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Genetic Analysis of Arowana (*Scleropages formosus,* Osteoglossidae) in Two Major Habitats at the Tasik Bera Lake, Pahang and at the Endau River, Johor, Malaysia

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Abstract

A study was carried out to monitor the genetic variability of three wild populations of the Asian green arowana (*Scleropages formosus*). Two populations of green arowana were obtained from the Tasik Bera Lake, Pahang in 2000 and 2002 while the other population was collected from the Endau River, Johor, in 2002. The average number of alleles per locus was low and departures from the Hardy-Weinberg Equilibrium were observed in all the populations depending on the locus. Among 11 microsatellite loci, eight significantly deviated from the Hardy-Weinberg Equilibrium (HWE) ($p<0.05$) in the Tasik Bera-2000 population whereas seven significantly departed from HWE ($p<0.05$) in the Tasik Bera-2002 and Endau River-2002 populations. Low level of genetic diversity and deficit in heterozygosity were observed from the analysis of these 11 loci. The differentiation among the three populations was significant ($p<0.05$). The estimates of effective population size ranged from 1043 to 1519. The F_{ST} and R_{ST} values ranged from 0.048 to 0.067 and 0.050 to 0.072, respectively. The genetic data indicate the occurrence of a bottleneck effect in the wild progeny and considerable division among the green arowana

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populations due to environmental destruction, over catching, breeding behavior as well as barriers to dispersal.

Introduction

The presence of the Asian arowana (*Scleropages formosus*) has been reported in Borneo, Cambodia, Malaysia, Sumatra and Thailand (Pouyaud et al. 2003). Previous studies have reported the presence of cross-back golden variety in the Pahang and Johor States while the blue-base variety was found in Bukit Merah, Perak (Zakaria-Ismail 1987; Pouyaud et al. 2003). It is believed that the wild populations of the golden variety are almost extirpated due to over collection and rapid habitat modification. The green variety however is still relatively common in areas such as Tasik Bera in Pahang, Endau River in Johor (Ng & Tan 1999; Sim 2002) and Terengganu drainage (Cramphorn 1983)(Fig. 1).

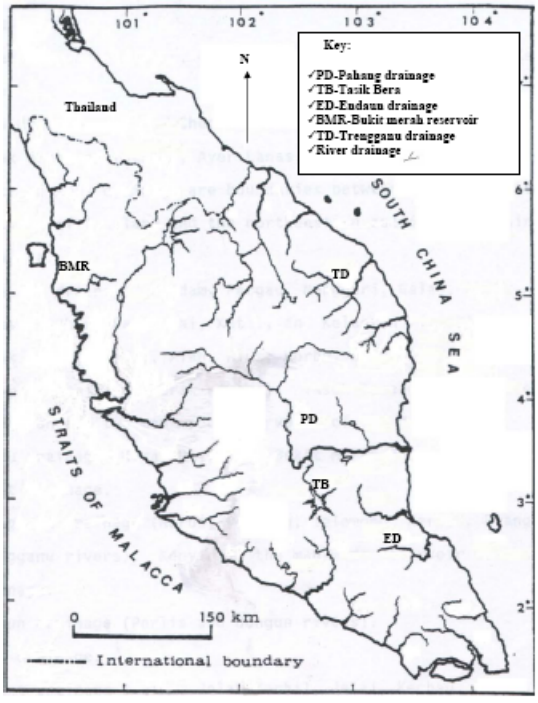

Fig. 1. Major river drainages and major habitats of arowana (*Scleropages formosus*) in Peninsular Malaysia

Fish populations are declining as in many parts of the world at an alarming rate (Safina 1995). Similarly in Malaysia habitat alteration, detrimental ways of fish catching and other human activities are threatening the arowana populations (Ismail 1989). The low fecundity rate, the oral brooding habit and open water spawning, further threaten the survivability of the arowana (Khan et al. 1996). In 1975 the species was classified as one of the most highly endangered fish by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in Appendix I (Greenwood et al. 1996).

A genetic assessment of the arowana has been undertaken using various kinds of molecular markers with a view to have a sound knowledge of its biology, ecology, biogeography and inter-populational genetic diversity. The use of microsatellites is well documented in fish (Sekino et al. 2002; Was & Wenne 2002; Elliot & Reily 2003). It is a powerful technique to detect genetic variability between and within strains of arowana (Yue et al. 2000; 2004). Conservation of the genetic diversity in such a declining species could not be achieved without the having necessary background knowledge on the pattern and amount of genetic diversity.

Although molecular markers have been used successfully for monitoring genetic diversity in captive stocks of arowana, genetic variation of the wild population has not been studied thus far. In this study, we used microsatellite markers to assess the genetic variability of three wild populations of green arowana in Tasik Bera. This study will provide a genetic background for the conservation of wild arowana in the Malaysian river systems.

Materials and Methods

Samples

Three populations of green arowana were used in this study. Sixty-nine and twenty-eight green arowana were collected from Tasik Bera, Pahang (Fig. 1) in 2000 and 2002, respectively. Thirty-eight green arowana were sampled from the Endau River (Fig. 1) in 2002. Scales and fin clips were preserved in 70% ethanol at 4°C until genomic DNA was extracted.

DNA Extraction and PCR

Samples were analysed using TNES-Urea buffers (Asahida et al. 1996) and genomic DNA was isolated with conventional phenol-chloroform extraction. Genetic variation within and between these populations was assessed using eleven microsatellite loci. Among these markers six loci were described by Yue et al. (2000), while five loci were isolated by Tang et al. (2004). Details of all the microsatellite loci and PCR conditions are given in table 1. The polymerase chain reaction (PCR) was performed on the HYBAID OmniGene thermal cycler in a total volume of 25 µl. Reactions contained 1x PCR buffer (Promega), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of each primer (Table 1), 1U *Taq* polymerase (Promega) and 20 ng of genomic DNA. Amplifications for the six loci

described by Yue et al. (2000), was carried out using 4 minutes (min) of initial denaturation followed by 30 cycles of 30 seconds (s) of denaturation at 94.5°C, 30 s of annealing at the temperature detailed in table 1 and 30 s extension at 72°C with a final extension of 5 min at 72°C. The other five loci were amplified with 3 min of initial denaturation followed by 40 cycles of 10 s of denaturation at 95°C, annealing at specific temperature (Table 1) for 10 and 30 s extensions at 72°C with a final extension of 5 min at 72°C. The PCR products were run on a 10% non-denaturing polyacrylamide gel (16cm x 20cm) at 250 V for 4-5 hours. A 20-bp DNA marker (Cambrex BioScience) was used to estimate the PCR fragment size. Gels were visualized using DNA silver staining system (Promega) and analyzed using the GelCompar the II Software (Applied Maths).

Statistical analysis

Microsatellite allele frequencies of each population at each locus were estimated using GENEPOP version 3.1c (Raymond & Rousset 1995). The observed (H_o) and expected heterozygosities (H_e), of the polymorphic loci of each population were estimated using the ARLEQUIN version 2.000 (Schneider et al. 2001) software. The HWE at each locus was assessed using the ARLEQUIN programme. The inbreeding coefficient, F_{IS} (Weir & Cockerham 1984) was estimated to measure the HWE departures for each population using the GENEPOP programme. The recent effective population size reduction was detected using the BOTTLENECK version 1.2.02 software (Cornuet & Luikart 1996). The two-phased model was tested using the Wilcoxon sign-rank test with default settings and 1000 iterations. The effective population sizes for these populations were estimated based on the unbiased expected heterozygosity under SMM (Nei 1987; Lehmann et al. 1998) and the microsatellite mutation rate of 2.5 x 10^{-4} (Yue et al. 2004). Population differentiation was measured by calculating pairwise weighted F_{ST} (Weir & Cockerham 1984) values over all loci. The R_{ST} values were calculated as sum of squared size differences based on number of repeats (Slatkin 1995). The probability associated with the F_{ST} and R_{ST} values was estimated through random permutation procedure (1000 permutation) using the ARLEQUIN programme.

Table 1. Primers sequences of 11 microsatellite loci

Locus	Repeat motif	Primer sequences (5'-3')	GenBank accession nos.	Annealing Temperature (°C)
D32	$(CA)_{13}$	F AGCACCCTGTTACTGGAAGAGA	AF219960	55
		R AGTGTGATGCTTTTGCTTTGAGAA		
D92	$(GT)_{13}$	F AGTCGCACACCACCACCTCAG	AF219969	55
		R TCAGCGATAACCCCACACCT		
D95	$(CA)_9$	F CCTGCGGAAGAAGAAAAGACT	AF219971	55
		R CATGGTGTTGGCTGTGAGGAG		
D27	$(CA)_{17}$	F GTGTCAGTATAGTGAATCTGTAG	AF219958	55
		R TGACAATGGCAGCATAATGAGAT		
D85	$(CA)_{10}$	F GTTCCACAGGGGCTGAGAAAAT	AF219967	55
		R GAGGACGGAACAAAAGCATTGG		
D11	$(GT)_{16}$	F TGGTTTCCACCTACAGTCCAAAGA	AF219953	55
		R GTTACGAGTATCTGGCCCAATGG		
K10	$(CA)_{20}$	F GCACCTAACTGAAGAGCATT	AY173130	57
		R AAAATTACCTGCTTGTGTGC		
K13	$(CA)_5CG(CA)_3$	F GCACTGTTAAGTTCTGGTGTC	AY173131	51
		R GATACGCATGACATTCTGTG		
K16	$(TG)_5$	F CAGTGGTTGCACACTTACAG	AY173136	50
		R AAAGTCGGCATGATGAAATA		
K27	$(CA)_{16}$	F CCATTAACCCCTTGTCCTCA	AY173135	50
		R AAGGATGCAGGAGAGCAAAA		
K37	$(CA)_4$	F CCATTAGCAAACCCATGCTT	AY173132	51
		R TGGAAATGTGTCATCCTTCAG		

Results

Microsatellite variability

All eleven polymorphic microsatellite loci exhibited variability in the three populations. Allele frequency distributions for these populations are listed in table 2. A total of 30 alleles were detected over

135 individuals. Twenty-nine alleles were detected in the Tasik Bera population collected in 2000, but the number of alleles was reduced to 28 two years later while 27 alleles were found in the Endau River population. The number of alleles per locus ranged from 2.45 in the Endau River population to 2.63 in the Tasik Bera-2000 population. Alleles $D92^{116}$ and $K13^{206}$ were found in the two Tasik Bera populations but undetected in the Endau River population. Among the 30 alleles detected, two population marker alleles were found. Allele $D27^{76}$ was only found in the Tasik Bera population sampled in 2000 while Allele $K13^{202}$ was only detected in the Endau River population. Most of the alleles found in this study showed high frequencies and rare alleles were undetected.

Hardy-Weinberg Equilibrium

The Tasik Bera-2002 population showed the highest average observed heterozygosity (0.338), followed by the Tasik Bera-2000 (0.306) and the Endau River (0.280) populations (Table 3). The average expected heterozygosity at the eleven loci was highest (0.427) in the Tasik Bera-2002 population, followed by the Endau River-2002 population (0.398) and the Tasik Bera-2000 population (0.395) (Table 3). Positive values of average inbreeding coefficients (F_{IS}) were estimated for all the three populations. The highest F_{IS} value (0.356) was observed in the Endau River-2002 population, whereas the lowest (0.296) was seen in the Tasik Bera-2002 population (Table 3).

Among the eleven loci screened in the Tasik Bera population collected in 2000, eight deviated significantly ($p<0.05$) from the HWE. Seven out of eleven loci from both the Tasik Bera-2002 and the Endau River-2002 populations departed significantly from HWE ($p<0.05$) (Table 3).

Bottleneck analysis

There were indications of recent bottlenecks in these populations. Two loci in the Tasik Bera-2000 population (D32 and D95) showed significant ($p< 0.05$) heterozygosity excess (Table 4). The allelic distribution shows a significant 'mode-shift' ($p< 0.05$) (lack of low frequency alleles) in the Tasik Bera and the Endau River populations (Fig.2).

Effective population size

The effective population size of the Tasik Bera populations was higher than the Endau River population. The estimates by the SMM were 1519 for the Tasik Bera-2000 population, 1471 for the Tasik Bera-2002 population and 1043 for the Endau River-2002 population (Table 5).

Table 2. Allele distributions for eleven microsatellite loci in three populations of green arowana at Tasik Bera and Endau River

Allels(bp)	Tasik Bera -2000	Tasik Bera -2002	Endau River -2002
D32			
201	0.333	0.500	0.711
208	0.159	0.179	0.118
244	0.268	0.214	0.145
285	0.239	0.107	0.026
D92			
110	0.210	0.160	0.066
116	0.029	0.036	0.000
120	0.493	0.518	0.631
151	0.268	0.286	0.303
D95			
140	0.312	0.304	0.105
148	0.688	0.696	0.895
D27			
72	0.029	0.018	0.184
76	0.072	0.000	0.000
82	0.754	0.678	0.671
103	0.145	0.304	0.145
D85			
114	0.188	0.214	0.263
129	0.812	0.786	0.737
D11			
127	0.275	0.768	0.368
131	0.725	0.232	0.632
K10			
193	0.181	0.643	0.776
225	0.819	0.357	0.224
K13			
185	0.840	0.839	0.776
202	0.000	0.000	0.158
206	0.109	0.125	0.000
229	0.051	0.036	0.066
K16			
149	0.920	0.964	0.763
164	0.080	0.036	0.237
K27			
180	0.732	0.661	0.671
220	0.268	0.339	0.329
K37			
138	0.899	0.857	0.947
146	0.101	0.143	0.053

Table 3. Number of alleles (N), observed heterozygosity (H_o), expected heterozygosity (H_e) and inbreeding coefficients (F_{is}) at eleven microsatellite loci for three populations of green arowana (*Indicates significance, $p<0.05$)

Microsatellite	Tasik Bera -2000	Tasik Bera-2002	Endau River-2002
D32			
N	4	4	4
H_o	0.507	0.429	0.289
H_e	0.745	0.694	0.484
P	0.000*	0.000*	0.000*
F_{is}	0.316	0.367	0.381
D92			
N	4	4	3
H_o	0.928	0.893	0.737
H_e	0.645	0.634	0.512
P	0.000*	0.000*	0.003*
F_{is}	-0.443	-0.418	-0.448
D95			
N	2	2	2
H_o	0.275	0.250	0.053
H_e	0.447	0.466	0.214
P	0.000*	0.063	0.001*
F_{is}	0.364	0.424	0.727
D27			
N	4	3	3
H_o	0.435	0.643	0.658
H_e	0.408	0.479	0.501
P	0.675	0.045	0.059
F_{is}	-0.066	-0.423	-0.318
D85			
N	2	2	2
H_o	0.000	0.000	0.000
H_e	0.322	0.378	0.419
P	0.000*	0.000*	0.000*
F_{is}	1.000	1.000	1.000
D11			
N	2	2	2
H_o	0.000	0.000	0.000
H_e	0.416	0.378	0.498
P	0.000*	0.000*	0.000*
F_{is}	1.000	1.000	1.000
K10			
N	2	2	2
H_o	0.362	0.714	0.447
H_e	0.310	0.490	0.372
P	0.163	0.005*	0.171
F_{is}	-0.214	-0.543	-0.276
K13			
N	3	3	3
H_o	0.319	0.321	0.342
H_e	0.293	0.314	0.373
P	0.495	1.000	0.502
F_{is}	-0.135	-0.136	0.084

Table 3. Number of alleles (N), observed heterozygosity (H_o), expected heterozygosity (H_e) and inbreeding coefficients (F_{is}) at eleven microsatellite loci for three populations of green arowana (*Indicates significance, p<0.05) (continued)

Microsatellite	Tasik Bera -2000	Tasik Bera-2002	Endau River-2002
K16			
N	2	2	2
H_o	0.000	0.000	0.000
H_e	0.150	0.105	0.392
P	0.000*	0.019*	0.000*
F_{is}	1.000	1.000	1.000
K27			
N	2	2	2
H_o	0.536	0.464	0.553
H_e	0.406	0.480	0.448
P	0.001*	1.000	0.262
F_{is}	-0.360	-0.017	-0.239
K37			
N	2	2	2
H_o	0.000	0.000	0.000
H_e	0.198	0.284	0.127
P	0.000*	0.000*	0.000*
F_{is}	1.000	1.000	1.000
Mean			
N	2.63	2.55	2.45
H_o	0.306	0.338	0.280
H_e	0.395	0.427	0.398
F_{is}	0.315	0.296	0.356

Table 4. Observed gene diversity (H_e, Hardy-Weinberg heterozygosity) and equilibrium gene diversity (H_{eq}) of three populations of green arowana under two-phased model (* Indicates significance, p<0.05)

	Tasik Bera- 2000			Tasik Bera- 2002			Endau River- 2002		
	H_e	H_{eq}	*P*	H_e	H_{eq}	*P*	H_e	H_{eq}	*P*
D32	0.745	0.481	0.000*	0.694	0.588	0.140	0.484	0.527	0.280
D92	0.645	0.490	0.160	0.634	0.543	0.280	0.512	0.402	0.420
D95	0.447	0.162	0.040*	0.466	0.277	0.320	0.214	0.248	0.260
D27	0.408	0.490	0.280	0.479	0.411	0.420	0.501	0.364	0.260
D85	0.322	0.206	0.320	0.378	0.262	0.380	0.419	0.212	0.220
D11	0.416	0.198	0.200	0.378	0.180	0.240	0.498	0.211	0.120
K10	0.310	0.194	0.300	0.490	0.227	0.140	0.372	0.191	0.220
K13	0.293	0.362	0.380	0.314	0.450	0.120	0.373	0.399	0.440
K16	0.150	0.173	0.500	0.105	0.283	0.140	0.392	0.200	0.240
K27	0.406	0.205	0.280	0.480	0.243	0.220	0.448	0.187	0.120
K37	0.198	0.213	0.480	0.284	0.232	0.420	0.127	0.193	0.420

Genetic differentiation

All pairwise F_{ST} values were significantly different from zero (p<0.05) (Table 6). This showed that there is a pronounced genetic differentiation among these three populations. Comparison of pairwise F_{ST} indicated that the Tasik Bera-2002 and the Endau River populations were the

most similar. The highest distance was found between the Tasik Bera-2002 and Endau River populations. Significant ($p<0.05$) pairwise R_{ST} values were observed among these three populations (Table 6). The population differentiations based on F_{ST} and R_{ST} values were similar but R_{ST} values were higher than F_{ST} values.

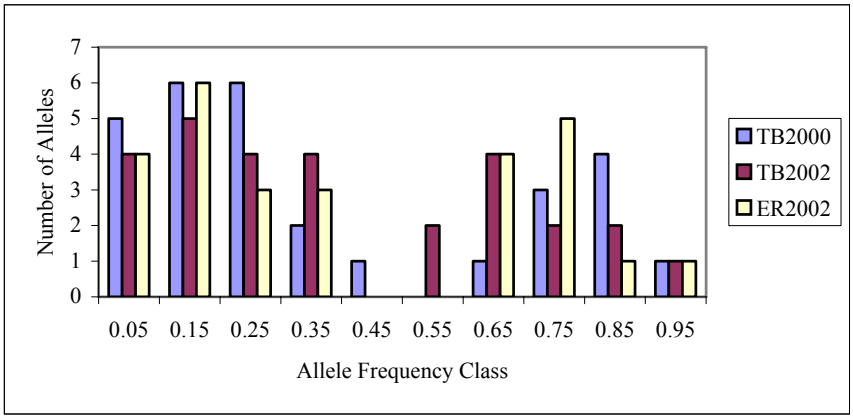

Figure 2. Allele frequency distributions from three populations of green arowana (TB2000; Tasik Bera-20000, TB-2002; Tasik Bera-2002 and ER2002; Endau River-2002 population)

Table 5. Effective population sizes for three populations of green arowana estimated using Stepwise Mutation Model (SMM) models

Locus	Tasik Bera- 2000 SMM	Tasik Bera- 2002 SMM	Endau River- 2002 SMM
D32	7189	4840	1380
D92	3470	3233	2100
D95	1135	1253	309
D27	927	1342	1508
D85	588	792	981
D11	966	792	1484
K10	550	1422	768
K13	500	562	797
K16	192	124	853
K27	917	1349	1141
K37	277	475	156
Mean	1519	1471	1043

Table 6. Pairwise comparisons of microsatellite F_{ST} (below diagonal) and R_{ST} (above diagonal) between 3 populations of green arowana (*indicates a significant genetic distance, $p<0.05$)

Populations	Tasik Bera-2000	Tasik Bera-2002	Endau River-2002
Tasik Bera 2000		0.068*	0.050*
Tasik Bera 2002	0.064*		0.072*
Endau Rompin	0.048*	0.067*	

Discussion

Deviation from Hardy-Weinberg Equilibrium

In this study, most of the loci screened deviated from HWE. Five loci showed significant deviations from HWE across all populations. Deficit in heterozygosity were observed from the analysis of eleven loci compared with those estimated by Yue et al. (2000; 2004). The deficiency in heterozygosity suggested that non-random mating in these populations was occurring. Inbreeding increases the proportion of homozygous individuals in a population. Due to the territorial behavior of arowana, the entire populations may be divided into several subpopulations and only mating between related individuals is likely to happen. Positive values in the inbreeding coefficient (F_{IS}) were observed for most of the loci across all three populations. An alternative explanation is that although the individuals were sampled randomly, they may not represent the true population due to the behavior of the arowana. The male arowana will build up its territory and fries could be collected in the mouth of the males or near the adults. Thus, individuals collected at the same location might often be siblings produced by relatively few adults.

Microsatellite variability

In the present study, less alleles were observed from the analysis of six loci (D11, D27, D32, D85, D92 and D95) compared with those observed by Yue et al. (2000; 2002; 2004). However, this comparison is considered biased due to the unequal representation of populations in different geographical regions and time frame. In the present study all of the samples were collected from a single region. The green arowana stock of Yue et al. (2000) was pooled from farms in Singapore and Indonesia and this could be expected to contribute to a wider gene pool. However, those wild-caught arowana in the early 1980s might not represent the recent natural populations (Yue et al. 2004). Our estimates of effective population size of the natural populations were very low compared with the results obtained by Yue et al. (2004).

Ruzzante (1998) concluded that 50 to 100 individuals are necessary for a precise estimation of population structure and genetic distance. The sample sizes of the Tasik Bera population collected in 2002 and the Endau River population were lower than what was recommended. However, suboptimal sample size should not be the main reason for the over all low genetic variability in this study. The sample size of the Tasik Bera popula-

tion collected in 2000 fell within the recommended range and was higher than the sample size used by Yue et al. (2000; 2002; 2004) but this population still displayed a very low level of genetic variability.

Our results showed that there was lack of private alleles to differentiate these three populations due to the low number of alleles per locus. The results suggested that the Tasik Bera and Endau River populations have been through a population bottleneck. Our results showed that none of the rare alleles was detected in these three populations. Besides, the Tasik Bera -2000 and Endau River-2002 populations showed significant 'mode-shifted' distributions indicating that there was a recent bottleneck. Although allelic diversity depends on the sample size, the sample size of the Tasik Bera- 2000 and Endau River-2002 populations were larger than the Tasik Bera-2002 and the population studied by Yue et al. (2004), which showed no sign of recent bottleneck.

Reduction in genetic diversity had been linked to decreases in growth and fecundity, changes in sex ratio and the ability to adapt to environmental changes (Chapman et al. 1999). Thus the solution is to widen the gene pool by means of introducing individuals from other populations although this will be a difficult task. To date, no survey has been carried out to determine the genetic diversity of other wild populations such as Kahang River and Kenyir Lake in Malaysia (Fig. 1). Another source of diversity will be the populations in captivity. While in captivity it is possible to develop diversified stocks by selection and breeding (Yue et al. 2000; 2002; 2004). However it must also be noted that the adverse genetic effects of stock transfer such as out breeding depression (Philipp 1991; Templeton et al. 1986) should be taken into consideration.

Genetic differentiation

Estimates of F_{ST} and R_{ST} were statistically greater than zero for each comparison and this suggested the existence of three genetically different populations. The pairwise genetic differentiation provides an evidence of the underlying geographic and temporal components of population divisions among these three populations. Although the Tasik Bera-2000 and Tasik Bera 2002 populations were collected from the same location, the former showed a closer genetic relationship with the Endau River population than the latter. This could be due to the sampling error because the sample size of the former was larger than the latter.

The Tasik Bera and Endau River populations which were collected from two geographically distant drainages had low levels of gene flow. The territorial behavior of arowana further eliminated long distance disper-

sal, thus making them more susceptible to genetic drift. Habitat modification and pollution load are thought to drastically reduce the population size precipitating genetic bottleneck (Frankel & Soule 1981). Furthermore, the size of these over-fished populations is expected to fall due to the disturbances in age structure and sex composition. Thus, when a population loses genetic variation from a bottleneck, the genetic distance between it and the ancestral population may increase very quickly (Chakraborty & Nei 1977; Hedrick 1999).

Conclusion

Data gathered show evidence that both the Tasik Bera and Endau River populations went through a genetic bottleneck. This phenomenon appears sufficient to explain the significant difference between the three populations. Thus microsatellite markers have important implications for arowana conservation and long-term population restoration. We anticipate the use of this data to encourage efforts to widen the genetic pool of this species. Though the introduction of automation and flourometric detection methods for microsatellite assays have now become the tool of choice for endangered wildlife and captive management applications but our protocol has been found to be effective, and can be used to genotype fish samples inexpensively. It is to be hoped that this will allow countries with limited financial resources, to address population and conservation issues of endangered fish as well as other aquatic organisms in nature.

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