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# Effective Population Size and Maintenance of Genetic Diversity in Captive-Bred Populations of a Lake Victoria Cichlid

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**Abstract:** We used microsatellite DNA markers to investigate the maintenance of genetic diversity within and between samples of subpopulations (spanning five captive-bred generations) of the haplochromine cichlid *Prognathochromis perrieri*. The subpopulations are maintained as part of the Lake Victoria Cichlid species survival plan. Changes in the frequencies of 24 alleles, over four polymorphic loci, were used to estimate effective population size ( $N_e$ ). Point estimates of  $N_e$  ranged from 2.5 to 7.7 individuals and were significantly smaller than the actual census size ( $N_{obs}$ ) for all subpopulations (32–243 individuals per generation), with the corresponding conservative  $N_e/N_{obs}$  ratios ranging from 0.01 to 0.12. Approximately 19% of the initial alleles were lost within the first four generations of captive breeding. Between-generation comparisons of expected heterozygosity showed significant losses ranging from 6% to 12% per generation. Seven private alleles were observed in the last sampled generation of four subpopulations, and analysis of population structure by  $F_{ST}$  indicated that approximately 33% of the total genetic diversity is maintained between the subpopulations from different institutions. To reduce the loss of genetic variation, we recommend that offspring production be equalized by periodically removing dominant males, which will encourage reproduction by additional males. Consideration should also be given to encouraging more institutions to maintain populations, because a significant fraction of the genetic variation exists as among-population differences resulting from random differentiation among subpopulations.

Tamaño Poblacional Efectivo y Mantenimiento de la Diversidad Genética en Poblaciones de un Cíclido del Lago Victoria Reproducidas en Cautiverio

**Resumen:** Utilizamos marcadores microsatélite de ADN para investigar el mantenimiento de la diversidad genética dentro y entre muestras de subpoblaciones (abarcando cinco generaciones reproducidas en cautiverio) del cíclido haplocrómico *Prognathochromis perrieri*. Las subpoblaciones son mantenidas como parte del Plan de Supervivencia de Especies de Cíclidos del Lago Victoria. Usamos cambios en las frecuencias de 24 alelos en cuatro loci polimórficos para estimar el tamaño poblacional efectivo ( $N_e$ ). Valores estimados de  $N_e$  variaron de 2.5 a 7.7 individuos y fueron significativamente más pequeños que el tamaño real de censo ( $N_{obs}$ ) para todas las subpoblaciones (32–243 individuos por generación) con las correspondientes proporciones conservadoras de  $N_e/N_{obs}$  variando de 0.01 a 0.12. Aproximadamente el 19% de los alelos iniciales fueron perdidos dentro de las primeras cuatro generaciones de reproducción en cautiverio. Comparaciones de la heterocigotidad esperada entre generaciones mostraron pérdidas significativas de entre 6 - 12% por generación. Siete alelos privados fueron observados en la última generación muestreada de cuatro subpoblaciones y los análisis de la estructura poblacional por  $F_{ST}$  indicaron que aproximadamente un 33% de la diversidad genética total es mantenida entre las subpoblaciones de diferentes instituciones. Para reducir la pérdida de variación genética recomendamos que la producción de crías sea equilibrada removiendo perió-

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dicamente a los machos dominantes, lo cual inducirá la reproducción entre los machos adicionales. Se deberá tomar en consideración el estimular a más instituciones para mantener poblaciones debido a que una fracción significativa de la variación genética existe como diferencias entre poblaciones resultando de la diferenciación azarosa entre subpoblaciones.

## Introduction

The maintenance of genetic variation can be critical to the success of a captive breeding and reintroduction program for endangered species (Hedrick et al. 1986). The detrimental effects of inbreeding have been documented in many captive populations (Senner 1980). In addition, the amount of genetic diversity represents the adaptive potential of captive populations and reintroduced populations derived from them (Lacy 1993).

When detailed pedigrees are not available, estimates of the effective population size ( $N_e$ ) can be used to predict the rate at which diversity will be lost to genetic drift (Wright 1931, 1938; for review see Caballero 1994). Although several methods exist to estimate  $N_e$  from demographic data (Caballero 1994), the data required for these models are often difficult to collect. Fortunately, molecular markers can also be used to estimate  $N_e$  (for review see Nunney & Elam 1994). The temporal method estimates  $N_e$  from the standardized variance of changes in allele frequencies between discrete generations (Nei & Tajima 1981; Pollak 1983; Waples 1989).

Population subdivision can also affect the maintenance of genetic variability (Kimura & Crow 1963; Lacy 1987). Because the effects of genetic drift are random, different subpopulations can move toward fixation for different alleles. Therefore, a larger number of different alleles can potentially be maintained within the total population, although any one subpopulation may be monomorphic.

The haplochromine cichlids of the Lake Victoria region of Africa are of great conservation interest (Kaufman 1992; Witte et al. 1992). A Lake Victoria cichlid species survival plan (SSP) has been developed through the Association of Zoos and Aquariums to guide conservation efforts. The Lake Victoria SSP details plans for habitat restoration and a captive breeding and reintroduction program for approximately 40 species, including the piscivore *Prognathochromis perrieri*. At the time of this study, August 1996, *P. perrieri* had been maintained through five captive generations.

The objective of our research was to estimate the maintenance of genetic diversity within a portion of the SSP captive-breeding program of the Lake Victoria cichlid *P. perrieri* through the use of microsatellite DNA markers. We estimated (1) the effective population size and the  $N_e/N_{\text{obs}}$  ratio, (2) the rate at which expected heterozygosity declined between generations, (3) the rate at which alleles were lost between generations, (4) the

number of private alleles present in subpopulations maintained at different institutions, and (5) the degree of partitioning of genetic diversity between subpopulations ( $F_{\text{ST}}$ ).

## Methods

### Background

The original captive population of *P. perrieri* was founded with between 10 and 20 adults. The administration of the breeding program for *P. perrieri* results in the maintenance of subpopulations at several different institutions. Depending on the number of individuals in a particular generation, these subpopulations may consist of one or several tanks. Approximately 15–30 adults, all from the same generation ( $F_n$ ), are housed within each breeding tank. Individuals are kept in mixed-sex groups. *P. perrieri* is a haplochromine cichlid with female mouthbrooding. When females reproduce, they are removed to separate brooding tanks; they are then returned to a tank of their generation after they finish mouthbrooding. Discrete generations are maintained by removing any progeny ( $F_{n+1}$  generation) into tanks separate from the adults. Individuals of different generations are kept separated in different tanks. When adults of a single generation are maintained in several different breeding tanks, progeny that hatch at approximately the same time in different tanks are placed together in a single tank. Individuals within a generation may be moved between tanks as the fish grow and mature. These protocols are designed to prevent smaller fry from being cannibalized by either the breeding adults or larger fry that were hatched earlier. This protocol also effectively prevents divergence between lineages maintained within a single institution. Additional information is available from the AZA website (<http://www.aza.org/programs/ssp/>).

Although the overall survey size and sex ratio of adults within an institution is recorded once per year, the exact number and sex ratio of adults varies slightly between breeding tanks and was not available. Overall, the sex ratio was approximately equal at all institutions. At the time of sampling, between August 1995 and August 1996, there were approximately 1100 individuals maintained at 11 different institutions within the *P. perrieri* captive-breeding program. Five of these institutions maintained multiple generations of fish and larger numbers of individuals, and they cooperated in the study.

### Sampling and Molecular Analysis

Seven different samples, representing five generations, were obtained from five cooperating AZA institutions (Fig. 1). Samples were classified by generation and institution. Tissue samples were obtained from 20 individuals of each generation from each subpopulation, except for samples F<sub>4</sub>C and F<sub>5</sub>LA, which each consisted of only 17 individuals. When a generation of a subpopulation at a single institution was maintained in several tanks, representative numbers of individuals were sampled from each tank.

Tissue samples (5 mm<sup>2</sup>) were collected from the caudal fin and stored in 100% ethanol prior to DNA analysis. Seven microsatellite loci were used for this study. Six of the loci (OSU9, OSU12a, OSU16, OSU19, OSU20, and OSU21) were developed from the related haplochromine cichlid *Astatoreochromis alluaudi* (Wu et al. 1999). Details of these microsatellite loci can be found in GENBANK (accession numbers U66810, U66809, U66812, U66813, U66815, and U66816, respectively). Additional information is available at the Fuerst lab internet site (<http://www.biosci.ohio-state.edu/~pfuerst>). The seventh locus was developed from the Lake Malawi cichlid *Pseudotropheus zebra* and designated DXTUCA3 (H. Sultmann, unpublished data, GENBANK accession number U94850).

The DNA was extracted from fin-clip samples by standard phenol-chloroform protocols, precipitated with 100% ethanol and resuspended in TE buffer (Maniatis et al. 1982). Microsatellite polymerase chain reaction (PCR)

amplifications were conducted according to the protocols of Wu et al. (1999). Annealing temperatures were 52° C for locus DXTUCA3, 55° C for OSU19, 58° C for OSU16, and 60° C for the remaining four loci. The PCR was performed for 22 cycles for loci OSU12a and OSU21, 28 cycles for OSU9, 32 cycles for OSU16, and 30 cycles for the remaining three loci. Allele frequencies and the number of alleles in each discrete generation were estimated from those observed in the samples. The expected heterozygosity ( $H_e$ ), tests for linkage disequilibrium (Goudet et al. 1996), heterozygote deficiency and excess (Rousset & Raymond 1995), and allele frequency heterogeneity for all pairwise subpopulation comparisons (Raymond & Rousset 1995a) were conducted with GENEPOP V3.1 (updated version of GENEPOP V1.2; Raymond & Rousset 1995b).

### Statistical Methods

The effective population sizes ( $N_e$ ) were estimated by the temporal method as described by Waples (1989; see also Nei & Tajima 1981; Pollak 1983). A measure of the standardized variance in allele frequency change ( $F_c$ ) at a locus between sample groups in different generations is given by

$$\hat{F}_c = \frac{1}{K} \sum_{i=1}^K \frac{(x_i - y_i)^2}{(x_i + y_i)/2 - x_i y_i},$$

where  $K$  is the number of segregating alleles, and  $x_i$  and  $y_i$  are the allele frequencies of the  $i$ th allele in the temporal samples (Nei & Tajima 1981; Waples 1989). When data for multiple loci are available, the weighted mean of the single locus ( $F_c$ ) values is calculated as

$$F_c = \frac{\sum K_j F_{cj}}{\sum K_j} \quad (1)$$

for  $K_j$  alleles at the  $j$ th locus (Tajima & Nei 1984; Waples 1989). Waples defines two sampling plans. Plan I involves sampling individuals after reproduction or sampling with replacement, as is the case of this study. Under sampling plan I, the effective population size is estimated as

$$\hat{N}_e = \frac{t}{2[F_c - (1/(2S_0)) - (1/(2S_t)) + (1/(N_{obs}))]}, \quad (2)$$

where  $F_c$  is given by equation 1, and  $S_0$  and  $S_t$  are the sample sizes for each generation,  $t$  is the number of generations separating the samples, and  $N_{obs}$  is the census size of the generation previous to the earliest sampled (Waples 1989). The value of  $N_{obs}$  can be obtained from records of the Lake Victoria cichlid SSP studbook.

Estimates of  $N_e$  were calculated for all appropriate pairwise comparisons in which a derived and the ancestral sample had been tested (Fig. 1). The 95% confidence limits of the estimates were also obtained. The  $N_e/N_{obs}$  ratios were calculated from the estimate of  $N_e$  and the

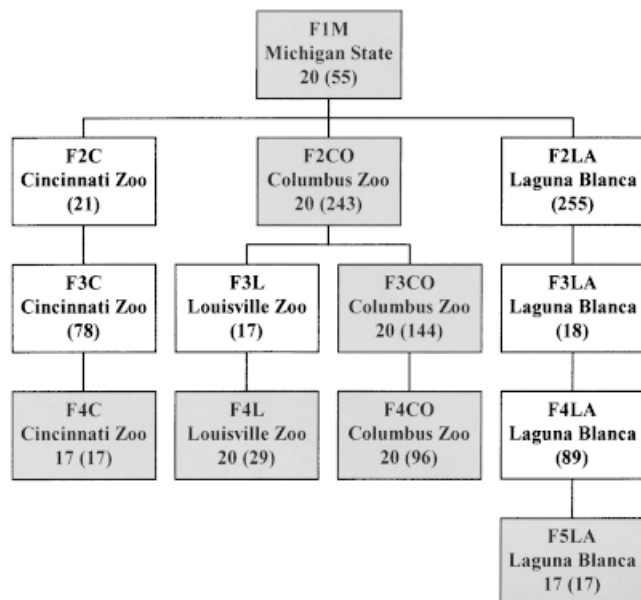


Figure 1. "Pedigree" of captive populations of *P. perrieri*. Shaded boxes indicate subpopulations that were sampled. The total survey number,  $N_{obs}$ , of adults within each subpopulation is in parentheses.

actual census size ( $N_{\text{obs}}$ ) obtained from the Lake Victoria cichlid SSP studbooks. The harmonic mean of the census sizes was used for multigeneration comparisons.

The loss of expected heterozygosity and loss of alleles were estimated by regression analysis using the natural logarithm of  $H_e$  or of the total number of alleles (Briscoe et al. 1992). All sampled generations were included in this regression. In addition, a large sample test between two sample proportions (Devore & Peck 1993) was conducted to compare  $H_e$  in the  $F_1M$  sample to the latest sample from each institution ( $F_4CO$ ,  $F_4C$ ,  $F_4L$ , and  $F_5LA$ ). The percent decline in expected heterozygosity was calculated as the difference in  $H_e$  between the earlier and later samples standardized by  $H_e$  in the earlier sample; this was then multiplied by 100 to convert to a percentage. The per-generation decline was calculated as the total decline in  $H_e$  calculated earlier, divided by the number of generations separating the two samples.

The numbers of private alleles within the latest sampled generation maintained at each institution were tabulated. The pairwise partitioning of diversity between subpopulations ( $F_{ST}$ ; Weir & Cockerham 1984) was calculated with GENEPOP V3.1 (Raymond & Rousset 1995b).

## Results

Five of the seven loci were polymorphic in *P. perrieri*. Loci OSU12a and OSU21 were monomorphic and are not included in any of the following analyses. Allelic variation at the remaining loci was tested for the presence of linkage disequilibrium. Locus DXTUCA3 showed evidence of significant linkage disequilibrium with locus OSU20 ( $p < 0.05$  in all seven comparisons). Loci OSU9, OSU16, OSU19, and OSU20 (Table 1) showed no linkage disequilibrium compared with one another and were treated as independent. Results from locus DXTUCA3 were excluded from further analyses. (Analyses that included locus DXTUCA3 produced estimates of  $N_e$  in all population comparisons that were not appreciably different.)

The loci were tested for departures from expected genotypic frequencies (Table 1). Of the 28 tests, only the test of locus OSU9 in the  $F_4CO$  sample suggested the occurrence of a significant deficiency of heterozygous individuals that would develop if null alleles were present at these loci. The tests of loci OSU9 in the  $F_3CO$  sample, OSU16 in the  $F_1M$  sample, and OSU20 in the  $F_4CO$  sample showed significant excesses of heterozygous individuals. Of the 84 possible pairwise comparisons among the seven different samples, 73 indicated significant differences in allele frequencies.

Point estimates for  $N_e$  obtained by the temporal method ranged from 2.5 to 7.7 individuals (Table 2). In each case, estimates were based on frequency changes for at least 12 independent alleles. The upper limit of the 95% confidence interval of  $N_e$  never exceeded 14 individuals and was much smaller than the actual census size for all comparisons. Estimated  $N_e/N_{\text{obs}}$  ratios ranged from 0.01 to 0.12, with only two exceeding 0.10.

Expected heterozygosity declined from 0.62 in the  $F_1M$  subpopulation to 0.39 in  $F_4C$  (Table 1). The decline in expected heterozygosity approached statistical significance based on the regression analysis ( $r^2 = 0.50$ ,  $p = 0.07$ ). Results from the test of the proportion of expected heterozygous individuals show a significant decline in  $H_e$  for all four comparisons (Table 3). The average per-generation decline in  $H_e$  ranged from 6% to 12%, resulting in an average overall loss of 25% of the initial expected heterozygosity.

The observed number of alleles declined from 16 in the initial  $F_1M$  subpopulation to 13 in the  $F_5LA$  sample (which included three alleles not observed in  $F_1M$ ). Similar to the analysis of  $H_e$ , this regression analysis only approached statistical significance ( $r^2 = 0.53$ ,  $p = 0.06$ ). It did show a strong trend, however, and suggests that approximately one allele is being lost per generation. In fact, four alleles were observed in earlier generations that were not observed in any of the latest subpopulation samples. The decline in the total number of observed alleles is 4% per generation, resulting in an overall loss of 19% of the initial allelic diversity.

**Table 1.** Number of observed alleles and expected heterozygosity (in parentheses) for each polymorphic microsatellite locus and number of private alleles in subpopulation samples of *P. perrieri*.

Subpopulation	OSU9	OSU16	OSU19	OSU20	Total	Private alleles <sup>a</sup>
$F_1M$	2 (0.46)	4 (0.62)	5 (0.60)	5 (0.78)	16 (0.62)	—
$F_2CO$	2 (0.50)	3 (0.48)	5 (0.67)	7 (0.76)	17 (0.60)	—
$F_3CO$	2 (0.50)	4 (0.65)	5 (0.73)	5 (0.78)	16 (0.65)	—
$F_4CO$	2 (0.46)	2 (0.38)	4 (0.51)	3 (0.55)	11 (0.48)	1
$F_4L$	2 (0.42)	2 (0.40)	5 (0.70)	2 (0.50)	11 (0.51)	0
$F_4C$	2 (0.11)	2 (0.44)	2 (0.36)	4 (0.65)	10 (0.39)	2
$F_5LA$	2 (0.06)	2 (0.47)	3 (0.61)	6 (0.77)	13 (0.48)	4
Total <sup>b</sup>	2 (0.37)	6 (0.49)	6 (0.60)	10 (0.68)	24 (0.54)	7

<sup>a</sup>Alleles present only in one specific subpopulation in the last generation sampled and not present in any other subpopulation.

<sup>b</sup>Total number of distinct alleles observed among all generations (not the sum of the columns). Total heterozygosity is the average of all individuals or loci.

**Table 2.** Actual population sizes ( $N_{\text{obs}}$ ), estimated number of independent alleles, and estimates of effective population size ( $N_e$ ) with 95% confidence intervals (C.I.) for  $N_e$  for pairwise sample comparisons of *P. perrieri*.

Pairwise comparison	Survey population size ( $N_{\text{obs}}$ )*	Number of independent alleles	Point estimate of $N_e$	95% C.I. for $N_e$	$N_e/N_{\text{obs}}$ ratio
F <sub>1</sub> M-F <sub>2</sub> CO	55	16	2.7	1.3, 4.6	0.05
F <sub>1</sub> M-F <sub>3</sub> CO	90	14	4.5	1.9, 7.9	0.05
F <sub>1</sub> M-F <sub>4</sub> CO	103	12	7.7	3.0, 13.8	0.07
F <sub>1</sub> M-F <sub>4</sub> C	38	16	4.6	2.0, 8.1	0.12
F <sub>1</sub> M-F <sub>4</sub> L	37	13	4.4	1.7, 8.0	0.12
F <sub>1</sub> M-F <sub>5</sub> LA	45	16	4.5	1.9, 8.0	0.10
F <sub>2</sub> CO-F <sub>3</sub> CO	243	15	3.1	1.1, 6.7	0.01
F <sub>2</sub> CO-F <sub>4</sub> CO	181	14	4.9	1.8, 10.8	0.03
F <sub>2</sub> CO-F <sub>4</sub> L	32	14	3.2	1.2, 6.6	0.10
F <sub>3</sub> CO-F <sub>4</sub> CO	144	12	2.5	0.8, 6.2	0.02

\*Survey size is the actual number of adults (or harmonic mean of the number of adults for multigeneration comparisons) and was derived from the Lake Victoria cichlids species survival plan studbooks.

The pairwise estimates of  $F_{\text{ST}}$  range from 0.26 to 0.47 (Table 4), indicating that 26–47% of the total genetic diversity, depending upon the pairwise comparison, exists as subpopulation differences between the different institutions. When all samples were compared, 33% of the total genetic diversity existed among subpopulations. All tests for allele frequency heterogeneity between the latest subpopulation samples were significant ( $p < 0.05$ ). This is reflected by the fact that seven private alleles were observed among the latest subpopulation samples (F<sub>4</sub>CO, F<sub>4</sub>C, F<sub>4</sub>L, F<sub>5</sub>LA) maintained at the different institutions (Table 1). In addition, pooling the allele frequencies across these subpopulations yielded an expected heterozygosity of 0.65, an estimate that is consistent with  $H_e$  in the F<sub>1</sub>M subpopulation (0.62).

## Discussion

The results obtained from the temporal method of estimation indicate that in the captive-bred population of *Prognathochromis perrieri* the effective population size was much smaller than the actual survey size. This suggests the potential for significant and rapid losses of genetic variability within this captive-breeding program. The observation of a reduced effective population size compared to census size is consistent with previous empirical studies (Frankham 1995), although most other empirical studies have found  $N_e/N_{\text{obs}}$  ratios that are much closer to 1.0 (Nunney 1993).

The regression over time for expected heterozygosity and for number of alleles suggest strong trends toward the loss of genetic diversity. With the small number of generations available, however, the power to detect significant declines in either  $H_e$  or the number of alleles was reduced greatly. Results from the test of the proportion of heterozygous individuals indicated a statistically significant decline in genetic diversity for all comparisons and were consistent with the estimates of small ef-

fective population sizes obtained from the temporal method.

Genetic variability appears to be partitioned between the different institutions. Estimates of  $F_{\text{ST}}$  indicated that 33% of the total genetic diversity within the captive-breeding program existed as differences in allele frequencies between different subpopulations. Seven of the alleles observed in early generations existed as private alleles between the latest subpopulation samples. Rare alleles often are not observed in relatively small samples (Brookes et al. 1997); so our results may be influenced by sampling error. We did, however, sample all available individuals for two of the four subpopulations and 25% and 70% of the individuals from the remaining two subpopulations. In addition, pooling the allele frequencies from the four latest subpopulation samples at the different institutions and recalculating  $H_e$  yielded an estimate consistent with the F<sub>1</sub>M subpopulation. Taken together, these results suggest that genetic diversity is being preserved between the subpopulations at different institutions, consistent with predictions regarding the potential to maintain genetic diversity between small subdivided populations (Kimura & Crow 1963; Lacy 1987).

Two of the seven microsatellite loci screened were monomorphic in the captive populations of *P. perrieri*, a

**Table 3.** Large sample test for equality in the proportion of heterozygotes in two samples and estimated loss of expected heterozygosity in *P. perrieri*.

Comparison	p	$H_e$	
		total percent decline	average per-generation percent decline
F <sub>1</sub> M-F <sub>4</sub> CO	<0.005	23	8
F <sub>1</sub> M-F <sub>4</sub> L	<0.025	18	6
F <sub>1</sub> M-F <sub>4</sub> C	<0.005	37	12
F <sub>1</sub> M-F <sub>5</sub> LA	<0.050	23	6
Average		25	8

**Table 4.** Pairwise partitioning of genic diversity ( $F_{ST}$ ) between the last sampled generations of four institutional subpopulations of *P. perrieri*.

Subpopulation	F4-Louisville	F4-Cincinnati	F5-Laguna Beach
F4-Columbus	0.27	0.28	0.26
F4-Louisville	—	0.36	0.34
F4-Cincinnati	—	—	0.47

possible result of the relatively small size of the founding population. These two loci, however, also showed reduced polymorphism compared with other loci in wild populations of related Lake Victoria cichlid species (Wu et al. 1999). Two other loci showed significant linkage disequilibrium. This does not necessarily indicate the existence of linkage, because it can also be expected from populations derived from a small number of parents (Bartley et al. 1992). To be conservative, we considered only four of the five polymorphic loci to be independent. In some samples, observed heterozygosity deviated significantly from Hardy-Weinberg expectations. This is also often observed in the offspring of a small number of parents (Haldane 1954). Although the overall trend was toward a loss of expected heterozygosity, a few instances occurred in which a locus showed increases in  $H_e$  from one generation to the next. This might be expected, in the short term, because the effects of genetic drift on allele frequencies are random.

Although we cannot identify the exact cause of low effective population size and the resulting loss of genetic diversity, the possibility of high variance in reproductive success among both males and females is a likely contributing factor. *P. perrieri* has a polygynous mating system, with only a few dominant males obtaining all or most of the matings within a breeding tank (S. Andromeda, personal communication). High variance in female reproductive success due to a small number of females contributing several broods to the next generation could also result in low effective population sizes (Wright 1938). Equalization of family size is recommended by Allendorf (1993) to delay adaptation to captivity and increase  $N_e$  within captive-breeding programs.

The results from the  $F_{ST}$  analysis of genic diversity indicate that a large proportion of the total genetic variation exists between subpopulations. This observation, along with the pooled  $H_e$  estimate, indicates that maintaining *P. perrieri* at several different institutions has effectively preserved some of the initial heterozygosity, and we strongly suggest that this management technique be continued. Nevertheless, population subdivision may not be adequately delaying the loss of allelic diversity because four alleles that were sampled in earlier generations were not observed in the latest subpopulation samples from any institution. Therefore, the fact that several different subpopulations exist does not eliminate the need to maintain large effective population sizes within each institution.

Our study suggests that the amount of genetic diversity within the captive-breeding program of *P. perrieri* is declining rapidly. High variances in male and female reproductive success are hypothesized as a possible cause. We recommend management strategies to encourage reproduction by more individuals. Although these management strategies may be more labor- and space-intensive, they warrant further consideration for the effective maintenance of genetic diversity within the Lake Victoria cichlid SSP and other freshwater fish captive-breeding programs.

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