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# High levels of genetic diversity and an absence of genetic structure among breeding populations of the endangered Rufous-backed Bunting in Inner Mongolia, China: implications for conservation

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## Abstract

**Background:** The Rufous-backed Bunting, *Emberiza jankowskii*, is an endangered species that is primarily distributed in Inner Mongolia, China. The main threats to the continued persistence of this species are habitat loss and degradation. However, the impact of population loss on genetic diversity remains unclear. To support future conservation and management efforts, we assessed the genetic diversity and population structure of *E. jankowskii* using mitochondrial DNA and microsatellites.

**Methods:** Blood samples were collected from 7–8-day-old nestlings in Inner Mongolia, China between May and August of 2012 and 2013. Mitochondrial DNA sequences and microsatellite markers were used to assess the genetic diversity, genetic structure and inbreeding of *E. jankowskii*. The results of genetic diversity and inbreeding were compared to other avian species.

**Results:** We found an unexpectedly high level of genetic diversity in terms of mitochondrial DNA and microsatellite compared to other avian species. However, there were high levels of gene flow and minimal genetic structuring, among the fragmented breeding populations of *E. jankowskii* in Inner Mongolia. These findings suggest that *E. jankowskii* in Inner Mongolia is a metapopulation. Despite the high genetic diversity of *E. jankowskii*, local populations in each small patch remain at risk of extinction due to habitat loss. In addition, the *E. jankowskii* population has a high risk of inbreeding.

**Conclusions:** To minimize further loss of genetic diversity of this endangered species, we suggest that the *E. jankowskii* in Inner Mongolia should be considered as a protected species for management purposes. Conservation efforts should concentrate on *E. jankowskii* habitat management. This may be most effectively achieved by protecting the current breeding habitats and prohibiting over-grazing.

**Keywords:** Conservation, *Emberiza jankowskii*, Genetic diversity, Genetic structure, Habitat fragmentation

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## Background

Genetic diversity is a prerequisite for the adaptation and survival of species (Schonewald-Cox et al. 1983; Lande 1988). Indeed, the level of genetic diversity exhibited by a species reflects its evolutionary potential (Frankel and Soulé 1981). Therefore, evaluations of genetic diversity are essential for species management and the development of conservation strategies, especially for endangered species (Hedrick 2004). Generally, endangered species exist in small populations and exhibit low rates of genetic diversity (Hinkson and Richter 2016). Small populations are more prone to genetic drift, which can randomly reduce genetic variability and population fitness by increasing homozygosity (Lande 1999; Willi et al. 2007; Freeland et al. 2011). Therefore, species with low genetic diversity may lack the ability to adapt to environmental changes, and their potential for long-term survival may be diminished (Liu et al. 2003). However, relatively ancient endangered species (e.g. those that have become endangered over the last 150 years due to habitat fragmentation) may retain high genetic diversity due to long generation times (Lippé et al. 2006).

The Rufous-backed Bunting (*Emberiza jankowskii*), a small grassland passerine with strict habitat requirements, was a common species throughout its historical breeding range. Historically, *E. jankowskii* has bred in Heilongjiang, western and eastern Jilin Province in China, extreme northeastern North Korea, and south of the Russian Far East (Wang et al. 2011). Because of habitat fragmentation, human activities, and low reproductive success, populations of this species have declined dramatically over the last 50 years (Jiang et al. 2008). *E. jankowskii* has been listed as endangered on the IUCN Red List since 2010 (BirdLife International 2018). From 2011–2016, we surveyed the breeding distribution of *E. jankowskii* in China and demonstrated that this species has vanished from most of its historic breeding sites (Wang et al. 2011; Han et al. 2018). Fortunately, we discovered 13 new *E. jankowskii* breeding sites in Inner Mongolia. However, these new breeding sites were seriously degraded because of over-grazing, urbanization, and agriculture (Han et al. 2018). During the course of our survey from 2012 to 2018, the populations of *E. jankowskii* at the new breeding sites began to decline and become increasingly fragmented (Han et al. 2018).

Experimental studies and field investigations have shown that fragmented populations may lose allelic richness and genetic diversity (Buza et al. 2000; Tomimatsu and Ohara 2003). Previously, major histocompatibility complex (MHC class II B) genes were used to examine the genetic diversity of *E. jankowskii*, and results showed relatively low levels of MHC polymorphisms (Li et al. 2017). MHC genes were mainly relevant to immune

function (Mak and Sanderson 1985; Schad et al. 2004). These findings indicate that MHC polymorphisms are affected by selection. The selection pressures on MHC genes can produce discrepancies between patterns of MHC and neutral variation in natural populations (Alcaide et al. 2010; Sutton et al. 2011). MHC polymorphisms will not provide a precise measure of genome-wide diversity. We suggest the use of neutral markers to evaluate the genetic diversity of *E. jankowskii*.

To investigate the genetic diversity and structure of *E. jankowskii* based on neutral markers, we (1) used mitochondrial DNA sequences and microsatellite markers to compare this level of genetic diversity to previously published levels of genetic diversity in other avian species, (2) estimated whether *E. jankowskii* was inbreeding because of low population sizes, (3) determined the genetic structure among populations of *E. jankowskii*, and (4) discussed the next stage of *E. jankowskii* conservation work.

## Methods

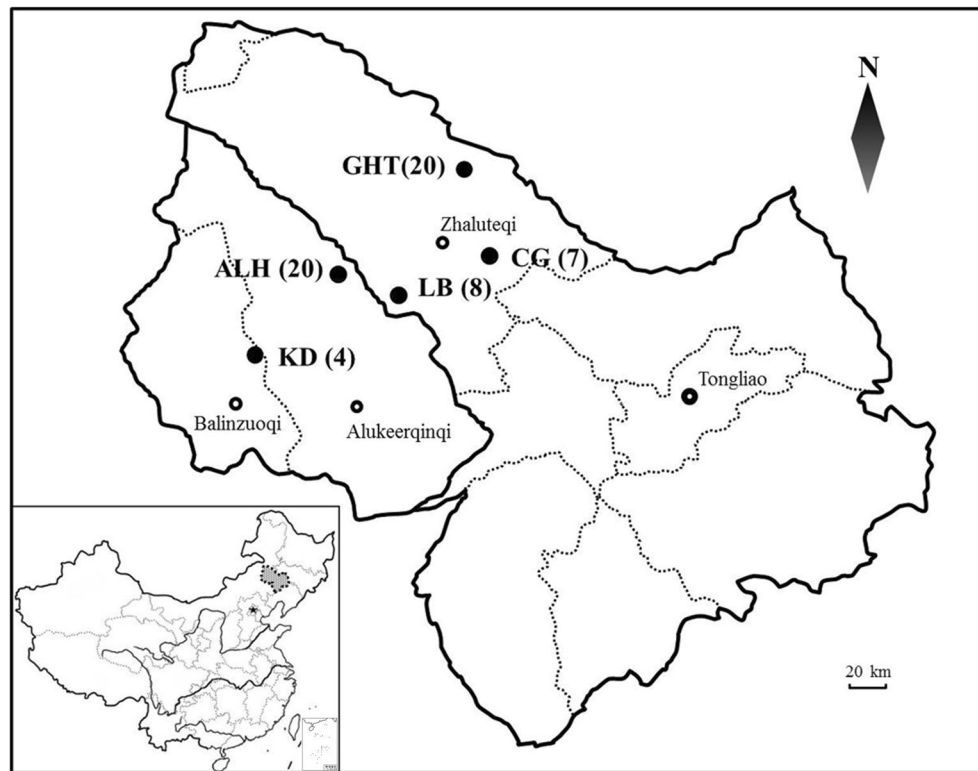
### Sample collection

We used a direct search method to find *E. jankowskii* nests in Gahaitu (GHT), Aolunhua (ALH), Lubei (LB), Chagantaoligao (CG), and Kundu (KD), in Inner Mongolia, China between May and August of 2012 and 2013 (Fig. 1). Blood samples were taken by venipuncture (brachial vein) under the wings of 7–8 day old nestlings. Samples were mixed with absolute ethyl alcohol in 1.5 mL tubes and preserved at  $-20^{\circ}\text{C}$ .

According to the population density of Han et al. (2018) and the area of our sample sites (GHT 3.87 km<sup>2</sup>, ALH 4.74 km<sup>2</sup>, LB 3.91 km<sup>2</sup>, CG 1.27 km<sup>2</sup>, and KD 2.55 km<sup>2</sup>), we assessed the population sizes of five sampling sites. The population size, sampling nests in each sample site, and the distance among sampling sites are presented in Table 1.

### Mitochondrial DNA amplification and microsatellite genotyping

Total genomic DNA was isolated using an AxyPrep Blood Genomic DNA Miniprep Kit (Aisijin, Hangzhou, China). All nestlings in the same nest have the same parents, we sampled only one randomly selected nestling per nest (a total of 59 individuals). We used polymerase chain reactions (PCRs) to amplify hypervariable region I (HVI) of the mitochondrial control region using the novel primers A629 (5'-GTATTCAACCGACCACTT-3') and S19 (5'-CTCCGAGACCTACGACCTG-3'). PCR amplifications were performed in 20  $\mu\text{L}$  volumes containing 10  $\mu\text{L}$  of 2  $\times$  Taq PCR Master Mix (Sangon Biotech), 0.25  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), and 1  $\mu\text{L}$  template DNA. The PCR cycling program was as follows: an initial denaturation step at 94  $^{\circ}\text{C}$  for 5 min; followed



**Fig. 1** Geographical distribution of *Emberiza jankowskii* in Inner Mongolia, China. Black dots represent sampling sites. Black circles indicate cities. The numbers of individuals sampled are given in parentheses

**Table 1** Population size, sampling nests, and distance of sample sites

Sample site	Sampling nests	Population size	Distance among sampling sites (km)				
			GHT	LB	ALH	CG	KD
GHT	20	152.28	–				
LB	8	153.86	70.31	–			
ALH	20	220.41	79.95	31.09	–		
CG	7	49.97	43.53	48.98	48.98	–	
KD	4	118.58	137.54	76.26	57.67	125.20	–

by 35 cycles of denaturation at 94 °C for 30 s, annealing at 53.7 °C for 30 s, and extension at 72 °C for 30 s; then a final extension at 72 °C for 10 min. All the sequences were deposited in GenBank under accession numbers MH670589–MH670647.

We amplified 10 microsatellite loci from each of the 59 individuals using 10 primer pairs: Ecit3, Dpu16, Escu1, Escu2, Escu3, Escu4, LS2, Ecit2, HXW20, and embc30 (Hanotte et al. 1994; Mundy and Woodruff 1996; Dawson et al. 1997; Wonke et al. 2007; Yu et al. 2011; Segelbacher and Schuphan 2014). We optimized the reaction volumes and PCR protocols for each primer pair. Microsatellite

loci were scored by Sangon Biotech (Shanghai, China). Final fragment sizes were calculated in GeneMarker v1.91 (SoftGenetics LLC, State College, PA, USA) and scored manually.

#### Data analysis

##### Genetic diversity, Hardy–Weinberg, and linkage disequilibrium tests

We calculated various mitochondrial diversity parameters in DnaSP v5.10 (Rozas et al. 2003), including haplotype diversity ( $H_d$ ), nucleotide diversity ( $\pi$ ), and the

number of polymorphic and phylogenetically-informative sites (Nei and Tajima 1981; Graur and Li 2000).

We used GENEPOP v4.6 (Rousset 2008) to test for deviations from the Hardy–Weinberg equilibrium and to identify alleles exhibiting linkage disequilibrium among all loci across all populations, using 10,000 dememorizations, 10,000 batches, and 10,000 iterations per batch. Critical significance levels were adjusted for multiple comparisons using the sequential Bonferroni correction ( $\alpha = p/n$ ; Rice 1989). Loci were checked for the likelihood of null alleles with Micro-checker (Van et al. 2004).

Genetic statistics were used to measure the genetic variability of the microsatellites, observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) under Hardy–Weinberg were also computed with GENEPOP 4.6. The number of alleles, the number of private alleles, and gene diversity were computed with FSTAT v2.9.3 (Goudet 2001).

#### Gene flow and inbreeding

We calculated the fixation index ( $F_{ST}$ ) in Arlequin v3.5 and Genetix v4.05 (Nei 1978; Excoffier et al. 1992; Belkhir et al. 1996; Schneider et al. 2000), and gene flow ( $N_m$ ) was estimated using the equation  $N_m = 0.25 (1 - F_{ST}) / F_{ST}$  (Excoffier et al. 1992; Schneider et al. 2000). The inbreeding coefficient ( $F_{IS}$ ) was calculated with GENEPOP 4.6.

#### Population structure

For our phylogenetic analyses, the D-loop sequences of *E. schoeniclus* (AJ243929), *E. cirrus* (AJ243928), and *Miliaria calandra* (AJ243925) were downloaded from GenBank and used as outgroups. The optimal models of nucleotide substitution were determined using the Akaike Information Criterion (AIC) in jModelTest v2.1.10 (Posada and Thomas 2004). The best model of nucleotide substitution, as selected by the AIC, was TVM+G. We used maximum likelihood (ML) in PHYML (Guindon et al. 2005), maximum parsimony (MP) and neighbor-joining (NJ) in PAUP\* v4.0 (Swofford 2001), and Bayesian inference (BI) in MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001; Ronquist and Huelsenbeck 2003) to construct phylogenies. For the ML analysis, we set the parameters of the custom model based on the AIC results. Networks were constructed by median-joining in Network 5.0 (Bandelt et al. 1999).

We assessed population structure based on a Bayesian analysis of microsatellites. We used STRUCTURE v2.3.3 (Pritchard et al. 2000) to assign individuals to  $K=1-5$  data clusters, with the higher number of clusters corresponding to the number of populations assessed (Flanders et al. 2009; Mao et al. 2010). We performed 10 replicate STRUCTURE runs for each  $K$  value. Monte Carlo Markov chains (MCMCs) were admixed

for 2,000,000 cycles using independent models of allele frequencies, with the first 20% of the cycles discarded as ‘burn-in.’ The best  $K$  value was defined using the  $\Delta K$  statistic as described by Evanno et al. (2005).

We used the Bayesian method of MIGRATE-N to assess the microsatellite migration rates (Beerli 2006). Five long chains of  $1.0 \times 10^6$  generations were used for all runs of MIGRATE-N. The same three-step process was conducted on the actual data to form the test statistic. To ensure adequate mixing of Markov chains, we used Markov-coupling, where 4 independent chains were allowed to mix according to an adaptive heating scheme (1.0, 1.2, 1.5, 6.0).

#### Demographic estimates

To reconstruct the demographic history of *E. jankowskii*, the historical demographic dynamics of *E. jankowskii* were inferred from Bayesian skyline analyses implemented in BEAST 2.6.2 (Bouckaert et al. 2019). We used D-loop sequences for this analysis and selected the mean substitution rate at 1.0 to estimate time in units of substitution/site. Independent MCMC analyses were run for  $1.0 \times 10^8$  steps, sampling every  $10^5$  steps, and discarding 1000 samples as burn-in. The results visualized skyline plots with TRACER 1.5 (Drummond and Rambaut 2007). Sequence divergence rate of 2%/Myr was applied to the mtDNA D-loop region (Shields and Wilson 1987).

## Results

### Genetic diversity

The genetic diversity of *E. jankowskii* based on mitochondrial DNA and microsatellites is shown in Table 2. Amplification of the control regions (544 bp) from 59 individuals yielded 16 haplotypes with 14 polymorphic sites and 8 parsimony informative sites. The total haplotype diversity ( $H_d$ ) was 0.874 (standard deviation (SD)=0.023), and the overall nucleotide diversity ( $\pi$ ) was 0.0032 (SD=0.0003).

We detected no significant departures from the Hardy–Weinberg equilibrium after correcting for the number of pairwise comparisons ( $n=49$ ;  $\alpha=0.001$ ), with the following exceptions: locus Ecit3 in GHT, LB, and ALH; locus Esc $\mu$ 4 in GHT; locus HXW20 in GHT and CG; and locus embc30 in GHT and ALH. After Bonferroni correction, Fisher’s exact test identified no significantly linked pairs of loci ( $n=225$ ,  $\alpha=0.0002$ ). The micro-checker analysis did not suggest any scoring errors due to stuttering, nor did it suggest any large allelic dropout in any loci across all populations. Null alleles may have been present in locus Ecit3 in GHT, LB, and ALH; locus Esc $\mu$ 4 in GHT; locus LS2 in LB; locus HXW20 in GHT, ALH, and CG; and locus embc30 in GHT and ALH. Because we identified departures from the Hardy–Weinberg equilibrium

**Table 2 Genetic diversity parameters of *E. jankowskii***

Population	N	mtDNA			Microsatellite		
		A	$H_d \pm SD$	$\pi \pm SD$	$H_e$	$H_o$	$F_{IS}$
GHT	20	10	0.879 ± 0.052	0.0034 ± 0.0006	0.7113	0.6714	0.0575
ALH	20	8	0.895 ± 0.034	0.0030 ± 0.0003	0.6634	0.5929	0.1088
LB	8	5	0.857 ± 0.108	0.0028 ± 0.0006	0.7107	0.6071	0.1545
CG	7	3	0.714 ± 0.127	0.0032 ± 0.0008	0.7551	0.7959	-0.0588
KD	4	3	0.833 ± 0.222	0.0034 ± 0.001	0.5970	0.6786	-0.1633
Total	59	16	0.874 ± 0.023	0.0032 ± 0.0003	0.6993	0.6513	0.0692

Details of the populations are given in the text. Number of *E. jankowskii* individuals sampled (N); number of haplotypes (A); mean haplotype diversity ( $H_d$ ); mean nucleotide diversity ( $\pi$ ), expected heterozygosity ( $H_e$ ); observed heterozygosity ( $H_o$ ); inbreeding coefficient ( $F_{IS}$ )

and null alleles in the loci Ecit3, HXW20, and embc30 in two or more populations, we deleted these three loci from subsequent analyses.

The number of alleles per locus ranged from 4 to 16 across all populations (mean ± SD: 9.00 ± 5.03), with the most alleles found in the GHT population (7.00 ± 3.87) and the least found in the KD population (3.29 ± 1.11). Private alleles with a frequency over 10% were observed in the KD ( $P_A=2$ ) and LB ( $P_A=1$ ) populations. The gene diversity per population was 0.71 ± 0.19 in the GHT population, 0.72 ± 0.14 in the LB population, 0.67 ± 0.15 in the ALH population, 0.75 ± 0.13 in the CG population, and 0.58 ± 0.21 in the KD population. The highest observed heterozygosity ( $H_o$ ) and expected heterozygosity ( $H_e$ ) were observed in the CG population (0.7959 and 0.7551, respectively), while observed heterozygosity was lowest in the ALH population (0.5929) and expected heterozygosity was lowest in the KD population (0.5970) (Table 2).

Average inbreeding coefficient ( $F_{IS}$ ) of *E. jankowskii* in Inner Mongolia was estimated as 6.92%. Within population,  $F_{IS}$  ranged from -0.1633 (KD) to 0.1545 (LB) (Table 2).

Based on mitochondrial DNA and microsatellite data, the fixation index ( $F_{ST}$ ) was highest between the CG and LB populations (0.18462) and the CG and KD populations (0.06296), and lowest between the GHT and LB populations (0.00546) and CG and LB populations (0.00411). Thus, gene flow was highest between the GHT and LB populations (45.5376) and CG and LB populations (60.5779), and lowest between the CG and LB populations (1.1041) and CG and KD populations (3.7206) (Tables 3, 4).

**Population structure**

The NJ, MP, ML, and BI phylogenies were highly congruent. All *E. jankowskii* haplotypes clustered together, in a clade marked by shallow sequence divergences with no well-supported basal haplotype (Fig. 2). Additional

**Table 3 Pairwise fixation index ( $F_{ST}$ ) (lower triangle) and gene flow ( $N_m$ ) (upper triangle) of five *E. jankowskii* populations based on D-loop sequences**

	GHT	LB	ALH	CG	KD
GHT	-	45.5376	20.0258	3.9630	15.2586
LB	0.00546	-	-16.5792	1.1041	-2.7573
ALH	0.01233	-0.01531	-	1.9470	-72.9244
CG	0.05934	0.18462*	0.11379*	-	2.2196
KD	0.01612	-0.09971	-0.00344	0.10123	-

Statistically significant results are indicated by asterisks. \*,  $P < 0.05$ . “-” indicates null. Details of the populations are given in the text. The explanations apply also to Tables 4, 5 and 6

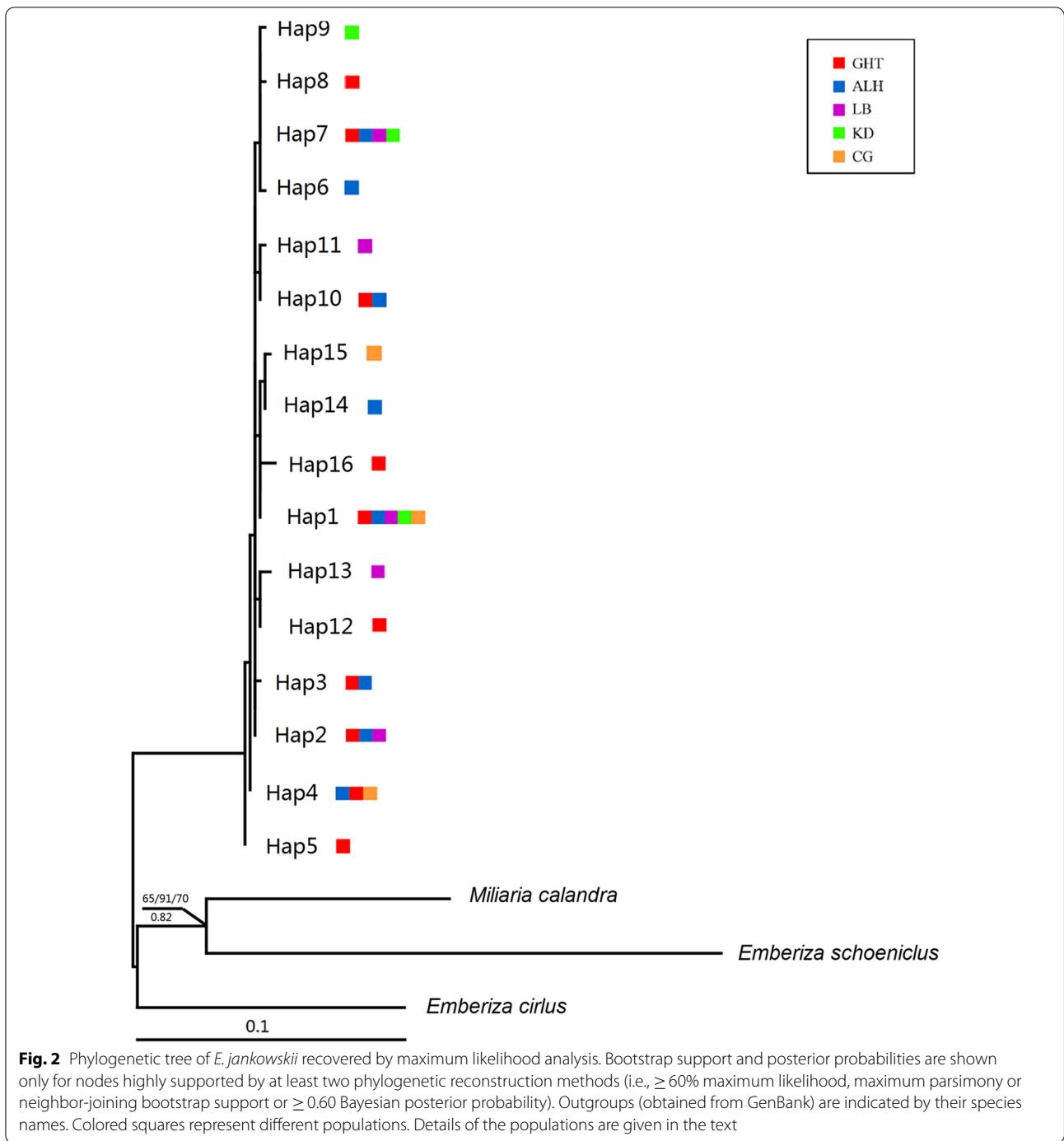
**Table 4 Pairwise fixation index ( $F_{ST}$ ) (lower triangle) and gene flow ( $N_m$ ) (upper triangle) of five *E. jankowskii* populations based on microsatellite data**

	GHT	LB	ALH	CG	KD
GHT	-	-18.2619	32.0097	-29.5228	9.5577
LB	-0.01388	-	22.8558	60.5779	9.2452
ALH	0.00775	0.01082	-	9.3654	6.8725
CG	-0.00854	0.00411	0.02600	-	3.7206
KD	0.02549	0.02633	0.03510	0.06296	-

analyses were conducted to assess the genetic structure of five populations by building a median-joining network. The network showed three different second levels (hap 1, 2 and 7), with some significant associations. Based on expectations from the coalescent theory, hap 2 was the haplotype with the highest frequency and most connections, and thus was the most likely ancestral haplotype (Fig. 3).

Although the best separation was obtained at  $K=2$  ( $\Delta K=1.68$ ), our STRUCTURE simulation indicated that homogenization was occurring across all *E. jankowskii* populations in Inner Mongolia; clear signs of admixture



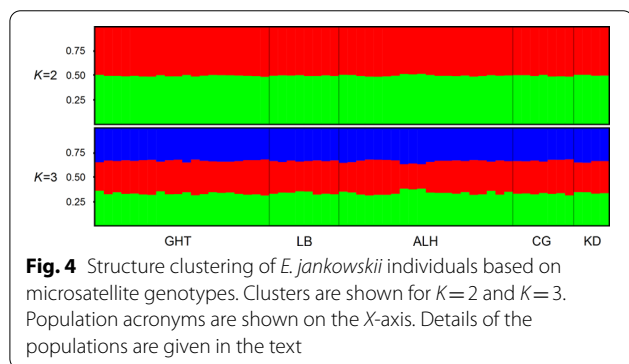
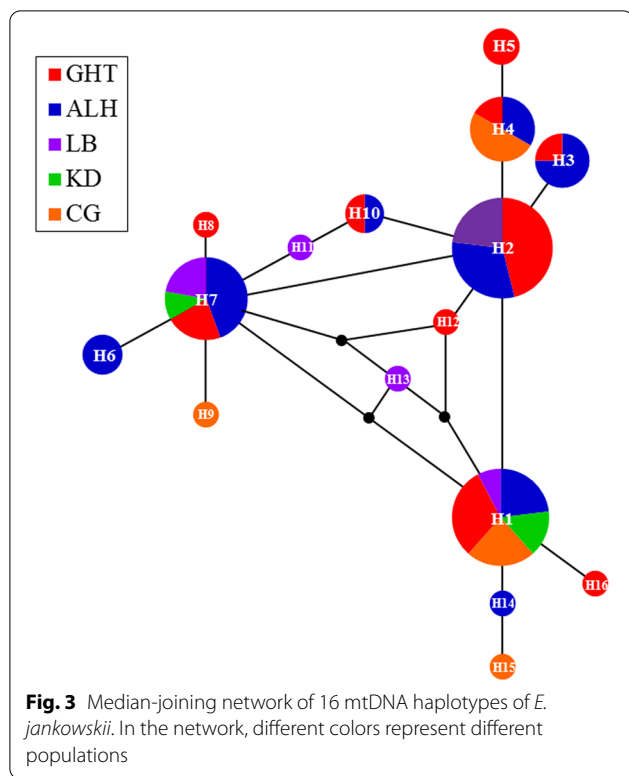


were observed in all subpopulations (Fig. 4, showing  $K=2$  and 3 only).

Our analysis of molecular variance (AMOVA), based on the distance matrices among individuals, indicated that the overall variation could be partitioned into two levels (Table 5). The proportion of variation attributable to within-population differences was particularly high

(mtDNA: 96.76%; microsatellites: 98.71%). The fixation indices ( $F_{ST}$ ) were 0.03 based on mtDNA, and 0.01 based on microsatellites ( $P>0.05$ ). All estimates of genetic differentiation indicated a relatively low level of genetic differentiation across all populations.

The results of Migrate-N show that there were significant gene flows between different populations. There



are migrations in and out among the five populations (Table 6).

**Demographic history**

Due to the existence of gene flow and the absence of genetic structure, we placed *E. jankowskii* in Inner Mongolia into one group for BSP analysis. The Bayesian skyline plots (BSP) revealed that the population size of *E. jankowskii* in Inner Mongolia is stable (Fig. 5).

**Discussion**

**High-level genetic diversity and inbreeding**

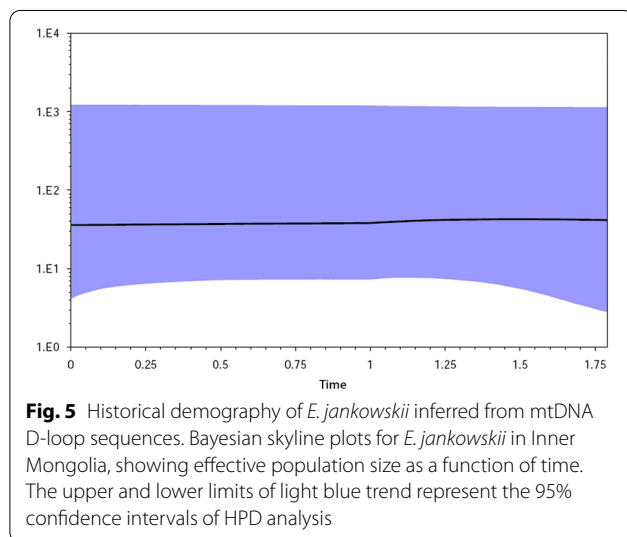
Surveys of genetic diversity in endangered species are essential for the evaluation of the evolutionary and adaptive potential of these species, such surveys are also vital for the development of effective conservation strategies to protect threatened populations, especially those that are fragmented (Zhang et al. 2017; Stevens et al. 2018). In this study, we investigated the genetic diversity of *E. jankowskii* for the first time, employing mitochondrial DNA sequences and microsatellite data. *E. jankowskii* had a higher level of haplotype diversity (0.874) and nucleotide diversity (0.0032) in its D-loop gene sequence than other endangered species, such as *Nipponia nippon* ( $H_d=0.386$ ,  $\pi=0.0007$ ) (Zhang et al. 2004) and *Megulus palustris sinensis* ( $H_d=0.772$ ,  $\pi=0.0014$ ) (Zhang 2007). Interestingly, the haplotype and nucleotide diversity of *E. jankowskii* was also higher than one species of least concern, *Parus varius* ( $H_d=0.5328$ ,  $\pi=0.0017$ ) (Du 2011). The microsatellite results also indicated that *E. jankowskii* had a higher degree of genetic diversity than all passerines birds ( $H_e=0.24-0.59$ ), which were listed in the article of Jamieson et al. (2006), and also higher than *N. nippon* ( $H_e=0.50$ ,  $H_o=0.47$ ) (He 2007), and *M. palustris sinensis* ( $H_e=0.53$ ,  $H_o=0.38$ ) (Zhao 2012). The observed heterozygosity of *E. jankowskii* was higher than *Gallus gallus* ( $H_o=0.37-0.41$ ) and *Perdix dauuricae* ( $H_o=0.58$ ), but the expected heterozygosity of *E. jankowskii* was a little lower than *Gallus gallus* ( $H_e=0.72-0.77$ ) and *Perdix dauuricae* ( $H_e=0.72$ ) (Cao et al. 2010; Masroor et al. 2012).

**Table 5 Analysis of molecular variance (AMOVA) among *E. jankowskii* mitochondrial D-loop sequences and microsatellite data across five populations**

Source of variation	df	Sum of squares	Fixation indices	Variance components	Percentage of variation
mtDNA					
Among populations	4	5.17	$F_{ST}$ : 0.03	0.03	3.24
Within populations	54	51.27	–	0.95	96.76
Microsatellites					
Among populations	4	12.44	$F_{ST}$ : 0.01	0.03	1.26
Within populations	113	273.95	–	2.42	98.71

**Table 6** Estimates of mutation-scaled effective population size ( $\theta$ ) and mutation-scaled effective immigration rate ( $M$ ) for the five populations of *E. jankowskii* using MIGRATE-N

Population	$\theta$	$M$ (mean)				
		GHT→	LB→	ALH→	CG→	KD→
GHT	0.044	–	174.12	165.75	174.53	69.65
LB	0.035	242.66	–	115.19	222.18	231.85
ALH	0.042	107.90	194.46	–	258.57	117.89
CG	0.038	173.60	177.42	233.25	–	36.08
KD	0.036	204.34	220.60	141.33	188.13	–



It is well established that decreases in genetic variation may reduce fitness and adaptability (Allentoft and O'Brien 2010). Generally, endangered species tend to have small population sizes and low levels of genetic diversity due to historical reasons, and reduce fitness (Matocq and Villablanca 2001). Unexpectedly, *E. jankowskii* exhibited high levels of genetic variability. This indicated that habitat fragmentation and degradation have not led to the rapid loss of genetic diversity in this species. There may be several explanations for this result. First, it is possible that *E. jankowskii* populations were historically very large (Fu and Chen 1966). Second, the observed reduction in *E. jankowskii* population sizes occurred during past 50 years (Jiang et al. 2008). This means that due to the small number of generations, *E. jankowskii* has not experienced a rapid decline in genetic diversity (Welch et al. 2012). Third, birds with higher dispersal abilities may therefore maintain high levels of genetic diversity due to gene flow. Fourth, hybridization and introgression with closely related species can also contribute to the maintenance of high levels of genetic diversity (Mallet 2005). However, whether hybridization

and introgression had occurred between *E. jankowskii* and *E. cioides* requires further investigation.

Although there are some methods available to minimize inbreeding (Pusey 1987), inbreeding may be inevitable in small, isolated populations and play a role in determining the probability of local extinction events (Frankham 2005). In this study, the average inbreeding coefficient of *E. jankowskii* in Inner Mongolia was estimated at 0.069 (Table 2), higher than other Passerine birds ( $F_{IS}=0.024-0.054$ ) and the captive panda population at the Chengdu ( $n=37$ ,  $F_{IS}=0.045$ ) (Sun et al. 2010; Abalaka et al. 2015). This indicated that a high inbreeding risk existed for *E. jankowskii* in Inner Mongolia or may have occurred to some extent. However, high levels of genetic diversity within *E. jankowskii* populations indicated that inbreeding depression did not occur.

#### Absence of genetic structure and high levels of gene flow

Genetic population structure imparts important information about species ecology and evolutionary history, including dispersal ability, adaptation, and speciation (Rousset 2004). Genetic population structure can also help to determine important species conservation units in patchy, fragmented habitats (Paetkau 1999). Here, no clear phylogeographic structure was observed across the five *E. jankowskii* populations in either the phylogenetic tree or the cluster analyses. The  $F_{ST}$  values of the five populations were relatively low (mtDNA: 0.00546–0.18462; microsatellites: 0.00411–0.06296). In addition, the  $F_{ST}$  values between populations ALH and LB; KD and LB; ALH and KD; GHT and LB; GHT and CG were negative (Tables 2, 3). Negative values suggest a lack of support for genetic differentiation between these population pairs (Foster et al. 2006).

Gene flow among populations is an important mechanism that maintains intraspecific genetic diversity (Slatkin 1994). Species with low levels of gene flow show more genetic differentiation among populations than do species with high levels of gene flow (Hamrick et al. 1991). In *E. jankowskii*, various levels of gene flow were calculated



among populations (Tables 3, 4). This was consistent with the AMOVA results, which identified more genetic variation within populations than between populations (Table 5). There was an asymmetric gene flow between certain populations (Table 6).

### Conservation implications

The five populations of *E. jankowskii* sampled here exhibited an absence of genetic structure and various levels of gene flow. Thus, *E. jankowskii* in Inner Mongolia is a metapopulation. We therefore suggest that, for the purposes of species management, *E. jankowskii* in Inner Mongolia should be defined as one protected unit.

Although *E. jankowskii* showed high genetic diversity, and *E. jankowskii* population sizes ranged between 9800 and 12,500 individuals (Han et al. 2018), but the number of individuals in every single patch is small (Table 1) and with high inbreeding risk. The local population in each patch might vanish due to habitat loss (Wang et al. 2011). Therefore, conservation policies limiting habitat destruction are necessary to mitigate the threat of species extinction.

The *E. jankowskii* in Inner Mongolia is still at risk of human disturbances, habitat loss, and habitat degradation (Han et al. 2018). The extremely limited habitat of this species (similar to the habitat requirements of *Prunus sibirica* and *Stipa baicalensis* on the Mongolian steppes) might make *E. jankowskii* very sensitive to habitat fragmentation, water pollution, global warming, and the degradation of habitats suitable for *P. sibirica* and *S. baicalensis*. Thus, habitat conservation and population management are urgently needed for this avian species. Indeed, the high genetic variability of *E. jankowskii* indicates the ability to recover if provided with effective protection (Evans and Sheldon 2008). Therefore, we suggest that management efforts for this species focus on the protection of the current breeding habitats and elimination of over-grazing. Establishing specific natural areas to preserve the breeding habitats of *E. jankowskii* and publicizing the importance of protecting this avian species to local residents might also be effective and should be considered. We hope that our work contributes to the establishment of a management and protection system for *E. jankowskii*. Our laboratory will continue to carry out the long-term monitoring of this species.

### Future prospects

This study has shown that *E. jankowskii* exhibits a high level of genetic diversity. We found that *E. cioides*, a closely related species (Alström et al. 2008), had overlapping distributions with *E. jankowskii* in Inner Mongolia. Several studies have indicated that species in nature are often incompletely isolated for millions of

years after speciation. There are many cases of hybridization and introgression occurred among closely related sympatric species (Mallet 2005). Whether hybridization and introgression had occurred between *E. jankowskii* and *E. cioides* and whether this contributes to the high level of genetic diversity of *E. jankowskii* require further investigation.

### Conclusions

*E. jankowskii* in Inner Mongolia is a metapopulation, so we suggest that the *E. jankowskii* in Inner Mongolia should be considered one protected unit for management purposes. The conservation efforts should concentrate on protecting the current breeding habitats and forbidding over-grazing.

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### Authors' contributions

SL and DL made equivalent contributions to this paper: both conceived and designed the experiments, carried out the molecular genetic analysis, participated in the manuscript's design, and drafted the manuscript. YJ developed the ideas and obtained funding support. LZ and BQ helped collect samples and classify species. WS helped perform the microsatellite experiments. All authors read and approved the final manuscript.

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### Availability of data and materials

The data on which this paper was made are fully available at the appendix of the manuscript.

### Ethics approval and consent to participate

This study conformed to the guidelines for the care and use of experimental animals established by the Ministry of Science and Technology of the People's Republic of China (Approval number: 2006–398). The research protocol was reviewed and approved by the Ethics Committee of Jilin Agriculture University.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

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