

Research article

Assessment of genetic diversity and inbreeding in the EAZA gibbon conservation breeding programmes using a novel panel of cross species microsatellites

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Abstract

We have developed a panel of 12 autosomal microsatellite loci to measure levels of genetic diversity and inbreeding within the EAZA gibbon conservation breeding programmes. Estimates of genetic diversity and inbreeding within captive populations are usually determined from studbook data. The assumption of unrelated founders in these calculations may be erroneous, resulting in inaccurate appraisals of populations. Genetic testing of individuals provides quantitative data to directly measure relatedness and inform on the genetic diversity of populations. Our panel of microsatellite loci successfully produced profiles in all species of gibbon tested (n=11 species, across three genera), offering excellent cross-species utility. Loss of alleles was observed for all examined EAZA populations across generations, and loss of heterozygosity was observed for all populations, except *Nomascus leucogenys*. Deviation from Hardy-Weinberg equilibrium was detected for the *Symphalangus syndactylus* EAZA population ($P<0.002$), but for no others. We were also able to compare the diversity of the EAZA gibbons with independent samples held at The University of Tokyo. This revealed unique alleles for each species in both sets of samples, indicating higher genetic diversity in wild populations, and lower in captive populations. Our data demonstrate the advantage of direct genetic testing to assess breeding populations, quickly highlighting where intervention may be necessary. These tools will assist gibbon conservation breeding programmes by providing robust information on the genetic diversity of populations, which can be used to guide future breeding decisions.

Introduction

Gibbons (Hylobatidae) are a family of primates, which together with the great apes (Hominidae), form the superfamily of apes (Hominoidea) (Groves 2001; Roos 2016). The family is divided into four genera, on the basis of chromosome complement / karyotype (ranging from $2n=38$ to $2n=52$) (Roos 2016). There are currently 20 recognised gibbon species (IUCN 2020; Mittermeier et al. 2013). All species of gibbon are listed as Vulnerable, Endangered, or Critically Endangered by the International Union for Conservation of Nature (IUCN) (IUCN 2020). As of September 2023, the European Association of

Zoos and Aquaria (EAZA) holds approximately 600 gibbons, a collection representing 10 species across three genera: *Symphalangus*, *Nomascus* (four species), and *Hylobates* (five species). Of these ten species, six are currently managed as EAZA Ex-Situ Programmes (EEPs): *Hylobates lar*, *Hylobates moloch*, *Hylobates pileatus*, *Symphalangus syndactylus*, *Nomascus leucogenys*, and *Nomascus gabriellae*. The EEPs are managed under the supervision of the EAZA gibbon Taxon Advisory Group (gibbon TAG), to enable oversight of captive population breeding (Lefaux et al. 2020).

Captive breeding has been recommended as a conservation measure for gibbons by the IUCN (Campbell et al. 2015; IUCN

2020). However, to maintain healthy populations, which remain viable in the long-term, conscientious management is required (Hosey et al. 2009; Montgomery et al. 1997; Soule et al. 1986; Witzemberger and Hochkirch 2011). Captive populations, being small and usually closed, are vulnerable to inbreeding (Frankham et al. 2010; Montgomery et al. 1997; Witzemberger and Hochkirch 2011). Of primary concern is that inbreeding increases the risk of the expression of deleterious phenotypes (Frankham et al. 2010). In a closed population, some level of inbreeding is inevitable (Dudash and Fenster 2000; Frankham 2008; Frankham et al. 2010). However, the rate at which inbreeding impacts genetic diversity can be reduced by appropriate management of captive breeding, by carefully planning pairings over the long-term (Frankham et al. 2010; Montgomery et al. 1997).

In order to reduce the rates of inbreeding and loss of genetic diversity, EEPs explicitly aim to pair mates based on minimising mean kinship (EAZA 2020). This strategy can also help reverse the effects of unequal breeding success in founder pairings (JBallou and Lacy 1995; Willoughby et al. 2015). Mean kinship is calculated from studbook data, and studbooks are normally maintained on the assumption that the founders were unrelated, which may be unsupported (EAZA 2020; Putnam et al. 2022; Russello and Amato 2004). Conventional management practices depend upon the validity of the unrelated founder assumption, leading to inaccurate estimates of relatedness when this assumption is violated (Putnam et al. 2022; Russello and Amato 2004). Genetic testing can provide more accurate calculations of mean kinship. These improved estimates allow for more appropriate decisions regarding pairings (EAZA 2020; Frankham et al. 2010; Russello and Amato 2004).

Genetic diversity is one of three forms of biodiversity recognised by the IUCN as requiring conservation (McNeely et al. 1990). Preservation of genetic diversity is also one of the primary goals of EAZA EEPs (EAZA 2020). Genetic variation is positively correlated with fitness (Reed and Frankham 2003), and seems to be pivotal for the long-term survival of sexually reproducing species (Frankham 1996; 2005; Frankham et al. 2010; Sacchi et al. 1998; Sherwin and Moritz 2000). Furthermore, reduced genetic diversity may reduce the chances of long-term success for captive populations

that are released back into the wild (Williams and Hoffman 2009; Willoughby et al. 2015). For the gibbon EEPs, the stated goal is to preserve at least 90% of the founding genetic diversity for 100–200 years (Lefaux et al. 2020).

Microsatellites are a type of hypervariable genetic marker, generated by replication slippage at simple sequence repeats (Ellegren 2004; Tautz 1989; Tautz and Renz 1984). Microsatellites have been used in the captive setting to monitor genetic variation, often with a focus on measuring heterozygosity, in a wide variety of captive species including giant pandas *Ailuropoda melanoleuca* (Shen et al. 2009), rhesus macaques *Macaca mulatta* (Kanthaswamy et al. 2006), Chinese water deer *Hydropotes inermis* (Hu et al. 2007), the Arabian oryx *Oryx leucoryx* (Arif et al. 2010), jaguars *Panthera onca* (Corner et al. 2019), snub-nosed monkeys *Rhinopithecus* spp. (Cai et al. 2020), lions *P. leo*, leopards *P. pardus*, and tigers *P. tigris* (Pandey et al. 2021), and the European ground squirrel *Spermophilus citellus* (Kovářík et al. 2026).

The aim of the present study was to develop a panel of microsatellites to investigate patterns of genetic diversity within EAZA gibbon populations. The particular aims were: to elucidate possible inbreeding, to estimate allelic diversity and heterozygosity, and to determine how these are changing across generations. With 20 species of gibbon currently recognised (IUCN 2020; Mittermeier et al. 2013), developing a single microsatellite panel which could be applied across species was crucial for our goal of creating a protocol which is broadly useful and practicable. To our knowledge, this is the first time a panel of microsatellites has been designed to work across multiple genera of gibbons. A further goal was to compare the genetic diversity present in the EAZA gibbon populations with that encompassed by gibbons outside of these collections. To this end, we were able to compare diversity with samples held at the University of Tokyo, Japan (maintained cell culture lines that most likely originated from captive animals in zoos and laboratories).

Materials and Methods

Samples

116 blood, tissue, hair, and FTA blood spot samples were generously donated by 20 EAZA institutions from seven countries across Europe (see Acknowledgements for contributing institutions). All invasive samples (i.e. blood, tissue) were residual material collected during medical procedures, or from autopsies. Hair samples provided for this study were collected by keepers in line with their institution's approved handling procedures. Approval for the project and sample collection was given by the EAZA Gibbon TAG (Taxon Advisory Group). No samples used in this study were collected by the authors. 51 samples from the University of Tokyo were tested. Table 1 lists the number of samples analysed for each species. All samples from EU countries were obtained prior to the UK's exit from the EU in 2020, and as a result did not require permits for transport. Samples at the University of Tokyo were tested in Japan, so no international transport of these samples was required. The samples used in this study had been collected previously by zoo professionals in line with their institution's approved handling procedures and as residual material collected opportunistically during routine medical procedures. The rationale for using these existing samples was reviewed and approved by the EAZA Gibbon TAG. Because the study involved only analysis of previously collected material, it did not undergo review by a full institutional ethics committee.

DNA Extraction

For blood and tissue samples, DNA was extracted using the Maxwell® 16 Automated Nucleic Acid and Protein Extraction System (Promega, Madison WI, USA), with the Maxwell® 16 Blood

Table 1. Number of samples obtained for each species from EAZA Institutions and the University of Tokyo.

Species	Number of samples		
	EAZA	Tokyo	Total
<i>Symphalangus syndactylus</i>	28	11	39
<i>Nomascus leucogenys</i>	13	2	15
<i>Nomascus gabriellae</i>	8	-	8
<i>N. leucogenys</i> x <i>N. gabriellae</i>	2	-	2
<i>Nomascus siki</i>	4	1	5
<i>Hylobates agilis</i>	8	5	13
<i>Hylobates lar</i>	13	13	26
<i>Hylobates pileatus</i>	11	9	20
<i>Hylobates moloch</i>	29	1	30
<i>Hylobates muelleri</i>	-	3	3
<i>Hylobates abbotti</i>	-	2	2
<i>Hylobates albibarbis</i>	-	4	4
Total	116	51	167

DNA Purification Kit, following the manufacturer's instructions. Samples were loaded into the cartridges in a dedicated pre-PCR Class II laminar flow hood (Walker Safety Cabinets, Glossop, UK). For hair samples, DNA extractions were performed in a dedicated pre-PCR clean room, in a laminar flow cabinet (Starlab, Milton Keynes, UK), which was UV irradiated prior to each use. All pipettes and consumables were kept exclusively in the laminar flow hood, and so were also regularly UV irradiated. Gloves were UV irradiated prior to use. DNA was extracted using the QIAamp (Qiagen, Manchester, UK) DNA Investigator Kit, following the manufacturer's instructions, with the following modifications: lysis at 56°C took place overnight (approximately 18 hours), and the DNA elution step was performed twice to increase yield. Samples were then concentrated using Amicon Ultra-0.5 mL Centrifugal Filters (Merck Millipore, Watford, UK), washing twice with 150µl TMT (10mM Tris-HCl, pH 8.0). For FTA blood-spot samples, DNA was extracted using the Qiagen DNA Investigator kit, following the manufacturer's instructions. For cell culture samples, DNA was extracted using the QIAamp DNA Mini Kit following the manufacturer's instructions.

Primer Design

12 microsatellites were selected to be amplified in three multiplex PCR reactions, each containing four amplicons. All primer sequences are listed in Table 2. The four microsatellites used in Multiplex 1 were identified using the programme GMATA V2.1 (Wang and Wang 2016), searching for tetra-, penta- and hexa-repeat motifs in the northern white-cheeked gibbon *Nomascus leucogenys* genome assembly (NomLeu3). The loci for Multiplex 2 were identified using RepeatMasker (Smit et al. 2015) annotation data acquired from the UCSC Table Browser (Karolchik 2004), for *Nomascus leucogenys* genome assembly (NomLeu3), filtering for tetra- and penta-repeat motifs. The UCSC Genome Browser (Kent et al. 2002) was also used to ensure the microsatellites were not within or adjacent to other repetitive regions. For Multiplex 3 four markers were taken from Hu et al. (2014). These authors designed primers for *Nomascus concolor*. Four primers were selected after preliminary laboratory testing showed they amplified and were variable across multiple gibbon species.

Table 2. Microsatellite primer sequences.

Primer	Sequence 5' - 3'	Microsatellite repeat motif	Multiplex
LLMK1A	CAGGTAGATGGGCAGAAAGG	AACC	1
LLMK1B	GAGAGGGCAAGCTAGAAGCA		
LLMK2A	GGGCCTGCAACTGACATATT	AACC	
LLMK2B	GAAGGGGAGGTGAGAAAAGG		
LLMK3A	AAGAGCCTCACCAGATAGCC	ACACAT	
LLMK3B	AGATTCAGGCTGTGGGTTGT		
LLMK12A	CCCAGCTGCACACTGTTCAAC	CCAA	2
LLMK12B	ACCATTGCATCAGCGGGAAGC		
833F	GCCCATTTAGCCAGTCAG	CACAG	
833R	TGGGAAATTGAAGGCAGTGT		
1124F	TGTCTCTCAAGTTCAGCCCT	TTCT	
1124R	CCTTCCAAATGTACCAAGGCA		
1216F	GCAGGACTGTTTGAAAGCCA	AAGC	3
1216R	GGAATGAAGGGAAGGGGTGT		
1316F	TGCCACCACTTTAGAGAGCA	ATCT	
1316R	TATGATTGTTCCAGGCGCTG		
NC20_F	GCGAGTTACCAGCGAGATTG	AC	
NC20_R	AGAACTGGAAGAACAGGACAAGC		
NC35_F	GGTAACATCCACAGCTTGCTAAC	AAAT	3
NC35_R	TAAATTGCACAGTCTTGGGTATG		
NC36_F	CTACTCTGAAGAACAATCAGGTC	AAAT	
NC36_R	GCAGGCAATTATTCCAACAACAG		
NC38_F	CCCCATCATGTGAGCTCAAAG	AAT	
NC38_R	TTACTGATAATGTGCCTGTCACT		

Table 3. Annealing temperatures, concentrations, and dyes used for each primer pair.

Multiplex	T _m (°C)	Marker	Primer concentration (Forward and Reverse) Eaza / Tokyo	Dye
1	64	LLMK1	0.48 µM / 0.2 µM	PET
		LLMK2	0.95 µM / 0.4 µM	6-FAM
		LLMK3	0.95 µM / 0.4 µM	VIC
		LLMK12	0.48 µM / 0.2 µM	NED
2	58	833	0.48 µM / 0.2 µM	PET
		1124	0.48 µM / 0.2 µM	VIC
		1216	0.48 µM / 0.2 µM	NED
		1316	0.48 µM / 0.2 µM	6-FAM
3	65	NC20	0.48 µM / 0.2 µM	6-FAM
		NC35	0.48 µM / 0.2 µM	VIC
		NC36	0.95 µM / 0.4 µM	NED
		NC38	0.48 µM / 0.2 µM	PET

PCR conditions

For Eaza DNA samples, all PCRs were performed in 10.5 µl reaction volumes: 0.95x KAPA Taq buffer A, 0.19µM each of dATP, dCTP, dGTP and dTTP, final MgCl₂ concentration 1.35mM, 5U Taq DNA polymerase, 5ng genomic DNA. See Table 3 for primer concentrations. All PCRs were performed with the following cycling conditions: 95°C for 2 minutes, 30 cycles of: 95°C for 30s, Annealing temperature X°C for 30s, and 70°C for 30s, and a final extension of 70°C for 7 minutes. See Table 3 for annealing temperatures. For the Tokyo samples, PCRs were performed in 15µl reactions: 1x KAPA2G Fast Multiplex Mix, 10ng genomic DNA, ddH₂O to a final volume of 15µl, with the following cycling conditions: 95°C for 3 minutes, 30 cycles of: 95°C for 15s, 64°C for 30s, and 72°C for 30s, and a final extension of 72°C for 7 minutes.

Fragment size determination

UK generated PCR products were run at the University of Leicester on either an Applied Biosystems 3130 Genetic Analyzer or a 3730 Genetic Analyzer, using POP-7™ Polymer. Samples were loaded into 96-well plates, each well containing: 1.5µl sample, 8µl Hi-Di™ formamide, and 0.5µl size standard (either GeneScan™ 600 LIZ or MapMarker® 1000 ROX_XL). The samples in Tokyo were sent to Fasmac Co., Ltd. (Japan) for capillary electrophoresis on a 3730xl DNA Analyzer, using POP-7™ Polymer. Allele sizes were called in Geneious Prime (Kearse et al. 2018). Occasionally allele sizes appeared discordant, with the same (Eaza) individual having allele sizes one or two base-pairs difference in length, when processed on different Analyzers. To resolve this, alleles for which individuals were homozygous were sequenced with Sanger Sequencing, and these confirmed sizes were then used for calibration.

Inbreeding Calculations

F_{IS} values for microsatellites and Chi-squared tests for detection of deviation from Hardy-Weinberg equilibrium were calculated using the online version of Genepop (Raymond and Rousset 1995; Rousset 2008) (<https://genepop.curtin.edu.au/>).

Results

Amplification success rates

From the 116 Eaza samples, full profiles were obtained for 73 samples (63%), partial profiles were obtained for 40 samples (35%), and three samples failed entirely (no microsatellite loci were amplified). The three DNA samples (2.6%) which failed were derived from hair (one *H. lar* and two *N. siki*). Samples which gave partial profiles (i.e. at least one locus failed to amplify) were from hair (n=12), FTA blood spots cards (n=23), or clotted blood (n=3). One good quality blood sample produced a partial profile, and one partial profile came from a very old DNA sample which had evaporated to dryness, despite storage at 4°C. For the Tokyo collection (DNA extracted from cultured gibbon cell lines), 51 samples were tested and all produced full profiles.

Heterozygosity

Figure 1 summarises the average heterozygosity observed in each species, for the Eaza and Tokyo collections, and the range of values observed. The number of loci evaluated varied between species, for several reasons. Markers which appear to be fixed (i.e. where only one allele was recorded in all individuals) were excluded for those species. In the Eaza collection, locus MK2 was only amplified from samples of *Nomascus* spp., so was not included for comparisons. Marker NC35 was excluded in *S. syndactylus* as Sanger sequencing of samples G68 and G69 revealed heterozygote genotypes with alleles differing by 1bp, but these could not be reliably distinguished from the peaks seen in the electropherograms. In Figure 1 'n' represents the final number of loci used for each species. No samples of *H. muelleri* or *H. albibarbis* were available in the Eaza collection. Samples of *N. leucogenys*, *N. siki*, and *H. moloch* from the Tokyo collection were not included for heterozygosity comparisons due to low sample numbers (n less than 3), with *N. siki* also being excluded from the Eaza collection for the same reason. Individuals with <8 successfully amplified loci (n=12) were also excluded. Finally, individuals with unique alleles, who were documented

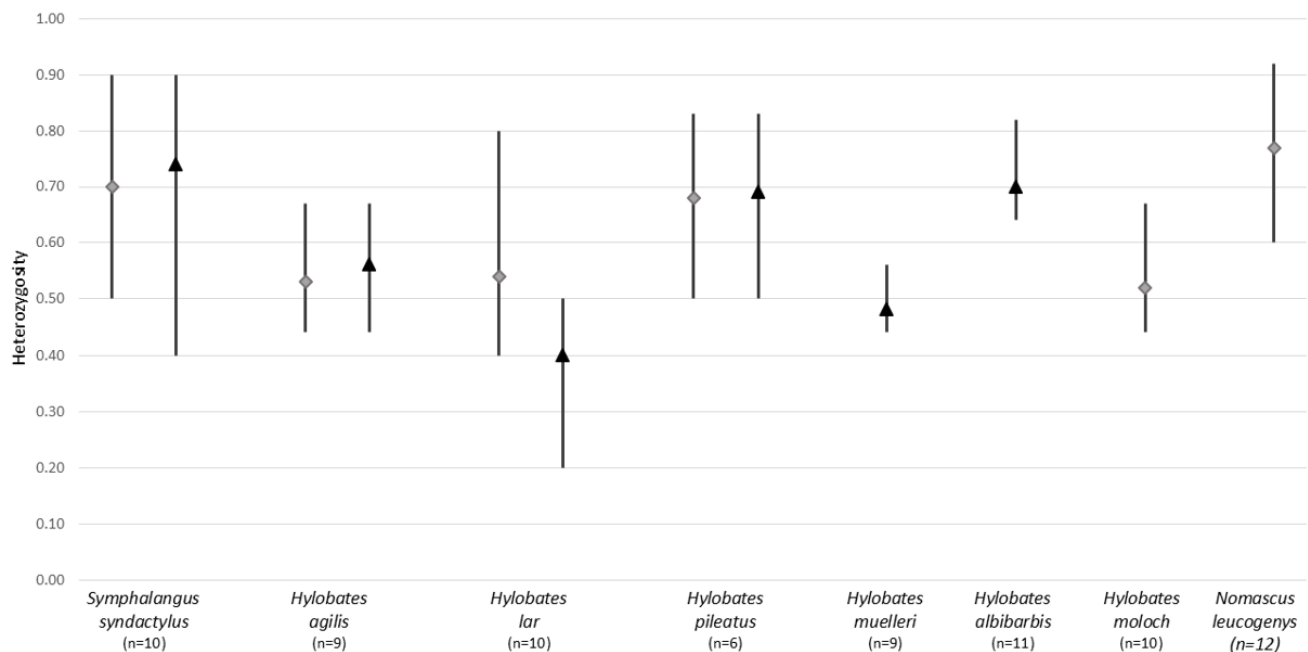


Figure 1. Comparison of levels of heterozygosity between the EAZA captive collection and the Tokyo samples. Lines indicate the minimum and maximum values, and markers show the mean across all loci. Lines with grey diamonds represent the EAZA collection, lines with black triangles the samples from Tokyo. The Tokyo samples of *H. moloch*, *H. abbotti* and *N. leucogenys* were excluded from the analysis as $n < 3$. There were no EAZA samples of *H. muelleri* or *H. albibarbis*. *Nomascus gabriellae* and *N. siki* were excluded from the analysis due to too few samples/incomplete profiles from both EAZA and Tokyo. Individuals of suspected mixed-species ancestry were also excluded from the analysis. n =number of loci for each species.

or suspected of being of mixed-species ancestry, were excluded ($n=7$). After exclusions, 112 samples were included in the analysis (70 from EAZA, and 40 from Tokyo).

Levels of heterozygosity varied considerably between individuals and species. The highest levels of mean heterozygosity observed in the EAZA collection were for *N. leucogenys*, while in the Tokyo collection the highest mean levels were for *S. syndactylus*. *Hylobates agilis* and *H. lar* had the lowest levels in both collections. (N.B. caution should be taken with the apparently low levels seen in *H. moloch*, as allele drop out (ADO) is present in some cases, likely resulting from lower quality DNA. ADO was confirmed, where possible, in individual cases, as offspring were missing parental alleles). Levels of heterozygosity between the EAZA and Tokyo

sample sets were broadly comparable between species. Only *H. lar* demonstrated a statistically significant difference in the levels of heterozygosity between the EAZA and Tokyo samples (average 0.54 and 0.40 respectively), in a two-tailed t-test ($P=0.04$).

Loss of heterozygosity

Complete assessment of changes in heterozygosity across generations within the EAZA populations is not possible due to incomplete sampling, and in some cases partial pedigrees. However, where DNA was available from complete family units, (i.e. both parents and their offspring), limited analyses could be performed. The following numbers of family units were amenable to analysis: *S. syndactylus* ($n=4$), *H. agilis* ($n=2$), *H. lar* ($n=1$), *H.*

Table 4. Changes in heterozygosity between generations in family units in the EAZA collection.

Species	Family units	No. parents	No. offspring	Change in heterozygosity (parent to offspring)
<i>S. syndactylus</i>	4	8	5	0.76-->0.63
<i>H. agilis</i>	2	3	3	0.56-->0.48
<i>H. lar</i>	1	2	1	0.55-->0.60
<i>H. pileatus</i>	2	4	4	0.71-->0.67
<i>N. leucogenys</i>	1	2	5	0.71-->0.89

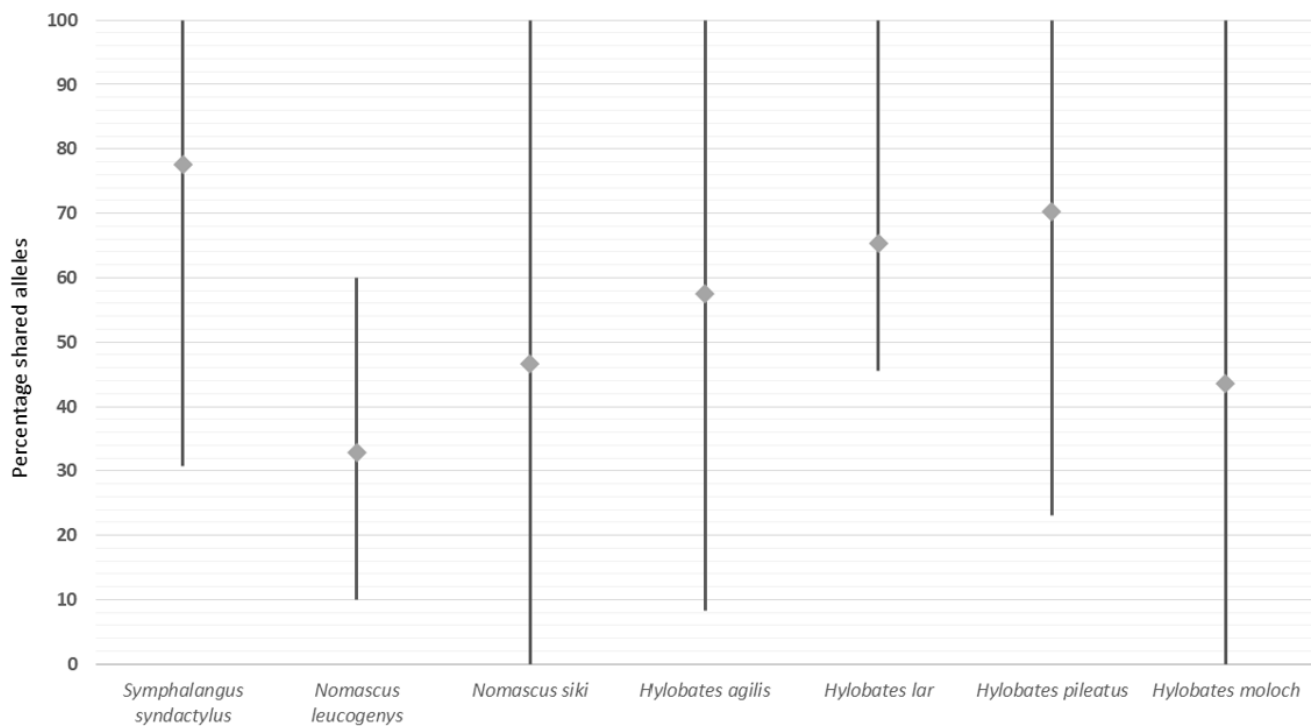


Figure 2. Ranges of percentage of shared alleles between EAZA and Tokyo sample sets, by species. Gray diamonds indicate the averages across all loci.

pileatus (n=2), and *N. leucogenys* (n=1). Table 4 provides the changes in heterozygosity for these groups. The general trend is loss of heterozygosity from parents to offspring, with the exception of *N. leucogenys* and *H. lar* where an increase in heterozygosity in the offspring was observed (*N. leucogenys* highlighted in bold, in Table 4). It is important to note that data for *H. lar* are from only a single triad, so the generality of this trend may be questioned.

Allele distribution

The number of alleles shared between the EAZA and Tokyo collections ranged from 0% to 100%, with significant variation when analysing by species or by locus. In both of these extremes (none or all alleles shared), the number of total alleles was low, ≤ 4 . Figure 2 provides a summary of the percentage of shared alleles, by species. *Nomascus leucogenys* is the only species for which no locus had 100% of alleles shared. The highest proportion of

alleles shared is 60% for marker NC35. This divergence suggests the *N. leucogenys* individuals which founded the EAZA population, and those that were sampled for the Tokyo collection were more genetically diverse than the other species investigated here.

Loss of alleles

As with heterozygosity, a thorough evaluation of loss of genetic variation within the EAZA populations is not possible due to incomplete sampling, and in some cases partial pedigrees. However, where DNA was available from complete family units, (i.e. both parents and at least two offspring), allele loss assessments could be made. Five such units were available in this data set (see Table 5). In the case of *H. agilis*, we have data for two parents and their single offspring, then their mate, and their three offspring. This group was considered as three 'parents/founders' and three offspring.

Table 5. Number of alleles retained in complete family groups. The * indicates those which meet the EAZA goal of retaining >90% genetic diversity (Lefaux et al. 2020).

Species/unit	Founders	Offspring	Alleles retained	% retained
<i>S. syndactylus</i>	2	2	19/25	75%
<i>N. leucogenys</i>	2	5	35/36*	97%*
<i>H. agilis</i>	3	3	16/20	75%
<i>H. pileatus</i> - group 1	2	2	11/16	69%
<i>H. pileatus</i> - group 2	2	2	15/18	83%
<i>H. pileatus</i> - combined	4	4	22/24*	92%*

Detection of possible inbreeding

The microsatellite data were examined for indications of inbreeding using the online version of Genepop (Raymond and Rousset 1995; Rousset 2008). F_{IS} values were calculated for each locus. Genotype frequencies within the populations were tested for deviation from Hardy-Weinberg equilibrium, with P values being calculated for Chi-squared tests. To avoid artificially increasing apparent heterozygosity, individuals with alleles that were suspected to be of hybrid origin were excluded. Individuals for which less than six loci successfully amplified were also omitted from the analysis, in order to avoid homozygous alleles being overly weighted. No evidence of inbreeding was detected for the *N. leucogenys* (n=13), *H. agilis* (n=8), *H. lar* (n=10), *H. pileatus* (n=11), or *H. moloch* (n=17) groups (non-significant P value range 0.07–0.69). However, inbreeding was strongly indicated for the *S. syndactylus* (n=27) group ($P=0.002$).

Comparison of EAZA and Tokyo collections

Comparisons of inbreeding and population differentiation between the EAZA and Tokyo sample sets were made for *S. syndactylus* (n=38), *H. agilis* (n=11), *H. lar* (n=23), and *H. pileatus* (n=20), and are shown in Table 6. F_{IS} Chi-squared P values are given for each sample set ($p<0.05$ is considered significant, and indicates inbreeding in the population). F_{IS} values are given for the two sets compared (values above 0 indicate inbreeding, higher values indicating higher levels of inbreeding to a maximum value of 1). F_{ST} values signify population differentiation (values above 0 indicate differentiation, values above 0.15 are considered to indicate significant differentiation (Frankham et al. 2010)). Comparing the two datasets, there is no indication of inbreeding for either the *H. agilis* groups or *H. pileatus* groups (negative F_{IS} values are treated as zero and no inbreeding detected). Modest inbreeding was detected for *S. syndactylus* ($F_{IS}=0.023$), derived from the EAZA sample set (Chi-squared P value=0.002).

Modest inbreeding was also detected for *H. lar* ($F_{IS}=0.122$) derived from the Tokyo sample set, although this was not significant (Chi-squared P value=0.077). Modest population differentiation was detected for *S. syndactylus*, *H. lar*, and *H. pileatus* (F_{ST} values ~0.05), while significant differentiation was detected between the two *H. agilis* sample sets ($F_{ST}=0.277$). This agrees with the *H. agilis* sample sets having the lowest proportion of shared alleles of these four species.

Discussion

The panel of 12 microsatellite loci, in three multiplexes, reported here has broad cross-species utility. To date, the panel has produced profiles for all species tested (n=11), across three gibbon genera. This is crucial for the panel to be of practical use in conservation / breeding management. Such broad utility allows a single laboratory protocol to be used for any individual, even in situations where species assignment is uncertain. To our knowledge, this is the first time a microsatellite panel has been developed for this number of gibbon species and genera. As a result, this development represents a significant contribution to the field of gibbon genetics, and should facilitate further studies of population genetics, as well as assisting genetically informed captive breeding.

A general trend of loss of genetic diversity and heterozygosity was indicated within the EAZA gibbon populations examined. Comparisons of the microsatellite alleles present in the EAZA samples with those in the Tokyo collection revealed that the EAZA populations have not retained the full range of genetic diversity for those species. Inbreeding was detected in the *S. syndactylus* samples, but not for any other species tested. Each of these aspects will be discussed below.

Loss of heterozygosity

With the exception of *N. leucogenys* (see below) we observed loss of heterozygosity between generations, which is expected in small, closed populations (Dudash and Fenster 2000; Frankham 2008; Frankham et al. 2010). For neutral variants, such as microsatellites, it is estimated that a population will lose half of its original heterozygosity in $1.4N_e$ generations, where N_e is the effective population size (Frankham et al. 2010). Without genetic data on the founding individuals, a precise estimate of N_e cannot be made. An accepted estimate is that N_e is approximately 1/10th of the census population size (N) (Frankham 1995; Frankham et al. 2010). However, species which maintain monogamous pairs - such as gibbons - may have an N_e closer to N (Frankham 1995; Frankham et al. 2010). Figure 3 shows the estimated time to loss of 50% heterozygosity for six species currently held by EAZA based on census population sizes as of September 2023. Two calculations of N_e have been made for comparison. N_e is estimated at 1/10th of N (the common estimate), and one half of N (as N_e for gibbons may be closer to N). The generation time

Table 6. Chi-squared P values, F_{IS} and F_{ST} values, for comparing EAZA and Tokyo sample sets. Chi-squared values of $P<0.05$ are considered significant and indicate differentiation from Hardy-Weinberg equilibrium (i.e. inbreeding), seen here for the EAZA *S. syndactylus* samples. $F_{IS}>0$ indicate inbreeding when both sets are compared (minus values are treated as zero and indicate no inbreeding). F_{ST} values >0 indicate differentiation between the two populations, values >0.15 indicate significant differentiation.

Species	Chi-squared P value		F_{IS} (combined data)	F_{ST} (combined data)
	EAZA	Tokyo		
<i>S. syndactylus</i>	0.002	0.662	0.023	0.056
<i>H. agilis</i>	0.578	0.833	-0.095	0.277
<i>H. lar</i>	0.621	0.077	0.122	0.051
<i>H. pileatus</i>	0.415	0.957	-0.078	0.044

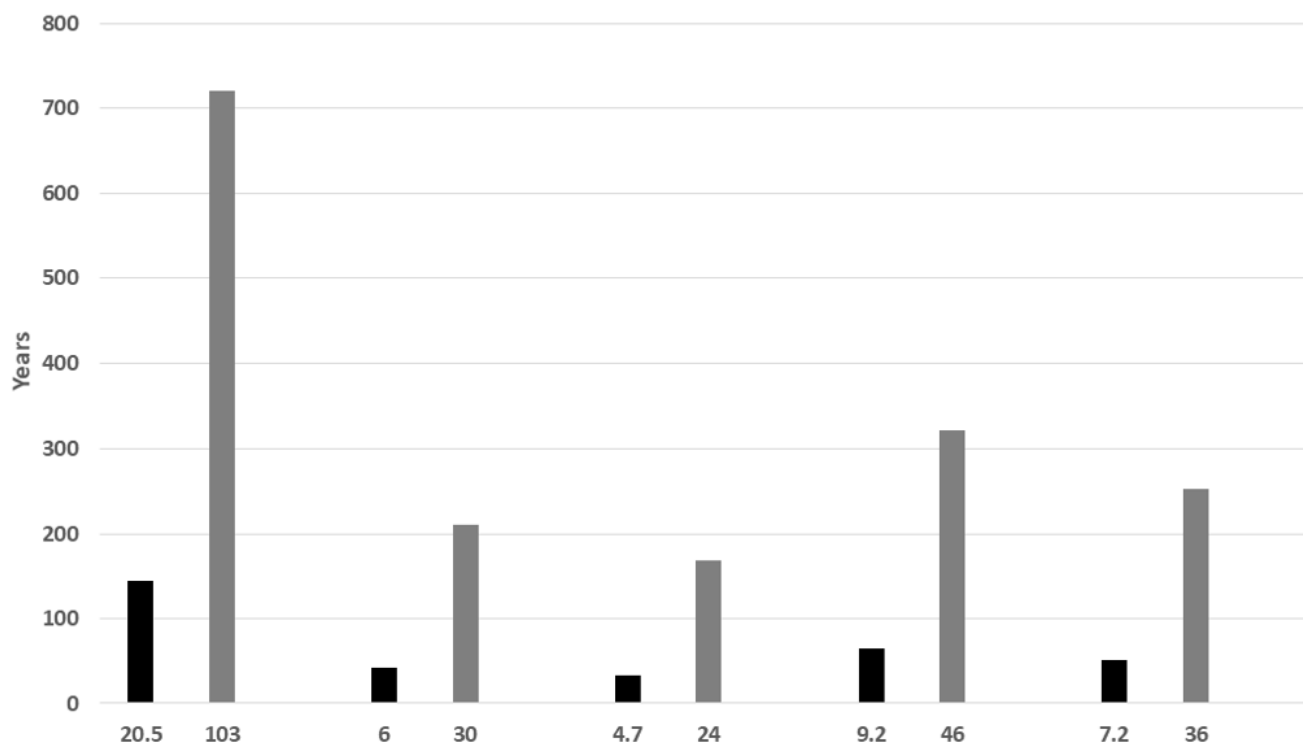


Figure 3. Estimated time (years) to loss of 50% of extant heterozygosity for the six EAZA gibbon EEPs. Time calculated as $1.4N_e$ generations (Frankham 1995; Frankham et al. 2010), with generation length estimated as 5 years, based on minimum age to sexual maturity of females in captivity (Geissmann 1991). N_e =census size as of September 2023. Horizontal axis labels indicate N_e , calculated as $1/10N$ (black bars) and $1/2N$ (grey bars).

is set as five years, as this is the approximate time for females to reach sexual maturity in captivity (Geissmann 1991). These two sets of calculations demonstrate how significantly the time to loss of 50% of heterozygosity can vary depending on the estimation of N_e , and so how critical this estimation is. Without direct genetic testing of founders, a detailed examination of the studbook data is required to estimate N_e , taking into account factors such as family size, sex ratio, and reproductive success. However, given the loss of heterozygosity and alleles observed in this study (mean loss approximately 18% in one or two generations), a more conservative estimation of N_e is recommended.

The exception to the trend of loss of heterozygosity is the family of *N. leucogenys* (two parents and their five offspring). Here we observed an increase in average heterozygosity from 0.71 to 0.89. Both parents are homozygous for different alleles, at three out of 12 loci. Consequently, all offspring from this pair will necessarily be heterozygous for these loci. This case illustrates how even when we see an increase in homozygosity in one generation, if the alleles retained by individuals differ, they can be mixed when breeding and heterozygosity regained in the following generation. Thus, a reduction in heterozygosity may not necessarily be cause for concern in the short-term, as with awareness and careful management, heterozygosity can be recovered. Genetic testing is crucial to achieve informed maintenance, by enabling identification of which individuals are homozygous for which alleles, and so which animals should be paired.

Loss of alleles

In all cases, we see loss of alleles from one generation to the next, with a range of 3–31% loss, in these groups. The goal set by EAZA is to retain at least 90% of genetic diversity for 100 or more years (Lefaux et al. 2020); the *N. leucogenys* family was the only group to be on track to achieve this, retaining 97% of alleles for the generation observed (though this is not 100 years). This is the family group with the most offspring (five). Some alleles however, are only retained in a single offspring. If those individuals do not breed, these may still be lost in the subsequent generation. This underlines the importance of all individuals breeding where possible, in order to maximise retention of genetic diversity.

The EAZA conservation breeding programmes consist of multiple family units, so examining only one or two for a species (as reported here) will not necessarily provide an accurate picture for the population as a whole. This is illustrated by the *H. pileatus* families in this study. Each unit consists of two parents and two offspring, and neither achieved >90% allele retention. However, when combining the data for the two families the retention rate increased to 92% (see Table 4). Furthermore, where alleles were shared between the two sets of parents, and an allele was lost from one family, it was sometimes retained in the other, thus being retained in the population as a whole. It is possible that similar scenarios will occur throughout the EAZA populations, and that where variation is lost in one breeding group it may be retained by others. However, in order to gain a full picture of the patterns of genetic diversity occurring, ongoing genetic monitoring of these captive populations is required.

For effective preservation of genetic diversity, individuals should have as many offspring as resources allow. While it may theoretically be possible for all genetic information from two parents to be passed on to two offspring, this is unlikely. Random segregation in meiosis often results in some alleles being passed on multiple times while others are lost. In this study, only the breeding pair with five offspring retained the target level of genetic variation, but retention was not 100%. However, breeding pairs producing so many offspring is often not practical in the captive setting, due to space and resource limitation. Therefore, having as many breeding pairs as possible is also key for maintaining diversity in the wider population, which requires careful planning between holders in order to maximise both genetic and physical resources. Global cooperation between institutions across geographic regions (such as Europe, Australia, the USA, and Asia) increases the size of the population (and therefore N_e), which can be critical to maintaining genetic diversity of ex-situ populations. Because the movement of animals around the world is a significant undertaking, in terms of cost, stress, and bureaucracy, genetic analyses can provide important data to inform decisions about which individuals to move, and where. While loss of heterozygosity can be reversed to some extent, by genetically informed breeding, as described above, this is not the case for loss of genetic variation. Once a particular genetic trait has been lost from a group, it cannot be recovered except by re-introducing it from outside the population, which may simply not be possible. Consequently, it is of critical importance to take all possible measures to maintain extant variation. To gain a more complete picture of the loss or retention of diversity in the EAZA populations, as many individuals as possible, from the oldest and youngest generations should be tested. A comprehensive genetic profile, perhaps using the marker panel assembled here, of the whole captive population, would provide valuable empirical data to aid conservation breeding decisions.

Evidence of inbreeding

The diversity of alleles observed has been adequate to assess levels of inbreeding within four species groups currently managed by EAZA, where a sufficient number of samples were available (range 8–27). Deviation from Hardy-Weinberg equilibrium indicates that the current *S. syndactylus* captive population is inbred ($p=0.002$). It is important to stress that this result may be an artefact of the incomplete sampling of the breeding population, but is nevertheless a cause for concern. Our observations recommend wider testing of the *S. syndactylus* captive population, in order to determine whether this result is representative of the global collection.

Levels of inbreeding are usually estimated from studbook data. However, even where records are well maintained, this can only ever be an ‘informed guess’. Founders are typically assumed to be unrelated in these analyses. If this assumption is violated, and assessments are based purely on studbook data, the result may be a higher estimate of genetic diversity than is actually present (Putnam et al. 2022). The data presented in this study provide empirical evidence of the genetic status of the EAZA gibbons, and highlight where intervention may be required to maintain the genetic diversity of the populations.

Future work

Having established the utility of the microsatellite panel, it is recommended that testing continue within the EAZA populations to confirm and expand these results. We recommend that further microsatellite testing be performed on as many of the oldest extant generation(s) (and preserved samples from older generations as available), and the youngest generation of breeding captive gibbons. This analysis would offer more comprehensive

insights into what genetic diversity was previously present, and what remains. It would also reveal how heterozygosity levels have changed, and provide more data for assessing possible instances of inbreeding. This is especially recommended for the *S. syndactylus* population, in light of the indication of inbreeding revealed in this work. Similar testing could be performed on other captive populations, such as those in the United States, Australia, and Asia, to elucidate inbreeding in those programmes.

This study also demonstrated that blood samples are ideal for deriving full microsatellite profiles, but that hair and preserved blood spots (FTA cards) are less informative. Where blood samples are not available (in non-captive settings) alternative sample sources such as fecal samples can be used (Markvirya et al. 2022). Lowering reliance of genetic monitoring on blood samples, by using non-invasively collected samples, would greatly improve the opportunity and welfare impacts of these interventions both in captive and wild populations.

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