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Biological Pollution: Molecular Identification of Non-Native Species in the Central Tyrrhenian Sea

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ABSTRACT

In the Mediterranean, numerous and frequent bio-invasions by non-native species have occurred in recent decades. Among the reasons for this biological pollution there is the recent trend of global warming, which led to the extension of distribution area of tropical and/or thermophilic species. The present investigation describes analyses of 12S and 16S mt-rRNA gene fragments conducted on fresh muscle tissues of Tyrrhenian Sea fishes that confirm the morphological records of *Fistularia commersonii*, *Sphoeroides pachygaster* and *Trachipterus trachipterus* and provides a 12S mt-rRNA fragments database for future investigations on *Remora osteochir, Tetragonurus cuvieri, Pomadasys incisus* and *Sudis hyalina*. Among the species examined, *Fistularia commersonii* and *Sphoeroides pachygaster* are allochthonous species penetrated into the Mediterranean through Suez Canal (Red Sea) and Gibraltar Strait respectively. *Pomadasys incisus* is thermophilus species basically restricted to the southern parts of the Mediterranean. The others, *Sudis hyalina*, *Tetragonurus cuvieri*, *Trachipterus trachipterus*, and *Remora osteochir* are all autochthonous species of sporadic occurrence. Accordingly, the present work proposes the unequivocal non-native species discrimination by polymerase chain reaction that may be used as an index of biological pollution.

Key words: Biological pollution, PCR, *Fistularia commersonii*, *Sphoeroides pachygaster*, *Trachipterus trachipterus*, *Remora osteochir, Tetragonurus cuvieri, Pomadasys incisus*, *Sudis hyalina*, Mediterranean Sea.

INTRODUCTION

Non-native species (NIS) introduced into the new environment result invaders when justified ecologically and/or economically harmful. One of the most debated questions about NIS has been the relative importance of biotic processes (e.g. competition and facilitation) of the native–invader relationship and the abundance of resources in the invaded habitat (Clavero and Garcıa-Berthou, 2005; García-Berthou and Moyle, in press). Those aspects in which introduced marine organisms can be regarded as being no different from chemical pollutants and as first, encouraged the use of the term "biological pollution" (Elliot, 2003). Among the reasons for this biological pollution is intrinsic to detect the recent trend of global warming (Walther *et al*., 2002). The effect of climate change and invasive species have been implicated in the decline and even collapse of several marine ecosystems (Frank *et al*., 2005) and are known to affect the presence of pathogens too (Campbell *et al*., 2007; Drake *et al*., 2007).

Changes in Mediterranean fish assemblages are a relatively well-studied phenomenon especially for its impact on marine organisms and on marine ecosystem (Occhipinti-Ambrogi, 2007). In the Mediterranean numerous and frequent bio-invasions have occurred in recent decades (Bianchi and Morri, 2000; Zenetos *et al*., 2005); in the same time, always in relation to the Mediterranean cold water, many species will not be able to migrate at higher latitudes, contrary to their Atlantic congeners, because cold areas (Gulf of Lyon, North Adriatic and North Aegean) are already located in the northern most parts of the basin and will be at higher risk of extinction, damaging the biodiversity (Carlton *et al*., 1999; Campbell *et al*., 2007). The actual Mediterranean biodiversity is mainly related to the basin geological evolution. However, the causes of this phenomenon and related biodiversity remain as hypotheses. One major hypothesis is the "meridionalization" of the northern coast of the western Mediterranean due to hydroclimatic changes resulting in an increase in the influx of warm Atlantic water masses via the Gibraltar Strait and tendency of thermophilic marine organisms typical of the southern coast of the Mediterranean Sea to expand or move their range to more temperate regions. The presence of fish due to this hypothesis is related to *Sparisoma cretense, Thalassoma pavo, Balistes carolinensis* and *Sphyraena viridensis* (Golani *et al*., 2002). Other hypothesis is due to the establishment of species from the tropics and sub-tropical as *Upeneus moluccensis, Stenopus hispidus and Musculista senhousia* (Golani *et al*., 2002).

Advances in sampling technology permit to survey previously unexplored areas and improve assessment of fish biodiversity, a fundamental step in defining the state of exploited fish and the environmental changes (Stachowicz and Byrnes, 2006; Vilà and García-Berthou, 2010). Several different approaches may be applied to discriminate among fish species, including analysis of geographic variations in morphometric and meristic characters (Bardamaskos *et al*., 2009), electrophoresis and isoelectric focusing (Berrini *et al*., 2005), immunological methods (Ochiai and Watabe, 2003) and, more recently, proteomic analysis (Mazzeo *et al*., 2008) and DNA microarrays (Kochzius *et al*., 2008). Generally, these techniques, especially isoelectric focusing, have been widely used and proved to be reliable and discriminative. Therefore, the application of DNA technology to fish species identification grew enormously during the last two decades (Barlow and Tzotzos, 1995). In fact, DNA is more thermostable than many proteins and present in almost all cells of the organism carrying the identical information making, therefore, all tissues suitable for the analysis (Haji Sulaiman and Ovenden, 2009). The advent of recombinant DNA techniques generated more reliable genetic markers useful to address the problem of genetic identification of species with high sensitivity and specificity. Although both nuclear and mitochondrial DNA are theoretically available for species identification, vertebrate mitochondrial genes present a high

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mutation rate which allows the discrimination of even closely related species (Kyle and Wilson, 2007). Nevertheless, the design of a great variety of universal primers for polymerase chain reaction (PCR) amplification of specific mitochondrial DNA (mtDNA) sequences, has promoted the use of mtDNA markers for fish species identification (Di Finizio *et al*., 2007) and FASTA, the sequences alignment discrimination (Mount, 2007). Most studies assume that invaders can affect negatively native Mediterranean biota, while a few others contend that allochthonous in coastal waters seem to play a beneficial role in ecosystem functioning (Sweijd *et al*., 2000; Leprieur *et al*., 2009).

In this context, two PCR amplicons have been sequenced from the mitochondrial 12S and 16S mt-rRNA gene fragments and aligned the sequences obtained of from some fishes from Tyrrhenian Sea. These fishes were morphologically identified as *Fistularia commersonii* (Rüppell, 1835), *Sphoeroides pachygaster* (Müller and Troschel, 1848), *Remora osteochir* (Cuvier, 1829), *Trachipterus trachipterus* (Gmelin, 1789), *Tetragonurus couvieri* (Risso, 1810), *Pomadasys incisus* (Bowdich, 1825) and *Sudis hyalina* (Rafinesque, 1810).

This study will provide a database for future biodiversity investigations and biological pollution monitoring on those species starting from an inequivocable their discrimination.

MATERIALS AND METHODS

Sample collection

Fish and fish fragments collected in 2008-2009 by fisherman and/or by a professional sub Adriano Madonna in the Tyrrhenian sea (Coordinates: 41° 13′ 0″ N, 13° 34′ 0″ E) and registered in the Comparative Endocrinology laboratories (EC*Lab*) archivium as *Fistularia commersonii* (10 m depth), *Sphoeroides pachygaster* (360 m depth), *Remora osteochir* (10 m depth), *Trachipterus trachypterus* (500-600 m depth), *Tetragonurus cuvieri* (5-6 m depth), *Pomadasys incisus* (10 m depth), *Sudis hyalina* (around 8 m depth) were analyzed immediately at their arrival in our laboratories. Genomic DNA was extracted from fish muscle (100 mg) and concentrated as previously published (Di Finizio *et al*., 2007).

PCR amplification and sequencing of 12S and 16S mt-rRNA gene fragments

PCR amplification and primers, named 16Sar and 16Sbr, for amplifying the 630 bp 16s rRNA region in whole species were performed as previously published (Di Finizio *et al*., 2007) using the following primers: 5'AAACTGGGATTAGATACCCCACTAT-3' (12Sa) and 5'-GAGGGTGACGGGCGGTGTGT-3' (12Sb) for 12S rRNA gene; 5'GCCTGTTTATCAAAAACAT-3' (16Sar) and 5' CCGGTCTGAACTCAGATCACGT- 3' (16Sbr) for 16S rRNA gene (Palumbi, 1996). PCR reaction was performed in a Techgene Thermal Cycler (Thecne Ltd., Cambridge, UK). Thirty-five cycles of amplification were carried out in a reaction buffer containing 50 mM KCl, 10 mM Tris/HCl, pH 9.0; 10 mM NaCl; 0.01 mM EDTA; 2.5 mM of each dNTP; 1 µM of each primer; 20 ng of template DNA; 0.5 unit of Taq DNA polymerase (Invitrogen, Milan, Italy). PCR amplification conditions for both genes were as follows: denaturation at 94 °C for 45 s, annealing at 52 °C for 55 s, and extension at 72 °C for 90 s. At the end of the incubation, 5 µl of PCR products were separated by electrophoresis through 2% agarose gel and visualized under UV light. A 100 bp ladder (Invitrogen, Milan, Italy, or Fermentas, M-Medical srl, Milan, Italy) was used to estimate the fragment size of the amplicons generated. Amplified DNA was desalted with Microcon 100 spin columns (Millipore-Amicon, Belford, MA, USA) according to the manufacturer's instructions and sequenced using Big Dye TM Terminator Cycle Sequencing Chemistry (Applied Biosystems, Foster City, CA, USA) in an automatic capillarity sequencer (ABI 310 Genetic Analizer; Applied Biosystems). Primers for sequencing were the same used for PCR amplification.

Sequence analysis

Resulted sequences were analyzed and aligned using Chromas 1.45 vs (Technelysium 186 Pty, Tewantin, Australia) and BioEdit (Tom Hall Ibis Therapeutics, Rutherford Road Carlsbad, CA) software. Genetic distances were calculated using the Tamura-Nei model (Grant and Utter, 1984). The obtained sequence fragments of 16S RNA were compared for control with GenBank sequences data for 16S RNA belonging to those species examined using FASTA (Mount, 2007, FASTA SIMILARITY SEARCH)

RESULTS

DNA extracted from fresh muscle of fish constitutes a more efficient template, indicating a good yield of PCR products. Figure 1 (A-B) shows electrophoretic analyses of PCR products generated after DNA amplification of mitochondrial 12S (Gel A) and 16S (Gel B) rRNA gene fragments from the fish muscle tissue studied. The described set of primers 12Sa; 12Sb and 16Sar; 16Sbr, successfully have amplified the mitochondrial region fragments examined, of approximately 630 bp and 420 bp long as reported in Figure 1. Therefore, a sufficient number of DNA molecules were isolated to be used as a suitable template, allowing their amplifications.

The PCR products were isolated from gel and sequenced in order to detect nucleotide substitutions useful to identify those species. The 16S mt-rRNA gene fragment sequences obtained are included in a FASTA analysis in order to confirm the attribution of species reporting as positive control the 16S mt-rRNA reference sequences (Table 1). The partial sequences of 12S mt-rRNA gene, referring to the PCR fragments amplified using 12Sa/12Sb primers, for the species analyzed in the present study, are available in GenBank and their accession numbers are reported in Table 1.

Analyses of 282 bp mitochondrial 12S rRNA gene segment and the sequence alignments reported in Figure 2 identified potential regions where nucleotide substitutions might allow discrimination among the species studied. A detailed comparison of the 282 bp 12S mt-rRNA fragments is reported in Table 2. For the 12S mt-rRNA PCR fragments, the differences among species ranged between 27 (11.84 % in *Remora*

osteochir) and 51 (19.86% in *Trachipterus trachypterus*) residues, compared to *Fistularia commersonii* (Table 2). The partial 282 bp obtained sequences of 12S mt-rRNA gene, referring to the PCR fragment amplified using 12Sa/12Sb primers, for the species analyzed in the present study, are representative of this domain features in considered species and are available in GenBank (see Table 1). We detected 108 polymorphic sites, including 57 parsimony informative sites with a nucleotide diversity (π) of 0.211. Distance values ranged from 0.133 to 0.289 in intra-specific pair wise comparisons with a mean value of 0.211.

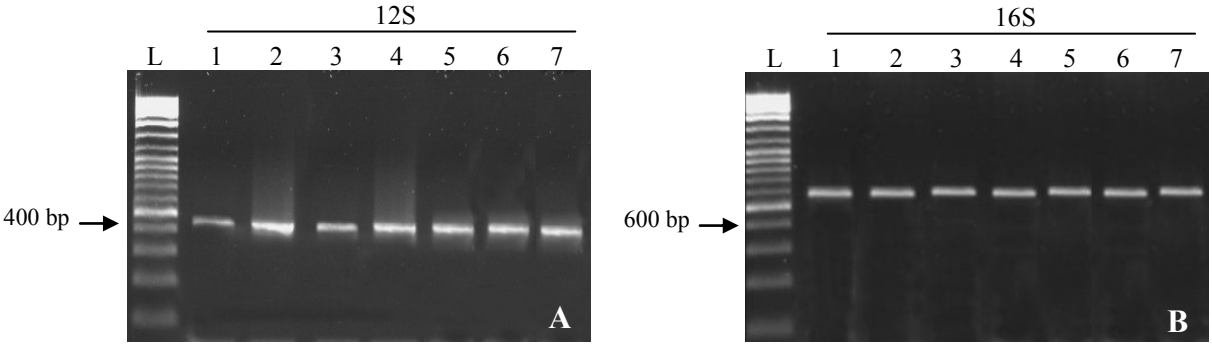

Figure (1): Electrophoretic analyses of PCR products generated after DNA amplification of mitochondrial 12S (Gel A) and 16S (Gel B) rRNA gene fragments from the studied species. Numbers at top indicate fish species as follows: 1, *Fistularia commersonii;* 2, *Sudis hyalina;* 3, *Remora osteochir;* 4, *Sphoeroides pachygaster;* 5, *Trachipterus trachypterus;* 6, *Tetragonurus couvieri;* **7**, *Pomadasys incisus.* Lane (L) indicates the 100 bp DNA ladder (Fermentas) used to assess the molecular size of fragments.

Table (1): GenBank accession numbers of the mitochondrial 12S and 16S rRNA gene sequences of fish species studied.

		GenBank accession number	
Scientific name	**Common name** [a]	**12S mt-rRNA**	**16S mt-rRNA**
Fistularia commersonii	Bluespotted cornetfish	NC003166 complete mitochondrial	
Sudis hyalina	Barracudina	EU574933*	
Remora osteochir	Marlin sucker	EU574934*	AY836584
Sphoeroides pachygaster	Blunthead puffer	AP006745 complete mitochondrial	
Trachipterus trachypterus	Ribbon fish	NC003166 complete mitochondrial	
Tetragonurus cuvieri	Smalleye squaretail	EU795693*	AB205429
Pomadasys incisus	Bastard grunt	EU795694*	EU410417

a. from the website www.fishbase.org
* this paper work

Table (2): Comparison of partial 12S mt-rRNA gene sequences obtained after PCR amplification of genomic DNA from the fish species studied.

Species		**Sequence data comparison** [a]		**Gaps**	**Gap Length**	**Identity**	**Similarity**	**Difference**	**% Change**
		Sequence Length	**Aligned Length**						
F. commersonii [b]	NC003166	282	285	4	4	245	0	36	14.035
S. hyalina	EU574933*	282	287	4	5	243	0	39	15.33
R. osteochir	EU574934*	282	287	5	6	253	1	27	11.84
S. pachygaster [b]	AP006745	282	288	7	7	241	0	40	16.31
T. trachypterus [b]	NC003166	282	287	6	6	230	0	51	19.86
T. cuvieri	EU795693*	282	287	5	6	247	0	34	13.93
P. incisus	EU795694*	282	287	6	6	241	0	40	16.02

[a] Data obtained using *infoalign* program enclosed in the EMBOSS software package available on the web (http://emboss.ch.embnet.org/Pise/).
* This paper work submitted in GenBank.
[b] Sequences from *F. commersonii* NC003166; *S. pachygaster* AP006745; *T. trachypterus* NC003166 have been used as references.

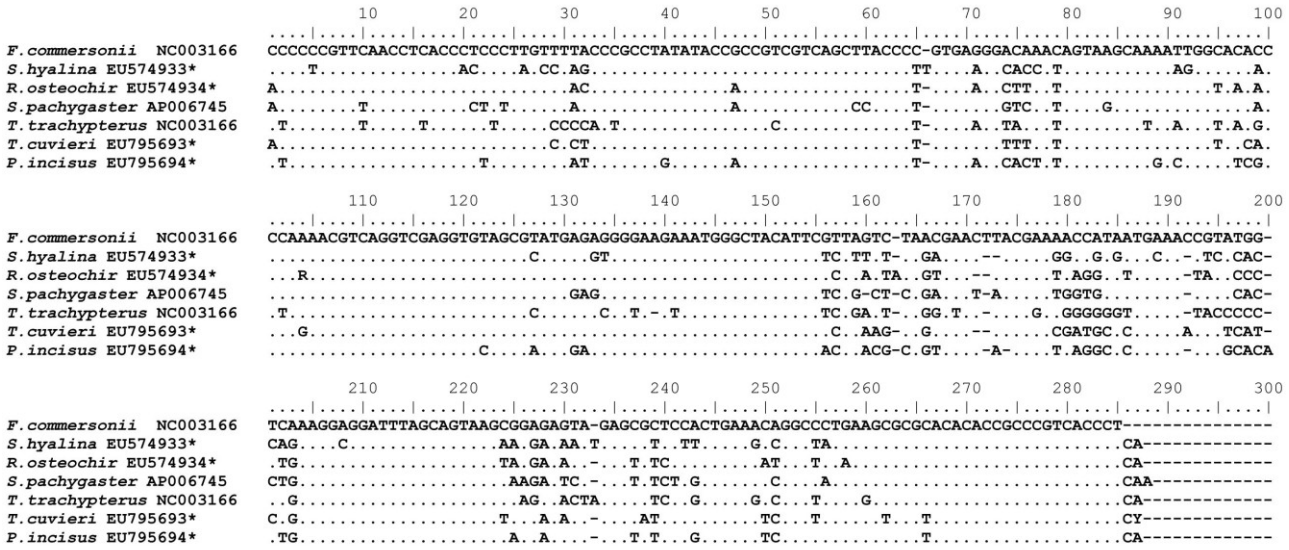

Figure (2): Alignment by ClustalW method of DNA sequences (282 bp) from a portion of the 12S rRNA gene fragment amplified by PCR with primers 12Sa and 12Sb using BIOEDIT software. * This paper work submitted in GenBank. Dots indicate residues that match the consensus.

DISCUSSION

Current investigations on the effects of climate warming on biodiversity and more specifically on biological pollution are relatively fragmented, temporally patchy and geographically limited. The need for long-term, basin-scale programs aiming to monitor the effects of climate change on Mediterranean species is necessary. The establishment of a systematic monitoring program on the tropicalization and meridionalization impacts across the basin will allow a proper interpretation of biodiversity changes (Vilà and García-Berthou, 2010). Furthermore, impacts of climate change on the preservation of Mediterranean biodiversity and its biological pollution have become a matter of great concern not only for specialists but also for the wide community in all countries (Navarro *et al*., 2009). As known, fishes have long been used as indicators of environmental changes (Mearns, 1988; Stephens *et al*., 1988; Roessig *et al*., 2004). Their high dispersal potential, ecological differentiation, general non-resilience, sensitivity to temperature, large size and ease of identification, make them excellent candidates for the study of the effects of climate variability (Wood and McDonald, 1997). In addition, the Mediterranean Sea, located in the temperate zone of the northern hemisphere, includes species with different origin and thermal tolerance, providing an excellent field of investigation.

The described set of primers 12Sa; 12Sb and 16 Sar; 16Sbr, successfully amplified a 12S mt-rRNA and 16S mt-rRNA mitochondrial region fragments, of approximately 630 bp and 420 bp long respectively using DNA extracts from all fresh fish and fish fragment analyzed in our research. Therefore, a sufficient number of DNA molecules were isolated to be used as a suitable template, allowing their amplifications. The reason why behind selecting the mitochondrial 12S and 16S rRNA genes, instead of the mostly widely used cytochrome b gene for species identification is the following: according to Palumbi (1996), these two genes seem to evolve more slowly than mitochondrial genome as a whole and other authors chose for fish species identification a similar strategy (Patarnello *et al*., 1993; Simons and Mayden, 1998; Cespedes *et al*., 2000; Di Finizio *et al*., 2007). Moreover, for *Remora osteochir, Tetragonurus couvieri, Pomadasys incisus, Sudis hyalina* fish species selected (Table 1) sequence data about 12S mt-rRNA gene were not previously published in the literature.

The 16S mt-rRNA gene fragment sequences obtained are included in a FASTA analysis in order to confirm the attribution of species reporting as positive control for the 16S mt-rRNA reference sequences of *Remora osteochir*, *Pomadasys incises* and *Tetragonurus cuvieri*. The complete mitochondrial DNA sequence from *Fistularia commersonii,* (GenBank accession number: NC010274) has been published by Kawahara *et al*. (2008) while *Sphoeroides pachygaster* (GenBank accession number: AP006745) and *Trachipterus trachypterus* (GenBank accession number: NC 003166) are present as complete mitochondrial sequence only in GenBank.

Sequence data comparison of partial 12S mt-rRNA gene sequences obtained after our PCR amplification of genomic DNA from the fish species studied allowed an unequivocal discrimination. The sequences 12S mt-rRNA gene fragment information represent a valid bio-indication of autochthonous and allochthonous fishes in the Tyrrhenian Sea, providing NIS identification. The continuous collections, inventory, systematic studies, and population genetic studies on all marine organisms are essential requirement to understand our marine ecosystem and to check the biodiversity as 'species richness'.

Therefore, correct identification of component species is the first step to study the ecosystem, useful to discriminate NIS. Among the species examined, *Fistularia commersonii* (Rüppell, 1835) and *Sphoeroides pachygaster* (Müller and Troschel, 1848) are allochthonous species penetrated into the Mediterranean through Suez Canal (Red Sea) and Gibraltar Strait, respectively. *Pomadasys incisus* (Bowdich, 1825) is thermophilus species basically restricted to the southern parts of the Mediterranean. The others, *Sudis hyalina* (Rafinesque, 1810); *Tetragonurus cuvieri* (Risso, 1810); *Trachipterus trachypetrus* (Gmelin, 1789), and *Remora osteochir* (Cuvier, 1829) are all autochthonous species of sporadic occurrence.

In particular, our data confirmed the *Fistularia commersonii, Sphoeroides pachygaster and Trachipterus trachypterus* morphological identifications, documenting for the first time the 12S mt-rRNA of *Remora osteochir* (GenBank accession number: EU574934), *Tetragonurus cuvieri* (GenBank accession number*:* EU795693)*, Pomadasys incisus* (GenBank accession number*:* EU 795694) and the 12S mt-rRNA of *Sudis hyalina* as new entry in GenBank. (accession number: EU574933) and enriched fish database permiting eggs, larvae and fish fragments identification and monitoring. The importance of studying and monitoring the NIS deals with the need of information to evaluate the ecological consequences of their invasion (Leprieur *et al*., 2009).

Gathering molecular information with enrichment of GenBank database may aid in the knowledge of the Mediterranean biodiversity, the historical affinities, as well as the origins and diversification patterns of species within different geographical sectors (Floeter *et al*., 2008). Furthermore, the molecular approach could be useful in the taxonomic identification of unmarketable fish species, as alien tetraodontids, which may cause food poisoning representing a potentially serious hazard for public health (Ragonese *et al*., 1992; García-Berthou, 2007) as well as to monitoring the real index of biological pollution.

In conclusion, the data presented in this study provide useful information on some interesting rare and thermophilic fish species of Tyrrhenian Sea and focus on the value of genetic biomarkers as indicators for potential changes occurring in the Mediterranean biodiversity as well in the biological pollution.

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