MOLECULAR ECOLOGY

Molecular Ecology (2016) **25**, 3540–3556 doi: 10.1111/mec.13716

Assessing current genetic status of the Hainan gibbon using historical and demographic baselines: implications for conservation management of species of extreme rarity

J. V. BRYANT,*† D. GOTTELLI,* X. ZENG,‡ X. HONG,‡ B. P. L. CHAN,§ J. R. FELLOWES,¶ Y. ZHANG,**†† J. LUO,†† C. DURRANT,* T. GEISSMANN,‡‡ H. J. CHATTERJEE† and S. T. TURVEY*

*Institute of Zoology, Zoological Society of London, Regent's Park, London NW1 4RY, UK, †Department of Genetics, Evolution & Environment, University College London, Gower Street, London WC1E 6BT, UK, ‡Bawangling National Nature Reserve Management Office, Changjiang Lizu Autonomous County, Hainan 572722, China, §Kadoorie Conservation China, Kadoorie Farm and Botanic Garden, Lam Kam Road, Tai Po, New Territories, Hong Kong, ¶The Kadoorie Institute, University of Hong Kong, Pokfulam Road, Hong Kong, **Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China, ††College of Life Sciences, Yunnan University, Kunming 650091, China, ‡‡Anthropological Institute, University Zurich-Irchel, Winterthurerstrasse 190, Zurich CH-8057, Switzerland*

Abstract

Evidence-based conservation planning is crucial for informing management decisions for species of extreme rarity, but collection of robust data on genetic status or other parameters can be extremely challenging for such species. The Hainan gibbon, possibly the world's rarest mammal, consists of a single population of ~25 individuals restricted to one protected area on Hainan Island, China, and has persisted for over 30 years at exceptionally low population size. Analysis of genotypes at 11 microsatellite loci from faecal samples for 36% of the current global population and tissue samples from 62% of existing historical museum specimens demonstrates limited current genetic diversity (Na = 2.27, Ar = 2.24, H_e = 0.43); diversity has declined since the 19th century and even further within the last 30 years, representing declines of ~30% from historical levels (Na = 3.36, Ar = 3.29, H_e = 0.63). Significant differentiation is seen between current and historical samples (F_{ST} = 0.156, P = 0.0315), and the current population exhibits extremely small N_e (current N_e = 2.16). There is evidence for both a recent population bottleneck and an earlier bottleneck, with population size already reasonably low by the late 19th century (historical N_e = 1162.96). Individuals in the current population are related at the level of half- to full-siblings between social groups, and full-siblings or parent–offspring within a social group, suggesting that inbreeding is likely to increase in the future. The species' current reduced genetic diversity must be considered during conservation planning, particularly for expectations of likely population recovery, indicating that intensive, carefully planned management is essential.

Keywords: bottleneck, conservation genetics, Critically Endangered, ghost alleles, *Nomascus hainanus*

Received 11 February 2015; revision received 13 May 2016; accepted 31 May 2016

Introduction

Conservation management decisions must be made rapidly to prevent species losses. For species of extreme rarity, which persist in single, geographically restricted populations reduced to handfuls of individuals, delays in decision-making can mean the difference between extinction and recovery (Groombridge *et al.* 2004; Turvey 2008; Grantham *et al.* 2009). As human pressures on global ecosystems intensify, more and more species are likely to decline to states of extreme rarity, making it

Correspondence: Jessica V. Bryant, Fax: +44 (0)20 7586 2870; E-mail: jessica.bryant@ioz.ac.uk

vital to develop methods that enable identification of appropriate and/or necessary management actions for such populations.

The need for evidence-based conservation, whereby robust empirical data on ecology, population dynamics and threats are used to guide management, is now widely accepted (Sutherland *et al.* 2004; Segan *et al.* 2011). However, for extremely rare species, which are most urgently in need of management action, robust data are often unavailable and collection of new data can be extremely challenging, as the very rarity of these species makes them difficult to study. It is therefore crucial that the suitability of the evidence-based approach is evaluated for such species.

A key consideration for effective conservation of small populations is the impact that a drastic reduction in population size, or bottleneck, can have upon genetic condition. Sudden population declines can lead to concomitant losses of genetic diversity, which can in turn impact long-term viability through reduced ability to withstand environmental change (Lindsey *et al.* 2013), compromised disease resistance (Siddle *et al.* 2007) and reduced survival/reproductive fitness (Swinnerton *et al.* 2004; Hemmings *et al.* 2012). Small populations are also more vulnerable to further diversity declines through processes which have minimal impacts in larger populations, notably chance loss of alleles through magnified effects of genetic drift, and increased probability of mating between related individuals (Frankham *et al.* 2009). A growing body of literature highlights the importance of contextualizing information on the current genetic condition of species of extreme rarity within the context of historical patterns of genetic status and a chronology of past population dynamics, to determine the implications of genetic factors for conservation management (Groombridge *et al.* 2009; Raisin *et al.* 2012; Bristol *et al.* 2013).

The Hainan gibbon (*Nomascus hainanus*) (Thomas, 1892) is the world's rarest ape, rarest primate and possibly rarest mammal species, consisting of a single population constrained to Bawangling National Nature Reserve (BNNR), Hainan Island, China (Appendix S1, Supporting information). Following a precipitous decline from ~2000 individuals in the 1950s due to habitat loss and hunting (Liu *et al.* 1984; Zhou *et al.* 2005), to a reported low of only 10 individuals by the early 1990s (Zhang 1992), the species has persisted as a single remnant population for over 30 years at exceptionally low population size, with estimates since the 1980s fluctuating between 10 and 25 individuals (Liu *et al.* 1989; Zhang & Sheeran 1993; Wu *et al.* 2004; Zhou *et al.* 2005; Li *et al.* 2010). The current population consists of ~25 individuals in three social groups: Group A (~11 individuals), Group B (seven individuals) and Group C (three individuals), together with a low, unknown number of solitary individuals (Turvey *et al.* 2015).

Despite the Hainan gibbon's Critically Endangered status, past genetic research has predominately consisted of inclusion in wider phylogenetic analyses (Su *et al.* 1995; Zhang 1995; Thinh *et al.* 2010a,b). Several authors have alluded to genetic consequences of its small population size, suggesting it may suffer from genetic constraints (Liu *et al.* 1989; Fellowes *et al.* 2008). Although the species exhibits no obvious sexual dimorphism before reproductive maturity, making it difficult to sex immature individuals visually, reports of nine of 12 offspring born between 1982 and 1989 being male have also led to concerns that the population exhibits an imbalanced sex ratio (Liu *et al.* 1989), which may limit mate availability, social group formation and population growth (Chan *et al.* 2005; Li *et al.* 2010). There has been speculation about incest and inbreeding constraining recovery, with concern that surviving individuals are likely closely related (Liu *et al.* 1989; Fellowes *et al.* 2008). However, there has only been one attempt to investigate the species' genetic status, through assessment of diversity in the mitochondrial DNA (mtDNA) control region (Li *et al.* 2010). Unfortunately, methodological issues associated with this study minimize its utility in understanding the species' current genetic health, including sampling limitations ($n = 6$ individuals, from one social group) which restrict representativeness, possible sequencing errors indicated by detection of four haplotypes in one social group, and failure to contextualize results against other gibbon species' genetic diversity or past Hainan gibbon diversity. Furthermore, the study provided no insights into fundamental demographic parameters of the current population, such as individual relatedness, inbreeding level or offspring sex ratio. These factors, which are crucial to understanding population viability, remain unknown.

The paucity of information on genetic health of the last Hainan gibbon population precludes accurate understanding of the role this factor may play in constraining population recovery. Comprehensive assessment of the species' current genetic status within the context of its genetic history is vital to inform conservation planning. Any genetic study of a species of such extreme rarity will inevitably be limited by sample size, although for tiny populations such as that of the Hainan gibbon, sampling even a handful of animals represents a substantial proportion of the total population and can provide important insights into demographic and population genetic status. On average, 52 species of mammals, birds and amphibians move one threat category closer to extinction each year (Hoffmann *et al.* 2010); therefore, studying species of extreme rarity will

likely become the norm rather than the exception for conservation managers and wildlife biologists; the challenge of assessing demographic and genetic status for such species will become increasingly commonplace and may require assessment of the relative effectiveness of multiple metrics. There is therefore a need to understand what information can feasibly be obtained from applying current analytical approaches to data sets and sample sizes that are restricted by species rarity.

Within these constraints, we therefore aimed to quantify genetic diversity of the current Hainan gibbon population and assess whether declines in diversity have occurred through known historical population reduction. We determined present and past genetic diversity of the species and any genetic differentiation between these temporal 'populations', evaluated genetic evidence for inbreeding and past genetic bottleneck, and estimated effective population size of the current and historical populations. Empirical data on population parameters that shed light on genetic and demographic factors that may affect population recovery are also required for accurate assessment of the species' current status. We therefore also assessed key genetic characteristics of the current population, including degree of relatedness and population sex ratio (including for offspring only). This comprehensive assessment represents a new baseline for understanding the possible influence that the Hainan gibbon's current genetic condition may have on long-term population viability, and assesses the extent to which standard scientific approaches can inform conservation planning for species of extreme rarity.

Materials and methods

Sample collection and DNA extraction

Faecal samples were collected opportunistically from the current population during fieldwork at BNNR in 2010–2011. Samples were collected immediately following observed defecations. All individuals of habituated Group B were sampled (samples B1–B7); single samples for one individual each were obtained from unhabituated groups A and C (samples A, C). Samples were preserved by adding silica gel beads (drying agent) to ~2–5 g of scat in 15-mL plastic tubes (Wasser *et al.* 1997; Goossens *et al.* 2003; Chambers *et al.* 2004); beads were regularly replaced upon saturation until samples were completely desiccated. Where available, additional sample material was kept in replicate silica-dried samples, with remaining material preserved in 70–90% ethanol. Samples were stored in cool dark conditions in the field and at 4 °C in the laboratory. DNA was extracted using QIAamp DNA Stool Kit (QIAGEN), with minor protocol modifications to enhance removal of impurities/inhibitors and increase DNA yield, and final elution volume of 120 μL to improve DNA concentration. For individuals where >1 sample was collected, multiple samples were extracted. DNA is not spread uniformly through faecal samples (Goossens *et al.* 2003), so where sample volume permitted, multiple independent extractions were taken to maximize probability of obtaining DNA.

Small samples (~5 × 2 mm) of skin, muscle or bone were obtained from 12 Hainan gibbon specimens in museum collections accessioned between 1899 and 1980, collected from Bawangling and elsewhere on Hainan (Appendix S2, Supporting information) and representing all but one of the historical specimens known at the time of this study (it was not possible to sample the holotype). DNA was extracted from skin/muscle using QIAamp DNA Micro Kit (QIAGEN) and from bone using QIAquick PCR Purification Kit (QIAGEN). A final elution volume of 100 μL and postelution addition of 5 μL of 1% Tween-20 (Sigma-Aldrich) was employed to increase DNA concentration.

While every precaution was taken to prevent contamination during sampling, to monitor possible human DNA contamination, hair samples (~10 freshly plucked hairs) were collected from all fieldworkers who collected samples and included as positive controls during DNA amplification. Blood samples from contemporary specimens of two gibbon species (*Hylobates lar*, *Nomascus concolor*: Zoological Society of London Blood and Tissue Bank) were also used as positive controls. DNA was extracted from control samples using DNeasy Blood & Tissue Kit (QIAGEN). Rigorous procedures were employed to minimize potential contamination throughout extraction, including stringent cleaning of surfaces/equipment with 10–40% bleach and/or exposure to ultraviolet radiation; extraction of samples for different individuals on separate occasions; museum sample processing in UV-irradiated fume hood to destroy contaminant DNA; and extraction of current, historical and control samples in physically separate laboratory areas in specialized facilities at Yunnan University, Kunming, China; Institute of Zoology, Zoological Society of London; and Royal Holloway, University of London.

Marker screening and genotyping

Gibbon-specific genetic markers are presently unavailable, so we amplified gibbon DNA using human-derived microsatellite primers via cross-species amplification (Goossens *et al.* 2000b, 2005; Vigilant & Bradley 2004). Thirty human microsatellite loci (Appendix S3, Supporting information) previously tested for gibbons

(*H. lar*, *Hylobates muelleri*; Clisson *et al.* 2000; Oka & Takenaka 2001; Chambers *et al.* 2004; Roeder *et al.* 2009) and <250 bp were screened; larger loci are problematic when amplifying DNA from noninvasive samples (Goossens *et al.* 2000b). Loci were tested using DNA extractions from three current population samples and both control gibbon samples.

Twenty-four microsatellite loci produced detectable PCR products for Hainan gibbon samples (Appendix S3, Supporting information) and were used for formal genotyping. Samples were amplified using fluorescently labelled forward sequences for each primer pair via PCR in a reaction volume of ~7 μL containing 2 μL (≤50 ng) template DNA, 1.5 μL (0.3 μM) primer, 0.02 μL bovine serum albumin (New England BioLabs) and 3.5 μL Multiplex PCR Mix (QIAGEN, final concentration 3 mM $MgCl_2$). The thermal profile for PCRs consisted of the following: denaturation and enzyme activation at 95 °C (15 min), 30–35 cycles of denaturation at 94 °C (30 s), annealing at relevant temperature (90 s) (Appendix S3, Supporting information), extension at 72 °C (60 s) and final extension at 72 °C (30 min). The 24 loci were divided into eight 'multiplex' mixes, each containing three loci. PCR products were visualized on an ABI PRISM 3130xl Genetic Analyser (Applied Biosystems) together with GeneScan 500 LIZ Size Standard (Applied Biosystems). Alleles were scored using GENEMAPPER V.4.1 (Applied Biosystems) against the internal size standard to derive individual genotypes at each locus.

Consensus genotypes were derived for each current and historical sample using a multitube, multisample approach and a strict set of a priori allele-scoring rules (Taberlet *et al.* 1996; Goossens *et al.* 2000a). At least five independent PCR replicates were genotyped for each extraction to minimize genotyping errors associated with low-quality template DNA (e.g. false alleles, allelic dropout) (Taberlet *et al.* 1999); multiple extractions/sample and samples/individual were genotyped where sample volume/number permitted. We calculated the mean quality index across samples and loci to assess genotyping reliability (Miquel *et al.* 2006).

To ensure standardized allele sizes between samples/replicates, PCRs were prepared in physically isolated areas to prevent cross-contamination but amplified simultaneously (same PCR), with reference samples (high-quality DNA extracted from a current sample) included in every PCR. Positive (human, gibbon) and negative controls from every stage (extraction blanks and PCR blanks) were also included during genotyping to monitor potential contamination and PCR failure. Current population sample genotypes were verified by checking for consistent allele sharing between individuals in the current population with known parentage. Loci monomorphic for the species and those that failed to amplify across both current and historical samples despite extensive replication were discounted, resulting in consensus genotypes from 13 polymorphic loci (Appendix S4, Supporting information).

Genotyping errors due to false alleles, allelic dropout, stutter and null alleles were checked using MICRO-CHECKER V.2.2.3 (van Oosterhout *et al.* 2004). LOSITAN (Antao *et al.* 2008) was used to detect loci under selection, using 100 000 simulations and a 0.95 confidence level for neutral markers; loci falling outside this confidence interval were considered non-neutral and excluded from analysis. Between-locus linkage disequilibrium was tested in F-STAT V.2.9.3.2 (Goudet 2002).

Sex determination

To investigate the remaining population's sex ratio, all current samples were genotyped using a fluorescently labelled Amelogenin primer (Sullivan *et al.* 1993). Amelogenin amplification products have short fragment lengths (<120 bp), making amplification viable for degraded DNA (Bradley *et al.* 2001). PCR amplification was conducted as previously described (with T_a = 55 °C). Human and gibbon controls were included in all PCR replicates, and the primer was incorporated into a multiplex mix (where allele sizes in humans vs. gibbons were obviously different for other primers) to ensure derived Amelogenin genotypes were from gibbon samples. PCR replication and genotype-scoring rules were applied as above to obtain consensus genotypes for each individual.

Temporal change in genetic diversity

Marker polymorphism was assessed by determining number of alleles/locus (*Na*), number of unique alleles/locus (*Pa*), observed heterozygosity (H_o) and expected heterozygosity (H_e) for each locus for each population and across loci for each population, using F-STAT V.2.9.3.2 (Goudet 2002). As sample size can effect estimates of allelic diversity (*Na* and *Pa*), per-locus and overall unbiased estimates of allelic richness (*Ar*) and unique allelic richness (*Pr*), taking into account small sample size, were calculated for each population using rarefaction within HP-RARE V.1.1 (Kalinowski 2004, 2005), applying a minimum sample of seven diploid individuals (i.e. number of genes, g = 14).

Differences in diversity between current and historical populations, and potential diversity loss over time, were assessed by comparing *Na* and H_e. *Ar* is more sensitive to bottleneck effects than other diversity measures using microsatellite data: alleles can be lost rapidly from these loci via genetic drift following a bottleneck,

with allelic diversity (dependent on effective population size and within-population allele number/frequency) declining more rapidly than heterozygosity (dependent only on effective population size) (Spencer *et al.* 2000; Leberg 2002; Keller *et al.* 2012). We further compared *Ar* (and *Pr*) between current and historical populations to determine whether declines were due to loss of alleles from the historical population. As samples were limited ($<2g = 28$) for both populations, the nonparametric Wilcoxon signed-rank test within R V.3.0.1 (R Development Core Team 2013) was used to investigate declines.

Genetic differentiation of current and historical populations

Differentiation between current and historical populations was examined with the fixation index (pairwise F_{ST}; Cockerham & Weir 1993), calculated using F-STAT V.2.9.3.2 with a randomization approach to test for significance (Goudet 2002); with principal coordinate analysis (PCoA) using pairwise genetic distances between all samples within GENALEX V.6.5 (Peakall & Smouse 2006); and with a Bayesian clustering approach within STRUCTURE V.2.3.4 (Pritchard *et al.* 2000). We assessed extent of partitioning by exploring a range of values for the number of populations prior, '*K*', with $K = 1$–8 and five replicates/*K*, using 100 000 iterations following a burn-in of 10 000 iterations, after which we obtained consistent and convergent results (Pritchard *et al.* 2000, 2010). An admixture model and independent allele frequencies were adopted, as appropriate for closely related populations but where allele frequencies may be reasonably different (Pritchard *et al.* 2000). Optimal *K* was determined using the Δ*K* approach (Evanno *et al.* 2005) implemented in STRUCTURE HARVESTER V.0.6.93 (Earl & vonHoldt 2012). Related individuals within a sample may create a false signal of population genetic structure or overestimate cluster number (Rodríguez-Ramilo & Wang 2012); we confirmed the pattern and extent of population structure using CLUSTER_DIST, which maximizes between-group genetic distances and does not make Hardy–Weinberg and linkage equilibrium assumptions (Rodríguez-Ramilo *et al.* 2014) (Appendix S5, Supporting information).

Population bottleneck

Genetic evidence for a past bottleneck was assessed via graphical investigation of mode allele frequency shift between historical and current populations (Luikart *et al.* 1998) by grouping alleles across polymorphic loci for each population into 10 frequency classes (0.001–0.100, 0.101–0.200 etc., until 1.0) and comparing resultant histograms. We also used BOTTLENECK V.1.2.02 (Cornuet & Luikart 1996) to assess mode-shift and evaluate heterozygosity excess under four mutation models, using three significance tests (sign, standardized differences, Wilcoxon signed-rank). Microsatellites rarely confirm strictly to either the infinite allele mutation (IAM) model or stepwise-mutation model (SMM); therefore, a two-phase model (TPM) accommodating both mutation types (Di Rienzo *et al.* 1994; Piry *et al.* 1999) was also adopted with 70% and 90% SMM, respectively (thus 30% and 10% IAM).

Effective population size

To determine current effective population size (N_e) and assess evidence for temporal change in N_e, we employed multiple 'single-sample' approaches: linkage disequilibrium, heterozygosity excess and molecular coancestry within NEESTIMATOR V.2.0 (Do *et al.* 2013), and full-likelihood sib-ship assignment (Wang 2009) within COLONY V.2.0.4.5 (Jones & Wang 2010). We also adopted a Bayesian approach implemented in TMVP (Beaumont 2003), sampling independent genealogical histories from temporally spaced gene frequency data (all samples, pooled) to give a posterior distribution of estimated historical N_e (time of oldest historical sample) and current N_e (time of youngest sample). Allele frequencies were calculated for dated historical and current samples, with time measured in gibbon generations (15 years; Chivers *et al.* 2013) since sample collection, and a rectangular (uniform) prior of (0, 5000) employed for estimation of historical and current N_e. We determined joint mode of the posterior distribution of historical and current N_e estimates, discarding the first 1% (100 estimates) of the simulated 10 000 estimate chain as burn-in, applying a smoothing parameter of $\alpha = 0.6$ (after exploring $\alpha = 0.3$–0.7) within R V.3.0.1 (R Development Core Team 2013). This α-value was subsequently employed to determine the 95% higher posterior density (HPD) limits of each N_e, as it produced a sharp joint mode located away from the upper limit of priors for either N_e.

Inbreeding and relatedness

Inbreeding within current and historical populations was assessed by comparing H_e to H_o and assessing the inbreeding coefficient (F_{IS}) (Weir & Cockerham 1984). Inbreeding-driven deviations from HWE were evaluated by estimating F_{IS} for each locus and across all loci for each population using F-STAT V.2.9.3.2 (Goudet 2002). Relationships between individuals in the current population were investigated using COLONY V.2.0.4.5 (Jones & Wang 2010) to infer parentage and full/half sib-ship relations over the entire population and determine the

best configuration of relationships under maximum likelihood (ML). We used a 0.01 genotyping error rate, polygamous mating system and 0.5 probability that an actual father/mother of an offspring was included in candidate father/mother data sets. As all current samples originated from one population, we retained all links from the best configuration. The coefficient of relatedness (r) was determined using ML-RELATE (Kalinowski *et al.* 2006) to estimate pairwise relatedness between all individuals in the current population. ML r estimates and ML configuration indicated degree and structure of relatedness within the current population over >1 generation to reveal probable shared parentages that likely produced observed relatedness/relationships.

Sex ratios

Sex-determination (Amelogenin) consensus genotypes were used to calculate sex ratios of all individuals sampled from the current population, all individuals in Group B and immature offspring within Group B.

Results

Marker characteristics

No evidence of null alleles, allelic dropout or scoring error due to stutter was detected for the final 13 loci. LOSITAN simulation results identified two loci (D17S804, D20S206) falling outside the 95% quantile for neutral markers; data for these loci were excluded from further analyses. Significant linkage disequilibrium was detected between two further pairs of loci in the current population and one pair in the historical population ($P < 0.05$; Appendix S6, Supporting information); these patterns were not consistent across both populations or the entire sample for any one pair of loci, so all 11 loci were retained given the small final number of markers. Consensus genotypes for these loci were obtained for nine living individuals and eight museum specimens (final data set: mean quality index = 0.78, missing data percentage = 2.67%), representing 36% of the current global population and 61.5% of known historical specimens.

Temporal change in genetic diversity

Genetic diversity in both temporal 'populations' was low, with small *Na*, *Ar* and H_e values observed for each locus and small overall average values for each population (current: *Na* = 2.273, *Ar* = 2.240, H_e = 0.431; historical: *Na* = 3.364, *Ar* = 3.290, H_e = 0.626; Table 1). Current diversity was lower than historical diversity for all metrics (Table 1); across all loci, *Na* was 32% lower in the current population (one-sided Wilcoxon $W = 99.5$, $P = 0.0031$), *Ar* was 32% lower ($W = 103$, $P = 0.0023$), and H_e was 31% lower ($W = 97$, $P = 0.0090$). 'Historical' alleles have been lost over time, with alleles in the historical population absent from the current population at seven of 11 loci, and a significantly lower current *Pr* ($W = 93$, $P = 0.0139$).

Table 1 Genetic diversity for 11 selected polymorphic microsatellite loci and summary statistics (overall mean or total ± SE) of genetic diversity of current and historical populations across all loci

	Historical population (n = 8)							Current population (n = 9)						
Locus	*Na*	*Pa*	*Ar*	*Pr*	H_o	H_e	F_{IS}	*Na*	*Pa*	*Ar*	*Pr*	H_o	H_e	F_{IS}
D7S817	5	4	4.750	4.000	0.625	0.750	0.176	2	1	2.000	1.000	1.000	0.529	−1.000
DQCar	4	2	3.867	2.000	0.750	0.592	−0.292	2	0	2.000	0.008	0.889	0.523	−0.778
D1S548	3	0	3.000	0	0.875	0.692	−0.289	3	0	2.961	0	0.778	0.621	−0.273
HPRT1	3	1	3.000	1.000	0.286	0.670	0.593	2	0	2.000	0	0.222	0.366	0.407
D9S302	2	0	2.000	0	0.286	0.440	0.368	2	0	2.000	0	0.222	0.366	0.407
DXYS156	2	0	2.000	0	0.857	0.527	−0.714	2	0	1.778	0	0.111	0.111	0.000
D5S1470	3	2	3.000	2.000	0.571	0.615	0.077	2	1	1.995	0.995	0.333	0.294	−0.143
DXS8043	3	1	3.000	1.000	0.429	0.692	0.400	2	0	2.000	0	0.444	0.471	0.059
D6S265	5	1	4.867	1.000	0.500	0.767	0.364	4	0	3.956	0.125	1.00	0.725	−0.412
D2S367	4	3	3.875	3.000	0.500	0.717	0.317	2	1	2.000	1.000	1.00	0.529	−1.000
D5S1457	3	1	2.875	1.000	0.375	0.425	0.125	2	0	1.961	0	0.222	0.209	−0.067
Overall	**3.364**	**1.364**	**3.290**	**1.340**	**0.550**	**0.626**	**0.129**	**2.273**	**0.273**	**2.240**	**0.280**	**0.566**	**0.431**	**−0.337**
S.E.	0.310	0.388	0.288	0.358	0.063	0.036	n/a	0.195	0.141	0.194	0.139	0.111	0.055	n/a

Diversity measures: number of alleles/locus (*Na*), number of unique alleles/locus (*Pa*), allelic richness (*Ar*), unique allelic richness (*Pr*), observed heterozygosity (H_o), expected heterozygosity (H_e). Inbreeding estimator (F_{IS}) was used to detect deviations from HWE for each locus and each population. Overall values (bold) represent population values: average *Na*, *Pa*, *Ar*, *Pr*, H_o, H_e, overall F_{IS}.

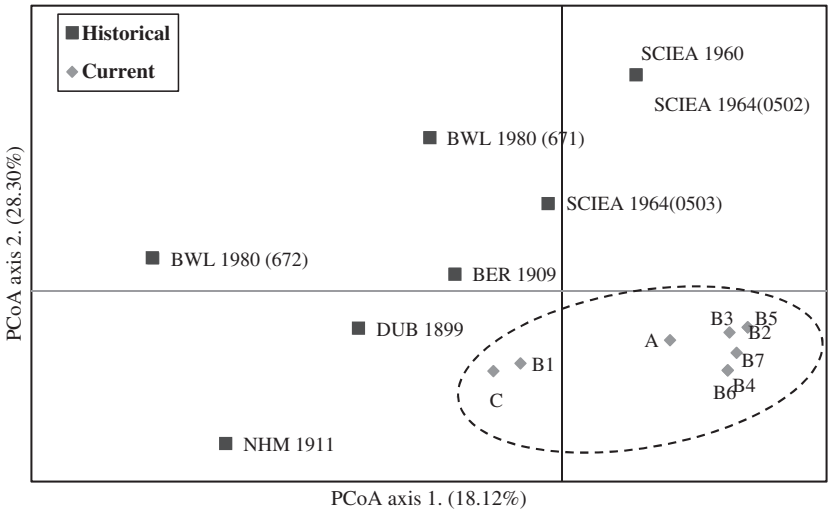

Fig. 1 PCoA based on pairwise genetic distances between historical (squares) and current (diamonds) samples. Proportion of total variance explained by each axis indicated in parentheses; dashed oval encircles current samples.

Genetic differentiation of current and historical populations

Current and historical populations showed significant differentiation, with 16% of total observed genetic variation distributed between populations and 84% within populations (pairwise F_{ST} = 0.156, P = 0.0315), suggesting substantial divergence of the current population. PCoA confirmed temporal differentiation between populations and revealed additional divergence patterns (Fig. 1). Current samples clustered along axis 2, but the eldest living individual (B1) and groups A and C individuals diverged slightly from other Group B individuals on axis 1. Historical samples dispersed along both axes, clustering loosely but away from current samples, indicating greater genetic variation compared to current samples and divergence between current and historical populations.

Bayesian cluster analysis distinguished three genetic populations (peak ΔK at K = 3 clusters) (Fig. 2). Ninety-five per cent of current samples fell into one cluster, indicating differentiation from historical samples (Fig. 3). Historical samples subdivided into two populations (47.2%, 49.2%) corresponding approximately to a split between older samples (1899–1911) and younger samples (1960–1980s); however, one sample from the 1980s (BWL672) clustered more often with older samples as this sample retained some 'older' alleles. Two current samples (B1, C) occasionally clustered with the population comprising samples from 1899 to 1911 plus BWL672, again due to these individuals retaining alleles otherwise only present in historical populations. This pattern was supported by CLUSTER_DIST analysis, which identified additional clusters in the historical sample (corresponding to further temporal partitioning of specimens) but grouped most current samples into one cluster/population, and assigned two current samples (B1, C) to another cluster along with two historical samples (Appendix S5, Supporting information), indicating that observed population structure was not solely the result of close relationships between individuals in the current population.

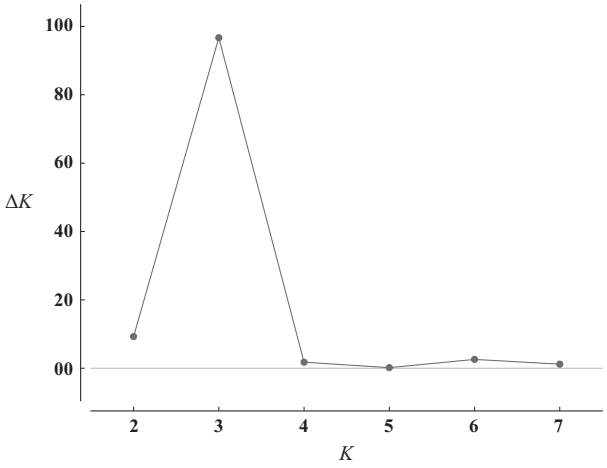

Fig. 2 Second-order rate of change of likelihood function with respect to K (ΔK) over successive K values. Peak indicates modal value of ΔK distribution corresponding to optimal K.

Population bottleneck

The historical population exhibited the L-shaped allele frequency distribution expected for nonbottlenecked populations; many alleles fall into low-frequency classes (0.001–0.2), and few fall into intermediate-frequency (0.201–0.8) or high-frequency (0.801–1.0) classes (Fig. 4). The current population showed fewer alleles in low-frequency classes and more in higher-frequency classes.

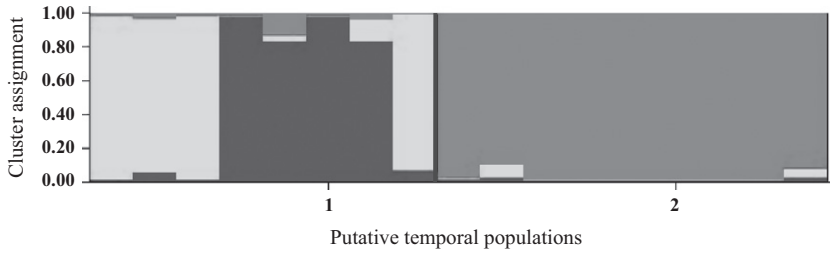

Fig. 3 Population assignment of current and historical samples by STRUCTURE into three genetically distinct populations (different shades of grey). Each individual represented as separate vertical bar sectioned into shaded segments representing different genetic units. Segment length is proportional to likelihood of assignment (proportion of times in 100 000 iterations) of individual to that population. Putative temporal populations shown as 1 = historical, 2 = current separated by black line, although this information was not used a priori for analysis.

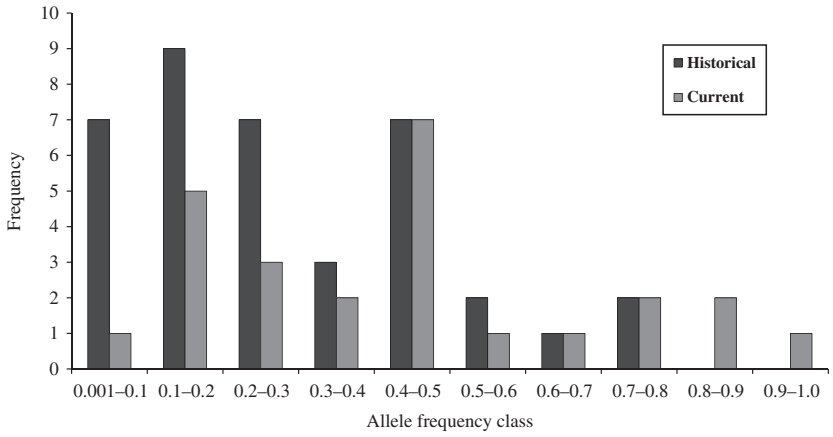

Fig. 4 Distribution of allele frequencies for historical and current populations across all loci.

Mode allele frequency across all loci in the historical population was 0.188, but was higher in the current population at 0.5; this mode-shift was confirmed within BOTTLENECK.

For the current population, all three significance tests indicated significant heterozygosity excess under IAM. Two tests indicated significant excess under the more conservative TPM with 70% SMM; TPM with 90% SMM indicated heterozygosity excess using the Wilcoxon test only (Table 2). Significant heterozygosity excess was also revealed in the historical population for two of three tests under IAM and TPM with 70% SMM and from the Wilcoxon test under TPM with 90% SMM. Although the test statistic only approaches a normal distribution if >20 loci are used (Cornuet & Luikart 1996), the nonparametric Wilcoxon test remains robust using few polymorphic loci (Piry *et al*. 1999) and indicates both current and historical populations show a genetic signal consistent with a bottleneck.

Effective population size

All single-sample estimates reported exceptionally low current N_e (Table 3). Estimates of historical N_e varied substantially, but generally showed slightly larger values. However, large confidence intervals for all estimates limit comparison between temporal populations. This apparent lack of difference in historical vs. current N_e could reflect limitations of single-sample approaches for small n, or stable low N_e over time pre-dating mid-20th-century decline. Accuracy of single-sample estimates is likely limited and may only approximate true N_e.

Bayesian estimation of N_e at the time of oldest and youngest samples (1899–2011) was more informative and indicated temporal change in N_e (Fig. 5a). Density of points in the posterior distribution is proportional to the probability density of historical and current N_e; the off-diagonal distribution of points indicates that current N_e is not equal to historical N_e. The exceptionally flat posterior distribution, with points densely concentrated along the x-axis (historical N_e), provides strong evidence of decline in N_e between 1899 and 2011 and indicates current N_e is very low. The joint mode (and 95% HPD limits) for the marginal from the density estimation is as follows: historical N_e = 1162.96 (95% HPD limits = 55.64–4129.95), current N_e = 2.16 (95% HPD limits = 0.98–4.18) (Fig. 5b). Estimation of historical N_e is

Table 2 Results from heterozygosity excess tests in BOTTLENECK using three significance tests and four models of allele mutation (IAM, SMM, two TPM variants)

		Historical population				Current population			
Test	Test statistic/probability variant	IAM	SMM	TPM (70% SMM)	TPM (90% SMM)	IAM	SMM	TPM (70% SMM)	TPM (90% SMM)
Sign test	Expected number loci with heterozygosity excess	6.16	6.50	6.36	6.60	5.66	5.72	5.29	5.56
	Observed number loci with heterozygosity excess	9	8	9	9	9	8	8	8
	Probability (*P*-value)	0.073	0.274	0.092	0.118	**0.040**	0.140	0.090	0.119
Standardized differences test	T2	2.65	1.15	1.92	1.54	2.32	1.37	1.75	1.54
	Probability (*P*-value)	**0.0040**	0.124	**0.028**	0.062	**0.010**	0.086	**0.040**	0.061
Wilcoxon signed-rank test	Probability (one tail for heterozygosity deficiency)	0.999	0.926	0.992	0.966	0.994	0.913	0.966	0.966
	Probability (one tail for heterozygosity excess)	**0.001**	0.087	**0.011**	**0.042**	**0.008**	0.103	**0.042**	**0.042**
	Probability (two tails for heterozygosity excess/deficiency)	**0.002**	0.175	**0.021**	0.083	**0.016**	0.206	0.083	0.083

IAM, infinite allele mutation; SMM, stepwise-mutation model; TPM, two-phase model.
Significant *P*-values (<0.05) indicated in bold.

Table 3 N_e estimates of current and historical populations inferred via linkage disequilibrium (LD), heterozygosity excess (HE), molecular coancestry (Coan.) and full-likelihood sib-ship assignment (FL); 95% confidence intervals in parentheses

Population	LD (95% CI)	HE (95% CI)	Coan. (95% CI)	FL (95% CI)
Historical	2.8 (1.5–16.5)	Infinity (8.5–infinity)	4.4 (2.1–7.6)	16 (7.0–86)
Current	3.1 (1.2–infinity)	2.6 (1.4–infinity)	1.8 (1.0–2.9)	4 (2.0–20)

somewhat uncertain, indicated by the large 95% HPD limit range; however, N_e was certainly larger in the late 19th century.

Inbreeding and relatedness

For the current population, H_o was generally greater than H_e at each locus (nine of 11 loci), producing an overall trend of mean H_o > mean H_e (Table 1). The opposite was true of the historical population ($H_e > H_o$ for eight of 11 loci). Although no estimated F_{IS} values were significant for any locus or overall for the historical population (all *P*-values > 0.05), F_{IS} values of three loci and the overall estimate for the current population were significant (all *P*-values < 0.05), indicating nonrandom mating (outbreeding) within the current population.

Overall, ML *r* estimates indicated a high level of relatedness between all individuals in the current population (Appendix S7, Supporting information). Average relatedness across sampled individuals was high, roughly between half- and full-sibs (mean $r = 0.34 \pm 0.05$). Average relatedness within Group B was approximately full-sibs or parent–offspring (mean $r = 0.45 \pm 0.07$). Between social groups, relatedness was slightly lower, between cousins and half-sibs. Relatedness of groups B and C (mean $r = 0.18 \pm 0.09$) was similar to that of groups A and B (mean $r = 0.16 \pm 0.07$), approximately at the level of cousins; relatedness of groups A and C was slightly higher ($r = 0.38$, no SE as comparison between two individuals only), at least half but almost full-sibs. ML configuration of relationships supported these results and revealed additional between-individual relationships, including some that suggest possible past inbreeding (Appendix S7, Supporting information).

Sex ratios

Amelogenin consensus genotypes were obtained for all sampled individuals from the current population and confirmed the sex in all cases where an individual's sex

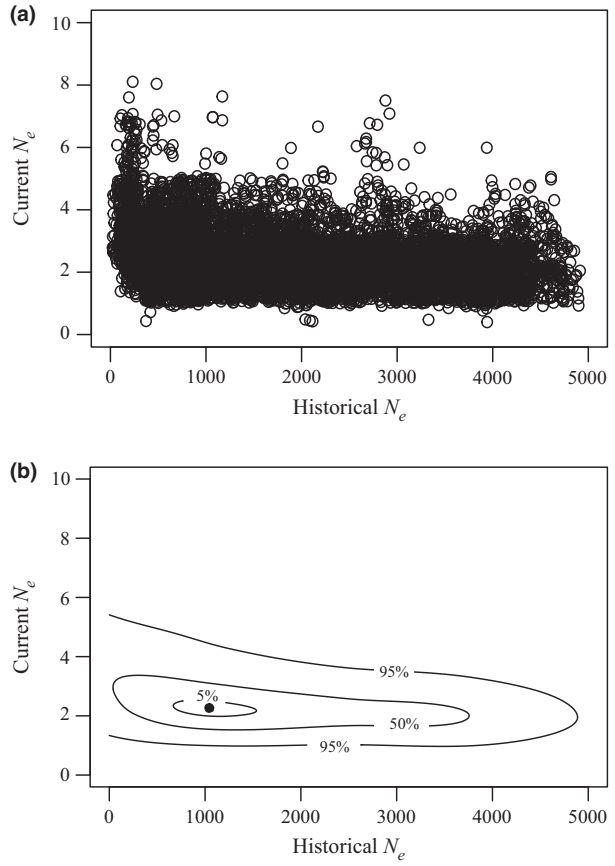

Fig. 5 Bayesian estimates of historical and current N_e using TMVP: (a) posterior distribution of historical and current N_e, with density of points proportional to probability density of N_e at time of oldest and youngest samples; (b) 5%, 50% and 95% higher posterior density limits of posterior distribution and joint mode of historical and current N_e (single solid circle).

was already known (three males, two females). The overall sampled population's sex ratio was two males per female (Table 4). Group B had a ratio of four males to three females; this group's immature offspring included three males and one female.

Discussion

This study represents the first investigation of the genetic status of the sole remaining Hainan gibbon population within the context of its genetic history, and demonstrates this population is genetically impoverished following a substantial crash in N_e consistent with the species' range decline. Both historical and current samples show low levels of polymorphism compared with limited available data on diversity in other gibbon populations (*Hylobates lar*: *Na* = 7.0, H_e = 0.725, Chambers *et al.* 2004; *H. muelleri*: *Na* = 14.8, Oka & Takenaka 2001; Appendix S3, Supporting information). Although this low diversity may partly reflect cross-amplification, these other studies also cross-amplified many of the same human microsatellites and found greater diversity. Furthermore, despite low diversity in both samples, we detected significant reductions in heterozygosity and allelic diversity over time, indicating that low diversity is not a long-term pattern; instead, diversity has declined since the 19th century and even further within recent decades. This decline was detected even with limitations to available data (sample size, number of loci amplified across all samples); these estimates are therefore probably conservative with respect to amount of diversity lost. This detected decline also corresponds with differences in diversity reported for many threatened species, which on average possess ~65% of microsatellite diversity of phylogenetically related, nonthreatened taxa (Frankham *et al.* 2009). Similar diversity declines following severe bottlenecks are known from other threatened species: H_e declined by 52% and *Na* by 32% in black-footed ferrets (*Mustela nigripes*) (Wisely *et al.* 2002), and H_e declined by 57% and *Na* by 55% in Mauritius kestrels (*Falco punctatus*) (Groombridge *et al.* 2000). 'Ghost' alleles (Bouzat *et al.* 1998) present in the historical gibbon population were absent from the current population for several loci, indicating loss of alleles over time due to a bottleneck. The overall pattern detected by PCoA, with broadly distributed historical samples and tightly clustered current samples, also supports the detected diversity decline, likely reflecting 20th-century gibbon range contraction across Hainan (Appendix S1, Supporting information; Liu *et al.* 1984; Zhou *et al.* 2005).

This decline has led to significant differentiation of current and historical populations. Observed

Table 4 Sex ratios for current population and results of tests for deviation from 1:1 sex ratio using Yates-corrected Pearson's chi-squared statistic

Sex ratio tested	Females	Males	Observed ratio (female:male)	Yates-corrected chi-square test (χ^2) against 1:1	
Sampled population (n = 9)	3	6	1:2	Yates χ^2	0.44
				P	0.50
Group B (n = 7)	3	4	3:4	Yates χ^2	0
				P	1
Group B offspring (n = 4)	1	3	1:3	Yates χ^2	0.25
				P	0.62

Degrees of freedom for all tests = 1; sample size used to calculate ratios indicated.

differentiation is virtually identical to differentiation in island populations of Kloss' gibbon (*Hylobates klossii*) that have been isolated for *c.* 7000 years (F_{ST} = 0.157; Whittaker 2009) and comparable to that in partially/completely disconnected subpopulations of other threatened mammals, for example Sumatran tiger (*Panthera tigris sumatrae*) (Smith 2012) and Ethiopian wolf (*Canis simensis*) (Gottelli *et al.* 2004). Assignment of 5% of the current population to inferred historical populations (Fig. 3) indicates that most current-day individuals have a different genetic signature to the historical population, but a small amount of historical genetic structure may persist today, contained within genotypes of two sampled individuals (B1, C). As B1 is already postreproductive, having not given birth since 2000 (based on ongoing field observations: Li *et al.* 2010; Bryant 2014), diversity will decline further following her death and current population structure may become increasingly distinct.

Island species may show diminished genetic diversity resulting from founder effects, evolutionary histories of sustained isolation and small N_e, or recent population crashes (Frankham 1997; Groombridge *et al.* 2009). Evidence of a recent bottleneck having produced the observed diversity reduction in the current gibbon population is compelling. There has been a clear shift in mode of allele frequencies between temporal populations and clear reduction in N_e from time of the oldest sample. The observed ratio of current N_e to current population size (2.16:25 = 0.086) compares closely to that found across 102 species (mean ratio = ~0.10; Frankham 1995), providing support for our N_e estimate. The acutely low N_e is particularly alarming, as previous studies indicate that $N_e < 50$ can significantly decrease population viability (Westemeier *et al.* 1998; Madsen *et al.* 1999), with $N_e \geq 1000$ required for long-term evolutionary potential (Frankham *et al.* 2014). Our analysis does not permit determination of exact timing of population crash, but the steepest decline probably occurred after collection of the youngest historical samples (1980), when Hainan experienced extensive deforestation (Li 2004) and BNNR received limited financial support due to changing political administration in the early 1990s, reportedly leading to forest loss and gibbon poaching (Zhang 1992; Zhang & Sheeran 1993).

Interestingly, there is also evidence for an older bottleneck, with significant heterozygosity excess and small N_e (estimated by single-sample metrics) detected for both temporal samples, and relatively low polymorphism already shown by historical samples. This could reflect limitations of approaches employed for given sample sizes: all single-sample N_e estimators lose power with small sample sizes ($n < 20$ individuals) and/or restricted numbers of markers (England *et al.* 2006; Wang 2009). Indeed, single-sample estimates of historical N_e (2.8–16) were lower than expected for the historical period (1899–1980); anecdotal population estimates suggest ~2000 individuals remained in the 1950s (Liu *et al.* 1984) and ~500 individuals in the 1970s (Zhou *et al.* 2005). Other factors beyond bottlenecks can generate heterozygote excess, especially in small populations (e.g. selection, dioecy, unequal sex ratios, polygamous mating; Storz *et al.* 2001; Balloux 2004), potentially explaining this pattern. However, Bayesian assessment of changing N_e over time revealed both temporal decline in N_e, supporting past population bottleneck, and a more realistic historical N_e, indicating that population size when the oldest sample was collected was already relatively small; the N_e of ~1000 implies a population of ~10 000 gibbons in the late 19th century, although the large HPD confidence limits show uncertainty around this estimation. Possible reduced Hainan-wide gibbon abundance >100 years ago is supported by contemporary historical accounts, which describe the species as already rare (Swinhoe 1870). Other gibbon species are also known to have declined across China by the 19th century (Wen 2009), and other mammals reportedly present in Hainan during the Ming-Qing dynasties (e.g. records possibly referring to dhole *Cuon alpinus* and Père David's deer *Elaphurus davidianus*) had disappeared by the 20th century (Dobroruka 1970; Wen 2009) likely due to historical persecution or overexploitation, suggesting that Hainan's mammal fauna was already being impacted by human activities. The Hainan gibbon may therefore have suffered substantial decline even before its 20th-century population crash.

We found evidence for a reduction in inbreeding due to nonrandom mating in the current population, with heterozygote excess ($F_{IS} < 0$), indicating that the species' mating behaviour may favour mating between less related individuals. Polygynous mating can generate heterozygote excess in populations characterized by such mating, which can produce negative F_{IS} values (Storz *et al.* 2001). The Hainan gibbon forms large polygynous groups which may be the normal social structure for this species (Bryant *et al.* 2015); this and/or other factors which create unequal sex-specific gene frequencies through binomial sampling error may have driven observed heterozygote excess (Balloux 2004). Inbreeding is lower than expected under random mating, but must still be high given evidence for mating between related individuals in the population pedigree. This is unsurprising if we consider the 'pedigree' definition of inbreeding (individuals are considered inbred when parents are related) instead of the 'nonrandom mating' (F_{IS}) definition (Keller & Waller 2002), as mating between relatives will occur in small populations even under random mating (Keller *et al.* 2012). Indeed,

individuals in the current population appear related at the level of half- to full-siblings between social groups, and full-siblings or parent–offspring within Group B. Crosses at even half-sibling level will theoretically increase nonrandom mating and the inbreeding coefficient by 0.15 after only two generations, reaching 1.0 (complete inbreeding) after ~20 generations (Hartl & Clark 1997). Li *et al.* (2010), using only six samples from one group, reported four haplotypes within Group B. Our results indicate higher levels of relatedness, with only two maternal lines present in this group's pedigree (Appendix S6, Supporting information). These results may reflect restricted sampling and/or low polymorphism for the few loci genotyped, implying closer relationships than actually exist (Kalinowski *et al.* 2006; Jones & Wang 2010). However, our pedigree was derived from more extensive sampling and is likely to represent relatedness more accurately than the previous assessment. Kenyon *et al.* (2011) detected full-sibling relationships between adults from neighbouring yellow-cheeked gibbon (*Nomascus gabriellae*) groups in Vietnam, suggesting levels of relatedness between Hainan gibbon social groups seem realistic; while direct comparisons are limited by differences in methodology and social/mating systems, studies of other bottlenecked populations of threatened taxa have revealed similarly elevated relatedness levels within social clusters (Taylor *et al.* 1997; Hagell *et al.* 2013). Data limitations for other gibbons make it difficult to assess whether our results indicate closer-than-average relatedness; however, as there are no unrelated potential mates within the remaining Hainan gibbon population, mating between individuals with high levels of relatedness and thus inbreeding, along with probability of genetic identity by descent, is already very high and will only increase.

Molecular sex determination suggested a male-biased offspring ratio in Group B, supporting previous suggestions based upon visual observations (Liu *et al.* 1989). Assuming equal probability to produce either sex, we might observe a ratio at least this male-biased 7.3% of the time by chance. However, several small ex situ gibbon populations display similarly male-skewed sex ratios at birth; Jago & Melfi (2010) detected male bias of 67–90% for three gibbon species kept in zoos despite captive management and demonstrated a significant statistical association with gross energy within captive diet, with females on lower-calorie diets more likely to produce male offspring. Given concerns regarding potentially suboptimal habitat quality at Bawangling, which may be close to gibbon elevational limits and ecologically marginal (Chan *et al.* 2005; Turvey *et al.* 2015), these findings have important management implications.

Our study possesses limitations inherent in all conservation genetics studies of extremely rare species: issues of sample size and potentially reduced statistical power, with inferential power of diversity analyses and detection of diversity declines constrained by small n, unavoidably biased sampling of the current population, and number of loci used to characterize past and present genetic status. However, n has been found to have little effect on H_e or pairwise F_{ST}, even with only five individuals genotyped at 10 loci (Smith 2012). Furthermore, we detected significant biological effects despite our reduced sample, meaning that these effects must be substantial to be detected. Although low diversity detected within the current population may partly reflect sampling bias (seven individuals from one group, but only one individual each from other groups), detection of a bottleneck during the last century suggests that observed diversity more likely reflects drastic population reduction, and our results are consistent with historical population size estimates (Liu *et al.* 1984, 1989; Zhou *et al.* 2005).

Detected diversity declines may reflect temporal and spatial sampling bias, both unavoidable constraints of limited sample availability. Such drawbacks to temporal comparisons in critically small populations are not unique to this study (Groombridge *et al.* 2000, 2009; Gottelli *et al.* 2004; Holbrook *et al.* 2012). Historical samples with successful DNA extraction ($n = 8$) spanned an 81-year period and were probably collected from localities across Hainan (Appendix S2, Supporting information). By comparison, current samples represent a temporal snapshot from one location. However, at least half the successfully amplified historical samples were definitely from the same location as current samples (Bawangling region), reducing spatial sampling bias; remaining samples lack adequate collection data to determine precise geographic provenance. Bayesian assessment of change in N_e, incorporating dates for each sample, supported a past population bottleneck. There was also clear evidence of genetic differentiation between current and historical samples, indicating a shift in genetic composition of the species over time. Additionally, even in the absence of historical context, remaining diversity in the current population is exceptionally low. This is unlikely to represent genotyping error, as conservative genotyping rules were employed to derive consensus genotypes. The population also shows other hallmarks of being genetically compromised (e.g. high level of relatedness).

Reduced genetic diversity and extremely low N_e in the current population may have important implications for long-term viability, potentially increasing vulnerability to disease, and hampering its ability to respond to

sudden environmental variation and potential future climate change effects at Bawangling (Lindsey *et al.* 2013). Highly threatened species can sometimes persist for long periods despite reduced genetic diversity [e.g. Iberian lynx *Lynx pardinus* (≥50 000 years) Rodríguez *et al.* 2011; koala *Phascolarctos cinereus* (≥120 years) Tsangaras *et al.* 2012]; however, such species have typically consisted of >1 population, making them less vulnerable to stochastic effects that could eliminate the last Hainan gibbon population. A strategy of 'genetic rescue', where genes are introduced from other wild or captive populations to improve the genetic state of a population with low genetic diversity (Hedrick & Fredrickson 2010), is also not an option, as the BNNR population constitutes the only known population. Thankfully, other species have managed to recover from critically low sizes without addition of new genetic variation, despite severe losses of genetic variability following extreme bottlenecks, for example Chatham Islands black robin (*Petroica traversi*) (Ardern & Lambert 1997), Mauritius kestrel (Groombridge *et al.* 2000), and Mauritius parakeet (*Psittacula echo*) (Raisin *et al.* 2012). Such recoveries have only been achieved through intensive, carefully planned management, indicating reduced genetic diversity may not preclude conservation success, but must be considered during conservation planning.

Long-term Hainan gibbon recovery will likely require intensive management, for example potential translocation of individuals to establish new founder populations, and our findings have important implications in this regard. As all sampled individuals are related at the level of half- to full-siblings, it is essential to consider data on relatedness when deciding potential management actions, although attempts to maintain genetic integrity must be coupled with maintenance of social integrity for gregarious, group-living species with complex social behaviours such as gibbons. The close observed relationships and evidence of inbreeding indicate it may be necessary to adjust potential expectations of likely population recovery rates, as lowered reproductive fitness and reduced survival are known in other populations experiencing inbreeding (Swinnerton *et al.* 2004; Hemmings *et al.* 2012), and a crucial next step is to incorporate our data on Hainan gibbon genetic diversity and relatedness into population viability analysis (Turvey *et al.* 2015). Attention should focus on preservation of all remaining gibbon individuals to prevent further diversity declines and losses to the breeding pool. Consequently, eliminating the threat of hunting is absolutely paramount, as is reduction of other anthropogenic activities currently degrading habitat at Bawangling (illegal forest clearance, nontimber forest product collection, livestock grazing, infrastructure development for tourism; Zhang *et al.* 2010; Turvey *et al.* 2015). Increasing available habitat may also support population growth, reduce environmental impacts to the offspring sex ratio, and allow the population to withstand localized environmental threats in the face of its reduced diversity.

Our study demonstrates that despite small sample sizes and challenges to data collection, it is possible to generate comprehensive new baseline data sets regarding the genetic status of Critically Endangered species through use of multiple analytical techniques, with resultant information providing crucial insights to inform conservation management. Our research also adds to a growing body of literature (Groombridge *et al.* 2009; Bristol *et al.* 2013; Tollington *et al.* 2013) demonstrating the importance of contextualizing measures of genetic condition for threatened populations against their historical genetic status and chronologies of past population dynamics and human impacts, to reveal nuanced insights required for conservation management. Moving forward, further assessment of the robustness of existing statistical methods to tiny samples sizes, and development of new metrics or analytical frameworks to accommodate these issues, will be crucial to facilitate genetic studies on species of extreme rarity.

Acknowledgements

This research was made possible through collaboration with Bawangling National Nature Reserve Management Office, Hainan Bawangling Forestry Bureau and Hainan Provincial Forestry Department. We thank the Hainan Gibbon Monitoring Team, Chungdong Wang, Selina Brace, Ian Barnes, Jim Groombridge, Linda Vigilant, Benoit Goossens, Guy Cowlishaw, Jinliang Wang and Jing Chai. We are also grateful to the editor and three anonymous reviewers for useful comments which helped improve and clarify our manuscript. Historical samples were provided by Natural History Museum, London; National Museum of Ireland, Dublin; Museum für Naturkunde, Berlin; South China Institute of Endangered Animals, Guangzhou. Funding was provided by Ocean Park Conservation Foundation, Hong Kong; Mohamed bin Zayed Species Conservation Fund; Daisy Balogh Memorial Travel Fund; Harold and Olga Fox Fund; UCL Overseas Research Scholarship; IRTSTYN Yunnan University; Royal Society University Research Fellowship (UF080320).

References

Antao T, Lopes A, Lopes R *et al.* (2008) LOSITAN: a workbench to detect molecular adaptation based on a F_{ST}-outlier method. *BMC Bioinformatics*, **9**, 323.

Ardern SL, Lambert DM (1997) Is the black robin in genetic peril? *Molecular Ecology*, **6**, 21–28.

Balloux F (2004) Heterozygote excess in small populations and the heterozygote-excess effective population size. *Evolution*, **58**, 1891–1900.

Beaumont MA (2003) Estimation of population growth or decline in genetically monitored populations. *Genetics*, **164**, 1139–1160.

Bouzat JL, Lewin HA, Paige KN (1998) The ghost of genetic diversity past: historical DNA analysis of the greater prairie chicken. *The American Naturalist*, **152**, 1–6.

Bradley BJ, Chambers KE, Vigilant L (2001) Accurate DNA-based sex identification of apes using non-invasive samples. *Conservation Genetics*, **2**, 179–181.

Bristol RM, Tucker R, Dawson DA *et al.* (2013) Comparison of historical bottleneck effects and genetic consequences of re-introduction in a Critically Endangered island passerine. *Molecular Ecology*, **22**, 4644–4662.

Bryant JV (2014) *Developing a Conservation Evidence-base for the Critically Endangered Hainan Gibbon (Nomascus hainanus)*. PhD Thesis, University College London, London, UK.

Bryant JV, Olson VA, Chatterjee HJ *et al.* (2015) Identifying environmental versus phylogenetic correlates of behavioural ecology in gibbons: implications for conservation management of the world's rarest ape. *BMC Evolutionary Biology*, **15**, 171–183.

Chambers KE, Reichard UH, Möller A *et al.* (2004) Cross-species amplification of human microsatellite markers using noninvasive samples from white-handed gibbons (*Hylobates lar*). *American Journal of Primatology*, **64**, 19–27.

Chan BPL, Fellowes JR, Geissmann T *et al.* (2005) *Hainan Gibbon Status Survey and Conservation Action Plan*. Kadoorie Farm and Botanic Garden, Hong Kong.

Chivers DJ, Anandam MV, Groves CP *et al.* (2013) Family Hylobatidae (gibbons). In: *Handbook of the Mammals of the World, Volume 3: Primates* (eds Mittermeier RA, Rylands AB, Wilson DE), pp. 754–791. Lynx Edicions, Barcelona.

Clisson I, Lathuilliere M, Crouau-Roy B (2000) Conservation and evolution of microsatellite loci in primate taxa. *American Journal of Primatology*, **50**, 205–214.

Cockerham CC, Weir BS (1993) Estimation of gene flow from F-statistics. *Evolution*, **47**, 855–863.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001–2014.

Di Rienzo AA, Peterson C, Garza JC *et al.* (1994) Mutational processes of simple-sequence repeat loci in human populations. *Proceedings of the National Academy of Sciences of the USA*, **91**, 3166–3170.

Do C, Waples RS, Peel D *et al.* (2013) NeEstimator v2.0: re-implementation of software for the estimation of contemporary effective population size (N_e) from genetic data. *Molecular Ecology Resources*, **14**, 209–214.

Dobroruka LJ (1970) To the supposed formerly occurrence of the David's deer *Elaphurus davidianus* Milne-Edwards, 1866, in Hainan. *Mammalia*, **34**, 162–164.

Earl D, vonHoldt B (2012) STRUCTURE HARVESTER: a website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conservation Genetics Resources*, **4**, 359–361.

England PR, Cornuet JM, Berthier P *et al.* (2006) Estimating effective population size from linkage disequilibrium: severe bias in small samples. *Conservation Genetics*, **7**, 303–308.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology*, **14**, 2611–2620.

Fellowes JR, Chan BPL, Zhou J *et al.* (2008) Current status of the Hainan gibbon (*Nomascus hainanus*): progress of population monitoring and other priority actions. *Asian Primates Journal*, **1**, 2–9.

Frankham R (1995) Effective population size/adult population size ratios in wildlife: a review. *Genetics Research*, **66**, 95–107.

Frankham R (1997) Do island populations have less genetic variation than mainland populations? *Heredity*, **78**, 311–327.

Frankham R, Ballou JD, Briscoe DA (2009) *An Introduction to Conservation Genetics*, 2nd edn, 644 pp. Cambridge University Press, Cambridge.

Frankham R, Bradshaw CJA, Brook BW (2014) Genetics in conservation management: revised recommendations for the 50/500 rules, Red List criteria and population viability analyses. *Biological Conservation*, **170**, 56–63.

Goossens B, Chikhi L, Utami S *et al.* (2000a) A multi-samples, multi-extracts approach for microsatellite analysis of faecal samples in an arboreal ape. *Conservation Genetics*, **1**, 157–162.

Goossens B, Latour S, Vidal C *et al.* (2000b) Twenty new microsatellite loci for use with hair and faecal samples in the chimpanzee (*Pan troglodytes troglodytes*). *Folia Primatologica*, **71**, 177–180.

Goossens B, Anthony N, Jeffery K *et al.* (2003) Collection, storage and analysis of non-invasive genetic material in primate biology. In: *Field and Laboratory Methods in Primatology: A Practical Guide* (eds Setchell JM, Curtis DJ), pp. 295–308. Cambridge University Press, Cambridge.

Goossens B, Chikhi L, Jalil MF *et al.* (2005) Patterns of genetic diversity and migration in increasingly fragmented and declining orang-utan (*Pongo pygmaeus*) populations from Sabah, Malaysia. *Molecular Ecology*, **14**, 441–456.

Gottelli D, Marino J, Sillero-Zubiri C *et al.* (2004) The effect of the last glacial age on speciation and population genetic structure of the endangered Ethiopian wolf (*Canis simensis*). *Molecular Ecology*, **13**, 2275–2286.

Goudet J (2002) *FSTAT (Version 2.9.3.2), Computer Program for PCs*. Institute of Ecology, Lausanne, Switzerland.

Grantham HS, Wilson KA, Moilanen A *et al.* (2009) Delaying conservation actions for improved knowledge: how long should we wait? *Ecology Letters*, **12**, 293–301.

Groombridge JJ, Jones CG, Bruford MW *et al.* (2000) Ghost alleles of the Mauritius kestrel. *Nature*, **403**, 616.

Groombridge JJ, Massey JG, Bruch JC *et al.* (2004) An attempt to recover the po'ouli by translocation and an appraisal of recovery strategy for bird species of extreme rarity. *Biological Conservation*, **118**, 365–375.

Groombridge JJ, Dawson DA, Burke T *et al.* (2009) Evaluating the demographic history of the Seychelles kestrel (*Falco araea*): genetic evidence for recovery from a population bottleneck following minimal conservation management. *Biological Conservation*, **142**, 2250–2257.

Hagell S, Whipple AV, Chambers CL (2013) Population genetic patterns among social groups of the endangered Central American spider monkey (*Ateles geoffroyi*) in a human-dominated landscape. *Ecology and Evolution*, **3**, 1388–1399.

Hartl DL, Clark AG (1997) *Principles of Population Genetics*, 4th edn, 565 pp. Sinauer Associates, Sunderland, Massachusetts.

Hedrick PW, Fredrickson R (2010) Genetic rescue guidelines with examples from Mexican wolves and Florida panthers. *Conservation Genetics*, **11**, 615–626.

Hemmings NL, Slate J, Birkhead TR (2012) Inbreeding causes early death in a passerine bird. *Nature Communications*, **3**, 863.

Hoffmann M, Hilton-Taylor C, Angulo A *et al.* (2010) The impact of conservation on the status of the world's vertebrates. *Science*, **330**, 1503–1509.

Holbrook JD, DeYoung RW, Tewes ME *et al.* (2012) Demographic history of an elusive carnivore: using museums to inform management. *Evolutionary Applications*, **5**, 619–628.

Jago N, Melfi VA (2010) Sex ratios in captive gibbons. *Zoo Research News*, **11**, 2–3.

Jones OR, Wang J (2010) COLONY: a program for parentage and sibship inference from multilocus genotype data. *Molecular Ecology Resources*, **10**, 551–555.

Kalinowski ST (2004) Counting alleles with rarefaction: private alleles and hierarchical sampling designs. *Conservation Genetics*, **5**, 539–543.

Kalinowski ST (2005) HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes*, **5**, 187–189.

Kalinowski ST, Wagner AP, Taper ML (2006) ML-RELATE: a computer program for maximum likelihood estimation of relatedness and relationship. *Molecular Ecology Notes*, **6**, 576–579.

Keller LF, Waller DM (2002) Inbreeding effects in wild populations. *Trends in Ecology and Evolution*, **17**, 230–241.

Keller LF, Biebach I, Ewing SR *et al.* (2012) The genetics of reintroductions: inbreeding and genetic drift. In: *Reintroduction Biology: Integrating Science and Management* (eds Ewen JG, Armstrong DP, Parker KA *et al.*), pp. 360–394. Wiley-Blackwell, Oxford.

Kenyon M, Roos C, Binh VT *et al.* (2011) Extrapair paternity in golden-cheeked gibbons (*Nomascus gabriellae*) in the secondary lowland forest of Cat Tien National Park, Vietnam. *Folia Primatologica*, **82**, 154–164.

Leberg PL (2002) Estimating allelic richness: effects of sample size and bottlenecks. *Molecular Ecology*, **11**, 2445–2449.

Li Y (2004) Past, present and future of Hainan tropical rainforests. *Living Forests*, **7**, 9–12.

Li Z, Wei F, Zhou J (2010) Mitochondrial DNA D-loop sequence analysis and population rejuvenation of Hainan gibbons (*Nomascus hainanus*). *Biodiversity Science*, **18**, 523–552.

Lindsey HA, Gallie J, Taylor S *et al.* (2013) Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature*, **494**, 463–467.

Liu Z, Yu S, Yuan X (1984) Resources of the Hainan black gibbon and its present situation. *Chinese Wildlife*, **6**, 1–4.

Liu Z, Zhang Y, Jiang H *et al.* (1989) Population structure of *Hylobates concolor* in Bawanglin Nature Reserve, Hainan, China. *American Journal of Primatology*, **19**, 247–254.

Luikart G, Allendorf FW, Cornuet JM *et al.* (1998) Distortion of allele frequency distributions provides a test for recent population bottlenecks. *Journal of Heredity*, **89**, 238–247.

Madsen T, Shine R, Olsson M *et al.* (1999) Conservation biology: restoration of an inbred adder population. *Nature*, **402**, 34–35.

Miquel C, Bellemain E, Poillot C *et al.* (2006) Quality indexes to assess the reliability of genotypes in studies using noninvasive sampling and multiple-tube approach. *Molecular Ecology Notes*, **6**, 985–988.

Oka T, Takenaka O (2001) Wild gibbons' parentage tested by non-invasive DNA sampling and PCR-amplified polymorphic microsatellites. *Primates*, **42**, 67–73.

van Oosterhout C, Hutchinson WF, Wills DP *et al.* (2004) MICRO-CHECKER: software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes*, **4**, 535–538.

Peakall R, Smouse PE (2006) GenAlEx 6. Genetic analysis in Excel: population genetic software for teaching and research. *Molecular Ecology Notes*, **6**, 288–295.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective size using allele frequency data. *Journal of Heredity*, **90**, 502–503.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945–959.

Pritchard JK, Wen X, Falush D (2010) *Documentation for Structure Software: Version 2.3*. University of Chicago, Chicago, Illinois.

R Development Core Team (2013) *R: A Language and Environment for Statistical Computing (V.3.0.1)*. R Foundation for Statistical Computing, Vienna, Austria.

Raisin C, Frantz AC, Kundu S *et al.* (2012) Genetic consequences of intensive conservation management for the Mauritius parakeet. *Conservation Genetics*, **13**, 707–715.

Rodríguez R, Ramírez O, Valdiosera CE *et al.* (2011) 50,000 years of genetic uniformity in the Critically Endangered Iberian lynx. *Molecular Ecology*, **20**, 3785–3795.

Rodríguez-Ramilo ST, Wang J (2012) The effect of close relatives on unsupervised Bayesian clustering algorithms in population genetic structure analysis. *Molecular Ecology Resources*, **12**, 873–884.

Rodríguez-Ramilo ST, Toro MA, Wang J *et al.* (2014) Improving the inference of population genetic structure in the presence of related individuals. *Genetics Research*, **96**, e003.

Roeder AD, Bonhomme M, Heijmans C *et al.* (2009) A large panel of microsatellite markers for genetic studies in the Infra-Order Catarrhini. *Folia Primatologica*, **80**, 63–69.

Segan DB, Bottrill MC, Baxter PWJ *et al.* (2011) Using conservation evidence to guide management. *Conservation Biology*, **25**, 200–202.

Siddle HV, Kreiss A, Eldridge MDB *et al.* (2007) Transmission of a fatal clonal tumor by biting occurs due to depleted MHC diversity in a threatened carnivorous marsupial. *Proceedings of the National Academy of Sciences of the USA*, **104**, 16221–16226.

Smith O (2012) *Population Genetics and Structure of the Sumatran Tiger*. PhD Thesis, Imperial College London, London, UK.

Spencer CC, Neigel JE, Leberg PL (2000) Experimental evaluation of the usefulness of microsatellite DNA for detecting demographic bottlenecks. *Molecular Ecology*, **9**, 1517–1528.

Storz JF, Bhat HR, Kunz TH (2001) Genetic consequences of polygyny and social structure in an Indian fruit bat,

Cynopterus sphinx. I. Inbreeding, outbreeding, and population subdivision. *Evolution*, **55**, 1215–1223.

Su B, Monda K, Wang W *et al.* (1995) Molecular phylogeny of Chinese concolor gibbons (subgenus *Nomascus*) using noninvasive DNA genotyping. In: *Primate Research and Conservation* (eds Xia W, Zhang Y), pp. 55–63. China Forestry Publishing House, Beijing.

Sullivan KM, Mannucci A, Kimpton CP *et al.* (1993) A rapid and quantitative DNA sex test-fluorescence-based PCR analysis of X-Y homologous gene amelogenin. *BioTechniques*, **15**, 636–641.

Sutherland WJ, Pullin AS, Dolman PM *et al.* (2004) The need for evidence-based conservation. *Trends in Ecology and Evolution*, **19**, 305–308.

Swinhoe R (1870) On the mammals of Hainan. *Proceedings of the Zoological Society of London*, **38**, 224–239.

Swinnerton KJ, Groombridge JJ, Jones CG *et al.* (2004) Inbreeding depression and founder diversity among captive and free-living populations of the endangered pink pigeon *Columba mayeri*. *Animal Conservation*, **7**, 353–364.

Taberlet P, Griffin S, Goossens B *et al.* (1996) Reliable genotyping of samples with very low DNA quantities using PCR. *Nucleic Acids Research*, **24**, 3189–3194.

Taberlet P, Waits LP, Luikart G (1999) Noninvasive genetic sampling: look before you leap. *Trends in Ecology and Evolution*, **14**, 323–327.

Taylor AC, Horsup A, Johnson CN *et al.* (1997) Relatedness structure detected by microsatellite analysis and attempted pedigree reconstruction in an endangered marsupial, the northern hairy-nosed wombat *Lasiorhinus krefftii*. *Molecular Ecology*, **6**, 9–19.

Thinh VN, Mootnick A, Geissmann T *et al.* (2010a) Mitochondrial evidence for multiple radiations in the evolutionary history of small apes. *BMC Evolutionary Biology*, **10**, 74.

Thinh VN, Rawson B, Hallam C *et al.* (2010b) Phylogeny and distribution of crested gibbons (genus *Nomascus*) based on mitochondrial cytochrome *b* gene sequence data. *American Journal of Primatology*, **72**, 1047–1054.

Tollington S, Jones CG, Greenwood A *et al.* (2013) Long-term, fine-scale temporal patterns of genetic diversity in the restored Mauritius parakeet reveal genetic impacts of management and associated demographic effects on reintroduction programmes. *Biological Conservation*, **161**, 28–38.

Tsangaras K, Ávila-Arcos MC, Ishida Y *et al.* (2012) Historically low mitochondrial DNA diversity in koalas (*Phascolarctos cinereus*). *BMC Genetics*, **13**, 92.

Turvey ST (2008) *Witness to Extinction: How We Failed to Save the Yangtze River Dolphin*, 234 pp. Oxford University Press, Oxford.

Turvey ST, Traylor-Holzer K, Bryant JV *et al.* (2015) *International Conservation Planning Workshop for the Hainan Gibbon: Final Report*. Zoological Society of London, London/IUCN SSC Conservation Breeding Specialist Group, Apple Valley, Minnesota (English and Chinese).

Vigilant L, Bradley BJ (2004) Genetic variation in gorillas. *American Journal of Primatology*, **64**, 161–172.

Wang J (2009) A new method for estimating effective population sizes from a single sample of multilocus genotypes. *Molecular Ecology*, **18**, 2148–2164.

Wasser SK, Houston CS, Koehler GM *et al.* (1997) Techniques for application of faecal DNA methods to field studies of ursids. *Molecular Ecology*, **6**, 1091–1097.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution*, **38**, 1358–1370.

Wen R (2009) *The Distributions and Changes of Rare Wild Animals in China*, 782 pp. Chongqing Science and Technology Press, Chongqing.

Westemeier RL, Brawn JD, Simpson SA *et al.* (1998) Tracking the long-term decline and recovery of an isolated population. *Science*, **282**, 1695–1698.

Whittaker DJ (2009) Phylogeography of Kloss's gibbon (*Hylobates klossii*) populations and implications for conservation planning in the Mentawai Islands. In: *The Gibbons: New Perspectives on Small Ape Socioecology and Population Biology* (eds Lappan S, Whittaker DJ), pp. 73–89. Springer-Verlag, New York, New York.

Wisely SM, Buskirk SW, Fleming MA *et al.* (2002) Genetic diversity and fitness in black-footed ferrets before and during a bottleneck. *Journal of Heredity*, **93**, 231–237.

Wu W, Wang X, Claro F *et al.* (2004) The current status of the Hainan black-crested gibbon *Nomascus* sp. cf. *nasutus hainanus* in Bawangling National Nature Reserve, Hainan, China. *Oryx*, **38**, 452–456.

Zhang Y (1992) Hainan gibbon (*Hylobates concolor hainanus*) threatened. *Asian Primates*, **2**, 6.

Zhang Y (1995) A molecular phylogeny of gibbons based on DNA sequences. In: *Primate Research and Conservation* (eds Xia W, Zhang Y), pp. 50–54. China Forestry Publishing House, Beijing.

Zhang Y, Sheeran L (1993) Current status of the Hainan black gibbon (*Hylobates concolor hainanus*). *Asian Primates*, **3**, 3.

Zhang M, Fellowes JR, Jiang X *et al.* (2010) Degradation of tropical forest in Hainan, China, 1991–2008: conservation implications for Hainan gibbon (*Nomascus hainanus*). *Biological Conservation*, **143**, 1397–1404.

Zhou J, Wei F, Li M *et al.* (2005) Hainan black-crested gibbon is headed for extinction. *International Journal of Primatology*, **26**, 453–465.

J.V.B. designed the research, conducted field research, collected, analysed and interpreted the data and wrote the manuscript. S.T.T. conceived and supervised the research and contributed to the manuscript. D.G. and C.D. provided laboratory and analytical guidance. Z.Y. and L.J. contributed laboratory space and reagents in China. T.G. provided key specimen samples. Z.X., H.X., B.P.L.C. and J.R.F. supported field research. H.J.C. supervised the research.

Data accessibility

A file containing information on individuals genotyped (sample years, locations, types, number of samples and

DNA extractions/individual, final locus genotypes and final multiplex mixes) is available from Dryad Digital Repository: doi: 10.5061/dryad.r1mc3. All analyses are described in sufficient detail to enable replication with data provided.

Supporting information

Additional supporting information may be found in the online version of this article.

Appendix S1 Map showing the location of the sole surviving population of the Hainan gibbon (*Nomascus hainanus*), which is constrained to Bawangling National Nature reserve (BNNR) on Hainan Island, China.

Appendix S2 Existing museum specimens of *Nomascus hainanus* sampled for assessment of species' historical genetic state, with details of samples collected.

Appendix S3 Details of human microsatellite loci screened.

Appendix S4 Thirteen successfully genotyped polymorphic microsatellite loci, with final annealing temperatures used and characterisation in *Nomascus hainanus* across all samples.

Appendix S5 Assessment of the impact of the incorporation of closely-related individuals upon population structure within the overall study sample.

Fig. S1 Rate of change in the average genetic distance between successive K values (ΔK) over increasing K values.

Table S1 Cluster assignment of all samples (current and historical) by CLUSTER_DIST for inferred number of clusters $K = 6$, based upon highest cluster probabilities.

Appendix S6 Tests of linkage disequilibrium between eleven successfully genotyped polymorphic microsatellite loci pairs, within each temporal population and across all samples.

Appendix S7 Population pedigree of current Hainan gibbon population.

Table S2 ML relatedness coefficients (r) indicating pairwise relatedness between all sampled individuals of current population, and between these individuals and the two youngest historical specimens (BWL 671–672).

Fig. S2 Pedigree constructed from ML configuration of relationships between individuals in current population.

Fig. S3 Pedigree constructed from ML configuration of relationships between individuals in current population and youngest historical specimens (BWL 671–672).