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Effective Population Size in the Captive Breeding Program of the Lake Victoria Cichlid *Paralabidochromis chilotes*

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Microsatellite DNA markers were used to monitor levels of genetic variation in 3 generations of the American Zoo and Aquarium Association [AZA] Species Survival Plan [SSP] captive breeding program for the Lake Victoria cichlid *Paralabidochromis chilotes*. Temporal changes in the frequency of 15 alleles, across four polymorphic loci, were used to estimate effective population size (N_e). The upper limit of the 95% confidence interval for N_e never exceeded eight individuals, with all of the corresponding N_e/N ratios falling below 0.15. A test of the proportion of expected heterozygous individuals between the F_1 and F_3 generations indicated a significant decline in expected heterozygosity of 5% per generation. Alternative husbandry protocols, including subdividing the captive population, are addressed to reduce the further loss of genetic variation. Zoo Biol 18:215–222, 1999. © 1999 Wiley-Liss, Inc.

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INTRODUCTION

To ensure the highest probability of success for a captive breeding and reintroduction program, the maintenance of genetic variation must be a priority [Hedrick et al., 1986]. Several factors such as founder effect, inbreeding, and genetic drift can have detrimental effects on the future adaptive potential of captive populations [Lacy, 1993]. Fortunately, conservation efforts are increasingly incorporating genetic as well as ecological concerns into recovery programs [Haig, 1998].

When detailed pedigrees are available, inbreeding coefficients can be easily calculated to estimate the maintenance of gene diversity [Wright, 1951]. However, due to

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limited time and resources, some captive breeding programs do not maintain complete pedigrees. Even without this information, molecular markers can be used to estimate the maintenance of genetic variation within conserved populations [Nunney and Elam, 1994]. One method, referred to as the temporal method, estimates the effective population size (N_e) based on the variance in changes in allele frequencies between discrete generations [Nei and Tajima, 1981; Pollak, 1983; Waples, 1989]. This relies on the observation that populations with larger effective sizes will have a lower variance in allele frequency change than populations with smaller effective sizes.

Molecular markers have been widely used to study a number of questions in ecology and conservation [Avise, 1994; Haig, 1998; Parker et al., 1998]. Among the various molecular techniques available, microsatellite DNA markers are quickly becoming one of the most useful and widely used. Microsatellites are short, tandem repeats of DNA that can be amplified through the use of the polymerase chain reaction (PCR) [Queller et al., 1993]. This allows the analysis of DNA variation from very small tissue samples [Avise, 1994]. Microsatellites are inherited as co-dominant markers, allowing homozygous individuals to be differentiated from heterozygous individuals and thus permitting the calculation of exact allele frequencies within each sample. The high polymorphism of microsatellite markers makes them particularly useful for studying captive populations of endangered species such as the Lake Victoria cichlids.

Lake Victoria has been the site of a major environmental disaster [Witte et al., 1992]. A Species Survival Plan (SSP) has been developed through the American Zoo and Aquarium Association (AZA) to guide conservation efforts aimed at preventing any further extinctions. This SSP details plans for habitat restoration and a captive breeding and reintroduction program for approximately 40 species of Lake Victoria cichlids, including the insectivore *Paralabidochromis chilotes*.

The husbandry protocol for maintaining the captive breeding population is summarized below [additional information can be obtained from the AZA web home page (http://www.aza.org/publications/)]. The captive population of *P. chilotes* was founded with one male and two females. During captive propagation, approximately 15 to 30 adults, all from the same generation, are housed within each breeding tank. Discrete generations are maintained by placing progeny into tanks separate from the adults. Progeny of the same generation that hatch at approximately the same time (and are therefore approximately the same size) are placed together in a single tank. These protocols are designed to prevent smaller fry from being cannibalized by larger individuals. The overall census size and the sex ratio of adults are recorded once per year. At the time of sampling (August 1996), there were approximately 140 individuals within the *P. chilotes* captive breeding program.

The purpose of this study was to monitor the level of genetic diversity within the captive breeding program populations of *P. chilotes* using DNA microsatellite markers. In doing this, we were able to 1) determine levels of polymorphism at several microsatellite DNA loci, 2) estimate the effective population size and N_e/N ratios, 3) estimate the loss of expected heterozygosity, and 4) estimate the loss of alleles.

METHODS

Twenty individuals were sampled from each generation (F_1, F_2, F_3). Tissue samples (5×5 mm) were collected from the caudal fin and stored in 100% ethanol

before DNA analysis. When a generation was maintained in several tanks, representative numbers of individuals were sampled from each tank. A total of nine microsatellite loci were screened for this study. Seven of the nine loci (OSU9, OSU12a, OSU14a, OSU16, OSU20, OSU21, and OSU24L2) were developed for *Astatoreochromis alluaudi* [Wu et al., 1999]. Details of these microsatellites are available at the Fuerst lab web home page [http://www.biosci.ohio-state.edu/~pfuerst]. The final two loci were developed from *Pseudotropeus zebra* and designated DXTUCA-3 and DXTUCA-9 [H. Sueltmann, unpublished, details at the web home page (http://tilapia.unh.edu) of the Tilapia genome project].

DNA was extracted from finsnips using standard phenol-chloroform protocols, precipitated with 100% ethanol and resuspended in tris-EDTA buffer [Maniatis et al., 1982]. Microsatellite PCR amplifications were conducted according to the protocols described in Wu et al. [1999]. 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], as well as tests for heterozygote deficiency and excess [Rousset and Raymond, 1995], were conducted using GENEPOP V3.1 [updated version of GENEPOP V1.2, Raymond and Rousset, 1995]

The effective population sizes (N_e) were estimated using the temporal method as described in Waples [1989]. Roughly, the temporal method uses the standardized variance in allele frequency changes between discrete generations to estimate effective population size. The accuracy of the estimates depend on the number of individuals sampled, the number of loci used, and, most important, the number of independent alleles surveyed. The high polymorphism traditionally associated with microsatellite DNA markers makes them an ideal molecular tool to address these types of conservation questions [see Brookes et al., 1997 for a more detailed discussion of the application of the temporal method]. The N_e/N ratios were calculated conservatively using the upper bound of the 95% confidence interval for the estimate of N_e and the actual census size (N) obtained from the Lake Victoria Cichlid Studbooks (available through Jay Hemdel, Toledo Zoo, Lake Victoria Cichlids SSP Studbook Keeper). The harmonic mean of the census sizes was used for the F_1–F_3 comparison.

The loss of expected heterozygosity and loss of alleles were estimated by regression analyses, using the natural logarithm (ln) of H_e or (ln) of the total number of alleles [Briscoe et al., 1982]. In addition, a large sample test between two sample proportions [Devore and Peck, 1993] was also conducted comparing H_e between the F_1 and F_3 generations. The percentage of decline in expected heterozygosity was calculated as the difference in H_e between the F_1 and F_3 generations standardized by H_e in the F_1 generation; this was then multiplied by 100 to convert to a percentage. The per generation decline was simply calculated as the total decline in H_e from above divided by 2, the number of generations separating the F_1 and F_3 generations.

RESULTS

Four of the nine microsatellite markers screened, OSU16, OSU24L2, DXTUCA-3, and DXTUCA-9, were polymorphic in *P. chilotes*; these loci were used for the following analyses. A total of 15 alleles was observed at the four polymorphic loci, with three to five alleles present at each locus (Table 1). The average expected het-

TABLE 1. Number of alleles and expected heterozygosities (in parentheses) at the four polymorphic loci within the captive breeding program of *P. chilotes*

Generation	Number of alleles (expected heterozygosity)				
	OSU16	OSU24L2	DXTUCA-3	DXTUCA-9	Total
F_1	5 (0.62)	3 (0.57)	3 (0.53)	4 (0.65)	15 (0.59)
F_2	4 (0.64)	3 (0.59)	3 (0.42)	4 (0.72)	14 (0.59)
F_3	3 (0.60)	3 (0.34)	2 (0.35)	3 (0.66)	11 (0.49)
Total[a]	5 (0.62)	3 (0.50)	3 (0.43)	4 (0.68)	15 (0.56)

[a]Total number of alleles is the total number of distinct alleles observed across all generations and is not the sum of the column.

erozygosities across all generations at each locus ranged from 0.43 [DXTUCA-3] to 0.68 [DXTUCA-9], and the overall observed heterozygosity across all generations and loci was 0.56 (Table 1). Tests for genotypic disequilibrium suggested random associations between all pairs of loci across all generations ($P > 0.05$), and, therefore, all loci were treated as independent for further analyses. None of the generations contained a significant deficiency of heterozygotes ($P > 0.05$ for all tests), suggesting that null alleles were not present at these loci.

Estimates of the effective population size using the temporal method are presented in Table 2. The census size (N) ranged from 34 to 60 individuals, and 10 or 11 independent alleles were present for each comparison. The upper limit on the 95% confidence intervals of N_e was less than eight individuals for all comparisons, and all N_e/N ratios were less than 0.15.

The expected heterozygosity across the four loci declined from 0.59 in the F_1 generation to 0.49 in generation F_3 (Table 1). The large sample test of population proportions for H_e between the F_1 and F_3 generations indicated a significant decline in expected heterozygosity ($P < 0.05$, one-tailed). Expected heterozygosity declined at an average rate of 5% per generation. However, the regression analysis of $\ln(H_e)$ failed to indicate a statistically significant loss of gene diversity ($P > 0.1$). The observed number of alleles declined from 15 in the F_1 generation to 11 in the F_3 generation (Table 1). However, the regression analysis of the loss of alleles is not statistically significant ($P > 0.05$). Because the regression analysis consisted of only two transitions between generations, it is unlikely to represent a powerful test.

DISCUSSION

All estimates of N_e indicate that the effective population size is much smaller than the actual census size of the captive populations *of P. chilotes*. These results

TABLE 2. Estimates of effective population size (N_e) and N_e/N ratios

Comparison	Census size (N)	Independent alleles (n)	Point estimate of N_e	95% CI for N_e	N_e/N ratio
F_1–F_2	34	11	1.0	(0.6, 1.3)	0.04
F_1–F_3	43	11	1.4	(0.6, 2.0)	0.05
F_2–F_3	60	10	3.2	(1.0, 7.8)	0.13

CI, confidence interval.

indicate the potential for very rapid losses of gene diversity within this captive breeding program. Previous empirical studies have also documented N_e/N ratios below 1.0 [Frankham, 1995], although others observed N_e/N ratios that approach or even exceed 1.0 [Nunney, 1993].

A decline in expected heterozygosity of 10% between generations F_1 and F_3 was observed with the large sample test of proportions. Even though four alleles [27%] present in the F_1 generation were not observed in the F_3 sample, the regression analysis of loss of alleles is not statistically significant. The lack of significance for this regression is clearly due to the small number of generations that were available. Given the significant decline detected by the large sample test of proportions, the results from the regression analyses should be utilized with extreme caution when making recommendations for the captive breeding program of *P. chilotes*.

Although microsatellite markers are considered to be one of the most variable types of molecular markers, only four of the nine loci that we screened were polymorphic in the captive populations of *P. chilotes*. This could have resulted from the fact that the entire captive population was founded with only three individuals. At most, only six alleles at any particular locus could have been present in the founding population. Wu et al. [1999] screened wild populations of five haplochromine cichlids for many of these microsatellite primers and observed approximately 20 alleles at each locus. Based on this observation, a founding population of three individuals would, at best, contain only 30% of the allelic diversity present in the wild populations.

Implications for the Lake Victoria Cichlid Breeding Program

The very low estimates of effective population size and the observation of a significant loss of gene diversity are consistent with the finding of a study on another species, *Prognathochromis perrieri*, of the Lake Victoria cichlid captive breeding program [Fiumera et al., in press]. This suggests that rapid losses of gene diversity may be occurring throughout the captive breeding populations of Lake Victoria cichlids, as well as other freshwater fish following similar management protocols.

Although the methods of analysis used in this study cannot identify the exact causes of the low observed effective population sizes, several possible suggestions can be made to increase N_e and increase the potential to maintain genetic variation. Naturally, the most effective method would be to maintain a complete pedigree of all matings and encourage reproduction by under-represented individuals. Unfortunately, this would be prohibitively costly, and, therefore, several alternative methods are suggested.

First, *P. chilotes* is thought to have a polygynous mating system with a few dominant males obtaining all or most of the matings within a breeding tank. Removal of dominant males from the breeding tank after successful fertilization could encourage reproduction by additional males. It has been observed that new males obtain breeding coloration within 1–2 days after the death of the dominant male. Such a management strategy does have a potential danger. It may unintentionally increase selection for individuals who become dominant under captive conditions. Those least able to exert behavioral dominance under captive breeding conditions may carry alternative alleles that could still be lost. Such arguments can, of course, be applied to genetic factors that predispose to any conditions that result in selection for domestication. However, selection is likely to affect only allelic variation closely linked to the locus under selection and should not affect the general levels of genetic

variation in the population. Second, effective population sizes could also be reduced if a small number of females contribute several broods, while some females fail to contribute any [Wright, 1938]. “Equalization of family size” was recommended by Allendorf [1993] and demonstrated by Borlase et al. [1993] to delay adaptation to captivity and increase effective population size within captive breeding programs. *P. chilotes* are female mouth brooders. Because current management protocols involve removal of brooding females from breeding tanks, it would not be highly labor intensive to prevent these females from contributing further. Although this management strategy may result in fewer broods being produced, the benefits in terms of maintaining higher amounts of genetic variation are theoretically sound and should be investigated further.

In addition to protocols designed to increase N_e, population subdivision may also help to maintain gene diversity. Kimura and Crow [1963] suggested that gene diversity could be maintained between subdivided populations, whereas Lacy [1987] and Lacy and Lindenmayer [1995] used computer simulations to demonstrate the effective maintenance of gene diversity between small populations. Although each subpopulation will lose genetic variation (i.e., heterozygosity and alleles) at a rate proportional to the effective size of that subpopulation, different subpopulations may become fixed for different alleles [Gilpin, 1991; Hedrick, 1996; Hedrick and Gilpin, 1997 for discussions]. This might result in a larger number of alleles being maintained within the total captive population. However, the benefits of increased variability are balanced by potential demographic events, such as population extinction, to reduce the overall effective population size. A large percentage of the total gene diversity present within the later generations of the captive populations of the Lake Victoria cichlid *Prognathochromis perrieri* existed between subpopulations maintained at different institutions [Fiumera et al., in press]. One must consider the possible effects of increased variance in family size that might be associated with the subdivision of the population into a few subpopulations. Maximal N_e will occur when the difference in offspring numbers between breeders is minimized, as pointed out above.

An anonymous review noted that the same effect can be achieved by dividing the current breeding adults into additional tanks within each institution, thereby increasing the number of breeders and decreasing the variance in family size. Although it may slightly increase the space requirements, it could encourage reproduction by non-dominant males and effectively result in population subdivision, thereby decreasing the rate at which genetic diversity is lost within the captive populations [Gilpin, 1991; Hedrick, 1996; Hedrick and Gilpin, 1997]. Therefore, subdividing the captive populations of *P. chilotes* should also be considered as a management strategy to help maintain genetic variation. The institutions cooperating in the Lake Victoria Cichlids SSP must carefully consider the economics and logistics of a more complex population structure and weigh the costs against the benefits of reduced loss of genetic variability.

In addition to preventing the loss of diversity within the captive breeding program, we suggest that attempts be made to obtain additional founder stock. Although all founders contributed, the entire *P. chilotes* captive breeding program was initiated with only three individuals. Attempts to incorporate additional genetic variability into the captive population should be made.

The methods described to reduce the variance in reproductive success among both males and females and subdivision of the captive population are reasonable

suggestions, with respect to cost and space limitations, to effectively maintain larger amounts of genetic variation within the *P. chilotes* captive breeding program. Although the exact success of these techniques cannot be predicted for all species, they warrant consideration and their potential benefits should be investigated empirically.

CONCLUSIONS

1. Estimates of the effective population size were much smaller than the actual census size, suggesting the potential for rapid losses of gene diversity, and were consistent with the statistically significant decline in expected heterozygosity.

2. The small number of founders may have contributed to the low proportion of polymorphic microsatellite markers observed in this study. Adding new founders may increase the amount of allelic diversity present within the captive population.

3. Equalizing the contribution of males and females to the next generation and subdividing the population are discussed as possible methods to increase the amount of genetic variation maintained within the captive breeding program of the Lake Victoria cichlid *P. chilotes* and other freshwater fish captive breeding programs following similar protocols.

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