

Sequence data on four genes suggest nominal *Gerres filamentosus* specimens from Nayband National Park in the Persian Gulf represent two distinct species

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Sequence data of a recent *COI* barcoding study on fish from the Nayband National Park in the Persian Gulf showed relatively high sequence divergence among nominal *Gerres filamentosus* specimens. The sequences grouped into two clusters, signaling the possible existence of cryptic species. The clustering pattern was corroborated by results of *16S rDNA* sequencing. We investigated the hypothesis that *G. filamentosus* specimens represent cryptic species by inspecting sequences of *Cyt b*, an additional mitochondrial gene, and *28S rDNA* a well-conserved nuclear marker. Based on sequence data of four genes (*COI*, *16S rDNA*, *28S rDNA* and *Cyt b*), we propose that nominal *Gerres filamentosus* specimens from Nayband National Park represent a species complex comprising two distinct but closely related species. The observation of a large deletion in the highly conserved *28S rDNA* gene of a representative specimen of one of the *G. filamentosus* clusters was considered particularly informative. *G. filamentosus* occurs through vast ranges of the Indo-Pacific, from the east coast of Africa to Japan and Australia. More comprehensive sampling and additional morphological and molecular analysis of members of this nominal species is required.

Key words: Endemic *Gerres filamentosus*, Nayband National Park, cryptic species, DNA barcoding, Persian Gulf

INTRODUCTION

DNA barcoding based on the sequence of a 650 base pair segment from the 5' region of the mitochondrial cytochrome c oxidase subunit 1 (*COI*) gene has been proposed for species identification in order to partly compensate for the shortcomings of morphology-based taxonomy (Hebert et al., 2003). The protocol is intended to allow species designation by sequencing the relevant portion of the *COI* gene in its genome and finding the best match for this sequence in an existing database of *COI* barcode sequences. A side benefit of the protocol is its potential for flagging potentially new species, for example when no close match to a barcode sequence can be found in the database or when there is notable divergence in barcode sequences of specimens

designated as the same species based on morphology. There have been many successful applications of this approach for species identification and discovery in the context of group-specific taxonomic queries or biodiversity inventories, and most of these are recorded on the Barcode of Life Data Systems (BOLD) website (<http://www.barcodeoflife.org>) (Jacques et al., 2010; Savolainen et al., 2005; Valdez-Moreno et al., 2009). Nevertheless, attention has been brought to aspects of barcoding that calls into question its efficacy on theoretical and empirical grounds (Dasmahapatra et al., 2010; Hickerson et al., 2006; Meyer and Paulay, 2005; Rubinoff et al., 2006; Will and Rubinoff, 2004). A consensus is emerging that views *COI* DNA barcoding, not as a replacement for traditional taxonomy, but as a valuable addition to the integrated taxonomic toolbox. While it is expected to contribute significantly to facilitation and standardization of species identification, conclusions may in some cases require verification by other markers other methods (Miller, 2007; Mitchell, 2008; Ward, 2009).

Fish are the most extensively *COI* barcoded group among the Chordata. The Chordata constitute the second largest library of barcodes among all animal phyla, surpassed only by the diverse and highly investigated Arthropoda. As of March 16, 2011, 11351 of the 19513 chordate species barcodes reported in BOLD belonged to either the Class Actinopterygii or Class Elasmobranchii. In a recent review of major fish barcoding projects, *COI* barcoding was reported to have identified unique sequences for 98% and 93%, respectively, of previously described marine and freshwater fish species (Ward et al., 2009). In addition to allowing molecular identification of specimens belonging to these species, the barcoding status of fish is of importance in relation to conservation, economic, and societal issues (Ardura et al., 2010; Costa et al., 2007; Yancy et al., 2007).

COI barcode data for 76 fish species, belonging to 32 families, captured in Nayband National Park (NNP), Persian Gulf, Iran, were recently recorded (Asgharian et al., 2011). These species were estimated to constitute one third to one half of the fish species of the park. Taxonomic and sequence data on the specimens, as well as photographs, can be accessed through the page for the "Nayband National Park Fish Barcoding" (NNPF) project on the BOLD website. All species can be distinguished by their barcode sequences; no barcode sharing between species was observed. However, remarkably high intraspecific divergences were observed in two species. The average intraspecific divergence in *COI* barcode sequences for *Gerres filamentosus* Cuvier 1829 and *Plectorhinchus schotaf* (Forsskål 1775) were 7.46% and 5.12%, respectively. The average intraspecific divergence for the remaining species was 0.18%. This figure is comparable to intraspecific distances previously reported for marine (0.25-0.39%) (Steinke et al., 2009; Ward et al., 2005) and fresh water (0.30-0.45%) fish species (Hubert et al., 2008; Valdez-Moreno et al., 2009). It was noted that the *COI* sequences of *G. filamentosus* specimens formed two clusters with average intracluster sequence divergences of 0% and 0.12% and average intercluster divergence of 13.84%, suggesting that this nominal species may represent two molecular operational taxonomic units. *Plectorhinchus schotaf* was represented by two specimens diverging by a mean of 5.12%, as stated above. Neighbor-joining analysis of *16S rDNA* sequences fully corroborated the *COI* clustering pattern for the eight specimens of *G. filamentosus*, but was inconclusive with respect to the two *P. schotaf* specimens. Here, we further investigate the hypothesis that *G. filamentosus* samples represent cryptic species by investigating sequences of *Cyt b* and of *28S rDNA*. *Cyt b* is another mitochondrial gene that codes cytochrome b, and *28S rDNA* is a nuclear marker.

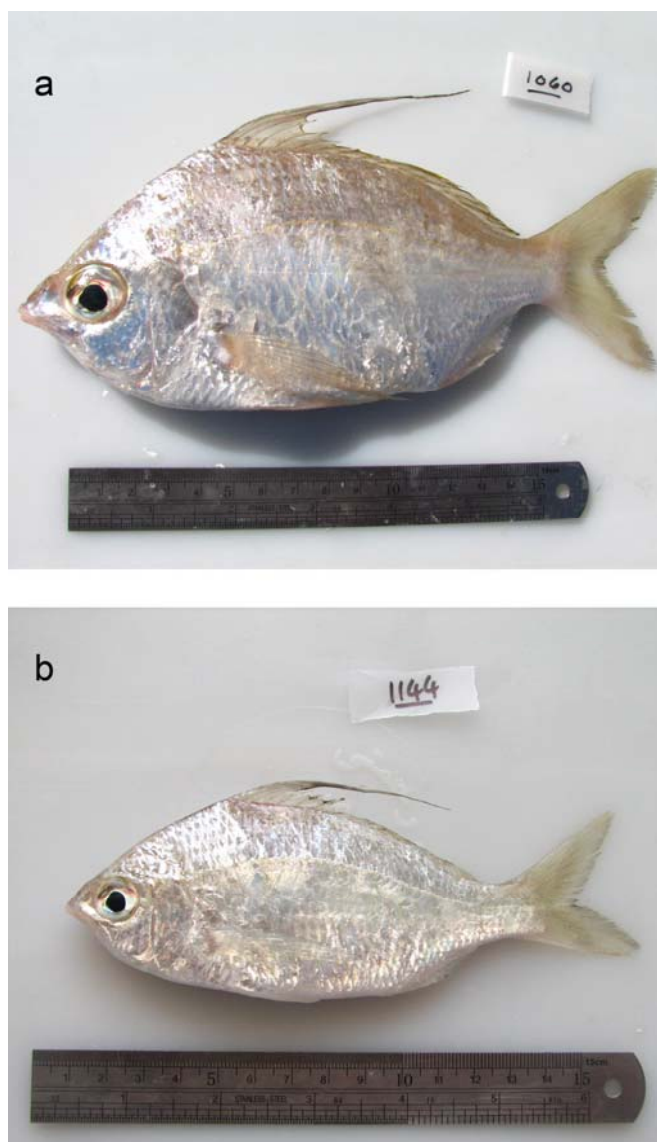


FIGURE 1. Representative specimens of the two *G. filamentosus* clusters from Nayband National Park. (a) specimen 1060 belongs to cluster 2 and (b) specimen 1144 belongs to cluster 1.

MATERIAL AND METHODS

Fish specimens

Ten specimens of fish belonging to the genus *Gerres* and two *Plectorhinchus schotaf* specimens from Nayband National Park were examined. Additionally, human and murine *28S rDNA* sequences and *COI* sequences of three *G. filamentosus* specimens from the South China Sea obtained from publicly available sources were used in the analysis. Table 1 provides BOLD ID numbers of the NNP specimens and source information pertaining to the downloaded sequences. Specimens 1144, 1150, and 1151 constituted cluster 1 and specimens 1002, 1060, 1076, 1077, and 1082 constituted cluster 2 of *G. filamentosus* in the previous study based on *COI* and *16S rDNA* sequence data. Figure 1 is a lateral image of a representative specimen of each cluster. Several ichthyologists unanimously identified all eight specimens as *G. filamentosus* (personal communication).

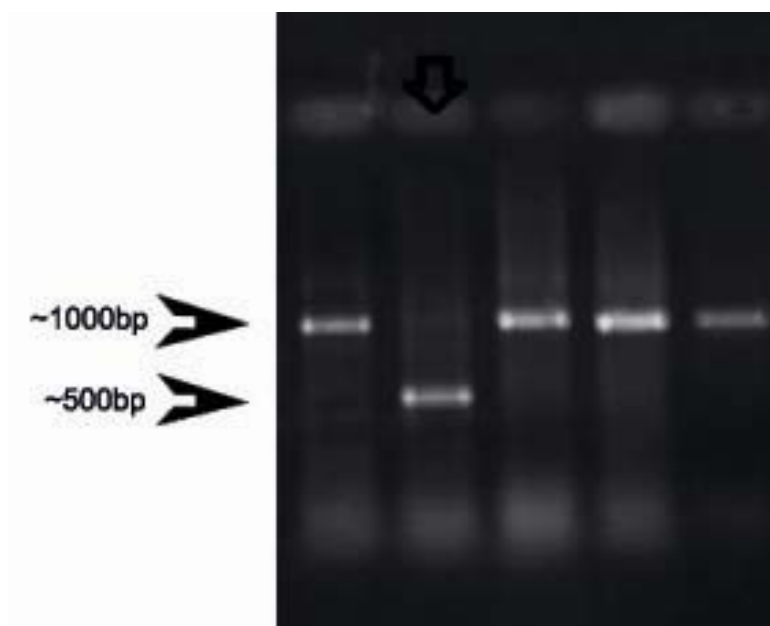


FIGURE 2. Gel electrophoresis evidence of deletion in *28S rDNA* of *G. filamentosus* specimen 1144. The 1144 amplicon is in the lane indicated by an arrow. The other lanes show migration of *28S rDNA* amplicons of other *G. filamentosus* specimens.

TABLE 1- Source information on fish analyzed or sequences used in this study

Samples*	Family	Species	Specimens	BOLD	Location	GenBank
				Project	Lat./Lon.	accession no.
1002, 1060, 1076, 1077, 1082, 1144, 1150, 1151	Gerreidae	<i>Gerres filamentosus</i>	8	NNPF	27.4/52.6	HQ149847-54
1135, 1137	Gerreidae	<i>Gerres oyena</i>	2	NNPF	27.4/52.6	HQ149845-6
222-06, 254-06, 312-06	Gerreidae	<i>Gerres filamentosus</i>	3	FSCS	20.4/110.7, 21.6/112.0, 22.7/115.2	EF607385-7
1105, 1024	Haemulidae	<i>Plectorbinchus schotaf</i>	2	NNPF	27.4/52.6	HQ149903-4
Human 28s rDNA		<i>Homo sapiens</i>				NR_003287.2
Murine 28S rDNA		<i>Mus musculus</i>				NR_003279.1

* Numbers are BOLD IDs assigned to the fish at www.boldsystems.org

28S rDNA and Cyt b sequence retrieval

DNA was extracted from 1-3 mm³ pieces of muscle using AccuPrep Genomic DNA Extraction kit (Bioneer, Daejeon, Korea) as previously described (Asgharian et al., 2011). The D1-D2 region of *28S rDNA* sequence was amplified using LSUD1, D2 fw1 (5'- AGCGGAGGAAAAGAACTA - 3') and LSUD1,D2 rev1 (5'- TACTAGAAGGTTCGA TTAGTC -3') primers (Sonnenberg et al., 2007). Partial *Cyt b* sequence amplification was performed using L14816 (5'-CCATCCAA

CATCTCAGCATGATGAAA-3') and H15173 (5'-CCCCTCAGAATGATATTTGTCCTCA-3') primers (Parson et al., 2000). Sequencing was conducted using the ABI Big Dye terminator chemistry and an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA, USA).

SEQUENCE ANALYSIS

Chromatograms were visually inspected for reading errors using the Sequencher software (Gene Codes Corporation, Ann Arbor, MI, USA). *Cyt b* sequences were checked for uninterrupted open reading frames using ChromasPro version 1.41 (Technelysium, Australia). Sequences were aligned in MEGA 4.0 (Tamura et al., 2007). Computation of pairwise p-distances and creation of Neighbor-Joining (NJ) trees were performed in MEGA 4.0.

RESULTS

28S rDNA

28S rDNA sequences were retrieved from four *G. filamentosus* (1002, 1060, 1077, 1144), one *G. oyena* (1135) and two *Plectorbinchus schotaf* (1024, 1105) specimens. Specimen 1144 belonged to cluster 1 of *G. filamentosus* COI sequences, and 1002, 1060, 1077 were members of cluster 2. The 28S rDNA PCR product of all specimens except 1144 contained approximately 1000 bp. The 28S primers used were originally designed based on the *D. melanogaster* 28S rDNA sequence, and the observed amplicon lengths were close to those expected (1056 bp; GenBank acc. m21017) using *D. melanogaster* DNA as template (Sonnenberg et al., 2007). Surprisingly, specimen 1144 repeatedly produced a notably shorter amplicon (Fig. 2). Bidirectional sequencing showed that it contained only 493 bp. Sequence alignment revealed that all but 45 nucleotides at the 3' terminus of the 1144 sequence were identical to the 5' terminus of the other three *G. filamentosus* 28S rDNA sequences, suggesting that 1144 28S rDNA contained a deletion of approximately 500 bp. The common 448 bp aligned well with a segment of 450 bp human 28S rDNA (positions 51-500 of human 28S rDNA reference sequence: GenBank acc. NR_003287.2), the difference in length being due to various small insertions and deletions. The 45bp segment at the 3' terminus of the 1144 sequence that was absent from the sequences of the other *G. filamentosus* specimens was a *bona fide* 28S rDNA sequence,

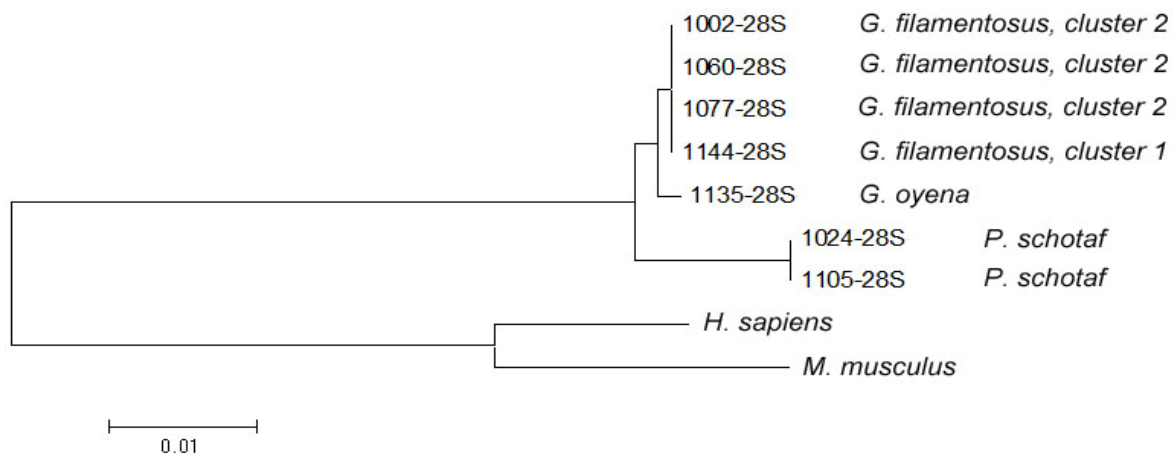


FIGURE 3. 28S rDNA NJ tree. The tree is based on sequences homologous to the ~450 bp segment of 28S rDNA present in all fish specimens sequenced. Homologous sequences from human and murine 28S rDNA are included.

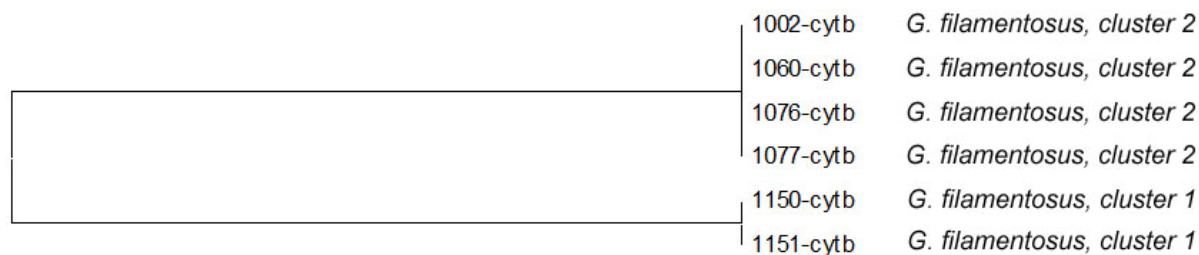


FIGURE 4. *Cyt b* NJ tree. The tree is based on *Cyt b* sequences of two *G. filamentosus* specimens belonging to cluster 1 (1150, 1151) and four *G. filamentosus* specimens belonging to cluster 2 (1002, 1060, 1076, 1077) as defined by *COI* sequences. Identical clustering based on *Cyt b* sequences is evident.

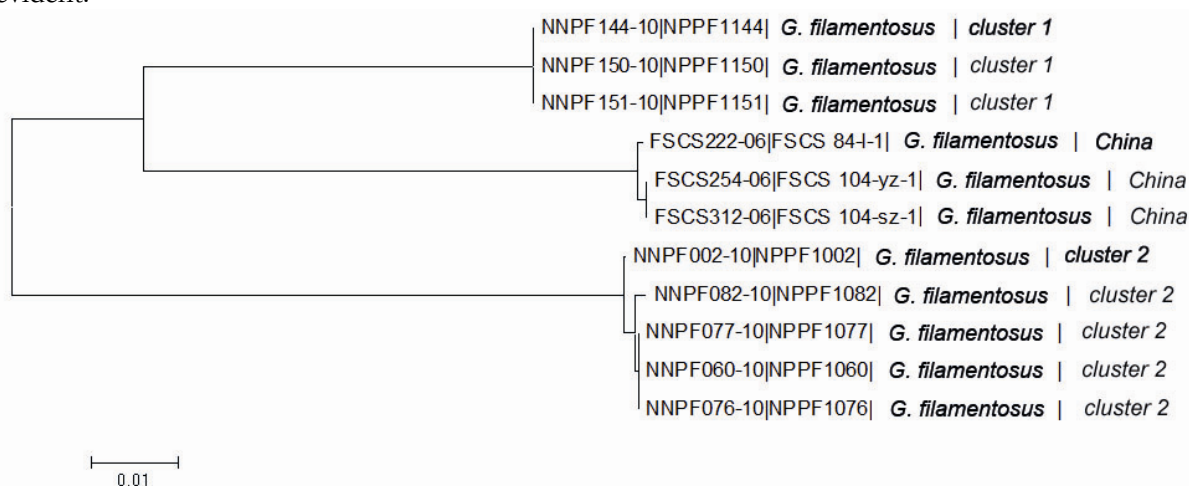


FIGURE 5. *COI* NJ tree. The tree is based on *COI* sequences of eight Nayband National Park (Persian Gulf) and three South China Sea *G. filamentosus* specimens.

as evidenced by its 100%, or nearly 100%, match to *28S rDNA* sequences of various species, including beetle, butterfly, fish, and human (BLAST search results, data not shown). The terminal 21 base of the 45 bp segment, as expected, matched the sequence of the 28S reverse primer used for amplification. As specimen 1144 was the only representative of *G. filamentosus* cluster 1 for which a *28S rDNA* sequence was retrieved, it was important to assess whether the deletion observed in this specimen was an aberration. The extent of conservation of *28S rDNA* sequences in the region homologous to the ~450 bp segment present in all the fish samples sequenced, including 1141, was compared to the extent of conservation of the sequence of the remaining 3' terminus constituting approximately 600 bp in all *G. filamentosus* specimens except 1144 and approximately 900 bp in humans. Pair-wise p-distances were calculated among all available *Gerres*, *P. schotaf*, human, and murine *28S rDNA* sequences (Table 2). Consistently, all *G. filamentosus*/*G. oyena*, *Gerres*/*P. schotaf*, and fish/mammal pair-wise p-distances for the ~600-900 bp downstream segment were two- to three-fold that of the ~500 bp upstream segment. Therefore, the deleted portion of *28S rDNA* in 1144 lies within a generally less conserved region of the gene. A NJ tree based on sequences homologous to the ~450 bp segment common to all the fish specimens sequenced is presented in Figure 3. The *Gerres* specimens form a cluster within which *G. filamentosus* specimens are slightly separated from the single *G. oyena* specimen. The *Gerres* cluster then joins the *P. schotaf*

TABLE 2. Pairwise *p*-distances between 28S rDNA sequences

450 bp segment present in all the fish samples sequenced and in <i>Homo sapiens</i> and <i>Mus musculus</i>								Remaining 28S rDNA sequence 3' to the 450 bp segment*								
Specimen no.	1002	1024	1060	1077	1105	1135	1144	Human	Specimen no.	1002	1024	1060	1077	1105	1135	Human
1024	0.01								1024	0.035						
1060	0	0.013							1060	0	0.035					
1077	0	0.013	0						1077	0	0.035	0				
1105	0.01	0	0.013	0.013					1105	0.035	0	0.035	0.035			
1135	0	0.015	0.003	0.003	0.015				1135	0.016	0.4	0.016	0.016	0.4		
1144	0	0.013	0	0	0.013	0.003			<i>H. sapiens</i>	0.232	0.214	0.232	0.232	0.214	0.213	
<i>H. sapiens</i>	0.09	0.097	0.092	0.092	0.097	0.09	0.092		<i>M. musculus</i>	0.234	0.227	0.234	0.234	0.227	0.228	0.1
<i>M. musculus</i>	0.1	0.108	0.097	0.097	0.108	0.097	0.097	0.094								

* The remaining segment is deleted in specimen 1144.

cluster to make a whole-fish cluster. This fish cluster shows notable divergence from the mammal outgroup cluster.

Cyt b

Cyt b sequences were retrieved from two *G. filamentosus* specimens belonging to cluster 1 (1150, 1151) and four *G. filamentosus* specimens belonging to cluster 2 (1002, 1060, 1076, 1077). The *Cyt b* sequences of *G. filamentosus* specimens of the two clusters differed, but within cluster sequences were identical. The *p*-distance between sequences of the two clusters was 29.4%. The NJ tree built resulting from these six *Cyt b* sequences is presented in Figure 4.

Inter-regional comparison of *COI* data

The NJ tree was built with *COI* sequences of NNP *G. filamentosus* specimens and of three conspecifics from the South China Sea (Fig.5). The NNP *G. filamentosus* cluster 1 specimens (1144, 1105, 1151) clustered more closely with the Chinese specimens than with the five sympatric NNP *G. filamentosus* cluster 2 specimens (1002, 1060, 1076, 1077, 1082).

DISCUSSION

The 28S rDNA data provide compelling evidence that *G. filamentosus* cluster 1 specimen 1144 has a deletion of approximately 0.5 kb as compared to the sequences of *G. filamentosus* cluster 2 specimens. The same region in other fish specimens exhibited two- to three- fold the rate of base substitutions/minor deletions as a more conserved region of 28S rDNA (Table 2). The deleted segment is part of the D2 domain of the 28S rDNA gene, known to be among the most divergent regions of the gene (Allard and Honeycutt, 1991; Sonnenberg et al., 2007; Zardoya and Meyer, 1996). Nevertheless, 28S rDNA is generally considered a conserved gene with little or no intraspecific variation. In a study of European ground beetles, no intraspecific or intrageneric variations in the D3 region of 28S rDNA were found (Raupach et al., 2010). Similarly, a 663 bp segment of the 28S rDNA of five species in the teleost family Leiognathidae showed zero intraspecific divergence (Sparks et al., 2005). Observation of sequence differentiation of 28S rDNA between specimens nominally considered conspecific strongly suggests the existence of cryptic species, and even a single mutational difference may be an indication of taxon delimitation (Sonnenberg et al., 2007). It is argued that, due to the high copy number of rRNA coding genes in the genome, any variant that arises through a new mutation must homogenize over at least most of the copies to become detectable by PCR and sequencing. This homogenization can only occur over

an adequate period of time in an interbreeding population. The sequence variation observed between 28S rDNA of *G. filamentosus* specimen 1144 and *G. filamentosus* specimens 1002, 1060, and 1077 strongly supports the contention that these specimens constitute two distinct *Gerres* species. Although based on the 28S rDNA sequence of only a single specimen of *G. filamentosus* cluster 1, the contention is credible because of corroboration by previously reported COI and 16S rDNA sequence data (Asgharian et al., 2011) along with the *Cyt b* data presented here.

The pattern of segregation of nominal *G. filamentosus* specimens into two clusters based on *Cyt b* sequences mirrors the pattern observed previously, based on COI and 16S rDNA sequences. All the patterns divide the nominal *G. filamentosus* specimens into two clusters. The average p-distance intercluster divergence for the *Cyt b* tree was 29.4%, while the average p-distance intercluster divergence for the COI tree of the same specimens was 12.5%. *Cyt b* partial sequences have been widely used for species identification in fish. However, sequences in previous studies were not taken from a unique standard area of the gene, making direct comparison of results difficult (Ward et al., 2009). Nevertheless, the 29.4% divergence observed here between the two NNP *G. filamentosus* clusters is to our knowledge much higher than values previously reported for intraspecific variation in any region of the *Cyt b* gene and is well within range of interspecific variations (Jérôme et al., 2003; Kochzius et al., 2010; Lin et al., 2005; Sotelo et al., 2001). For example, Kochzius et al. (2010) reported a mean intraspecific distance of 0.57 (SE=0.22) among 50 European marine fish species, and Jerome et al. (2003) found interspecific divergence among nine clupeiform fishes to fall in the range of 4–28%. In the study of four *Thunnus* species by Lin et al. (2005), maximum intraspecific variation was 1.06%. Although Sotelo et al. (2001) did not explicitly report divergence values, the lengths of branches in the phylogenetic tree they provided indicate extremely small intraspecific variations. The observation that COI sequences of NNP *G. filamentosus* cluster 1/*G. filamentosus* South China Sea specimens were more similar than NNP *G. filamentosus* cluster 1 /NNP *G. filamentosus* cluster 2 specimens supports the contention that the *G. filamentosus* population in NNP comprises two genetically divergent lineages.

In conclusion, on the basis of the sequence data of four genes (COI, 16S rDNA, 28S rDNA, and *Cyt b*), we propose that nominal *Gerres filamentosus* specimens from NNP probably represent a species complex comprising two distinct, but closely related, species. The level of genetic divergence between these two lineages is higher than values usually taken as indications of existence of novel species (Baldwin et al., 2011; Järnegren et al., 2007; Smith et al., 2008). *Gerres filamentosus* occurs through vast ranges of the Indo-Pacific, from the east coast of Africa to Japan and Australia (www.fishbase.org). We suggest that more comprehensive sampling and additional morphological and molecular analysis of members of this nominal species are needed. Additionally, our results indicate the benefits of using multiple markers for molecular taxonomy. Although only two specimens were studied, initial clustering results of *P. schotaf*, based on COI data, were not reproduced with 16S and 28S rDNA sequence data. Finally, it was unfortunate that we could not retrieve acceptable *Cyt b* and 28S rDNA sequence data for all specimens studied. Improved protocols for amplification by PCR using DNA of stored samples are necessary.

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