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Low genetic diversity in captive populations of the critically endangered Blue-crowned Laughingthrush (*Garrulax courtoisi*) revealed by a panel of novel microsatellites

Guoling Chen [1], **Chenqing Zheng** [2], **Nelson Wan** [3], **Daoqiang Liu** [4], **Vivian Wing Kan Fu** [5], **Xu Yang** [2], **Yat-tung Yu** [5], **Yang Liu** [Corresp. 1]

[1] Department of Ecology, School of Life Sciences, The State Key Lab of BioControl and Sun Yet-sen University, Guangzhou, Guangdong, China

[2] Shenzhen Realomics Biological Technology Ltd, Shenzhen, Guangdong, China

[3] Ocean Park Hong Kong Corporation, Aberdeen, Hong Kong S.A.R., China

[4] Nanchang Zoo, Nanchang, Nanchang, Jiangxi, China

[5] The Hong Kong Bird Watching Society, Kowloon, Hong Kong S.A.R., China

Corresponding Author: Yang Liu
Email address: liuy353@mail.sysu.edu.cn

Background. Understanding genetic diversity and population structure is critically important for the conservation and management of endangered species. These factors are particularly relevant for species with small populations and/or restricted ranges, such as the critically endangered Blue-crowned Laughingthrush, *Garrulax courtoisi*, which has only two wild populations left in Wuyuan, Jiangxi and Simao, Yunnan, China.

Methods. In this study, novel microsatellites markers were developed using whole-genome sequencing of the target species. We genotyped 14 and nine individuals from the Oceanic Park of Hong Kong, which are of unknown origin, and the Nanchang Zoo, which were introduced from the wild Wuyuan population, respectively, using the novel microsatellite markers. The genetic diversity of captive Blue-crowned Laughingthrush populations was estimated based on genetic polymorphisms revealed by a new microsatellite data set and mitochondrial sequences.Then, we characterised the population structure using STRUCTURE, principal coordinates analysis, population assignment test using the microsatellite data, and haplotype analysis of mitochondrial data. Additionally, we quantified genetic relatednessbased on the microsatellite data with ML-Relate.

Results. This is the first study to describe this novel set of 12 microsatellite markers for Blue-crowned Laughingthrush. Our results based on the microsatellite dataset and mitochondrial sequences showed equally low levels of genetic diversity of the two captive Blue-crowned Laughingthrush populations. The population structure analysis, population assignment test using the microsatellite data, and haplotype analysis of the mitochondrial data showed some population structuring between these two populations. The average pairwise relatedness coefficient was not significant, and their genetic relatedness was quantified.

Discussion. This study provided a genetic tool which allowed the first estimate of captive population genetic diversity and relatedness for a critically endangered bird species. Furthermore, our results indicate that we cannot exclude the possibility that the origin of the Hong Kong captive population was the wild Wuyuan population. These results provide valuable knowledge that can help improve conservation management and planning for both captive and wild Blue-crowned Laughingthrush populations.

Original paper

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Guoling Chen[1], Chenqing Zheng[2], Nelson Wan[3], Daoqiang Liu[4], Vivian Wing Kan, Fu[5], Xu Yang[2], Yat-tung Yu[5], Yang Liu[1]*

[1] State Key Laboratory of Biocontrol, Department of Ecology and School of Life Sciences, Sun Yat-sen University, Guangzhou, 510275, China

[2] Shenzhen Realomics Biological Technology Ltd, Shenzhen, 518000, P. R. China

[3] Ocean Park Hong Kong Corporation, Aberdeen Hong Kong S.A.R., China

[4] Nanchang Zoo, Nanchang, 330025, China

[5] The Hong Kong Bird Watching Society, Kowloon Hong Kong S.A.R., China

***Correspondence:** Dr. Yang Liu, Email: liuy353@mail.sysu.edu.cn; Fax: +86 20 8411 0436

Abstract

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Results. This is the first study to describe this novel set of 12 microsatellite markers for Blue-crowned Laughingthrush. Our results based on the microsatellite dataset and mitochondrial sequences showed equally low levels of genetic diversity of the two captive Blue-crowned Laughingthrush populations. The population structure analysis, population assignment test using the microsatellite data, and haplotype analysis of the mitochondrial data showed some population structuring between these two populations. The average pairwise relatedness coefficient was not significant, and their genetic relatedness was quantified.

Discussion. This study provided a genetic tool which allowed the first estimate of captive population genetic diversity and relatedness for a critically endangered bird species. Furthermore, our results indicate that we cannot exclude the possibility that the origin of the

Hong Kong captive population was the wild Wuyuan population. These results provide valuable knowledge that can help improve conservation management and planning for both captive and wild Blue-crowned Laughingthrush populations.

Introduction

Conservation genetics applies genetic theories and techniques to assist with the conservation and management of endangered populations and reduce extinction risk (Frankham et al., 2010). Genetic factors increase species' extinction risk through inbreeding depression, loss of genetic diversity, and loss of evolutionary potential (Frankham, 2005). Conservation biologists have increasingly recognised that successful management of threatened species requires focus on both wild and captive populations (Canessa et al., 2016).

Many endangered species require captive breeding to save them from extinction through genetic rescue and reintroduction, as they are incapable of surviving in inhospitable natural environments because of direct or indirect human impacts in the form of habitat loss, overexploitation, pollution, or introduced predators, competitors, or diseases (Frankham, 2008). Furthermore, captive populations provide ideal systems for ecological, evolutionary, and genetic research of endangered species. For captive populations, it is easier and safer to obtain DNA samples and observe the ecological habit of endangered species, because there is easier and more controlled access to individuals (Ballou & Foose, 1996; Frankham, 2010; Frankham et al., 2010). Therefore, it is both extremely important and efficient to use captive populations for elucidating the genetic status of endangered species, and appropriate genetic management can help conserve the entire population (Frankham, 2008; Frankham et al., 2010).

The Blue-crowned Laughingthrush (Figure 1), *Garrulax courtoisi*, is listed as "Critically Endangered" by the IUCN Red Data Book (Birdlife International, 2017) and has an extremely

restricted distribution in southeastern China. The entire known wild population of the nominate subspecies, which consists of approximately 300 individuals, is restricted to six fragmented sites in Wuyuan County, Jiangxi Province, China (He et al., 2017). Historical records indicate a disjunct population of the subspecies *G. c. simaoensis* in southern Yunnan Province (He et al., 2017). However, its current status is not known, and it has not been encountered in the wild since 1956 (Wilkinson & He, 2010a). In addition, around 200 captive Blue-crowned Laughingthrush individuals are kept in several zoos in China, Europe, and America without known subspecies origin (Wilkinson & Gardner, 2011; Wilkinson et al., 2004). The worryingly low population size and severely restricted distribution strongly indicates that this species is of high conservation concern.

Most recent studies on this rare species focused on habitat use and ecology in the wild Wuyuan population (He et al., 2017; Wilkinson & He, 2010a; Zhang et al., 2017), breeding ecology of captive populations (Liu et al., 2017; Liu et al., 2016; Wilkinson et al., 2004), and its taxonomic status and conservation management (Cheng & Tang, 1982; Collar et al., 2016; Wilkinson & He, 2010b; Wilkinson & He, 2010c). However, there is no information on the genetic diversity, population structure, or other important issues pertaining to conservation genetics in either the wild or captive Blue-crowned Laughingthrush populations. In fact, most taxa are not driven to extinction before genetic factors adversely affect them (Spielman et al., 2004). Recent conservation genetic studies have shown that a loss of genetic diversity in small populations is expected to increase extinction risk by reducing population fitness. This pattern may adversely affect the capacity of populations to evolve to cope with environmental change (i.e. by decreasing its evolutionary potential) (Frankham, 2005; Spielman et al., 2004). Elucidating genetic diversity is important for producing a better understanding of microevolutionary processes and developing appropriate conservation and management strategies. Therefore, it is extremely necessary to obtain basic information on the genetic diversity and population

structure of captive Blue-crowned Laughingthrush populations for effective *ex situ* conservation. Moreover, this research provides valuable information that can benefit effective *ex situ* conservation efforts and can help conserve the critically endangered wild Blue-crowned Laughingthrush populations.

Here, we present the first conservation genetic analysis of Blue-crowned Laughingthrush using mitochondrial DNA and a novel set of microsatellite markers developed by next-generation sequencing using the Illumina high-throughput sequencing platform. Specifically, we characterised the genetic diversity, genetic structure, and relatedness and origins of two captive populations in Ocean Park Hong Kong and Nanchang Zoo. These efforts can objectively help relevant conservation agencies, such as governments, zoos, and NGOs, develop appropriate conservation and management strategies for the Blue-crowned Laughingthrush.

Materials and Methods

Sample collection and DNA extraction

The Ocean Park Hong Kong (OPHK) population was introduced in 1998, and there are 16 individuals in the existing population. Both the number and source of Blue-crowned Laughingthrush they introduced in 1989 are unknown because of foot ring loss and lack of records. The Nanchang Zoo (NCZ) population in Jiangxi was introduced in 2010 and 2011 from the wild Blue-crowned Laughingthrush population in Wuyuan County, Jiangxi Province, and is the only captive population that has a confirmed source. In this population, six individuals are from the wild population and the other seven individuals are their descendants.

Since this study involved sampling of endangered species, all the animal operations were approved by the Institutional Ethical Committee of Animal Experimentation of Sun Yat-sen University and strictly complied with the ethical conditions by the Chinese Animal Welfare Act

(20090606). And all sampling procedures were performed with assistance of veterinarians or zoo keepers.

We collected Blue-crowned Laughingthrush samples from 23 individuals of two captive populations: 14 individuals from the long-established OPHK population and nine individuals from the recently established NCZ population. Fresh blood samples from the 14 OPHK individuals were obtained in a non-invasive manner during regular veterinary examinations, and muscle samples from dead individuals and three egg remains were obtained from the NCZ population. All samples were stored in 95% ethanol at −80°C. We extracted total genomic DNA using the QIAamp DNA Mini Kit (Qiagen, GmbH, Hilden, Germany) following the manufacturer's protocol, and quantified DNA quality with a NanoDrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA).

Genome sequencing, microsatellite loci identification, and primer design

We sequenced a Blue-crowned Laughingthrush draft genome from an OPHK specimen. P5 and P7 adapters were ligated to the fragments after the genomic DNA was digested. The P5 adapter contains a forward amplification primer site, an Illumina sequencing primer site, and a barcode. The selected fragments were end-repaired and 3' adenylated, and these fragments were PCR amplified with P5- and P7-specific primers. Our library was validated using the Agilent Technologies 2100 Bio-analyzer and ABI StepOnePlus Real-Time PCR System. After adapter ligation and DNA cluster preparation, the samples were sequenced using a Hiseq X-ten sequencer (BGI, Shenzhen, China).

Raw data from this single individual were processed by removing adapter sequences and subsequently removing the reads. Sequences with a low-quality rate [quality value ≤ 5 (E)] greater than or equal to 50% and with more than 10% unknown ('N') bases were removed. The

final read length was trimmed to 82 nucleotides (minimum length). Then, the high-quality sequences were selected to assemble the reference scaffolds. The genome was assembled using a short-read assembly method in SOAPdenovo2 (Li et al., 2010). A de Bruijn graph was built by splitting the reads into K-mers from the short-insert libraries (270 bp) without using pairing information. After a series of graph simplifications, the reads were assembled into contigs. All available paired-end reads were realigned onto the contig sequences to infer linkage between contigs. The linkage was removed if it was supported by unreliable paired-end reads. To simplify the contig linkage graph, we used subgraph linearisation, which extracted information on unambiguously linear paths. Iterative scaffolding was carried out to estimate insert size. Finally, to fill the intra-scaffold gaps, a local assembly was performed to locate the reads in the gap region, thus ensuring the other end of a scaffold was uniquely mapped to the linked contig.

We identified the microsatellites by screening the sequence data for di-, tri-, tetra-, and penta-nucleotide motifs with a minimum of six, five, five, and five repeats, respectively, by using the polymorphism information from the Blue-crowned Laughingthrush draft genome. Then we designed the primers in MSATCOMMANDER v.1.0.8 (Faircloth, 2008) and Primer 3 (Rozen & Skaletsky, 2000) to minimize potential structural or functional defects. After these procedures, we arbitrarily selected a panel of 20 novel di-nucleotide markers and 10 tri-nucleotide markers.

Microsatellite genotyping

The 30 selected loci were arranged into eight PCR multiplex sets (2–4 loci per set); each forward primer was labelled with fluorescent dye on the 5’ end of the forward primers, and the sequence GTTTCTT was placed on the 5’ end of the reverse primer (Brownstein et al., 1996). PCR amplifications were performed in a reaction volume of 10 µl, containing, 5 µL 2× PCR mix (QIAGEN Multiplex Kit), 2 µL 5× Q-Solution, 1 µL of a primer mix and 1 µL of template DNA. The

cycling conditions were as follows: initial denaturation at 95 °C for 15 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 90 s and at 72 °C for 90 s, and a final extension at 72 °C for 10 min. Products were isolated and detected on an ABI Prism 3730XL Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), and the fragment lengths were determined against an internal size standard (GeneScan™ 500 LIZ Size Standard, Applied Biosystems, Carlsbad, CA, USA) with GeneMapper v.3.7 (Applied Biosystems, Carlsbad, CA, USA). All samples were genotyped at the 30 microsatellite loci that were developed from the Blue-crowned Laughingthrush draft genome. We ultimately selected 19 microsatellite loci for our study and discarded 11 microsatellite loci because they had low polymorphism levels.

Mitochondrial DNA sequencing

To infer maternal relatedness of sampled individuals, partial mitochondrial cytochrome b (cytb) sequences were amplified and sequenced using the primers L14995 and H16065 (Groth, 1998). PCR amplifications were performed in a 20-μL reaction volume that contained 1–2 μL template DNA (50–100 ng), 10 μL 2× buffer, 2 μL dNTPs (2 mM), 0.5 μL $MgCl_2$ (2.5 mM), 0.5 μL of each primer (10 mM), and 0.5 μL (1 unit/μL) KOD DNA polymerase (Toyobo, Osaka, Japan). The PCR cycling conditions were as follows: an initial denaturation step of 4 min at 94 °C followed by 35 cycles of 40 s denaturation at 94 °C, 40 s annealing at 56 °C, and 90 s extension at 72 °C, followed by a final 10 min extension at 72 °C. The purified products were sequenced with both forward and reverse primers using a BigDye Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer's guidelines. The products were sequenced on an ABI Prism 3730 Automated DNA sequencer (Shanghai Majorbio Bio-pharm Technology Co., Ltd., Shanghai, China). Additional PCR profile information is provided as supplementary information (Supplementary Material Appendix 1, Table S2).

Genetic diversity estimates

For each microsatellite locus, we calculated the frequency of null alleles using Cervus v.3.0.7 (Kalinowski et al., 2010); then, we used Arlequin v.3.5 (Excoffier & Lischer, 2010) to further test for Hardy–Weinberg equilibrium using 1000 permutations and pairwise linkage disequilibrium by performing 100,000 Markov chain steps. Based on these three tests, seven out of 19 loci were removed from the dataset. To obtain genetic diversity estimates, we calculated the number of different alleles (Na), average allelic richness (A_R), observed heterozygosity (Ho), and expected heterozygosity (H_E) using the remaining 12 loci in GenAlEx v.6.5.1 (Peakall & Smouse, 2012). We also calculated the inbreeding index (F_{IS}) for each population and assessed the significance of this index based on 10,000 permutations in Arlequin v.3.5.

In addition, we carried out rarefication analysis using POWSIM v.4.0 (Ryman & Palm, 2006) to assess the statistical power of our microsatellite markers to detect levels of population differentiation and relatedness (e.g. Liu et al., 2013). Using an estimated effective size (Ne) of 1000 for the base population, we performed 1000 runs and generated eight predefined levels of population differentiation (F_{ST} = 0.001, 0.0025, 0.005, 0.01, 0.02, 0.025, 0.05, 0.075), with sample sizes, numbers of markers, and allele frequencies corresponding to the empirical data. The proportion of significant outcomes ($P < 0.05$) then corresponded to an estimate of power. The HO at each locus was tested for equal allele frequencies by both Pearson's traditional contingency chi-square and Fisher's exact tests. The information from all loci was then combined by summing the data from chi-square and Fisher's methods (Ryman & Jorde, 2001; Ryman & Palm, 2006).

Genetic population structure

For the microsatellite dataset, we applied four methods to estimate genetic population structure. First, we calculated pairwise F_{ST} between these two populations, and derived significance levels using 10,000 permutations in Arlequin v.3.5. Sequential Bonferroni

correction (Rice, 1989) was used to adjust the significance levels for multiple testing. Second, we further tested the genetic structure using the Bayesian clustering method in STRUCTURE v2.3 (Falush et al., 2003; Pritchard et al., 2000). Using the Bayesian admixture model with the correlated allele frequencies option, we performed 1,000,000 Markov chain Monte Carlo (MCMC) iterations, with the first 200,000 discarded as burn-in. We conducted 10 independent runs for each K-value (K = 1–4) for the entire dataset. We then used Structure Harvester v.0.6.8 (Earl & Vonholdt, 2012) to identify the most likely number of genetic clusters based on the ad hoc statistics described in Evanno et al. (2005). The final results for individual memberships were visualised by bar plot in DISTRUCT v.1.1 (Rosenberg, 2004). Third, principal coordinates analysis (PCoA) with pairwise Euclidian distances was carried out in GenAlEx v.6.5.1 (Peakall & Smouse, 2012) to visualise genetic relationships among individuals. Last, the biplots of pairwise population assignment likelihood values was computed using gamete-based Monte Carlo resampling method with a threshold of 0.01 in GenAlEx v.6.5.1 (Paetkau et al., 2004; Peakall & Smouse, 2012). This method uses genotype likelihoods to assign the possible population origins of individuals and allows estimation of dispersal events (Paetkau et al., 2004).

For DNA sequence data, we aligned the mitochondrial sequences using the Clustal W algorithm (Thompson et al., 1994) in MEGA v.6.06 (Tamura et al., 2013) with default parameters. The alignment was checked and manually adjusted when needed. To estimate the level of genetic polymorphism, basic genetic polymorphism statistics, such as haplotype number (h), haplotype diversity (Hd), number of segregating sites (S), and nucleotide diversity (π), of each population were calculated in DnaSP v.5.10.1 (Librado & Rozas, 2009). Then, this gene was analysed by haplotype network analysis using the reduced median-joining method (Bandelt et al., 1999) in PopART v.4.8.4 (Leigh & Bryant, 2015).

Relatedness analysis

For all relatedness estimates, the individuals from the OPHK and NCZ populations were separately analysed. For each pair of individuals from the OPHK or NCZ population, we calculated the Queller and Goodnight (1989) estimator of relatedness (R_{QG}) using GenAlEx v.6.5.1 (Peakall & Smouse, 2012). The genetic relatedness coefficient is defined as the proportion of ancestral alleles that are shared between descendants (Lynch & Walsh, 1998).

Then, the maximum likelihood estimates of relatedness (R) was calculated in ML-Relate (Kalinowski et al., 2006), and the likelihood of four relatedness categories [unrelated: R = 0; close kin (e.g. half-siblings, aunt–niece): R = 0.25; full-siblings: R = 0.5; parent–offspring: R = 0.5] was used to determine the proportion of a specific relatedness category. To assess the likelihood of a given relatedness category relative to the other three categories, a likelihood ratio test using a 95% confidence level and 1000 simulations was carried out in ML-Relate (Kalinowski et al., 2006).

Results

Genome sequencing, microsatellite loci identification, and primer design

The whole genome of the endangered Blue-crowned Laughingthrush was first assembled using the high-coverage (approximately 40×) sequence reads. After processing of the approximately 41.18 Gb of raw data and removal of ambiguous barcodes, about 39.1 Gb of clean data were retained. The assembly generated 1089819 scaffolds larger than 100 bp, with an N50 contig size of 1297 bp and an N50 scaffold size of 4548 bp. The microsatellite detection generated 70310 markers with 31216 di-nucleotide repeats, 21195 tri-nucleotide repeats, 10892 tetra-nucleotide repeats, and 7007 penta-nucleotide repeats.

Genetic diversity

For the microsatellite dataset, we obtained the 12 loci from 14 individuals from the OPHK population and nine individuals from the NCZ population (Table 1). We found no evidence of genotypic disequilibrium after Bonferroni correction. The number of alleles per locus ranged from 2 to 5, and the polymorphic information content per locus ranged between 0.16 and 0.70. For all microsatellite loci, Na for each population was approximately 3.25, and Na of the NCZ population was slightly higher than that of the OPHK population. We found low to moderate genetic diversity with a mean H_O of 0.36–0.45 and a mean H_E of 0.34–0.44. Moreover, we found no sign of inbreeding at the loci, with F_{IS} ranging from −0.283 (p = 0.923) to −0.214 (p = 0. 784) (Table 2).

Genetic population structure

The simulations performed in POWSIM using our particular microsatellite data and selected sample size showed that the statistical power was sufficient (> 90%) to detect genetic substructure if the true $F_{ST} \geq 0.075$ (Figure S1).

Using multiple approaches, we found evidence of genetic differentiation between OPHK and NCZ populations. First, significant genetic differentiation in the microsatellite dataset was revealed by a significant F_{ST} value (0.065, p = 0.003) between the two populations (Table 1); this was probably caused by two loci which have significant genetic differentiation between the two populations (BCLT_L5: F_{ST} = 0.154, p = 0.007; BCLT_L6: F_{ST} = 0.143, p = 0.006) (Table 3). The remining 10 loci had non-significant F_{ST} values (range, −0.032–0.137). The STRUCTURE analysis revealed that there are most likely two genetic clusters (Figure 2) based on the mean posterior probabilities (Figure 2b) and ΔK estimator (Figure 2c). Under the two genetic cluster scenario, it was shown that the genetic background of the NCZ population was more uniform than that of the OPHK population, whereas the OPHK population had an uneven genetic background (Figure 2a).

For the PCoA plot based on the 12 microsatellite sites of these two populations, we found that there was no differentiation between the OPHK and NCZ populations in the first principal coordinate but some differentiation in the second principal coordinate (Figure 3a). Assignment test results showed that, for the OPHK population, 100% of the individuals (n = 14) were assigned to the OPHK population. For the NCZ population, 77.8% of the individuals (n = 7) were assigned to the NCZ population, whereas 22.2% of the individuals (n = 2, individuals 6041 and 6043) were assigned to the OPHK population (Figure 3b).

For the mitochondrial cytb dataset, we obtained 1027 bp from each individual (GenBank Accession No. MH423582–MH423604). The average level of genetic diversity was similar between OPHK and NCZ populations (Table 2). Haplotype networks showed that there were five haplotypes among these individuals, and the most frequent haplotype was shared by 18 individuals. Three and one private haplotypes were shared by OPHK and NCZ populations, respectively (Figure 4).

Relatedness analysis

The average pairwise relatedness coefficient based on the 12 microsatellite loci within populations ranged from −0.013 to −0.05, and none of these values significantly differed from zero (Table 2). Such a low level of relatedness probably results from the fact that a small proportion of individuals from both populations are closely related (Figure 5). A dominant proportion of dyads came from unrelated individuals (72.53% in the OPHK population, 86.11% in the NCZ population).

Discussion

Newly developed polymorphic genetic markers of Blue-crowned Laughingthrush

In this study, we provided a set of polymorphic genetic markers for the Blue-crowned Laughingthrush, which is intended to be a useful genetic tool for efficient conservation of this species. Consequently, we were able to estimate the genetic diversity, population structure, and genetic relatedness of wild and captive populations of this endangered bird species. Furthermore, for the first time, we provided a set of markers that can be broadly applied to conservation genetic and evolutionary studies of *Garrulax* laughingthrushes, which is a popular group of captive species in zoos. We found that there is a lack of research on the genetic diversity and other important issues pertaining to conservation genetics of *Garrulax* (Collar & van Balen, 2013; Li, 2009; Wu et al., 2012).

Microsatellites are commonly used genetic markers in evolutionary, behavioural, and conservation genetics, because of their relatively high level of polymorphism and repeatability in genotyping, which is extremely advantageous for threatened species (Faria et al., 2016; Wang et al., 2017). Additionally, with the development of next-generation sequencing, the development of reliable microsatellites are no longer inefficient or time-consuming, even for birds that have few known microsatellite loci in their genomes (Castoe et al., 2012; Yang et al., 2017). Whole-genome sequencing determines the complete DNA sequence of an organism's genome and provides unprecedented genomic information that is orders of magnitude larger than alternative strategies that use high-throughput sequencing methods, such as reduced-representation and transcriptome sequencing, by which only a subset of information is obtained (Yue et al., 2012).

Low genetic diversity of two captive populations and its implications

We documented low genetic diversity of two captive populations of the critically endangered species Blue-crowned Laughingthrush. Our results showed that genetic diversity of the OPHK population was slightly lower than that of the NCZ population, but there was no significant

difference between them. Because the genetic diversity of species is likely to vary considerably among different taxonomic units (Frankham et al., 2010; Li et al., 2014), we compared genetic diversity of the Blue-crowned Laughingthrush with other closely related songbird species with different endangerment statuses (Table 3). Because this inference was based on genetic diversity genotyped from different microsatellites, we caution over-interpretation of our results. We found that level of genetic diversity in Blue-crowned Laughingthrush at microsatellite and mitochondrial DNA (cytb) are lower than those in the threatened Jankowski's Bunting, *Emberiza jankowskii* (Li, 2017), and much lower than those of non-threatened species (Li, 2009; McKay et al., 2010; Wu et al., 2012; Yang et al., 2017) (Table 2). This finding is consistent with the idea that threatened species usually have lower levels of genetic diversity than non-threatened species (Evans & Sheldon, 2008; Frankham et al., 2010), and probably results from the long-term decreased effective population size of Blue-crowned Laughingthrush (He et al., 2017).

Recent conservation genetic studies have shown that loss of genetic diversity can aggravate the risk of extinction in small populations (Frankham, 2005; Spielman et al., 2004). Both theoretical and empirical studies have revealed that loss of genetic diversity is related to inbreeding and thus leads to reduced fitness in small and endangered populations (Frankham, 2005; Hedrick & Garcia-Dorado, 2016). Unexpectedly, we found no evidence of inbreeding among closely related individuals in both Hong Kong and Nanchang captive populations. For the OPHK population, the best explanation is that this captive population likely originated from different wild sources (as described in the section below), and this might provide genetic rescue that prevented loss of genetic diversity. The results of the NCZ population may be related to the short amount of time (< 5 years) that this population has been captive. And our result of relatedness estimator may also support these results, most value of the pairwise relatedness (R_{QG}) are negative in both OPHK population and NCZ population (Table S2). The more negative value means the more confident of unrelated of that two individuals, and it further indicate the

390 detection of recent immigrants that carry novel alleles (Konovalov & Heg, 2008; Queller &
391 Goodnight, 1989). Another factor that must be considered is the statistical power of the
392 microsatellite data: although this power was high (90%), the probability still exists that the data
393 underestimated the inbreeding and relatedness (Liu et al., 2013).
394
395 Overall, results of this study have important conservation implications. First, understanding
396 genetic diversity and relatedness is a critical step for complementing effective management of
397 endangered animal species (Frankham, 2005; Seymour et al., 2001). Captive population
398 research provides a valuable opportunity to estimate genetic diversity in the Blue-crowned
399 Laughingthrush. Second, although we found no evidence of inbreeding in the two Blue-crowned
400 Laughingthrush populations, larger surveys of genetic diversity and relatedness may reveal
401 inbreeding in other captive populations in different zoos in Europe and North America
402 (Wilkinson & Gardner, 2011; Wilkinson et al., 2004). Using such resulting information,
403 management action plans can be made to maintain genetic health and avoid inbreeding by
404 exchanging non-related individuals among zoos worldwide. Finally, it is also necessary to carry
405 out *in situ* conservation efforts (Zhang et al., 2017) for species which are facing different
406 conservation challenges. This study provides genetic tools for conservation and evolutionary
407 genetic studies, and baseline information that can be compared between wild and captive
408 populations if needed by future reintroduction programs.
409
410 **Uncertainty of OPHK population origin**
411 Our sampling design also facilitated the first evaluation of OPHK population origin. Previous
412 studies hypothesised that captive OPHK individuals may belong to the subspecies from Simao
413 but without any clear evidence (Wilkinson & Gardner, 2011; Wilkinson et al., 2004). Using
414 multilocus analysis, we found evidence of genetic differentiation between OPHK and NCZ
415 populations based on significant F_{ST}, Bayesian-clustering analysis in STRUCTURE, and PCoA.

Mitochondrial DNA data analysis also supported this result, although most individuals shared the same haplotype, but both the OPHK and NCZ populations had private haplotypes. Moreover, assignment analysis revealed that two individuals of the NCZ population were inferred to be from the OPHK population; this result, which may be due to the low sample size of the NCZ population, further implies that higher genetic variation is present among individuals in the OPHK population.

It is known that the NCZ population was from the wild Wuyuan population; therefore, we are unable to exclude a single source of individuals in the OPHK population. The Simao population, which is represented by the subspecies *G. c. simaoensis*, was the only known wild source other than the Wuyuan population. Consequently, individuals from both Wuyuan and Simao populations may have contributed to the OPHK captive population. However, it is impossible to diagnose subspecies without having samples of *G. c. simaoensis*. Because the population status of *G. c. simaoensis* is unclear and it may have already been regionally extirpated (Wilkinson & He, 2010a), research on DNA extracted from museum specimens of *G. c. simaoensis* has great potential to resolve this issue.

Conclusions

The Global Species Management Plan for the Blue-crowned Laughingthrush has already been approved and facilitates management of Blue-crowned Laughingthrush zoo populations as a single unit worldwide (Gardner, 2013; WAZA, 2017). A European studbook of Blue-crowned Laughingthrush is maintained to manage captive populations in this region, but the genetic diversity and population structure of these populations are not clear, and no relatedness analysis based on genetic data has been conducted. Absence of such information can hinder management and conservation of this species, and understanding of other genetic problems, such as inbreeding depression (Frankham et al., 2010). With the new set of markers we

proposed, we estimated genetic diversity and relatedness of captive Blue-crowned Laughingthrush populations for the first time, which provides important information that can help prevent implementation of inappropriate recovery strategies and population reintroduction programs (Frankman, 2005). Information obtained from this study can benefit effective *ex situ* and *in situ* conservation efforts to recover bird species from the brink of extinction.

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References

Ballou JD, and Foose TJ. 1996. In: Kleiman DG, Lumpkin S, Allen M, Harris H, and Thompson K, eds. *Demographic and genetic management of captive populations In: Wild Mammals in Captivity*. University of Chicago Press, Chicago.

Bandelt HJ, Forster P, and Rohl A. 1999. Median-joining networks for inferring intraspecific phylogenies. *Molecular Biology and Evolution* 16:37-48.

Brownstein MJ, Carpten JD, and Smith JR. 1996. Modulation of non-templated nucleotide addition by Taq DNA polymerase: primer modifications that facilitate genotyping. *BioTechniques* 20:1004-1006, 1008-1010.

Canessa S, Guillera-Arroita G, Lahoz-Monfort JJ, Southwell DM, Armstrong DP, Chades I, Lacy RC, and Converse SJ. 2016. Adaptive management for improving species conservation across the captive-wild spectrum. *Biological Conservation* 199:123-131. 10.1016/j.biocon.2016.04.026

Castoe TA, Poole AW, de Koning APJ, Jones KL, Tomback DF, Oyler-McCance SJ, Fike JA, Lance SL, Streicher JW, Smith EN, and Pollock DD. 2012. Rapid Microsatellite Identification from Illumina Paired-End Genomic Sequencing in Two Birds and a Snake. *Plos One* 7. 10.1371/journal.pone.0030953

Cheng T, and Tang R. 1982. A new subspecies of *Garrulax galbanus* from Yunnan, China - *Garrulax galbanus simaoensis*. *Sinozoologica* 2:1-2.

Collar N, Robson C, and Sharpe CJ. 2016. Blue-crowned Laughingthrush (*Dryonastes courtoisi*). In: del Hoyo J, Elliott A, Sargatal J, Christie DA, and de Juana E, eds. *Handbook of the Birds of the World Alive*. Lynx Edicions, Barcelona.

475 Collar NJ, and van Balen S. 2013. Notes for the conservation of the Rufous-fronted Laughingthrush *Garrulax*
476 *rufifrons*. *Forktail* 1:15-18.
477 Evans SR, and Sheldon BC. 2008. Interspecific patterns of genetic diversity in birds: Correlations with extinction
478 risk. *Conservation Biology* 22:1016-1025. 10.1111/j.1523-1739.2008.00972.x
479 Excoffier L, and Lischer HEL. 2010. Arlequin suite ver 3.5: a new series of programs to perform population
480 genetics analyses under Linux and Windows. *Molecular Ecology Resources* 10:564-567. 10.1111/j.1755-
481 0998.2010.02847.x
482 Faircloth BC. 2008. MSATCOMMANDER: detection of microsatellite repeat arrays and automated, locus-specific
483 primer design. *Molecular Ecology Resources* 8:92-94. 10.1111/j.1471-8286.2007.01884.x
484 Faria J, Pita A, Rivas M, Martins GM, Hawkins SJ, Ribeiro P, Neto AI, and Presa P. 2016. A multiplex
485 microsatellite tool for conservation genetics of the endemic limpet *Patella candei* in the Macaronesian
486 archipelagos. *Aquatic Conservation-Marine and Freshwater Ecosystems* 26:775-781. 10.1002/aqc.2651
487 Frankham R. 2005. Genetics and extinction. *Biological Conservation* 126:131-140. 10.1016/j.biocon.2005.05.002
488 Frankham R. 2008. Genetic adaptation to captivity in species conservation programs. *Molecular Ecology* 17:325-
489 333. 10.1111/j.1365-294X.2007.03399.x
490 Frankham R. 2010. Challenges and opportunities of genetic approaches to biological conservation. *Biological*
491 *Conservation* 143:1919-1927. 10.1016/j.biocon.2010.05.011
492 Frankham R, Ballou JD, and Briscoe DA. 2010. Introduction to Conservation Genetics 2nd edn. *Cambridge*
493 *University Press, Cambridge, UK*.
494 Gardner L. 2013. *International Blue-crowned Laughingthrush Dryonastes courtoisi Studbook - No.1 Date current to*
495 *31st December 2013*. ZSL London Zoo.
496 Groth JG. 1998. Molecular phylogenetics of finches and sparrows: consequences of character state removal in
497 cytochrome b sequences. *Molecular phylogenetics and evolution* 10:377-390. 10.1006/mpev.1998.0540
498 He FQ, Lin JS, Wen C, Lin Z, Shi QH, Huang HQ, Cheng SL, and Xiao H. 2017. Prelim of Biology of the Blue-
499 crowned Laughingthrush *Garrulax courtoisi* in Wuyuan of NE Jiangxi, SE China. *Chinese Journal of*
500 *Zoology* 52:167-175.
501 Hedrick PW, and Garcia-Dorado A. 2016. Understanding Inbreeding Depression, Purging, and Genetic Rescue.
502 *Trends in Ecology & Evolution* 31:940-952. 10.1016/j.tree.2016.09.005
503 International B. 2017. Species factsheet: *Garrulax courtoisi*.
504 Kalinowski ST, Taper ML, and Marshall TC. 2010. Revising how the computer program CERVUS accommodates
505 genotyping error increases success in paternity assignment (vol 16, pg 1099, 2007). *Molecular Ecology*
506 19:1512-1512. 10.1111/j.1365-294X.2010.04544.x
507 Kalinowski ST, Wagner AP, and Taper ML. 2006. MLrelate: a computer program for maximum likelihood
508 estimation of relatedness and relationship. *Molecular Ecology Notes* 6:576-579.
509 Konovalov DA, and Heg D. 2008. A maximum-likelihood relatedness estimator allowing for negative relatedness
510 values. *Molecular Ecology Resources* 8:256-263. 10.1111/j.1471-8286.2007.01940.x
511 Leigh JW, and Bryant D. 2015. POPART: full-feature software for haplotype network construction. *Methods in*
512 *Ecology and Evolution* 6:1110-1116. 10.1111/2041-210x.12410
513 Li C. 2009. Sailing through the Late Pleistocene: unusual historical demography of an East Asian endemic, the

Chinese Hwamei (*Leucodioptron canorum canorum*), during the last glacial period (vol 18, pg 622, 2009). *Molecular Ecology* 18:2921-2921. 10.1111/j.1365-294X.2009.04241.x

Li D. 2017. The study on conservation genetics of the endangered *Emberiza jankowskii* Doctoral degree. Northeast Normal University.

Li RQ, Zhu HM, Ruan J, Qian WB, Fang XD, Shi ZB, Li YR, Li ST, Shan G, Kristiansen K, Li SG, Yang HM, Wang J, and Wang J. 2010. De novo assembly of human genomes with massively parallel short read sequencing. *Genome Research* 20:265-272. 10.1101/gr.097261.109

Li SB, Li B, Cheng C, Xiong ZJ, Liu QB, Lai JH, Carey HV, Zhang Q, Zheng HB, Wei SG, Zhang HB, Chang L, Liu SP, Zhang SX, Yu B, Zeng XF, Hou Y, Nie WH, Guo YM, Chen T, Han JQ, Wang J, Wang J, Chen C, Liu JK, Stambrook PJ, Xu M, Zhang GJ, Gilbert MTP, Yang HM, Jarvis ED, Yu J, and Yan JQ. 2014. Genomic signatures of near-extinction and rebirth of the crested ibis and other endangered bird species. *Genome Biology* 15. 10.1186/S13059-014-0557-1

Librado P, and Rozas J. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25:1451-1452. 10.1093/bioinformatics/btp187

Liu DQ, Wan CC, Fu WK, Lin JS, and Wu ZY. 2017. Observation of Blue － Crowned Laughingthrush Nestling Behaviors in Wuyuan, Hong Kong and Nanchang. *Chinese Journal of Wildlife* 38:249-253.

Liu DQ, Wu ZY, Wang XH, Huang HL, and Li DT. 2016. Cooperative Breeding behavior of captive Blue-crowned Laughingthrush (*Garrulax courtoisi*). *Chinese Journal of Wildlife* 37:228-233.

Liu Y, Keller I, and Heckel G. 2013. Temporal genetic structure and relatedness in the Tufted Duck *Aythya fuligula* suggests imited kin association in winter. *Ibis* 155:499-507.

Lynch M, and Walsh B. 1998. *Genetics and Analysis of Quantitative Traits*. Sinauer Associates, Inc. Sunderland, MA.

McKay BD, Mays HL, Peng YW, Kozak KH, Yao CT, and Yuan HW. 2010. Recent range-wide demographic expansion in a Taiwan endemic montane bird, Steere's Liocichla (Liocichla steerii). *Bmc Evolutionary Biology* 10. Artn 7110.1186/1471-2148-10-71

Paetkau D, Slade R, Burden M, and Estoup A. 2004. Genetic assignment methods for the direct, real - time estimation of migration rate: a simulation-based exploration of accuracy and power. *Molecular Ecology* 13:55-65. 10.1046/j.1365-294X.2003.02008.x

Peakall R, and Smouse PE. 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research-an update. *Bioinformatics* 28:2537-2539. 10.1093/bioinformatics/bts460

Queller DC, and Goodnight KF. 1989. Estimating relatedness using genetic markers. *Evolution* 43:258-275.

Rozen S, and Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, and Misener S, eds. *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, 365-386.

Ryman N, and Jorde PE. 2001. Statistical power when testing for genetic differentiation. *Molecular Ecology* 10:2361-2373. DOI 10.1046/j.0962-1083.2001.01345.x

Ryman N, and Palm S. 2006. POWSIM: a computer program for assessing statistical power when testing for genetic differentiation. *Molecular Ecology Notes* 6:600-602. 10.1111/j.1365-294X.2006.01378.x

Seymour AM, Montgomery ME, Costello BH, Ihle S, Johnsson G, St John B, Taggart D, and Houlden BA. 2001.

High effective inbreeding coefficients correlate with morphological abnormalities in populations of South Australian koalas (*Phascolarctos cinereus*). *Animal Conservation* 4:211-219.

Spielman D, Brook BW, and Frankham R. 2004. Most species are not driven to extinction before genetic factors impact them. *Proceedings of the National Academy of Sciences* 101:15261-15264.

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S. 2013. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30:2725-2729. 10.1093/molbev/mst197

Thompson JD, Higgins DG, and Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22:4673-4680.

Wang B, Xie X, Liu SM, Wang XJ, Pang H, and Liu Y. 2017. Development and characterization of novel microsatellite markers for the Common Pheasant (*Phasianus colchicus*) using RAD-seq. *Avian Research* 8. 10.1186/s40657-017-0060-y

WAZA. 2017. Global Species Management Plan.

Wilkinson R, and Gardner L. 2011. No laughing matter. *Zooquaria*:12-13.

Wilkinson R, and He FQ. 2010a. Conservation of Blue-crowned Laughing-thrush *Garrulax courtoisi* in Wuyuan, Jiangxi, and the search for 'lost' populations in Yunnan and Guanxi, China. *BirdingASIA* 13:100-105.

Wilkinson R, and He FQ. 2010b. Le garrulaxe de Courtois: conservation in situ. *CEPA Magazine*:12-17.

Wilkinson R, and He FQ. 2010c. Yellow-throated Laughingthrush *Garrulax galbanus* and Blue-crowned Laughingthrush *G. courtoisi* - new observations and interpretations on their taxonomy. *BirdingASIA*:73-82.

Wilkinson R, He FQ, Gardner L, and Wirth R. 2004. A highly threatened bird - Chinese Yellow-throated Laughing thrushes in China and in zoos. *International Zoo News* 51:456-469.

Wu YC, Huang JH, Zhang M, Luo ST, Zhang YH, Lei FM, Sheldon FH, and Zou FS. 2012. Genetic divergence and population demography of the Hainan endemic Black-throated Laughingthrush (Ayes: Timaliidae, *Garrulax chinensis monachus*) and adjacent mainland subspecies. *Molecular Phylogenetics and Evolution* 65:482-489. 10.1016/j.ympev.2012.07.005

Yang AL, Chen D, Wang PC, Fu YQ, and Zhang ZW. 2017. Characterization of novel microsatellite markers of the Emei Shan Liocichla using restriction site-associated DNA sequencing. *Avian Research* 8. 10.1186/s40657-017-0071-8

Yue GD, Gao Q, Luo LH, Wang JY, Xu JH, and Yin Y. 2012. The application of High-throughput sequencing technology in plant and animal research. *Scientia Sinica Vitae* 42:107-124. 10.1360/052011-634

Zhang WW, Shi JZ, Huang HQ, and Liu T. 2017. The impact of disturbance from photographers on the Blue-crowned Laughingthrush (*Garrulax courtoisi*). *Avian Conservation and Ecology* 12. 10.5751/Ace-01007-120115

Figure legends

Figure 1. A Blue-crowned Laughingthrush (*Garrulax courtoisi*) and its fledglings observed in Wuyuan, Jiangxi Province, China. Photographed by Lanhua Wang.

Figure 2. (a) Population structure results for Blue-crowned Laughingthrush. Each line represents one individual, and the possibility of individual that assigned to the given genetic cluster is represent by the length of each line. Abbreviations indicate different Blue-crowned Laughingthrush populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo). **(b)** Posterior probability means; Ln P(D) (± SD) increased per K. **(c)** Two genetic clusters were revealed based on the maximum value of ΔK and the order rate of change in posterior probability per K.

Figure 3. (a) Principal coordinates analysis results for 12 novel microsatellite loci of Blue-crowned Laughingthrush individuals genotyped at 12 microsatellite loci. Different colours represent postulated populations. **(b)** A biplot of the respective log-likelihood values for individuals from two populations. With log-likelihoods converted to positive values, the lowest value indicates the most likely population of origin. The abbreviations represent the different Blue-crowned Laughingthrush populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo).

Figure 4. Haplotype network analysis results for the cytb gene dataset (1017 bp) of Blue-crowned Laughingthrush. Black lines on branches indicate the inferred number of mutation steps between haplotypes or ancestral haplotypes. Circle size is proportional to the number of individuals with a particular haplotype. Abbreviations indicate different Blue-crowned Laughingthrush populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo).

Figure 5. Pairwise genetic relatedness among Blue-crowned Laughingthrush individuals. Different colours represent the two study populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo). Abbreviations indicate different levels of genetic relatedness (U: unrelated, HS: half-siblings, FS: full-siblings, PO: parent–offspring).

SUPPORTING INFORMATION

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

Table S1. Sample voucher numbers and sequence GenBank accession numbers of two captive Blue-crowned Laughingthrush (*Garrulax courtoisi*) populations used in this article.

Table S2. Genetic relatedness among analysed pairs of Blue-crowned Laughingthrush individuals.

Table S3. Reads of the 12 microsatellite loci in a sample set of two captive Blue-crowned Laughingthrush populations.

Figure S1. Analysis of statistical power to detect significant differentiation based on the 12 microsatellite markers used in this study. Datasets with eight predefined levels of population differentiation (F_{ST} values of 0.001, 0.0025, 0.005, 0.01, 0.02, 0.05, 0.075) were generated using POWSIM. Statistical power was defined as the proportion of times the null hypothesis of equal allele frequencies across populations was rejected using a chi-square test or a Fisher's exact test.

Figure 1

A Blue-crowned Laughingthrush (*Garrulax courtoisi*) and its fledglings observed in Wuyuan, Jiangxi Province, China.

Photographed by Lanhua Wang.

Figure 2

Population structure results for Blue-crowned Laughingthrush.

(a) Population structure results for Blue-crowned Laughingthrush. Each line represents one individual, and the possibility of individual that assigned to the given genetic cluster is represent by the length of each line. Abbreviations indicate different Blue-crowned Laughingthrush populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo). **(b)** Posterior probability means; Ln P(D) (± SD) increased per K. **(c)** Two genetic clusters were revealed based on the maximum value of ΔK and the order rate of change in posterior probability per K.

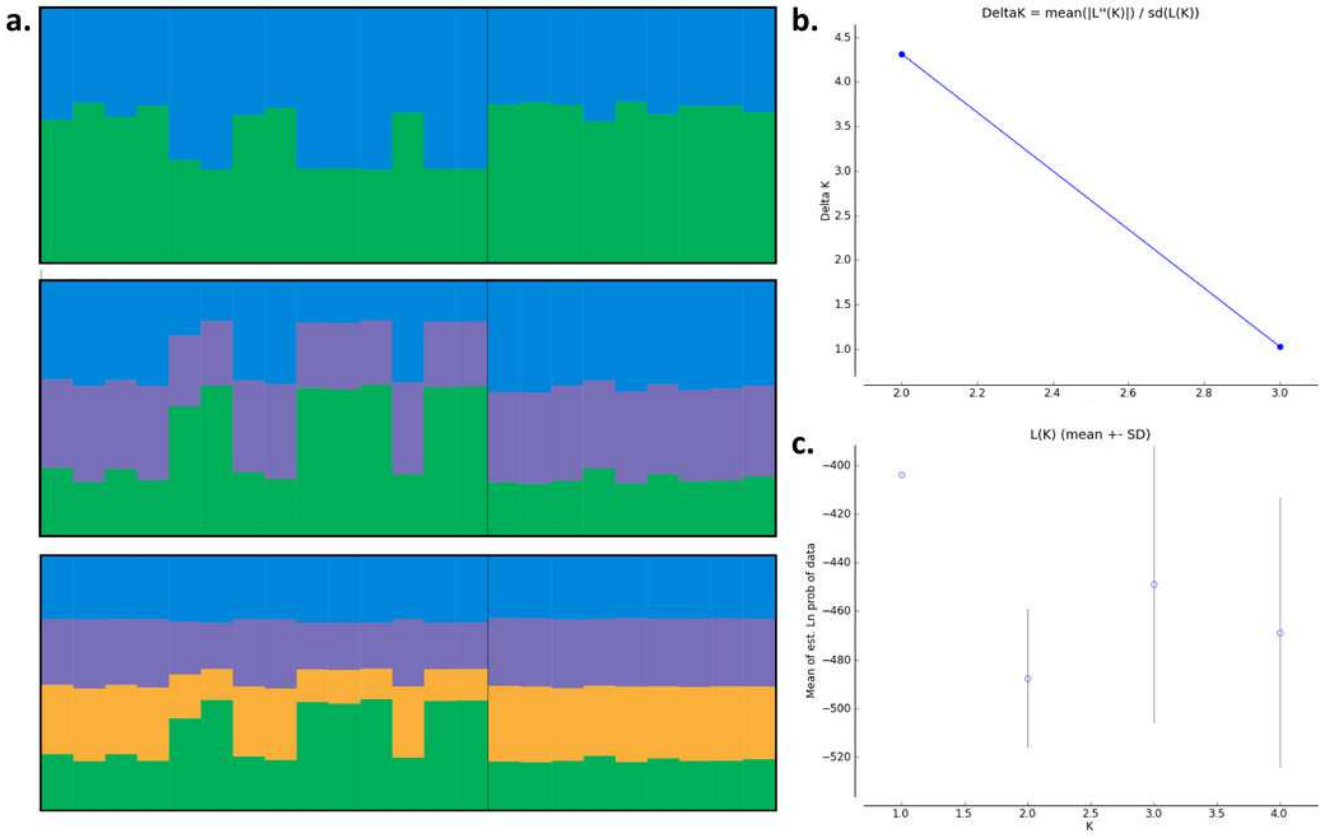
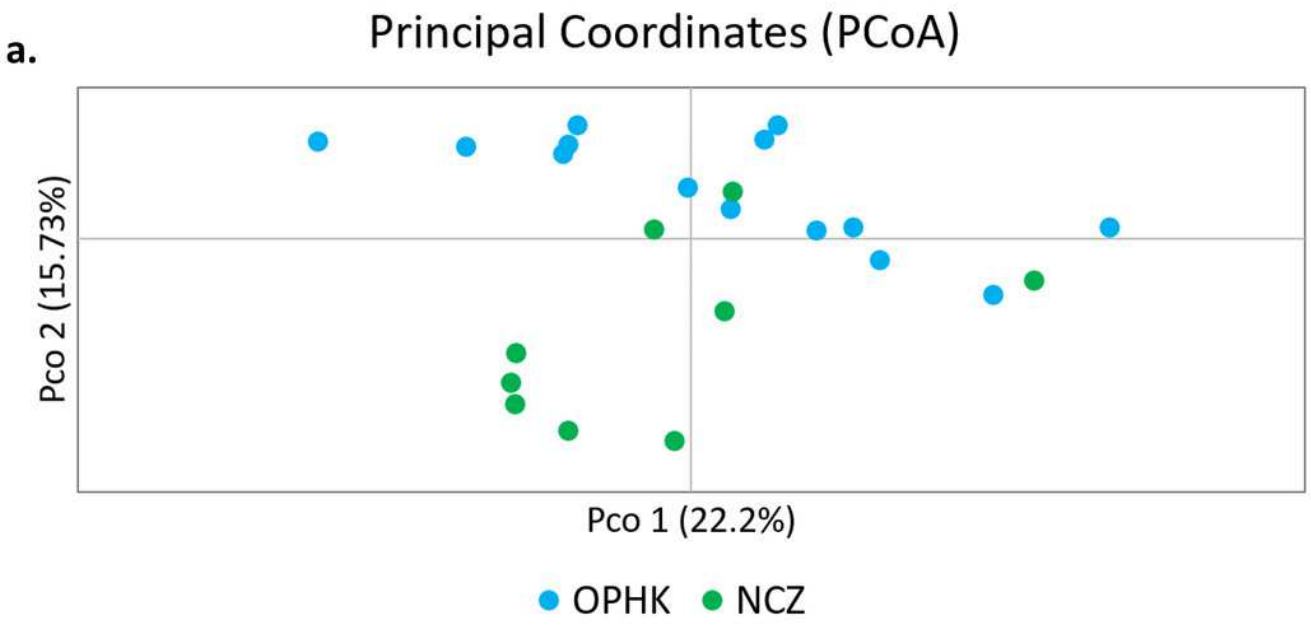


Figure 3

Principal coordinates analysis results ? and the respective log-likelihood values for individuals from two populations.

(a) Principal coordinates analysis results for 12 novel microsatellite loci of Blue-crowned Laughingthrush individuals genotyped at 12 microsatellite loci. Different colours represent postulated populations. **(b)** A biplot of the respective log-likelihood values for individuals from two populations. With log-likelihoods converted to positive values, the lowest value indicates the most likely population of origin. The abbreviations represent the different Blue-crowned Laughingthrush populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo).



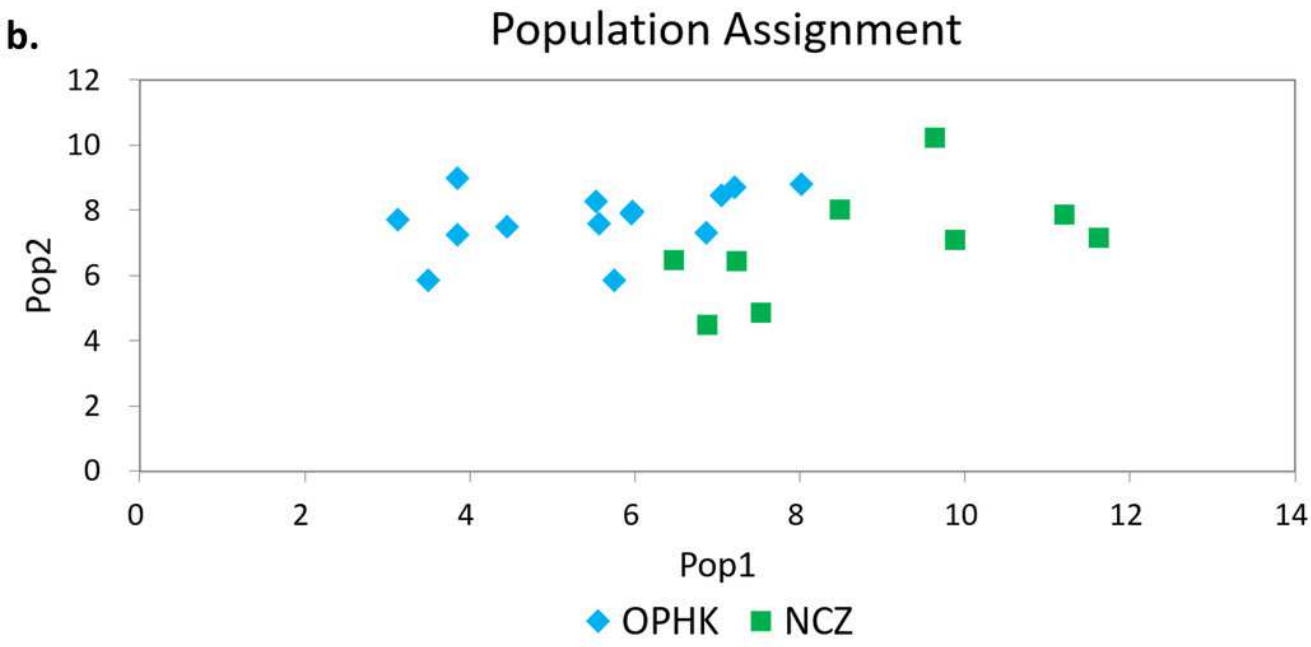


Figure 4

Haplotype network analysis results for the cytb gene dataset (1017 bp) of Blue-crowned Laughingthrush.

Black lines on branches indicate the inferred number of mutation steps between haplotypes or ancestral haplotypes. Circle size is proportional to the number of individuals with a particular haplotype. Abbreviations indicate different Blue-crowned Laughingthrush populations (OPHK: Ocean Park Hong Kong, NCZ: Nanchang Zoo).

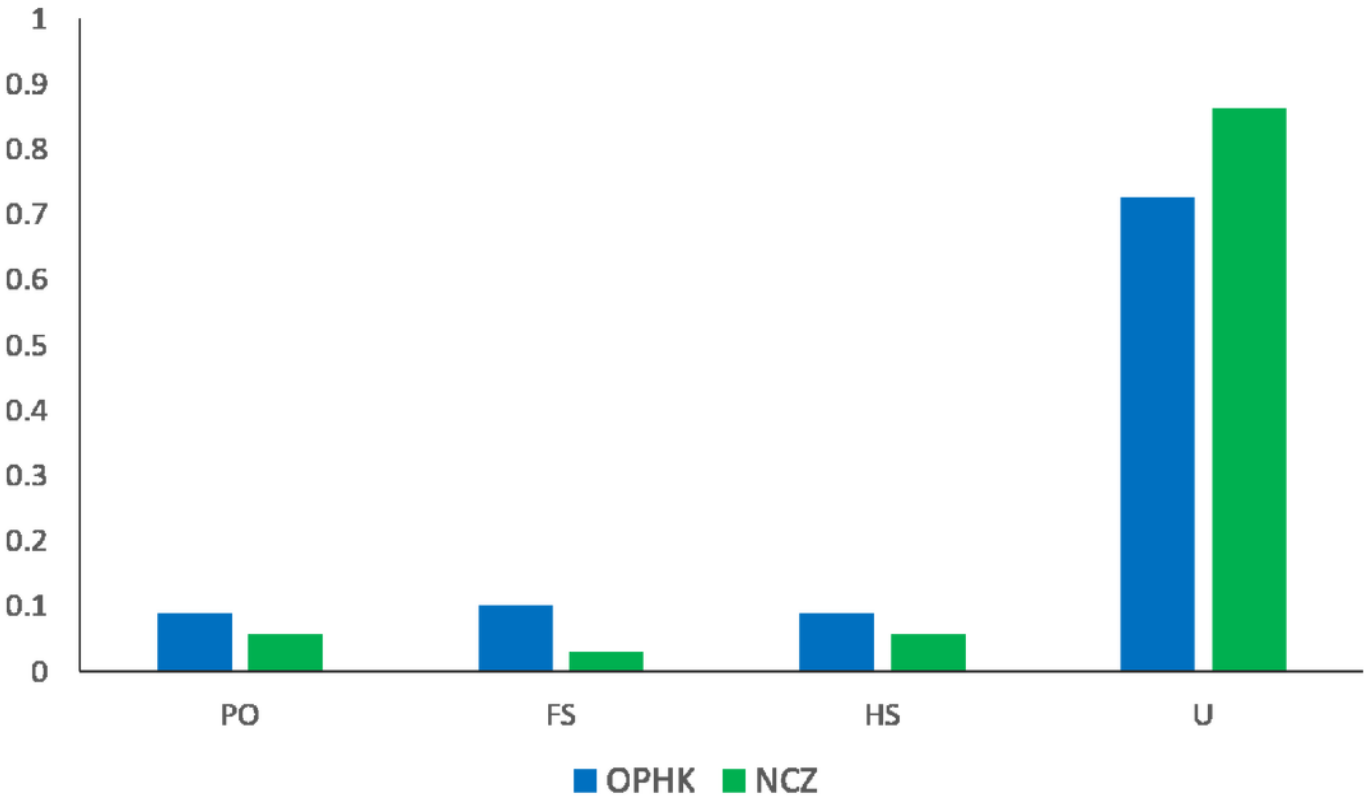


Table 1(on next page)

Characteristics of 12 microsatellite loci in a sample set of two captive Blue-crowned Laughingthrush (*Garrulax courtoisi*) populations.

The average number of different alleles (N_A), average allelic richness (A_R), mean observed (H_O±SD), mean expected (H_E±SD), polymorphism information content (PIC), and genetic differentiation index (F_{ST}, * indicates $p < 0.05$) for the OPHK and NCZ populations were estimated for each locus, and values in bold indicate significant deviations from Hardy–Weinberg equilibrium after Bonferroni correction.

Table 1. Characteristics of 12 microsatellite loci in a sample set of two captive Blue-crowned Laughingthrush (*Garrulax courtoisi*) populations.

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Locus	Repeat motif	Primer sequence	Annealing temperature (°C)	Size range(bp)	N_A	A_R	H_O	H_E	PIC	F_{ST}
BCLT_L1	(TG)6	F: CCAAATTCCCCCAGTCCTCC R: ATGTCAGACACAGCCCGAAC	58-60	101	3	3	0.261	0.241	0.222	-0.008
BCLT_L2	(AC)7	F: CCTGCGCATTACCTTGCATC R: GCAGACACACAGCATTGCAA	58-60	101	3	4	0.087	0.086	0.082	-0.019
BCLT_L3	(GT)7	F: ACAAGTCCCACGTGCTTTCA R: AAACAGTATCCCCTCCCTGC	58-60	102	3	4	0.261	0.300	0.262	0.137
BCLT_L4	(CT)6	F: TGACAAACTCTCCCAAGGCC R: GCTTTAGCAGGGATGTGGGT	58-60	121	3	4	0.391	0.341	0.308	0.075
BCLT_L5	(AC)7	F: TCCTCAGCTTTCAACCAGGT R: TCCAGGTGTTGTTCAGTGCA	58-60	131	4	16	0.696	0.764	0.700	0.154*
BCLT_L6	(TA)8	F: AAACCAGCCCTCGACCAAAA R: TCGAGGCTTAATCTGGGTGC	58-60	188	5	12	0.739	0.705	0.633	0.143*
BCLT_L7	(TA)6	F: CCCTTCATTAGCCCTGTGCA R: TTGTGTGTGTGCATGCCATG	58-60	216	3	4	0.522	0.565	0.456	0.024
BCLT_L8	(GT)6	F: AGCAGACCAGAGAGCAACAC R: TGGCAAAGAAGTTGGGGGTT	58-60	238	2	2	0.304	0.264	0.225	0.005
BCLT_L9	(TA)6	F: TGGAAGCATACACCACACAGA	58-60	258	5	5	0.565	0.538	0.484	0.028

		R: GCATTTTCTTCTTGGCTCTCAGT								
BCLT_L10	(TAT)5	F: GACAGACACGTGCTTCTCCA	58-60	137	3	6	0.478	0.530	0.405	-0.024
		R: GCAGGTCACCTCCTGAACTC								
BCLT_L11	(GCA)6	F: GGTTCACAGCCTCTGGTCTC	58-60	184	2	6	0.261	0.232	0.201	-0.032
		R: AGTTCTGGTTGGGAGTGCTG								
BCLT_L12	(TAG)5	F: TCCACTTCAGTCCCAGGTCA	58-60	254	3	9	0.174	0.165	0.154	0.007
		R: ATGGCAGTTGGGTTGGAACT								

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Table 2 (on next page)

Genetic variability in two captive Blue-crowned Laughingthrush (*Garrulax courtoisi*) populations of the analysed mitochondrial cytochrome b and 12 microsatellite loci.

The number of individuals for which mtDNA (N_{mt}) and microsatellites (N_{mi}) were analysed are shown. For mtDNA, the average number of nucleotide differences (K), number of haplotypes (N_H), haplotype diversity (H ± SD), and nucleotide diversity (π ± SD, in percent) were calculated. For microsatellites, the average number of different alleles (N_A ± SD), average allelic richness (A_R ± SD), mean observed heterozygosity (H_O ± SD), and mean expected heterozygosity (H_E ± SD) were quantified. The multilocus inbreeding coefficients (F_{IS}, none of the coefficients were significant) and average pairwise relatedness based on the Queller and Goodnight estimator (R_{QG} ± SD) are provided for each population, and values in bold indicate significant deviations from Hardy–Weinberg equilibrium after Bonferroni correction.

Table 2. Genetic variability in two captive Blue-crowned Laughingthrush (*Garrulax courtoisi*) populations of the analysed mitochondrial cytochrome b and 12 microsatellite loci.

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	Mitochondrial DNA					Microsatellites						
Population	*n*	*K*	N_H	H ± SD	π ± SD (%)	*n*	*Na* ± sd	A_R ± sd	H_O ±s d	H_E ± sd	F_{IS}	R_{QG}
OPHK	14	1.06	4	0.38±0.11	0.10±0.12	14	2.50±0.80	4.42±3.92	0.36±0.27	0.34±0.22	-0.214	-0.08±0.52
NCZ	9	1.10	2	0.37±0.11	0.11±0.17	9	2.75±0.97	5.42±4.10	0.45±0.24	0.44±0.22	-0.283	-0.13±0.29
Total	23	1.11	5	0.38±0.09	0.11±0.13	23	3.25±0.97	6.25±4.11	0.41±0.25	0.39±0.21	-0.231	-0.05±0.33

Table 3 (on next page)

Genetic diversity measured by mitochondrial and microsatellite loci in different songbird species (order Passeriformes).

For mtDNA, the haplotype diversity (H) and nucleotide diversity (π) are presented. For microsatellites, the average number of different alleles (N_A), and mean observed (H_O) and mean expected (H_E) heterozygosity are presented. CR: Critically endangered, EN: endangered, VU: vulnerable, LC: least concern.

Table 3. Genetic diversity measured by mitochondrial and microsatellite loci in different songbird species (order Passeriformes). For mtDNA, the haplotype diversity (H) and nucleotide diversity (π) are presented. For microsatellites, the average number of different alleles (N_A), and mean observed (H_O) and mean expected (H_E) heterozygosity are presented. CR: Critically endangered, EN: endangered, VU: vulnerable, LC: least concern.

Common name	Scientific name	Conservation status	Mitochondrial DNA		Microsatellites			References
			H	π	N_A	H_O	H_E	
Blue-crowned Laughingthrush	*Garrulax courtoisi*	CR	0.38	0.0011	2.58	0.40	0.37	This study
Jankowski's Bunting	*Emberiza jankowskii*	EN	0.75	0.009	14.2	0.61	0.76	(Li, 2017)
Emei Shan Liocichla	*Liocichla omeiensis*	VU	——	——	6.08	0.66	0.71	(Yang et al., 2017)
Steere's Liocichla	*Liocichla steeriia*	LC	——	——	19.6	0.76	0.79	(McKay et al., 2010)
Chinese Hwamei	*Garrulax canorus*	LC	0.96	0.003	——	——	——	(Li et al., 2009)
			0.943	0.004				
Black-throated Laughingthrush	*Garrulax chinensis*	LC	0.971	0.006	——	——	——	(Hurt, 2004)