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Short Communication

Genetic characterisation of populations of the critically endangered Goliath grouper (*Epinephelus itajara,* Serranidae) from the Northern Brazilian coast through analyses of mtDNA

Gláucia C. Silva-Oliveira[1], Péricles S. do Rêgo[1], Horacio Schneider[1], Iracilda Sampaio[1] and Marcelo Vallinoto[1,2]

[1]*Laboratório de Genética e Biologia Molecular, Instituto de Estudos Costeiros, Universidade Federal do Pará, Bragança, PA, Brazil.*
[2]*Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, Universidade do Porto, Vairão, Portugal.*

Abstract

The Goliath grouper (*Epinephelus itajara*) is one of the most endangered species of fish of the subfamily Epinephelinae. Slow to develop and mature, and dependent on mangrove habitats for breeding, the species also suffers intense harvesting, which has reduced drastically in numbers in many areas. To contribute to the understanding of the characteristics of *E. itajara* populations, we conducted a molecular genetics study of the species, focusing on populations from the Northern Brazilian coast. The mtDNA control region (D-loop) of 116 individuals from five localities (Bragança, Ajuruteua, Parnaíba, Fortaleza and Natal) was analysed, and a sequence of 499 base pairs identified. Analyses of the sequences indicated that genetic variability was generally lower in *E. itajara* than in other endangered species of the genus. AMOVA found no significant grouping structure among the populations. Nested Clade Analysis revealed a significant association between genetic variability and geographic distribution among only three populations (Ajuruteua, Parnaíba and Natal). Genetic diversity was higher in populations from the Amazon region, which may be related to the better conservation of mangrove habitats in this area. Therefore, the present study could be used for the implementation of conservation and management measures in order to protect and consolidate these populations.

Key words: Goliath grouper, *Epinephelus itajara,* control region, mtDNA, population genetics.

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The subfamily Epinephelinae encompasses the largest-bodied serranids, the groupers, with a total of 159 species distributed among 15 genera (Heemstra and Randall, 1993). The largest member of this subfamily is the Goliath grouper, *Epinephelus itajara* (known as the "mero" in Brazil), which can reach around 2.5 m in length and weigh almost 400 kg (Szpilman, 2000). This species is found in the tropical and subtropical waters of the Atlantic Ocean and western Pacific. In the western Atlantic, it ranges between North Carolina, through the Gulf of Mexico and the Caribbean to the southern Brazilian state of Santa Catarina (Sadovy and Eklund, 1999; Francesconi and Schwartz, 2000). The Goliath grouper is found in coastal waters at depths of 40 to 200 meters, where it seeks refuge in caves, shipwrecks, rock crevices and coral reefs (Heemstra and Randall, 1993; Sadovy and Eklund, 1999). Sedentary and highly territorial, the adults normally remain in their lairs until the beginning of the breeding season, when they migrate to the estuaries of large rivers to spawn, forming breeding shoals (Coleman and Koening Laboratory Research). Other important characteristics of the biology of the Goliath grouper include its fidelity to shoaling sites and dependence on mangrove habitats, where it spends the first five years of its life cycle (Frias-Torres, 2006; Coleman and Koening Laboratory Research). Subsequently, the adults migrate to the open sea, where they remain for the rest of their lives, only returning to the mangroves when they are sexually mature, at around six and a half years of age (Bullock *et al.*, 1992; Sadovy and Eklund, 1999).

The Goliath grouper is much sought after by both sport and commercial fishermen (Szpilman, 2000). In general, the species is targeted during the shoaling phase, which permits the harvesting of large numbers of individuals. This was the main cause of the decline *E. itajara* populations in Central America in the 1950s (Randall, 1983; Coleman and Koening Laboratory Research). The forma-

Send Correspondence to Marcelo Vallinoto. Laboratório de Genética e Biologia Molecular, Instituto de Estudos Costeiros, Universidade Federal do Pará, Al. Leandro Ribeiro s/n, 68.600-000 Bragança, PA, Brazil. E-mail: mvallino@ufpa.br.

tion of reproductive shoals in predictable periods, the dependence of the species on mangrove habitats and its slow maturation rates all contribute to its vulnerability (Sadovy and Eklund, 1999; Frias-Torres, 2006). According to Morris *et al.* (2000), around 40% of the epinephelids are currently listed at some level of extinction risk by the International Union for Conservation of Nature and Natural Resources (IUCN, 2007). At the present time, the Goliath grouper is listed as critically endangered, and is potentially vulnerable to drastic reductions in numbers in the near future (Tak-Chuen and Ferrera, 2006, in IUCN, 2007). Given its conservation status, *E. itajara* was the first species of marine fish to be protected under Brazilian law, starting in 2002 (IBAMA, portaria nº 121 20/09/2002 and portaria n. 42, 19/09/2007). Similar measures have already been taken since 1990 in the United States and 1993 in the Caribbean (Tak-Chuen and Ferrera, 2006, in IUCN, 2007).

In the light of the present status of *E. itajara*, there is an urgent need for population studies, in particular on genetic variability. However, no data are available on the genetic characteristics of this species, but some information is available for closely-related species. Three studies of population genetics are available for other members of the genus *Epinephelus* (Rivera *et al.*, 2004; Zatcoff *et al.*, 2004; Maggio *et al.*, 2006), although they are based on different methods and molecular markers. Given this situation, the aims of the present study were to estimate the genetic variability of *E. itajara* populations from northern Brazilian coast based on the analyses of the control region (D-loop) of the mitochondrial genome (mtDNA), investigate migratory patterns and demographic and structural traits of the different populations.

In the present study, 116 tissue samples of western Atlantic *E. itajara* were collected from five sites on the northern Brazilian coast, through donations from fishermen and fishmongers. Amazonian populations are represented by samples from the Ajuruteua peninsula (00°56'33" S and 47°06'58" W; n = 20) and the municipal fish market in Bragança (1°3'46" S and 46°46'22" W; n = 73), both in the state of Pará (Figure 1). In addition, samples were also obtained from the cities of Fortaleza, in Ceará state (3°43'1" S and 38°32'34" W; n = 2), Parnaíba, Piauí (2°54'18" S and 41°46'37" W; n = 12) and Natal, in Rio Grande do Norte (Rio Potengí, 5°45' 22" S and 35°12'5" W; n = 9). Samples were stored in 95% ethanol until processing for the isolation of genetic material.

Total DNA was extracted from the samples following a phenol-chloroform method (Sambrook *et al.*, 1989). Taxon identification was confirmed with the assistance of a species-specific molecular marker, in this case, a segment of 16S rRNA from the mitochondrial DNA (Palumbi *et al.*, 1991). The sequences of all samples were compared with the complete sequence of a juvenile *E. itajara* deposited in the tissue collection of the Genetic and Molecular Biology Laboratory of the Bragança campus of the Universidade

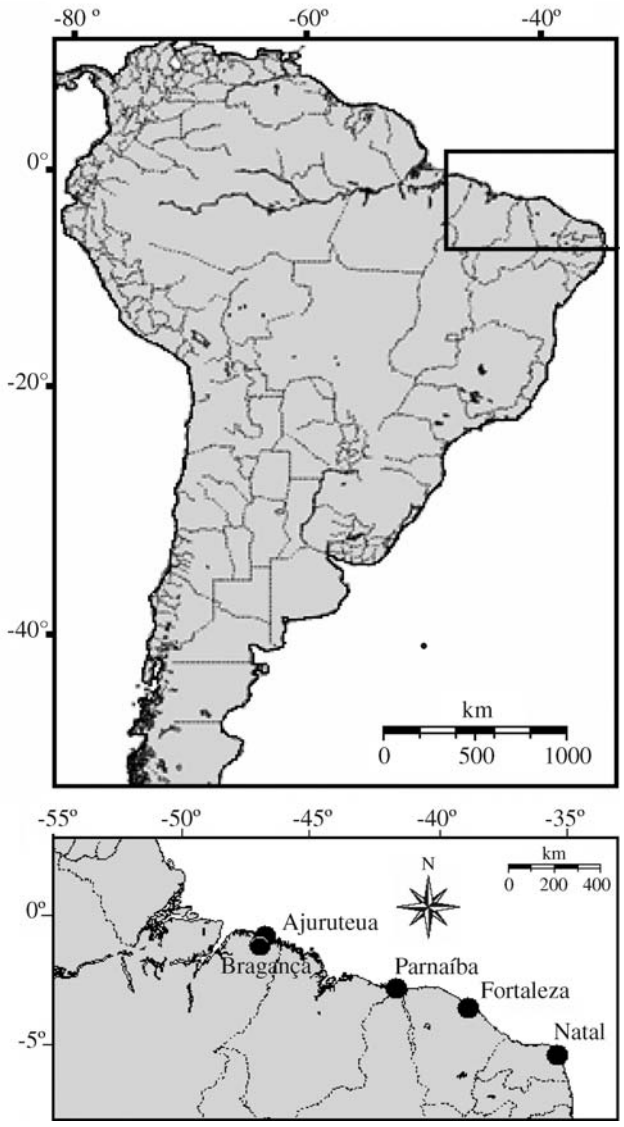

Figure 1 - Location of the collecting localities for the Goliath grouper on the Northern Brazilian coast (base map was created at Online Map Creations, www.aquarius.geomar.de/omc).

Federal do Pará (UFPA). Polymerase chain reactions (PCR) were conducted in order to isolate the D-loop fragment, using primers A-1 (Lee *et al.*, 1995) and Perc12S1R (Santa Brígida *et al.*, 2007). Approximately 100 ng of total DNA were used for each reaction, together with 4 µL dNTP (1.25 mM), 2.5 µL of 10X buffer (Invitrogen - Tris-HCl and KCl, pH 7.8), 1.5 µL $MgCl_2$ (50 mM), 0.25 µL of each primer (200 ng/µL), 0.25 µL of Taq polymerase (5 U/µL - Invitrogen) and extra-purified water to complete a final volume of 25 µL. The conditions required for amplification were 94 °C for 3 min, followed by 30 cycles at 94 °C for 1 min, 47.5 °C for 1 min, and 72 °C for 1 min, with an additional final extension cycle at 72 °C for 5 min. The PCR products were purified using an ExoSAP-IT kit (Amersham Pharmacia Biotech, Inc., UK), following the

manufacturer's protocol. The samples were sequenced separately on the basis of the primers used for PCR, as well as an internal primer (EIT-INT 5'-GAATATTCCTTCAAC ATTAC-3') designed for the present study. The dideoxyterminal method was used for sequencing, with the commercial DYEnamicTM ET dye Terminator kit (Amersham Pharmacia Biotech, Inc., UK) in a MegaBACE 1000 sequencer (Amersham Pharmacia Biotech, Inc., UK).

Sequences were aligned using the BIOEDIT program (Hall, 1999). For analysis, the sequences were grouped in two distinct files. The first file was used for the calculation of genetic variability, and contained all the samples from the five localities (n = 116), whereas the second (n = 41) was reserved for the analyses of population structuring, and excluded the samples from Bragança (73 individuals), the origin of which was unknown, and Fortaleza, which included only two specimens.

The first group was used to calculate the percentage of variable and informative sites for parsimony analysis, obtained with the MEGA program, version 3.1 (Kumar *et al.*, 2004). The number and respective frequencies of the different haplotypes were estimated using DnaSP, version 4.0 (Rozas *et al.*, 2003). Haplotypic (*h*; Nei, 1987) and nucleotidic (π; Nei, 1987) diversities were also calculated in Arlequin, version 3.1 (Excoffier *et al.*, 2005).

Population structuring and demographic history were analysed in the DnaSP and Arlequin programs. The genetic differentiation among the three selected populations (Ajuruteua - AJU, Parnaíba - PAR and Natal - NAT) was analysed through pairwise estimates of *Fst*, the significance of which was tested in 1000 permutations.

Among-population partitioning of genetic diversity was tested using the hierarchical analysis of molecular variance, AMOVA (Excoffier *et al.*, 1992). Deviations from theoretical neutral evolution were verified through *Fs* test (Fu 1997), based on 1000 permutations. A variety of tests were used to detect possible processes of demographic fluctuation: pairwise mismatch distribution of the haplotypes (Rogers and Harpending, 1992); the sum of the squared deviations (SSD) and its respective *p* value, which represents the probability of observing a match between the model of expansion and the random distribution; estimate of the expansion time of the population (t), following the Rogers and Harpending (1992) model; and the parameters τ (unit of population growth), θ_0 and θ_1 (θ values before and after expansion, respectively) based on the mismatch distribution, according to the equation $t = \tau/2u$, where *u* is the mutation rate per sequence per generation (Rogers, 1995; Schneider and Excoffier, 1999). The D-loop mutation rate adopted here was 3.6×10^{-8} (Donaldson and Wilson, 1999), which was the same one used for other epinephilids (Rivera *et al.*, 2004) and generation time for *E. itajara* was considered to be six and a half years (Bullock *et al.*, 1992). Effective population size (*Ne*) was inferred given the estimate of diversity index ($\theta = 2Ne\mu$) based on Watterson (1975) and Tajima (1983) estimates of θ (θ_S and θ_π, respectively) in Arlequin 3.1.

The haplotype network was defined using the TCS program, version 1.17 (Clement *et al.*, 2000), in which it was nested manually into increasingly inclusive clades (or nestings) following the rules described by Templeton *et al.* (1987) and Templeton and Sing (1993). This nested haplotype network was used in a nested clade analysis (NCA: Templeton *et al.*, 1987; Templeton and Sing, 1993). Interpretation of the results was based on the inference key available at the site http://darwin.uvigo.es/download/geodisKey_14Jul04.pdf. An analysis based on the correlation between the genetic and geographic distances among populations was conducted using the Mantel test run in Arlequin 3.1.

A total of 499 base pairs were obtained starting from the 3' region of the mtDNA control region in 116 *E. itajara* specimens from the northern Brazilian coast. This fragment of the mtDNA presented only 17 variable sites with just eight informative. The simple deletions were used in the analyses, although a deletion of eight base pairs was observed in one individual (position 254-261), and was considered as a single substitution. A total of 27 haplotypes were identified on the basis of these variable sites and gaps (Table 1). Four of these (H1, H2, H3, and H11) were well represented, whereas 15 were singletons. The population from Parnaíba presented only two haplotypes (H1 and H2), both of which were shared with Ajuruteua, Natal and Bragança.

As expected due to the largest sample size among the studied areas, Bragança had the largest number of haplotypes (22), 17 of which were exclusive to this locality, and 14 were singletons. However, it is important to note here that the samples from this site were obtained from a fish market which receives catches from a wide area surrounding the Amazon estuary, from the state of Amapá in the north, to Maranhão in the east (Braga CF Msc Thesis, Universidade Federal do Pará, Belém-PA, 2002). Because of its legally protected status, fishermen are reluctant to provide information on their fishing sites of this species, but it seems likely that the samples collected in this study were originated from a relatively wide geographic area. This also supports the idea that the Amazon region is an important refuge for *E. itajara*, given its extensive areas of well-preserved mangrove habitat, and the high productivity of local fisheries in Pará state (Braga CF Msc Thesis, Universidade Federal do Pará, Belém-PA, 2002; Tak-Chuen and Ferrera, 2006).

Haplotypic diversity varied from 0.53 in Parnaíba to 0.86 in Natal (Table 2). The values recorded here for the Goliath grouper are generally lower than those recorded in studies of other grouper species - *Epinephelus marginatus* (Maggio *et al.*, 2006), and *Epinephelus labriformis* and *Epinephelus clippertonensis* (Craig *et al.*, 2006) - however, based on a marker (the mitochondrial *cyt b* gene) which

Table 1 - Haplotypes of the control region of the mtDNA of the five populations of *Epinephelus itajara* analysed in the present study.

Haplotype	Variable sites	Number of specimens in population[1]					GenBank Access number
	12222222233333334444 60024568913346788002 9 31952442140207058675 6	BRA	AJU	PAR	FOR	NAT	
H1	ATCTAATTGATACTTTAA-CG	27	13	5		2	FJ176303
H2	........A.........-..	8	1	7		1	FJ176304
H3	G............-.G.-..	8	3				FJ176305
H4	G.T.............-..	1	1				FJ176306
H5	................-T.		1				FJ176307
H6	..............G.-..		1				FJ176308
H7	.........G........-..				2		FJ176309
H8	..............—..					3	FJ176310
H9	G..............-..	1				2	FJ176311
H10	G............G.-..					1	FJ176312
H11	G.T..........-.G.-..	8					FJ176313
H12	........A.....-.G.-..	1					FJ176314
H13	G.....C.......-.G.-..	1					FJ176315
H14	G...G.........-.G.-..	1					FJ176316
H15	G.T........C..-.G.-..	1					FJ176317
H16	............C....-T.	1					FJ176318
H17	..............-.A	2					FJ176319
H18	............-.G.-..	2					FJ176320
H19	G..C........T.-.G.-..	1					FJ176321
H20	.....-..........-..	1					FJ176322
H21	............-.G—..	2					FJ176323
H22	..............A..	1					FJ176324
H23	G.........C...-.G.-..	2					FJ176325
H24	.C.............-..	1					FJ176326
H25	G.T...........G.-..	1					FJ176327
H26	.......C.........-..	1					FJ176328
H27	............C..-..	1					FJ176329

[1]AJU = Ajuruteua; BRA = Bragança; FOR = Fortaleza; NAT = Natal; PAR = Parnaíba.

evolves more slowly than the control region. Nucleotide diversity is low (0.1% to 0.5%) in all *E. itajara* populations, which may reflect anthropogenic pressures - not just harvesting, but also the loss of mangrove habitat - or other evolutionary process, and will be an extremely important information to plan the management and conservation of the species. Conversely, the high diversity found in Natal could also be due to gene flow from populations from further south, which can be confirmed, in the future, by enlarging the sampling area.

The pairwise *Fst* values were significant when comparing Parnaíba with Ajuruteua (0.24971, $p < 0.01$) and Natal (0.28711, $p < 0.01$), while between Ajuruteua and Natal low and not significant value was observed (0.04891, $p > 0.05$). These three populations were used in AMOVA, which indicated that 19% of the observed variation was the result of variation among populations. Despite the significant Φst value observed in this analysis (0.185, $p < 0.01$), no significant structuring was found in the three populations (data not shown).

All populations except Parnaíba showed negative values of *Fs* (Table 2), although the values were only significant for the sample from Bragança, and the general population, which is probably due to the excess of uncommon haplotypes, a characteristic of populations in expansion (Fu, 1997). The unimodal curve of the mismatch distributions of these populations (data not shown), the Raggedness (0.03 in both cases) and SSD ($p > 0.05$) values, and the different values of θ are all typical of populations in expansion. The τ values recorded for Bragança (3.55) and the general population (3.11) indicate that they have been expanding over the past 197 thousand and 173 thousand

Table 2 - Indexes of genetic variability and results of the neutrality test for the different populations of *E. itajara* analysed in the present study.

Population	N	Number of haplotypes	Haplotypic diversity (*h*)	Nucleotidic diversity (π)	Fu's *Fs*
BRA	73	22	0.83 ± 0.04	0.005 ± 0.003	-12.874*
AJU	20	6	0.57 ± 0.12	0.002 ± 0.002	-1.61327
PAR	12	2	0.53 ± 0.08	0.001 ± 0.001	1.15205
FOR	2	1	n/a	n/a	n/a
NAT	9	5	0.86 ± 0.09	0.003 ± 0.002	-1.59315
Total	116	27	0.80 ± 0.03	0.004 ± 0.003	-19.477*

*Significant value ($p < 0,05$).

years, respectively. The estimates of the effective population size for the general population were approximately 27,300 and 17,660 using the Watterson (1975) and Tajima (1983) estimates of $\theta \pm SD$, respectively $\theta_S \pm SD = 3.192 \pm 1.059$ and $\theta_\pi \pm SD = 2.062 \pm 1.289$.

The nested clade analysis (Figure 2) only revealed a significant association between genetic variability and geographic distribution. The category at the 1-1 nested clade level showed significant differences ($p < 0.001$) for clade (Dc) and nested clade (Dn) distances, which indicates restricted gene flow with isolation by distance. However, the Mantel test found no association between the *Fst* values and geographic distances ($p > 0.05$). These results may be explained by the small number of haplotypes (two) recorded in the population from Parnaíba, and the fact that the one of these haplotypes is probably the ancestral form. While haplotypic diversity is moderate in this case ($h = 0.53$), it is important to remember that where a population is declining, the number of alleles will be affected before any changes in genetic diversity (Frankham *et al.*, 2002). These results indicate that *E. itajara* populations may suffer greater pressures in northeastern Brazil (Fortaleza, Parnaíba and Natal), where both fishery activity and habitat loss are more intense. However, a much wider range of populations must be studied throughout the geographic distribution of the species, as well as the use of nuclear markers, such as microsatellites, before it will be possible to analyse recent gene flow and population structure and dynamics more reliably. The sub-structuring of populations observed in the present study may be favoured by life history traits of the species, such as its territoriality, habitat preferences, reproductive shoaling and fidelity to shoaling sites (Heemstra and Randall, 1993).

The present study provides the first data on the genetic variability and population structure of the Goliath grouper, *Epinephelus itajara*, a critically endangered New World fish species considered to be an important fishery resource. The analyses of populations from the northern Bra-

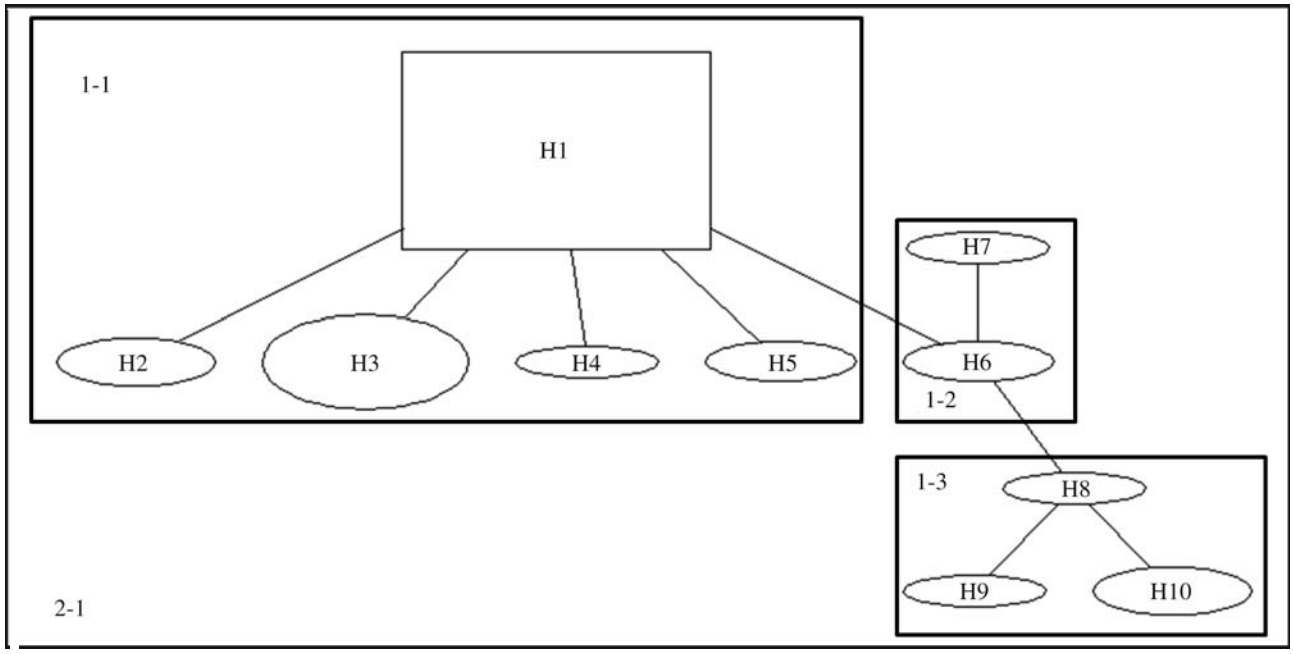

Figure 2 - Minimum-spanning network of *Epinephelus itajara* haplotypes and the nested clade design on which the nested clade analysis was based. The two specimens from Fortaleza (H7) were added to this analysis.

zilian coast indicated overfishing, especially in the Northeast. It is important to remember that a reduction in the effective size of populations of species such as *E. itajara* may leave the species extremely vulnerable to evolutionary processes. While samples of a larger number of individuals and localities will be necessary for a more reliable characterisation of the population dynamics of this species, the levels of genetic variability and structuring of stocks recorded here indicate an urgent need for the implementation of conservation and management measures in order to protect and consolidate these populations.

Many different fish species present clear signs of decline in abundance, which includes many different sharks (Baum *et al.*, 2003). Therefore, more effective controls of fishery activity are essential here, but the preservation of mangrove habitats and shoaling areas will be equally important for the long-term conservation of the species.

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