Genetic diversity of yellow grouper (*Epinephelus awoara*) determined by random amplified polymorphic DNA (RAPD) analysis

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The development of molecular techniques has enhanced our ability to identify fish species. A few molecular markers, such as mitochondrial DNA and ribosomal DNA, have been used to assist in the identification of fish species. One such technique now routinely used is the random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR; Williams et al., 1990). Genetic analysis with RAPD markers is relatively easy, fast, and efficient. RAPD analysis, however, may not be practical for identifing species that interbreed (Martinez et al., 1997). Although the major limitation of RAPD technique for identification of intraspecific specimens is its repeatability, adherence to protocol and standardized reactions can improve the method (Jones et al., 1997). This technique has been used for identification and detection of genetic diversity in various fish species (tilapia; Naish et al., 1995; striped bass; Bielawski and Pumo, 1997; grouper; Asensio et al., 2002). Most other DNA-based methods are more laborious and time consuming than RAPD, making them less suitable for large population or genetic diversity studies. Suitable loci with high reproducibility allow the identification of unequivocally distinct species (i.e., where there is large genetic differentiation between species, compared to within species; Greig et al., 2005).

Groupers (Epinephelinae: Serranidae) are among the most important and highly valued demersal species of tropical and subtropical coastal areas worldwide. In general, grouper species lack distinct morphological specializations, but color patterns and geographic location are used in the field. Some confusion exists over the names of some important Indo-Pacific grouper species (Heemstra and Randall, 1993; Sadovy, 1997) and little work has been done to establish species identities and their genetic diversity. One alternative to gathering information on these species is the use of molecular genetic markers. In the present study, RAPD analysis has been used to investigate the genetic variation in two populations of yellow grouper (*Epinephelus awoara*) from the South China Sea.

Materials and methods

Sample preparation and DNA extraction

Yellow grouper were obtained from two populations (30 individuals from Xiamen and 26 individuals from Guangdong) of the South China Sea. Muscle samples from fish were taken, placed in 95% ethanol, transported to the laboratory, and stored at –20°C until analysis. Genomic DNA was extracted according to the DNA extraction method of DeSalle et al. (1993).

Primer selection and RAPD reaction profile

Twenty primers (Table 1) were selected on the basis of presence of intense, well-distinguished, and reproducible bands for further analysis. PCRs for each population were performed in 2-μL volumes containing 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 1 μL dNTPs, 0.4 unit rTaq polymerase and approximately 20 ng of template DNA and 1 μL of each primer. Thermocycling conditions were as follows: initial denaturation step of 95°C for 5 min., 30 cycles of 45 s at 93°C, annealing temperature of 45 s, primary extension of 72°C for 2 min., and a final extension of 72°C for 5 minutes.

Detection of amplified DNA

Electrophoresis of a 10-μL portion of the amplification reaction was performed for 45 minutes at 100 V in a 1.2 % agarose gel containing ethidium bromide (1 μg/mL) in Tris-acetate buffer (0.004 M Tris acetate, 0.001 M EDTA, pH 8.0). DNA fragments were viewed with UV transillumination and analyzed with Genescan (Gene-Geneius Bio-imaging System, Cambridge, England). Their sizes were estimated by comparison with a commercial 1-kb plus DNA ladder.

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Table 1

Primer details and pattern of polymorphism in two sampled populations (Xiamen and Guangdong) of *Epinephelus awoara*. SN=serial number. XM = Xiamen; GD = Guangdong.

SN	Primers	DNA sequence	No. of bands	Total no. of polymorphic loci	Polymorphic loci in the two populations		Percentage of polymorphic loci
					XM	GD	
1	S117	CACTCTCCTC	8	7	7	4	87.5
2	S465	CCCCGGTAAC	9	8	8	2	88.9
3	S513	GGACGACAAG	6	5	0	5	83.4
4	S514	CAGGATTCCC	9	5	5	4	55.6
5	S515	GGACAACGAG	7	5	5	3	71.4
6	S517	CCGTACGTAG	10	10	10	5	100.0
7	S520	ACGGCAAGGA	7	6	6	6	85.71
8	S1021	GGCATCGGCT	10	7	7	3	70.0
9	S1023	GGGTCCAAAG	8	7	2	7	87.5
10	S1026	TGCCGCACTT	8	8	8	1	100.0
11	S1036	AAGGCACGAG	10	6	6	5	60.0
12	S1037	CCTCACGTCC	7	5	5	1	71.4
13	S1040	CCTGTTCCCT	10	7	7	2	70.0
14	S1042	TCGCACAGTC	6	2	2	2	33.3
15	S1043	AGCACCTCGT	11	11	11	6	100.0
16	S1044	GAATGCGACC	7	5	5	1	71.4
17	S1045	CAGCGTTGCC	10	7	5	7	70.0
18	S1049	ACGGCACGCA	5	3	3	3	60.0
19	S1053	CAGCCGTTCC	6	4	1	4	66.7
20	S1055	GAATCCGGCA	5	3	3	3	60.0
	Total			159	121	106	74
	Percentage of polymorphic loci			76.10	66.66	46.54	

Data analysis

The presence (1) or absence (0) of RAPD products was scored for all loci based on a minimum of two replicates. Only those characters that could be reliably scored from replicate RAPD reactions were included in the analysis. Loci that failed to generate RAPD amplification products were excluded from the analysis. Probability (Fischer's exact test) was carried out to evaluate differences in homogeneity of gene frequencies across populations for each loci. Test of heterozygosity (H_T), expected heterozygosity (H_S), population differentiation (G_{ST}), and gene flow (N_m) were also conducted between two populations of *Epinephelus awoara*.

Results

Two populations of *Epinephelus awoara* were analyzed to characterize the RAPD marker for genetic variation.

Useful RAPD primers and markers for genetic variation

The first group of 20 primers (Table 1) produced a total of 159 bands, among which 121 polymorphic bands (76.10%) were observed. The 38 monomorphic bands (23.90%) could be considered, on a preliminary basis, as population diagnostic bands that allow clear differentiation among populations of *Epinephelus awoara* because they were present in all individuals analyzed. Primers S514, S1021, S1036, S1040 and S1042 generated more diagnostic bands than did other primers. The absence of these markers in related populations must, however, still be confirmed.

Primers that showed high levels of reproducibility also produced more polymorphic bands. Over five polymorphic bands (5.76) per primer were produced from 20 primers on average. All 20 primers (Table 1) yielded satisfactory amplification products for all specimens tested. Each primer produced a unique band pattern of amplified DNA. For a given primer and DNA template, a number of bands appeared intense and reproducible in all the replicates, and bands of weaker stain appeared occasionally. All primers were able to distinguish two populations of *E. awoara*, reproducing different and well-characterized banding patterns. Banding pattern variations observed with some primers were not consistent for all individuals within or between populations and therefore these variations should be attributed to intraspecific polymorphism.

High rates of RAPD polymorphism

Polymorphic rates of RAPD bands were much higher between two populations of *Epinephelus awoara*. The number of reproducible and well-resolved bands analyzed in the two populations ranged from 5 to 11. Monomorphic bands, constantly present in all individuals, varied between primers, whereas total polymorphic bands were observed 76.10% of the time for the two populations of *E. awoara*. The Xiamen population of *E. awoara* exhibited 66.66% polymorphism, whereas polymorphism was 46.54% in the Guangdong population (Table 1). RAPD fragments of fewer than 1000 base pairs were found to be more polymorphic than larger fragments.

Genetic diversity

Genetic diversity between populations was clearly illustrated in Table 2 and Table 3. The genetic diversity statistics (H_T, H_S, G_{ST}, N_m) estimated between the two populations of *Epinephelus awoara* for each primer are listed in Table 3. Nei's (1973) unbiased measures of genetic identity and genetic distance between two populations were 0.6216 and 0.4755, respectively. Within the populations, total heterozygosity varied from 0.2189 to 0.4616 and expected heterozygosity ranged between 0.1232 and 0.2920. Probability (Fisher's exact test) of homogeneity was significant at $P>0.05$ for all primers except S465, S513, S1026, S1044, and S1055.

Discussion

This study showed a considerable amount of genetic variation present in *Epinephelus awoara*. RAPD analysis generated a large number of polymorphic DNA bands (Table 1) between the populations of *E. awoara*, thus making it one of the most efficient systems for generating DNA markers. However, low levels of interspecific variation were found in RAPD profiles among strains of grouper. Therefore, it was an inefficient system for generating molecular markers for gene mapping (Liu et al., 1999).

Several authors (Welsh and McClelland, 1990; Bardakci and Skibinski, 1994; Naish et al., 1995) have demonstrated that the RAPD PCR method is a powerful tool for the assessment of genetic markers that are capable of discriminating between species or subspecies in a wide range of organisms, including fishes. This capacity was confirmed by the results of the present study because, for all screened primers, different RAPD banding patterns were observed between the two populations (Table 1). A limitation arising with the application of the RAPD technique is the homology between comigrating bands produced by the same primer in different individuals (Hadrys et al., 1992). Nevertheless, in the present study, homology constitutes a valid assumption because all individuals belong to the same species.

Despite the fact that no specific markers were found to identify *Epinephelus awoara* species, data analysis of the observed and effective number of alleles revealed a degree of divergence (Table 2). The Nei (1973) gene diversity was also illustrated in Table 2. The two populations of *E. awoara* (Table 3) showed high level of genetic variation (i.e., Shannon's information index; Shannon-Weaver, 1949, average genetic index [H_{POP}] and genetic diversity index between populations [H_{SP}]). Similarly a high level of genetic variation and genetic distance (0.617–0.949) was observed in dinoflagellates (Baillie et al., 2000). This finding supports a high genetic distance for *E. awoara* (0.4755) in the present study. This may be due to the selection pressure of pollutants (Nadig et al., 1998) or to overexploitation of groupers in the South China Sea.

Total heterozygosity (H_T) values ranged from 0.2189 to 0.4616, expected heterozygosity (H_S) from 0.1232 to 0.3157, and an estimate of gene flow (N_m) from 0.3714 to 4.1722. The proportion of total genetic variation within species due to population differentiation (G_{ST}) ranged from 0.1070 to 0.5344 (Table 3). Carvalho et al. (1991) observed a very high differentiation ($G_{ST}=0.648$) in the guppy (*Poecilia reticulata*) in northern Trinidad. Similarly, Ward et al. (1994) also observed high G_{ST} values (>0.2000). In any case, the divergence in G_{ST} values indicates that, on average, marine subpopulations exchange between one and two orders of magnitude more migrants per generation. Genetic divergence between areas originates when populations are formed or through the restriction of gene flow (Lage et al., 2004). Higher H_S in marine fish is at least consistent with these fish having, on average, larger population sizes. Homogeneity of gene frequencies was found to be significant ($P>0.05$) across populations of *Epinephelus awoara* (Fig. 1) for most of the primers except S465, S513, S1026, S1044,and S1055 ($P>0.001$).

The factor that could influence the genetic variation of the grouper population geographically is the movement of adults, which can be extensive and cover considerable distances (Harding et al., 1997). The otherwise shelter-habituated adults travel long distances necessary to populate remote oceanic islands. The Bermuda fauna include more than 75% of the known west Indian groupers even though Bermuda lies more than 800 miles from other coral reefs. Such distributional patterns are best explained as the result of passive transport of larvae by oceanic currents. Similar patterns are also expected in the South China Sea. Adult movement of marine fish is relatively unfettered by physical barriers. In addition to gene flow through adult migration, marine fish frequently have a planktonic larval stage of several months duration, which can be expected to further enhance gene flow.

Estimation of genetic diversity of *Epinephelus awoara* by RAPD analysis (i.e., by using the mean of observed number of alleles [*Na*], effective number of alleles [*Ne*] and Nei's gene diversity [*H*]) was found to be 1.6563, 1.4169, and 0.2915, respectively for Xiamen populations, whereas they were 1.4824, 1.2713, 0.2167, respectively for Guangdong populations. Our results provide evidence for a loss (10.5%, 11.3%, and 25.7%, respec-

Table 2

Genetic variation in two sampled populations (Xiamen and Guangdong) of *Epinephelus awoara*. Na = Observed number of alleles; *Ne* = Effective number of alleles; *H* = Nei's (1973) gene diversity; *Ho* (I) = observed heterozygosity (Shannon's information index).

	Na		*Ne*		*H*		*Ho* (I)	
Loci	Xiamen	Guangdong	Xiamen	Guangdong	Xiamen	Guangdong	Xiamen	Guangdong
S117	1.8750	1.5000	1.5318	1.2272	0.3145	0.1449	0.4675	0.2254
S465	1.8889	1.1111	1.6659	1.0927	0.3673	0.0505	0.5324	0.0719
S513	1.0000	1.8333	1.0000	1.5851	0.0000	0.3298	0.0000	0.4819
S514	1.5556	1.4444	1.3886	1.1905	0.2231	0.1310	0.3269	0.2074
S515	1.7142	1.4285	1.2984	1.1368	0.1924	0.1019	0.3076	0.1565
S517	2.0000	1.5000	1.6602	1.2678	0.3886	1.1696	0.5808	0.2593
S520	1.7142	1.8571	1.5315	1.5871	0.2877	0.3580	0.4162	0.5048
S1021	1.8000	1.3000	1.3246	1.1497	0.1959	0.0946	0.3077	0.1463
S1023	1.2500	1.8750	1.1780	1.4712	0.1004	0.3229	0.1469	0.4806
S1026	2.0000	1.1250	1.6661	1.0575	0.3859	0.0394	0.5691	0.0618
S1036	1.7000	1.4000	1.3534	1.3771	0.3038	0.1940	0.2334	0.2712
S1037	1.7142	1.1428	1.4307	1.0318	0.2254	0.0260	0.3769	0.1039
S1040	1.7000	1.2000	1.5307	1.0605	0.3011	0.0464	0.4348	0.0786
S1042	1.3333	1.3333	1.2429	1.1814	0.1358	0.1107	0.1945	0.1689
S1043	2.0000	1.4545	1.7466	1.1649	0.4081	0.1044	0.5911	0.1694
S1044	1.7142	1.1428	1.4483	1.0057	0.2689	0.0054	0.3997	0.0136
S1045	1.5000	1.8000	1.4107	1.5349	0.2189	0.3146	0.3126	0.4642
S1049	1.6000	1.6000	1.5417	1.3333	0.2839	0.2124	0.3994	0.3228
S1053	1.6666	2.0000	1.1519	1.4657	0.0795	0.3059	0.1116	0.4780
S1055	1.4000	1.6000	1.2359	1.5058	1.1478	0.2722	0.2223	0.3871
Mean	1.6563	1.4824	1.4169	1.2713	0.2915	0.2167	0.3466	0.2527

tively for *Na, Ne*, and *H*) of genetic diversity; this loss may be due to high fishing pressure of the grouper population in Guangdong.

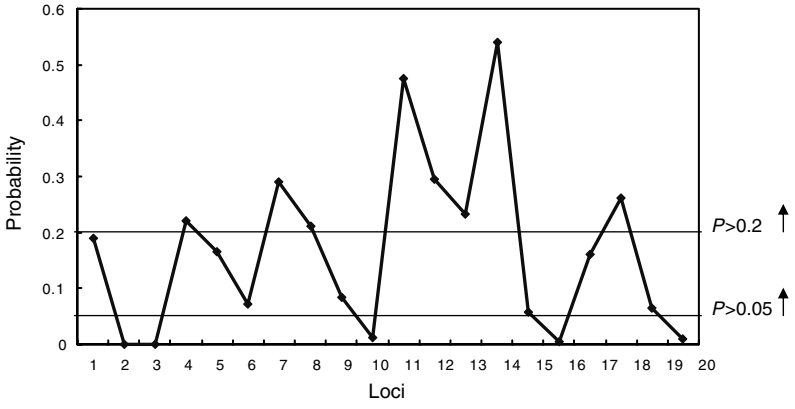

Figure 1

Probabilities ($P>0.05$) that show the significance of homogeneity of gene frequencies across populations of yellow grouper (*Epinephelus awoara*). Arrows show the range of probability.

Conclusion

These results indicate that commercial fishing may result not only in selective genetic changes in exploited stocks but also in reduced genetic diversity due to genetic drift. The loss of genetic diversity may be particularly pronounced during the initial stages of exploitation, and investigations into the advanced stages of exploitation may be less likely to detect significant changes in allelic diversity. This loss can also be tested for other commercially exploited species of grouper in genetic and demographic context. In the present study, the RAPD method proved useful and technically convenient for the study of genetic diversity of yellow grouper (*Epinephelus awoara*); therefore, it can also be applied to other species of grouper and enable the analysis of many samples in a short time. The present work also indicates that molecular markers used

in present study are a practical approach for studies of genetic diversity

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