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SHORT COMMUNICATION

Multi-generational evaluation of genetic diversity and parentage in captive southern pygmy perch (*Nannoperca australis*)

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Abstract Maintaining genetic diversity within captive breeding populations is a key challenge for conservation managers. We applied a multi-generational genetic approach to the captive breeding program of an endangered Australian freshwater fish, the southern pygmy perch (*Nannoperca australis*). During previous work, fish from the lower Murray-Darling Basin were rescued before drought exacerbated by irrigation resulted in local extinction. This endemic lineage of the species was captive-bred in genetically designed groups, and equal numbers of F1 individuals were reintroduced to the wild with the return of favourable habitat. Here, we implemented a contingency plan by continuing the genetic-based captive breeding in the event that a self-sustaining wild population was not established. F1 individuals were available as putative breeders from the subset of groups that produced an excess of fish in the original restoration program. We used microsatellite-based parentage analyses of these F1 fish to form breeding groups that minimized inbreeding. We assessed their subsequent parental contribution to F2 individuals and the maintenance of genetic diversity. We found skewed parental contribution to F2 individuals, yet minimal loss of genetic diversity from their parents. However, the diversity was substantially less than that of the original rescued population. We attribute this to the unavoidable use of F1 individuals from a limited number of the original breeding groups. Alternative genetic sources for supplementation or reintroduction should be assessed to determine their suitability. The genetic fate of the captive-bred population highlights the strong need to integrate DNA-based tools for monitoring and adaptive management of captive breeding programs.

Keywords Restoration genetics · Pedigree · Kinship · Relatedness · Fish · Biodiversity extinction

Introduction

Captive breeding programs aim to ensure the persistence of a population when it is endangered in the wild (Frankham et al. 2009). Captive-bred individuals may be used to supplement, reintroduce, or establish new wild populations. However, maintaining genetic diversity in captivity and successful supplementation or reintroduction is fraught with difficulties and compromises (Fraser 2008; Williams and Hoffman 2009), such as competing ecological and anthropogenic interests (Hobbs et al. 2009). A decline in genetic diversity and therefore evolutionary potential, or the detrimental consequences of inbreeding depression in captive, supplemented or reintroduced populations, can lead to the extinction of a population or species (Frankham 2005).

The Murray-Darling Basin (MDB) in Australia is a fundamentally altered and highly threatened ecosystem that is of great economic importance for agriculture. Human-made structures such as dams have been constructed since European settlement to regulate the naturally variable water flow for irrigation purposes (Kingsford et al. 2011).

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This is in addition to other human impacts such as the introduction of invasive species and habitat degradation. Over half of the MDB's native fish species are consequently listed in state legislations as threatened (Lintermans 2007). This crisis reached a tipping point when the MDB was heavily affected by an extended drought that started in 1997. This led to increased salinity, fragmentation of habitat, and eventually complete desiccation of much habitat by 2008 in the lower MDB (Wedderburn et al. 2012). Many freshwater fish species would have become locally extinct without intervention. This was especially the case for small habitat-specialist fishes with poor dispersal capabilities as they are especially sensitive to these anthropogenic changes and would be highly unlikely to re-colonize the lower MDB (Brauer et al. 2013; Saddlier et al. 2013).

A large-scale collaborative effort between governmental and non-governmental agencies and other stakeholders occurred to rescue several small freshwater fishes. Target species were rescued from the wild in 2008 and 2009, maintained in captivity by multiple organizations, and reintroduced in 2010 when rainfall and favorable habitat returned (Hammer et al. 2013). At Flinders University, South Australia, a genetic-based captive breeding program was implemented for an endemic lineage of southern pygmy perch (*Nannoperca australis*) rescued from littoral habitats of the lower MDB. This lineage is recognized as a distinct Management Unit (MU) (Cole et al. 2016). The breeding program successfully minimized the loss of genetic diversity in captivity by breeding 11 small groups of genetically-determined unrelated individuals (Attard et al. 2016). An equal number of F1 fish from each breeding group were released into the lower MDB. Monitoring of these individuals showed their survival and breeding in the wild, however continued monitoring is needed to determine whether the population becomes self-sustaining (Attard et al. 2016).

Here, we continue the genetic-based captive breeding program and assess its genetic potential as a supplementary or primary source of fish for the lower MDB if the reintroduction effort fails to establish a self-sustaining population. These captive born individuals could also be used to establish other wild populations in more secure habitats. We conducted microsatellite-based parentage analyses of F1 individuals that were not released into the wild and used the results to form breeding groups of unrelated individuals. After breeding, we assessed their individual contribution to the F2 generation and the potential maintenance of genetic diversity. The results directly inform an applied conservation project, and provide broader empirical data to demonstrate the potential benefits of genetic monitoring in captive breeding programs and therein test the theoretical basis for their design, which is a burgeoning field due to ever-increasing environmental change (Witzenberger and Hochkirch 2011).

Methods

Thirty-seven putative breeders were available from across three of the 11 original breeding groups (breeding groups 5, 6 and 10, Attard et al. (2016)) that produced an excess of F1 individuals relative to the other breeding groups. These excess individuals were not released to the wild to prevent a bias in the genetic composition of the reintroduced population. The formation of the second generation of breeding groups, including genetic data collection and analyses, followed that of Attard et al. (2016) as briefly described below (see Supplementary Information (SI) for differences).

Fourteen microsatellite loci previously developed for the species (Carvalho et al. 2012) were used for analyses. Parentage analyses of the excess F1 individuals were conducted using PAPA 2.0 (Duchesne et al. 2002). Individuals were chosen for breeding groups so that potential female-male pairs within breeding groups were unrelated. Breeding groups of two males and two females were established according to the species' natural breeding system and our previous success using this composition (Attard et al. 2016). Approximately 30 of the resulting F2 individuals from each breeding group were genotyped. We assessed their parentage using PAPA, including confirming accuracy by conducting a power analysis. We determined whether there was an unequal contribution of dams or sires of each breeding group to offspring using chi-square goodness-of-fit tests in R 3.2.3 (R Core Team 2015). The potential maintenance of genetic diversity was assessed by calculating the number of alleles, observed heterozygosity (H_o) and expected heterozygosity (H_E) of breeders and offspring using GENALEX 6.502 (Peakall and Smouse 2006, 2012).

Results

Five breeding groups were generated based on available broodstock composition and resources, with four successfully producing offspring (Table 1). These totalled approximately 500 F2 individuals. There were minimal genotyping errors in the offspring based on Mendelian inheritance (SI). There was significant evidence for skewed contribution of brooders in the successful breeding groups to offspring (Table 1). The genetic diversity lost between the F2 offspring and their F1 progenitors was minimal, but was large between the F2 offspring and the full original complement of F1 individuals (Table 2).

Table 1 Parentage analysis of F2 generation for each breeding group

	Sires	Dams		Total	%
Group 1		**(1) 5,2,2**	**(2) 10,2,1**		
	(3) 6,2,1	14	0	14	50
	(4) 6,2,1	14	0	14	50
		28	0	28	–
		100	0	–	100
Group 2		**(5) 10,1,1**	**(6) 10,1,1**		
	(7) 6,2,1	18	2	20	61
	(8) 6,2,1	10	3	13	39
		28	5	33	–
		85	15	–	100
Group 3		(9) 10,1,1	(10) 10,1,1		
	(11) 6,2,1	12	13	25	100
	(12) 6,2,1	0	0	0	0
		12	12	25	–
		48	52	–	100
Group 4		(13) 6,2,1	(14) 6,2,1		
	(15) 5,1,2	18	18	36	100
	(16) 5,2,1	0	0	0	0
		18	18	36	–
		50	50	–	100

Shown are the number of offspring from each parent-pair where the relevant dam column and sire row intersect, the total offspring for each breeder, and their percentage contribution to the offspring in the breeding group. An identification number for each F1 individual used as a breeder is provided in parentheses, and their parentage is indicated beside this as the breeding group, sire and dam numbers from Attard et al. (2016) separated by commas. Those in bold have significant ($P < 0.05$) evidence of skewed contribution to offspring based on chi-square tests

Discussion

Molecular markers were used to form a second generation in a captive breeding program of the endangered southern pygmy perch, assess the potential maintenance of genetic diversity across multiple generations, and evaluate the captive population's viability as a reservoir of genetic diversity. We successfully produced hundreds of F2 individuals. This involved rarely conducted microsatellite-based parentage analysis of F1 breeders to prevent inbreeding, and parentage analyses of F2 offspring to assess parental contribution. This differs from the first generation of this captive breeding program (Attard et al. 2016), where wild individuals had unknown parentage and so breeding groups were chosen based on pairwise estimates of relatedness. We found minimal loss of genetic diversity between the original breeders and the F1 generation, and between the subset of F1 individuals used as breeders and the F2 generation. However, the subset of F1 individuals was found to hold dramatically less genetic diversity than the full complement of F1 individuals. As such, the captive individuals are an insufficient reservoir of genetic diversity and would be inappropriate as a sole source of individuals for a wild population.

The loss of diversity is attributed to the use of F1 individuals from a limited number of the original breeding groups, combined with the skewed reproductive contribution of breeders in the original breeding groups. Due to the accumulation across generations of adaptations to captivity (Frankham 2008) and the loss of genetic diversity in small populations (Lacy 2013), the number of generations in captivity in our original restoration genetics program was kept to the smallest possible—one generation. Equal numbers of individuals were released from the original breeding groups to prevent a bias in the genetic composition of the reintroduced population. This was prior to knowing the skewed parental contribution, and so individuals kept in captivity were not representative of the original founders. Breeding groups 6 and 10 of the previous generation mostly consisted of full-siblings, and fewer individuals were available from breeding group 5. The F2 captive-bred generation provided additional evidence of reproductive skew in the species. The use of multiple breeding groups minimized the loss of diversity due to this skew between the F1 breeders and F2 individuals. The reproductive skew could be due to sexual selection given that the nuptial colour in males of this species during the breeding season is associated with body size, body condition, and male dominance (Mitchell 1976; Morrongiello et al. 2010). Keeping offspring from across as many breeding groups as possible or genetic selection before release of offspring to continue captive breeding will be required in any future captive breeding programs to maintain a genetically viable ex situ population. This will need to be balanced against increasing the likelihood of reintroduction success by releasing a sufficient number of individuals that, together, are representative of the genetic make-up of the natural population.

If monitoring reveals that the reintroduced population requires more individuals or more genetic diversity, which is possible given the continuing anthropogenic threats in the MDB, these should predominately, or only, be from sources other than the current captive stock. Translocations from other wild populations is an option that may aid this population by increasing adaptive potential (Weeks et al. 2011) given that the natural genetic diversity of the target population has already reduced due to European settlement (Attard et al. 2016). The only other remaining populations of this species in the MDB are in lower MDB stream habitats and the upper reaches of the basin. A basin-wide population genetic study based on allozymes, mitochondrial DNA and microsatellites (Cole et al. 2016) indicates

Table 2 Genetic variation based on 14 microsatellite loci of the wild-caught, F1 captive-bred and F2 captive-bred individuals

			n	*A*	H_O	H_E
Attard et al. (2016)		All wild-caught	64	7.57 (2.90)	0.643 (0.159)	0.653 (0.150)
		All F1 offspring	581	7.29 (2.92)	0.702 (0.139)	0.667 (0.131)
		Groups 5, 6, and 10 F1 offspring	152	5.14 (1.83)	0.697 (0.145)	0.627 (0.138)
Current study		All F2 offspring	113	4.43 (1.60)	0.689 (0.204)	0.601 (0.168)
	Group 1	F1 brooders	4	3.50 (1.40)	0.625 (0.273)	0.597 (0.250)
		F2 offspring	24	2.93 (1.33)	0.600 (0.314)	0.492 (0.255)
	Group 2	F1 brooders	4	3.43 (1.34)	0.702 (0.253)	0.634 (0.194)
		F2 offspring	32	3.43 (1.34)	0.686 (0.233)	0.572 (0.183)
	Group 3	F1 brooders	4	3.50 (1.45)	0.661 (0.334)	0.653 (0.208)
		F2 offspring	23	3.29 (1.38)	0.764 (0.287)	0.576 (0.220)
	Group 4	F1 brooders	4	3.50 (1.22)	0.714 (0.237)	0.640 (0.197)
		F2 offspring	34	3.07 (1.00)	0.704 (0.185)	0.563 (0.149)

Further details of the genetic variation in each first generation breeding group are in Table S7 of Attard et al. (2016)

Standard deviations are in parentheses

n number of samples; *A* mean number of alleles; H_O mean observed heterozygosity; H_E mean unbiased expected heterozygosity

that southern pygmy perch throughout the MDB are one evolutionarily significant unit (ESU) that was relatively recently connected by gene flow across the MDB. As such, the risks of reduced fitness due to outbreeding depression are predicted to be low, enabling the potential sourcing of individuals for translocations from other suitable MDB locations. The species has anthropogenic threats throughout its range of south-east Australia, so the demographic stability of potential source populations needs to be assessed before performing translocations.

Multi-generational genetic evaluation of the captive breeding program in relation to the natural population led to the realization of a large loss of diversity between the F1 and F2 individuals. Such genetic evaluations are key to determining the success of a breeding program but are often absent or poorly conducted (Witzenberger and Hochkirch 2011). Pedigrees based on direct observation are often used to choose breeders and minimize the loss of genetic diversity, however DNA-based evaluations may reveal incorrect pedigree records, relatedness in founders and, possibly also due to incomplete pedigrees, inbreeding (e.g. Hammerly et al. 2016; Knief et al. 2015; Mitchell et al. 2011). A common issue is also unequal breeder contribution to offspring and accompanying loss of diversity, such as in fish shown here and in aquaculture studies (e.g. Brown et al. 2005; Liu et al. 2012; Loughnan et al. 2013). We advocate the routine integration of DNA-based tools to determine pedigrees, inbreeding and diversity in all generations of captive breeding programs.

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