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ORIGINAL ARTICLE

Assessing the genetic integrity of captive and wild populations for reintroduction programs: the case of Cabot's Tragopan in China

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Abstract Reintroduction of captive-bred animals into suitable habitats is an important technique for the long-term conservation and recovery of populations of endangered species in fragmented landscapes. Inbreeding depression is an inherent risk when using captive populations for reintroduction programs and needs to be carefully assessed prior to reintroduction. In this study, we evaluated inbreeding levels within a captive breeding program and one remnant wild population of Cabot's Tragopan (*Tragopan caboti*), an endangered pheasant species endemic to China, for which reintroduction is now an essential conservation strategy for long-term population persistence. Fifteen highly polymorphic microsatellite loci were developed to genotype individuals. Inbreeding coefficients (F_{IS}) reveal that there is no evidence of inbreeding within the Tragopan Breeding Center of Beijing Normal University (TBCBNU) captive population and the remnant population from the Wuyi-Yandang Mountains. Diversity of origin, large founder population size and a rational breeding strategy are the most critical factors preventing inbreeding depression within the TBCBNU captive population. We suggest that the TBCBNU population is a suitable candidate stock for *T. caboti* reintroduction programs and that there is an urgent need to better coordinate and strengthen reproduction management of captive *T. caboti* populations to sustain the long-term *ex situ* conservation of the species.

Keywords *Tragopan caboti*, reintroduction, inbreeding, microsatellites

Introduction

Reintroduction of animals into an existing remnant wild population is an important technique for the successful conservation management of endangered species and locally declining populations (Frankham et al., 2002; Armstrong and Seddon, 2008). Many endangered species are often incapable of long-term survival in highly fragmented and isolated, human-disturbed natural habitats and *ex situ* conservation programs such as captive breeding are required to preserve them and provide suitable animals to supplement wild remnant populations (Tenhumberg et al., 2004; Robert, 2009). The success of these reintroduction programs is difficult to assess, but ideally they should be judged on the re-establishment of a long-term viable (self-sustaining) wild population (Griffith et al., 1989; Fischer and Lindenmayer, 2000). However, the use of captive propagation for reintroduction programs is not without risk. Generally, most captive populations are significantly smaller than remnant wild populations (Jiang et al., 2005)

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and evidence suggests that captive-bred populations tend to have lower genetic variability than that of the remnant populations (Jiang et al., 2005). This could result in genetic changes that may reduce the long-term viability of remnant wild populations (Lynch and O'Hely, 2001; Reed et al., 2007; Miller et al., 2009). The genetic integrity of both the remnant wild populations and a captive population therefore needs to be carefully assessed (Araki et al., 2007) prior to reintroduction.

Cabot's Tragopan (*Tragopan caboti*) is a globally threatened species, restricted to montane (subtropical) forests in southeastern China (800–1800 m in elevation) (Zhang and Zheng, 2007; Zhang, 2010). Currently listed as 'Vulnerable' (BirdLife International, 2008), CITES Appendix I (IUCN, 2008) and the first category of nationally protected wildlife species in China (Zheng and Wang, 1998), this species has been the subject of a long-term monitoring program since 1983 at one of its remaining strongholds in the Wuyi-Yandang Mountain range (e.g. Zheng et al., 1985; Young et al., 1991; Qian and Zheng, 1993; Ding and Zheng, 1997; Deng and Zheng, 2004; Zhang, 2005; Sun et al., 2009). Subtropical forest habitat continues to be reduced and degraded in the area through agricultural expansion and consequently, these forests now represent isolated population refugia (Zhang and Zheng, 2007). Cabot's Tragopan appears intolerant of the surrounding matrix habitat, consisting largely of secondary bamboo forests and agricultural land forms. The probability of its dispersal is directly related to the degree of subtropical forest fragmentation (Zhang and Zheng, 2007). Suitable habitat patches separated by distances of > 500 m appear to represent a threshold in the dispersal probabilities of the species, beyond which movement and dispersal is significantly reduced (Deng and Zheng, 2004). This raises important concerns regarding the risk of genetic isolation and inbreeding within the Wuyi-Yandang population and therefore the ability of the species to persist in fragmented landscapes. A recent population viability analysis (PVA) has demonstrated that the probability of the remnant Wuyi-Yandang population persisting over the long term will decrease markedly even with a reduction in the rate of deforestation (Zhang and Zheng, 2007).

Since 1985 and simultaneous with the long-term monitoring program of the wild population, a captive breeding program for *T. caboti* was established by the Tragopan Breeding Center of Beijing Normal University (TBCBNU; Zhang, 2006). The aim of this program is to maintain and propagate a captive population of Cabot's Tragopan for re-establishing new populations into areas where the species formerly occurred, or for remnant wild population supplementation. A pilot supplementation test was carried out in the Wuyanling Nature Reserve in November 1991 (Ding and Zheng, 1996), but the long-term project was suspended due to the lack of stable source of breeding populations and genetic evaluation. It is now acknowledged that populations will not persist in the long term without reintroduction efforts using captive-bred individuals, to complement land management strategies aimed at increasing connectivity between isolated remnant populations and to improve the quality of breeding habitats at known sites (e.g. Zhang and Zheng, 2007). Assessing the genetic integrity of the remnant Wuyi-Yandang tragopan population and that of the captive population is therefore an important step for the long-term conservation management of this threatened species.

We used nuclear genotypes to examine the genetic variation of both captive-bred and a remnant wild population of Cabot's Tragopan to help inform reintroduction strategies. Microsatellites represent a kind of highly polymorphic nuclear markers to make a comprehensive risk assessment of genetic variation for endangered species (Ellegren, 2004). First, we developed a polymorphic primer set including three novel loci isolated from Cabot's Tragopan and 12 loci developed from cross-species amplification of three Galliformes species. Then 158 individuals were genotyped on these loci to compare inbreeding coefficients between target populations.

Materials and methods

Sampling

A total of 142 blood and feather samples were obtained from the TBCBNU. As well, 16 feather samples were collected from the remnant wild Wuyi-Yandang population. The smaller sample size from the wild population was simply a reflection of the difficulty in capturing a larger number of this furtive and low density species — a situation typical for many of China's endemic Galliformes. All samples were stored in 95% ethanol solutions.

Development of microsatellite loci and genotyping

Genomic DNA was extracted from all samples using a standard protocol involving proteinase K digestion, followed by phenol/chloroform separation and precipitation with ethanol (Sambrook et al., 1989). We evaluated microsatellite loci, originally identified from 56 published primers of three Galliformes species: Turkey (*Meleagris gallopavo*) (Burt et al., 2003), Red Junglefowl (*Gallus gallus*)

(Crooijmans et al., 1997) and the Brown Eared-pheasant (*Crossoptilon mantchuricum*) (Zhao, 2007). Meanwhile, microsatellites were isolated using an enriched genomic library from muscle sample of an adult male at TBCBNU. Linked genomic fragments were enriched using $(CAG)_{10}$, $(GAAA)_{10}$ or $(GATA)_{10}$ biotinylated probes and plasmid DNAs from 51 positive clones were sequenced following Hsu et al. (2003). Primer pairs were designed for nine sequences, where tandem repeat sequences existed.

Polymerase chain reactions (PCR) was carried out using DNA from one adult male from TBCBNU for the nine primer sets isolated from the genomic library and 56 published primer sets together. PCR was performed using a 10 μL reaction mixture consisting of 1 μL genomic template DNA (about 15 ng), 1 × PCR buffer containing 0.3 mmol·L^{-1} each dNTP, 1.5 mmol·L^{-1} $MgCl_2$, 0.2 μmol·L^{-1} each primer and 1 U Taq polymerase (Trans Gene). PCR amplification was conducted in a MJ Research PT-200 thermo cycler for 5 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at gradient annealing temperature (45–55°C), 30 s at 72°C and completed with 10 min elongation at 72°C. We successfully amplified PCR products from 44 primer pairs. After loci were successfully sequenced, 26 of them were found to contain at least five repeat motifs. We screened these loci in 16 wild Cabot's Tragopans using ABI (Applied Biosystems) 3100 automated sequencer with forward primers labeled with 6-FAM or HEX fluorescent dye. Genotyping was carried out using GeneMapper 3.7 (Applied Biosystems), with ET-ROX 500 as internal size standard. In total, we identified three novel variable microsatellite loci for Cabot's Tragopan and a further 12 polymorphic microsatellite loci by cross-species amplification.

All 15 primer pairs were used to amplify the samples from both populations. All PCRs were carried out in a 10 μL reaction mixture consisting of 50 ng of template, 0.2 mmol·L^{-1} each primer, 1 × PCR amplification buffer (Takara), 2 mmol·L^{-1} $MgCl_2$, 0.15 mmol·L^{-1} each dNTP and 1 U Taq DNA polymerase (Takara), using a PTC-200 thermocycler (MJ Research). The following conditions prevailed: denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 30 s, annealing temperature (see Table 1) for 30 s and 72°C for 30 s, with a final extension at 72°C for 10 min. Genotyping of the PCR products was conducted on an ABI (Applied Biosystems) 3100 automated sequencer. The results were analyzed using ABI PRISM GeneMapper software, version 3.0 (Applied Biosystems).

Statistical analyses

We assessed the number of alleles (N_a), observed (H_O), expected heterozygosity (H_E) and exact tests for possible deviations from the Hardy-Weinberg equilibrium (HWE) for each of the fifteen loci by ARLEQUIN version 3.1 (Excoffier et al., 2005). We used MICRO-CHECKER version 2.2.3 (Van Oosterhout et al., 2004), to estimate null allele frequencies following Brookfield (1996). The pairwise linkage (genotypic) disequilibrium among microsatellite loci was evaluated by ARLEQUIN v3.1 as well. To reduce the probability of Type 1 errors in multiple tests, Bonferroni corrections (Rice, 1989) were applied in the calibration to reject the null hypothesis. Polymorphic information content (PIC) was used as a general estimate of polymorphism for all microsatellite loci used in the linkage analysis (e.g. Botstein et al., 1980; Shete et al., 2000). We used the inbreeding coefficient index F_{IS} (Weir and Cockerham, 1984) as a measure of the degree of inbreeding from the Hardy-Weinberg equilibrium within population by estimating homozygosity excess ($F_{IS} = 1 - H_O/H_E$, where H_O is the average observed heterozygosity within a subpopulation and H_E is the average expected heterozygosity within a subpopulation). For a population which is in Hardy-Weinberg equilibrium, the estimates of observed and expected heterozygosity are equal ($F_{IS} = 0$), whereas the value of F_{IS} should be positive for populations that have experienced inbreeding which induced a certain extant deficiency of heterozygosity. F_{IS} for all three populations was calculated using locus-by-locus AMOVA block implemented in ARLEQUIN version 3.1.

Results

Microsatellite polymorphism

The principal genetic characteristics of 15 microsatellite loci in the Wuyi-Yandang population are shown in Table 1. The number of alleles per locus ranged from 2 to 12, with a mean of 5.13 alleles. There was considerable variation in the estimates of polymorphism across loci based on PIC values, ranging from 0.27 to 0.87. Three loci, 5H7, MCW98 and ADL184 significantly deviated from the HWE with Bonferroni correction ($p = 0.0033$) and were removed from subsequent analyses. MICRO-CHECKER analysis revealed that the three loci with null allele, 5H7, MCW98 and ADL184, in accord with the three loci which deviated from HWE. Of the remaining 12 loci, there was no evidence of linkage disequilibrium with Bonferroni corrections, confirming the suitability of this suite of polymorphic microsatellites to assess the genetic differentiation between the captive populations and the wild population.

Table 1 Principal characteristics of microsatellite loci in wild *Tragopan caboti* individuals (n = 16). T_a = optimum annealing temperature; Size range = allele size range; N_a = number of observed alleles; PIC = polymorphic information content; p = probability test for deviation from Hardy-Weinberg equilibrium (HWE); Loci with significant deviation from HWE (p = 0.003 with Bonferroni correction) are highlighted in bold.

Locus	Repeat motif	Primer sequence (5′→ 3′)	T_a (°C)	Size range (bp)	N_a	PIC	p	Reference
C52	$(TAGA)_{12}$	F: TCCTGATGTGGCAGAGCAGAGG R: CTAACAAACAGTGTGTTATTGTGG	49	174–198	7	0.74	0.835	This study
C76	$(TATC)_9$	F: CAGACTGCATATGGCACTGTTG R: GCATGTCAGAATTCACCTGC	55	240–252	4	0.59	0.426	This study
C228	$(CTCTTT)_3(GAA)_9$	F: CAGAGAATAGATTAAGGGATAAGA R: AAACATAGATGGTAGCACTCCCT	55	174–179	2	0.27	1.000	This study
5A8	$(AAAC)_5$	F: GATCCAGGGGGTGTGCACACG R: CAGCCCGCACGGATGGTG	48	258–262	2	0.35	1.000	Zhao, 2007
5H7	$(TG)_{27}$	F: CCAAGAGGGAGGCACACGTTC R: AGCCATAAATAAGCAAACGC	48	185–219	12	0.87	< **0.003**	Zhao, 2007
5H8	$(TAGAA)_8$	F: TCCCTGCCAAATACATTGCA R: CTGTTTCCATACCAAGACGCTG	44	129–169	9	0.81	0.869	Zhao, 2007
RHT0010	$(AC)_{10}$	F: TTAACCTATCAGGTCGTTGCG R: GCATGTCAGAATTCACCTGC	55	185–187	2	0.34	1.000	Burt et al., 2003
RHT0047	$(AC)_{13}$	F: AGACCAGGTGGACCCAGAG R: TTTGGCATCTACATTTGCTCC	57	225–241	7	0.73	0.605	Burt et al.,2003
RHT0079	$(AC)_9$	F: TGTTCTTCACTGTTCTTGCAGG R: AGCAGTAATTCACAGCCTTCTC	55	208–216	4	0.46	1.000	Burt et al.,2003
RHT0263	$(AC)_9$	F: AGAACTGCACACAATGCAGC R: TCCACATGACAAACTGGATAGC	55	305–317	3	0.31	0.398	Burt et al.,2003
ADL142	$(AC)_9TA(AC)_7$	F: CAGCCAATAGGGATAAAAGC R: CTGTAGATGCCAAGGAGTGC	55	202–206	3	0.53	0.412	http://www.ncbi.nlm.nih.gov
ADL184	$(AC)_{13}$	F: GCCTCCTCACCCACAAAACC R: TCAGTAACACCACGAATGCC	55	122–142	10	0.81	< **0.003**	http://www.ncbi.nlm.nih.gov
ADL230	$(AC)_9$	F: GCCAAATAGTAATCCACTGC R: TCGCTCTTGCCATTGTAAGT	51	103–115	5	0.64	0.960	http://www.ncbi.nlm.nih.gov
MCW98	$(AC)_{17}$	F: GGCTGCTTTGTGCTCTTCTCG R: CGATGGTCGTAATTCTCACGT	53	244–258	4	0.67	< **0.003**	http://www.ncbi.nlm.nih.gov
MCW146	$(AC)_9$	F: CCGTGTGGTGAACAACGATGA R: CAAATCTGCCCTGACGTCAGC	55	134–142	3	0.29	0.524	Crooijmans et al., 1997
Mean/Total	–	–	–	–	5.13	0.56	–	–

Comparison of inbreeding coefficient index (F_{IS})

Both observed and expected heterozygosity were similar across loci for the TBCBNU population and the Wuyi-Yandang population (Table 2). There were no significant differences between H_O and H_E for each locus across the two populations (Table 2). The value of F_{IS} of the TBCBNU captive population was negative (–0.05), but was not significantly different from zero ($p = 0.996$), suggesting that there was no inbreeding within the TBCBNU captive population. Neither was there any evidence of inbreeding within the wild Wuyi-Yandang population ($F_{IS} = 0.05$, $p = 0.192$).

Discussion

In this study we developed twelve microsatellite primers with high polymorphism for Cabot's Tragopan that greatly improved our capacity to understand many ecological characteristics of this threatened species (mating systems, dispersal ability, sperm competition, etc). These primers are also beneficial for future research in conservation genetics, such as the effect of habitat fragmentation on genetic diversity and gene flow restriction between wild populations, but also for monitoring and evaluation of conservation program implementation. We used two different approaches to develop polymorphic microsatellite loci in Cabot's Tragopan, but both were less efficient than those used in other species (Barbará et al., 2007). However, the same loci did show higher levels of polymorphism in 15 Temminck's Tragopans (*T. temminckii*) collected from one population (unpublished data), which revealed lower genetic diversity in Cabot's Tragopan. We suggest that more primers with higher polymorphism should be developed that would enable more comprehensive genetic diversity monitoring protocols for captive stock and remnant wild populations.

Table 2 Genetic diversity comparison of 12 microsatellite loci in two populations of *Tragopan caboti*: H_O = observed heterozygosity; H_E = expected heterozygosity; F_{IS} = inbreeding coefficient index. Numbers in parenthesis indicate sample size for each population.

	Captive population (TBCBNU, $n = 142$)		Wild population (Wuyi-Yandang, $n = 16$)	
	H_O	H_E	H_O	H_E
C52	0.81	0.76	0.71	0.76
C76	0.30	0.47	0.46	0.58
C228	0.21	0.22	0.47	0.47
5A8	0.52	0.46	0.44	0.42
5H8	0.89	0.77	0.79	0.83
RHT0010	0.48	0.44	0.21	0.20
RHT0047	0.58	0.59	0.77	0.76
RHT0079	0.45	0.49	0.46	0.52
RHT0263	0.28	0.33	0.42	0.54
ADL142	0.66	0.58	0.71	0.58
ADL230	0.78	0.70	0.79	0.70
MCW146	0.31	0.31	0.31	0.38
Overall	0.56	0.54	0.51	0.60
F_{IS}	–0.05 ($p = 0.996$)		0.05 ($p = 0.192$)	

We have shown that assessing the genetic integrity of wild and captive bred populations is an essential component of reintroduction programs of threatened species. Our non-significant F_{IS} value in the TBCBNU and Wuyi-Yandang populations (Table 2) revealed no evidence of inbreeding in either the captive or wild target populations in the present study. For the TBCBNU population, the outcome could be attributed to the origins of captive population establishment and subsequent management methods. The number of TBCBNU founders was large ($n = 19$) and were introduced in stages from three different wild populations: Hunan, Wuyi-Yandang and Guangxi. Efforts were also made to increase the chances of random mating at TBCBNU by artificial fertilization (Zhang, 2006), which to a degree, helped to reduce the probability of inbreeding. Thus, the individuals from the TBCBNU stock are suitable candidates for Cabot's Tragopan reintroduction programs, whether the purpose is to re-establish a new population in an area where Cabot's Tragopan once existed, or to supplement an existing remnant wild population, since both projects require the selection and use of healthy individuals with similar genetic backgrounds. We stress the importance of determining whether the 'source' of birds for reintroduction will be detrimental to the genetic basis of either the new founder or the extant remnant population (World Pheasant Association and IUCN/SSC Reintroduction Specialist Group, 2009).

For the small and isolated remnant wild Wuyi-Yandang population, the high F_{IS} values are encouraging since previous PVA analysis has shown that removing the risk of inbreeding depression from this population significantly reduces the risk of its local extinction (Zhang and Zheng, 2007). Nevertheless, we exercise some concern over whether the degree of ongoing habitat fragmentation in the region may have had a 'debt' impact on the genetic diversity of *T. caboti,* which has yet to manifest itself. Although microsatellite markers have faster mutation rates than the large majority genetic markers (Ellegren, 2004), detection of reduced genetic variation in small isolated populations via genetic drift may not be detectable until the popula-

tion has undergone at least ten or twenty generations.

These studies should also explicitly consider using both mitochondrial DNA as a scale for the evolutionary history of the species and microsatellite loci as a scale for isolation and gene flow during recent geological periods. Such an approach would enable better monitoring of the captive population to establish clear genetic lineages amongst them and allow a more comprehensive assessment of the potential for genetic restoration and/or maintenance of isolated remnant wild populations.

Implications for conservation management

Our results have a number of implications for conservation management for Cabot's Tragopan. First, we have shown that any future establishment of captive *T. caboti* populations for the purpose of reintroduction should focus on careful screening and use of wild birds as potential founders. Second, wild individuals from e.g. the Wuyi-Yandang population could also be introduced to supplement the genetic integrity of captive populations, but we stress that this should not be done at the expense of efforts to conserve the wild population.

In this study we have identified that a particular captive stock — the TBCBNU population — is a suitable candidate for *T. caboti* reintroduction programs. Currently there is a proposal for re-establishing a *T. caboti* population at one of the few remaining isolated locations. We strongly encourage the proponents of this proposal to follow closely the current IUCN reintroduction guidelines (World Pheasant Association and IUCN/SSC Re-introduction Specialist Group, 2009): appropriate project 'success' indicators must be identified; suitable monitoring protocols must be agreed upon to enable monitoring the genetic diversity of wild populations using non-invasive sampling; ensure that the principal causes of local population extinction (over-hunting and selective logging) are no longer relevant, prior to releasing individuals into the wild.

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黄腹角雉笼养种群与野外种群的遗传多样性
——为再引入项目进行的种群内近交水平评估

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摘要：利用笼养种群在适宜栖息地开展再引入工作是保护和恢复濒危动物野外种群数量的有效方法。近交衰退是影响笼养种群健康程度的主要风险，因此需要在开展再引入项目前对笼养种群的近交程度进行评估。本研究以微卫星为遗传标记，对我国特有濒危雉类——黄腹角雉（*Tragopan caboti*）的一个笼养种群和一个野外种群的近交水平进行了评估和比较，从而为即将开始的再引入项目和长期保护提供保障。利用富集文库法和跨物种扩增法共筛选出15对多态性较高的微卫星位点。近交系数（F_{IS}）的分析结果表明，北京师范大学角雉繁育中心的笼养种群和江西武夷山的野外种群均不存在显著的近交现象。笼养种群来源地的多样化，较大的建群者数量和科学的繁殖管理方式是防止所研究的笼养种群发生近交衰退的有效途径。因此，我们认为北京师范大学角雉繁育中心的笼养个体是开展再引入项目的理想种源。当前急需解决的问题是：如何将笼养种群的健康管理与野外种群的长期保护政策有效结合起来。

关键词：黄腹角雉，再引入，近交，微卫星