology and Ecology* 291, 219–232.

Hemida F. and Capapé C. (2009) On the occurrence of a lessepsian migrant teleost off the Algerian coast (south-western Mediterranean): the bluespotted cornetfish, *Fistularia commersonii* (Fistularidae). *Cybium* 33, 81–82.

Karachle P.K., Triantaphyllidis C. and Stergiou K.I. (2004) Bluespotted cornetfish, *Fistularia commersonii* Rüppell, 1838: a lessepsian sprinter. *Acta Ichthyologica et Piscatoria* 34, 103–108.

Kocher T.D., Thomas W.K., Meyer A, Edwards S.V., Pääbo S., Villablanca F.X. and Wilson A.C. (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America* 86, 6196–6200.

Merella P., Casu M., Garippa G. and Pais A. (2010) Lessepsian fish migration: genetic bottlenecks and parasitological evidence. *Journal of Biogeography* 37, 978–980.

Neigel J.E. (1997) A comparison of alternative strategies for estimating gene flow from genetic markers. *Annual Review of Ecology and Systematics* 28, 105–128.

Pääbo S., Thomas W.K., Whitfield K.M., Kumazawa Y. and Wilson A.C. (1991) Rearrangements of mitochondrial transfer RNA genes in marsupials. *Journal of Molecular Evolution* 33, 426–430.

Pais A., Merella P., Follesa M.C. and Garippa G. (2007) Westward range expansion of the Lessepsian migrant *Fistularia commersonii* (Fistulariidae) in the Mediterranean Sea, with notes on its parasites. *Journal of Fish Biology* 70, 269–277.

Pogson G.H., Mesa K.A. and Boutilier R.G. (1995) Genetic population structure and gene flow in the Atlantic cod *Gadus morhua*: a comparison of allozyme and nuclear RFLP loci. *Genetics* 139, 375–385.

Por F.D. (1971) One hundred years of Suez Canal—a century of lessepsian migration: retrospect and viewpoints. *Systematic Zoology* 20, 138–159.

Posada D. and Crandall K.A. (1998) ModelTest: testing the model of DNA substitution, version 3.7. *Bioinformatics* 14, 817–818.

Redd A.J. and Stoneking M. (1999) Peopling of Sahul: mtDNA variation in aboriginal Australian and Papua New Guinean populations. *American Journal of Human Genetics* 65, 808–828.

Rilov G. and Galil B.S. (2009) Marine bioinvasions in the Mediterranean Sea—history, distribution and ecology. In Rilov G. and Crooks J.A. (eds) *Biological invasions in marine ecosystems*. Berlin and Heidelberg: Springer-Verlag, pp. 549–575.

Rozas J. and Rozas R. (1999) DnaSP version 3: an integrated program for molecular population genetics and molecular evolution analysis. *Bioinformatics* 15, 174–175.

Saccone C., Pesole G. and Sbisà E. (1991) The main regulatory region of mammalian mitochondrial DNA: structure–function model and evolutionary pattern. *Journal of Molecular Evolution* 33, 83–91.

Sanna D., Addis A., Biagi F., Motzo C., Carcupino M. and Francalacci P. (2008) mtDNA control region and D-HPLC analysis: a method to evaluate the mating system in Syngnathidae (Teleostei). *Marine Biology* 153, 269–275.

Sax D.F., Stachowicz J.J., Brown J.H., Bruno J.F., Dawson M.N., Gaines S.D., Grosberg R.K., Hastings A., Holt R.D., Mayfield M.M., O'Connor M.I. and Rice W.R. (2007) Ecological and evolutionary insights from species invasions. *Trends in Ecology and Evolution* 22, 465–471.

Spalding M.D., Fox H.E., Allen G.R., Davidson N., Ferdaña Z.A., Finlayson M., Halpern B.S., Jorge M.A., Lombana A., Lourie S.A., Martin K.D., McManus E., Molnar J., Recchia C.A. and Robertson J. (2007) Marine ecoregions of the world: a bioregionalization of coastal and shelf areas. *Bioscience* 57, 573–583.

Spencer C., Neigel J.E. and Leberg P.L. (2000) Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. *Molecular Ecology* 9, 1517–1528.

Sullivan J. (1996) Combining data with different distributions of among-site rate variation. *Systematic Biology* 45, 375–380.

Swofford D.L. (2003) *PAUP* Phylogenetic Analysis Using Parsimony (*and other methods), Version 4*. Sunderland, MA: Sinauer Associates.

Tang Q., Liu H., Maydenc R. and Xiong B. (2006) Comparison of evolutionary rates in the mitochondrial DNA cytochrome b gene and control region and their implications for phylogeny of the Cobitoidea (Teleostei: Cypriniformes). *Molecular Phylogenetetics and Evolution* 39, 347–357.

Teske P.R., Cherry M.I. and Matthee C.A. (2003) Population genetics of the endangered Knysna seahorse, *Hippocampus capensis*. *Molecular Ecology* 12, 1703–1715.

and

Wilson A.B., Vincent A., Ahnesjo I. and Meyer A. (2001) Male pregnancy in seahorses and pipefishes (Family Syngnathidae): rapid diversification of paternal brood pouch morphology inferred from a molecular phylogeny. *Journal of Heredity* 92, 159–166.

Correspondence should be addressed to:
M. Casu
Dipartimento di Zoologia e Genetica Evoluzionistica
Università di Sassari, Via F. Muroni 25
07100 Sassari (Italy)
email: marcasu@uniss.it

https://doi.org/10.1017/S0025315410001451 Published online by Cambridge University Press