Journal of Biogeography (J. Biogeogr.) (2013) 40, 1156–1169

ORIGINAL ARTICLE

Glacial expansion and diversification of an East Asian montane bird, the green-backed tit (*Parus monticolus*)

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ABSTRACT

Aim We combined genetic sequence data and ecological niche modelling to resolve the impacts of past climatic fluctuations on the distribution, genetic diversification, and demographic dynamics of an East Asian montane bird, the green-backed tit (*Parus monticolus*).

Location East Asia.

Methods Phylogenetic analyses were carried out using four mitochondrial fragments and seven nuclear loci from 161 birds sampled from 29 localities spanning the entire geographical range of the green-backed tit. We used *BEAST to estimate the species tree and calculate divergence times. Extended Bayesian skyline plots were used to infer potential historical shifts in population size. We used MaxEnt to predict potential distributions during three periods: the present day, the Last Glacial Maximum and the Last Interglacial.

Results The mitochondrial DNA (mtDNA) gene tree showed strong support for three reciprocally monophyletic groups: a south-western clade, a central clade and a Taiwanese clade. Taiwanese and Vietnamese samples had fixed differences at several nuclear loci, but the south-western and central samples shared haplotypes at all nuclear loci. The mtDNA gene tree topology differed from the species tree topology. The species tree suggested sister relationships between Taiwanese and Vietnamese operational taxonomic units (OTUs) and between south-western and central OTUs. Diversification within the green-backed tit was relatively recent, probably within the last 0.9 million years. Extended Bayesian skyline plots suggested rapid population expansion in the south-western and central phylogroups after the Last Interglacial, and this result was consistent with ecological niche models.

Main conclusions Our results suggest that genetic diversification within the green-backed tit was affected by the later Pleistocene climate fluctuations. Ecological niche models indicated that the present-day vegetation distribution was, in many ways, more similar to that of the Last Glacial Maximum than it was to that of the Last Interglacial. Continental populations of the green-backed tit experienced unusual demographic and range expansion that is likely to have occurred during the cooling transition between the Last Interglacial and the Last Glacial Maximum. We found incongruence between the mtDNA gene tree and the species tree, which underscores the importance of using both mitochondrial and nuclear markers when estimating the evolutionary history of populations.

Keywords

*BEAST, birds, East Asia, ecological niche modelling, green-backed tit, historical demography, *Parus monticolus*, phylogeography, spatial dynamics, species tree.

http://wileyonlinelibrary.com/journal/jbi
doi:10.1111/jbi.12055

INTRODUCTION

Pleistocene climate fluctuations have greatly influenced species distributions, genetic diversification and demography (Hewitt, 2000). Studies of European and North American taxa have found that species generally retracted to low-latitudinal refugia during the Last Glacial Maximum (LGM; 0.021–0.018 million years ago, Ma) and expanded northwards as the environment warmed (Hewitt, 2000). This latitude shift leaves a pattern of high genetic diversity within southern refugial populations and low genetic variation within populations newly established from the expansion (Hewitt, 1999). Compared to the many studies of European and North American taxa, there has been relatively little work focused on other parts of the world, such as East Asia (Beheregaray, 2008), although East Asia is an important biogeographical area, with high species diversity (Myers *et al.*, 2000; Qian & Ricklefs, 2000) and many endemic species (Lei *et al.*, 2003). Climate fluctuations are thought to have been milder in East Asia than in Europe and North America (Pinot *et al.*, 1999; Shi & Yao, 2002). Temperate East Asia was dominated by summer precipitation and did not develop large glaciers during major glacial stages (Zhang *et al.*, 2006). Milder climate cycles may have produced different phylogeographical patterns in East Asian species from those in Europe and North America.

Coincident with the spatial retraction, most European and North American species showed demographic contraction (i.e. population size decline) during the LGM, and expansion afterwards (Kvist *et al.*, 2004; Milá *et al.*, 2006). The idea is that geographical distributions during the Last Interglacial (LIG; 0.13–0.07 Ma) were similar to their present states, and that, as global climate cooled leading up to the LGM, temperate species retracted into smaller refugial areas. As temperatures warmed following the LGM, species expanded into their larger present-day distributions, leaving a pattern of demographic expansion that can be dated post-LGM. Several recent studies of East Asian species have, however, revealed an unusual pre-LGM expansion, with demographic expansion occurring during the LIG. For example, this pattern has been detected in the Chinese hwamei (*Leucodioptron canorum canorum*; Li *et al.*, 2009), the Chinese bamboo partridge (*Bambusicola thoracica thoracica*; Huang *et al.*, 2010) and Elliot's laughing thrush (*Garrulax elliotii*; Qu *et al.*, 2011). The recent development of high-resolution LGM and LIG environmental layers (Hijmans *et al.*, 2005; Otto-Bliesner *et al.*, 2006), combined with ecological niche modelling, provide a new opportunity to shed light on the possible causes of the unexpected pattern of pre-LGM expansion in temperate East Asia.

Ecological niche modelling uses species occurrence data and environmental variables to predict the potential geographical distribution of species (Guisan & Thuiller, 2005). It is based on three assumptions (Elith & Leathwick, 2009; Nogués-Bravo, 2009). First, the ecological niches of species are conserved over relatively long timespans. Second, the species distribution is at equilibrium with the present-day climatic conditions. Third, the distribution of the species is mainly determined by environmental variables, and not by biotic factors (e.g. competitors or predators). Although these assumptions are still debated (Kozak *et al.*, 2008; Peterson, 2011), incorporating ecological niche modelling into phylogeographical studies can potentially provide additional insights, such as locating Pleistocene refugia (Peterson & Nyári, 2007; Waltari *et al.*, 2007) and detecting environmental barriers to dispersal (Kozak *et al.*, 2008).

Here, we selected a montane bird – the green-backed tit, *Parus monticolus* Vigors, 1831 – to investigate the impacts of Pleistocene climate fluctuations in East Asia. The geographical distribution of the green-backed tit extends from the foothills of the Himalayas in Pakistan and northern India into south-western and central China, with disjunct populations in southern Vietnam on the Da Lat Plateau and in Taiwan (Harrap & Quinn, 1995). It generally occupies wooded habitats, including subtropical evergreen, deciduous, mixed and coniferous forests within an elevational range of 1000–3000 m (Li *et al.*, 1982). Four subspecies are recognized: *P. m. monticolus*, *P. m. yunnanensis*, *P. m. insperatus* and *P. m. legendrei*. *Parus m. monticolus* is distributed from the western Himalayas to the eastern Himalayas where it intergrades with *P. m. yunnanensis*. *Parus m. yunnanensis* is distributed from the eastern Himalayas to south-western and central China and northern Myanmar and Vietnam. *Parus m. legendrei* is distributed in southern Vietnam on the Da Lat Plateau. *Parus m. insperatus* is endemic to Taiwan Island (Fig. 1) (Harrap & Quinn, 1995).

In this study, we integrated both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) sequence data with ecological niche modelling to: (1) describe the phylogeographical structure and the timing of genetic diversification within the green-backed tit; (2) explore the relationship between estimated historical population size changes and climate fluctuations; and (3) test whether historical population size changes were consistent with historical distributions predicted by ecological niche modelling.

MATERIALS AND METHODS

Sample collection and laboratory methods

We obtained 161 samples from 29 sites spanning the entire geographical range of the green-backed tit (Fig. 1). Samples included a combination of fresh tissues (blood and muscle) as well as toepads from museum skins. There were eight toepads, including three from southern Tibet, two from north-eastern India and three from southern Vietnam. Genomic DNA was extracted using the DNeasy Tissue kit (Qiagen, Hilden, Germany). We followed the manufacturer's instructions to extract DNA from fresh tissues, and followed a modified extraction protocol described by Irestedt *et al.* (2006) to extract DNA from toepads. We sequenced four mitochondrial fragments and seven nuclear loci via

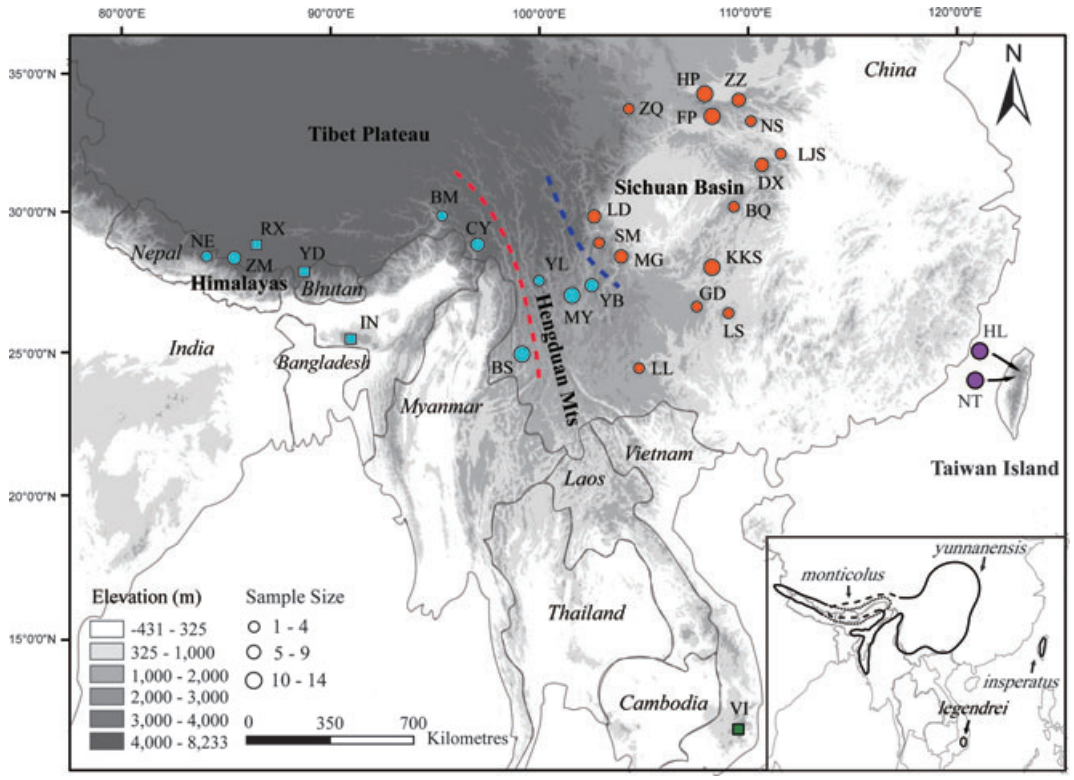

Figure 1 Topographical map of East Asia showing sampling sites and the distribution of mitochondrial DNA (mtDNA) phylogroups within the green-backed tit (*Parus monticolus*). The inset shows the approximate distribution of the four subspecies. Circles represent sampling sites of fresh tissue samples and the size of the circles is proportional to the sample size. Squares represent sampling sites of toepad samples. The colours of the circles and squares correspond to operational taxonomic units (OTUs): orange, central OTU; blue, south-western OTU; purple, Taiwanese OTU; green, Vietnamese OTU. The dashed lines between the distributions of *P. m. monticolus* and *P. m. yunnanensis* represent the contentious boundary. The red dashed line represents the Mekong–Salween Divide and the blue dashed line shows the Daxue Mountains. Elevation is represented by shading: lower elevations are shown lighter and higher elevations are shown darker. The green-backed tit is found at approximately 1000–3000 m in elevation.

polymerase chain reaction (PCR). The mtDNA genes were cytochrome *b* (*cytb*), cytochrome *c* oxidase subunit I (*COI*), NADH dehydrogenase subunit 2 (*ND2*) and the control region. These mtDNA regions were sequenced completely for all fresh tissue samples using the following primer pairs: *cytb*, H16065/L14990; *COI*, H7956/L6615; *ND2*, H6313/L5219 (Sorenson *et al.*, 1999). For the toepads, a number of new primers were designed to amplify smaller fragments (approximately 250–350 bp). PCR conditions for fresh tissues were as follows: denaturation at 94 °C for 2 min, followed by 40 cycles at 93 °C for 1 min, annealing temperature (46 °C for *COI*, 53 °C for *ND2* and 49 °C for *cytb*) for 45 s, and 72 °C for 1 min, and a final 8 min at 72 °C. The primers and amplification conditions used for the control region were as described in Kvist *et al.* (2003). A touchdown PCR was used to amplify DNA from toepads with annealing temperature ranging from 49 °C to 64 °C.

We sequenced nDNA loci for a subset of individuals. This included four autosomal and three Z-linked loci. The four autosomal markers were *FGB* (beta-fibrinogen), *MB* (myoglobin), *TGFB2* (transforming growth factor beta-2) and *ARNTL* (aryl hydrocarbon receptor nuclear translocator-like). The three Z-linked markers were *ACO1* (aconitase 1), *MUSK* (muscle-specific tyrosine kinase) and *Z-185* (similar to transient receptor potential cation channel, subfamily M, member 3, Shou-Hsien Li, unpublished data). We subsampled about five individuals from each sampling site for three nuclear loci (*MB*, *ACO1* and *MUSK*) sequencing, and about two individuals from each sampling site for the remaining four nuclear loci (*FGB*, *TGFB2*, *ARNTL* and *Z-185*) sequencing. In total, we sequenced about 100 individuals. The primer pairs used to amplify these seven loci were Fib.5F2/Fib.6R2, Myo3F/Myo2, TGFB2.5F/TGFB2.6R, ARNTL12F/ARNTL13R, ACO1-19F/ACO1-19R, MUSK-13F/MUSK-13R (Kimball *et al.*, 2009), and Z-185F2 (GGCAGAGTAGCTTCAACAGTCA)/Z-185R (AGTCTCCTCATGGACAGGTTGT). PCR conditions were as follows: an initial denaturation at 94 °C for 2 min, 35 cycles of 94 °C for 30 s, annealing temperature (56 °C for *Z-185* and following Kimball *et al.*, 2009; for others) for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 10 min. The amplification conditions used for the toepads were as described in Irestedt *et al.* (2006). All PCR products were purified using a QIAquick PCR purification kit (Qiagen) and sequenced using the BigDye

Terminator method (Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl Analyzer. Two individuals of the great tit (*Parus major*), the purported closest relative of the green-backed tit (Gill *et al.*, 2005), were sequenced for all loci and used as outgroup. Complete sequences were assembled using SEQMAN II (DNASTAR, Madison, WI, USA) and compared visually to the original chromatograms to avoid reading errors. We phased heterozygous nuclear sequences using the program PHASE (Stephens *et al.*, 2001). Only individuals with resulting phase probabilities greater than 0.7 were used in analyses (Harrigan *et al.*, 2008; Carling *et al.*, 2010). All sequences have been deposited in GenBank (accession numbers JQ323616–JQ324515, JX849669–JX849875).

Genetic diversity

Sequences were aligned using CLUSTAL W implemented in MEGA 4 (Tamura *et al.*, 2007). We concatenated sequences of the four mtDNA fragments to a single sequence of 3555 bp. For each nDNA locus, we tested for recombination using the Φ_w-statistic (Bruen *et al.*, 2006) implemented in SPLITSTREE 4 (Huson & Bryant, 2006). We used PAUP* 4.0b10 (Swofford, 2003) to generate maximum likelihood gene trees to test the molecular clock hypothesis for each locus using likelihood ratio tests. The McDonald–Kreitman test (McDonald & Kreitman, 1991) implemented in DNASP 5.0 (Librado & Rozas, 2009) was used to examine the selective neutrality of the three mitochondrial coding fragments by comparing the polymorphism within the green-backed tit to that in the great tit. The Ewens–Watterson test (Ewens, 1972; Watterson, 1974) and Chakraborty's amalgamation test (Chakraborty, 1990) implemented in ARLEQUIN 3.5 (Excoffier *et al.*, 2005) were used to test if nuclear sequence data deviated from neutral expectations. For mtDNA data, we calculated the nucleotide diversity (π), the number of haplotypes (*nh*) and haplotype diversity (*h*) for each population and for each phylogroup. For nDNA data, we calculated genetic diversity statistics for each locus. All these calculations were performed using DNASP.

Phylogenetic analyses

We first produced a mtDNA and seven nDNA median-joining networks using the program NETWORK 4.610 (Bandelt *et al.*, 1999). For the fresh tissue samples, we sequenced the four mitochondrial fragments and seven nuclear loci completely. However, we only sequenced partial mtDNA, partial *ACO1* and partial *MUSK* for the toepad samples because the age of these samples made it difficult to sequence many of these gene regions. The partial toepad sequence data set was combined with the complete fresh tissue sequence data set to construct the mtDNA, *ACO1* and *MUSK* haplotype networks. The mtDNA data resolved three genetic groups with high posterior probability. Therefore, we used these three clades as operational taxonomic units (OTUs) in a species tree analysis using the program *BEAST (Heled & Drummond, 2010). Although the mtDNA failed to resolve the monophyly of the Vietnamese population (this may be because we only successfully sequenced one individual with a *c.* 1600-bp fragment which did not contain enough variable sites), the Vietnamese population had fixed differences in both mtDNA and nDNA (see Results for details). Therefore, we also treated the Vietnamese population as an OTU and included it in our species tree analysis. The species tree approach implemented in *BEAST uses a Bayesian Markov chain Monte Carlo (MCMC) method to jointly estimate multiple gene trees embedded in a shared species tree.

We estimated divergence times in *BEAST using the molecular clock. The mtDNA alignment was the only locus to reject the molecular clock hypothesis, so an uncorrelated log-normal relaxed clock (Drummond *et al.*, 2006) was applied to the mtDNA alignment. We applied a strict clock to all the nDNA loci. We assumed a standard avian *cytb* substitution rate of 0.0105 per site per million years (2.1% per million years divergence rate; Weir & Schluter, 2008). The net average genetic distance between the green-backed tit and great tit was 1.3 times greater than the net average genetic distance of *cytb* alone. Thus, we multiplied the 0.0105 per site per million years substitution rate for *cytb* by 1.3 to arrive at a substitution rate of 0.01365 per site per million years for the entire mtDNA alignment. Nuclear substitution rates were scaled to the mtDNA alignment rate in *BEAST.

We selected the best-fit model of nucleotide substitution for each locus using the Akaike information criterion (AIC) in the program JMODELTEST 0.1.1(Posada, 2008). We used a UPGMA (unweighted pair-group method with arithmetic mean) topology as a starting tree and set all priors to their default values. We ran MCMC chains for 100 million generations (sampling every 10,000 generations and discarding the first 10% as burn-in) and assessed convergence of the MCMC chain in the program TRACER 1.5 (Rambaut & Drummond, 2007). We combined the partial toepad sequence data set and the complete fresh tissue sequence data set to conduct four *BEAST analyses, one with all eight independent sequence alignments, one with mtDNA, *ACO1* and *MUSK* (but adding Vietnam toepad sequences), one with only the seven nDNA alignments, and one with only the mtDNA alignment.

Demographic reconstruction

We used three common methods to explore the demographic histories of the three phylogroups that had large enough sample sizes (i.e. the south-western, central and Taiwanese phylogroups). First, two statistics, Fu's F_S (Fu, 1997) and Tajima's *D* (Tajima, 1989), were used to assess whether nucleotide polymorphisms deviated from expectations under neutral theory. We calculated these statistics for each population and for each phylogroup. Significance was tested with 10,000 coalescent simulations in ARLEQUIN. Second, mismatch distribution analysis was conducted for the three phylogroups using ARLEQUIN. The statistical tests and

mismatch distribution analysis were performed with mtDNA alone. Integrating multilocus data and coalescent-based methods can help to recover population size dynamics (Heled & Drummond, 2008; Lascoux & Petit, 2010). Thus, we also used the extended Bayesian skyline plots (EBSPs; Heled & Drummond, 2008) implemented in BEAST (Drummond & Rambaut, 2007) to assess historical changes in effective population size. This method has an important advantage over previous implementations of the Bayesian skyline, in that it incorporates data from multiple independent loci (Heled & Drummond, 2008). Analyses were conducted separately for the three mtDNA phylogroups. We ran EBSP analyses with clock models, substitution models and trees unlinked. The mtDNA substitution rate was the same as above, and, again, substitution rates for the nuclear loci were scaled relative to the mtDNA rate in BEAST. We used an uncorrelated lognormal relaxed clock for the mtDNA alignment and strict clocks for the seven nDNA alignments. All analyses were run for 200 million steps and sampled every 20,000 generations. MCMC convergence was assessed in TRACER.

Ecological niche modelling

We assessed potential historical shifts in the geographical distribution of the green-backed tit using MAXENT 3.3.2 (Phillips *et al.*, 2006) and climate data from three periods: the present day, the LGM and the LIG. MAXENT was selected because it has been shown to perform well compared with alternative modelling methods (Elith *et al.*, 2006) and is robust to low sample sizes (Pearson *et al.*, 2007).

Nineteen bioclimatic variables were downloaded from WorldClim (Hijmans *et al.*, 2005; http://www.worldclim.org/), and included data on annual trends, seasonality and extremes of temperature and precipitation. These bioclimatic variables were at a resolution of 2.5 arc-minutes. Species occurrence data included sampling sites, museum records (data from China were provided by the National Zoological Museum of China; data from adjacent countries were downloaded from http://data.gbif.org/) and birdwatching locations (Chinese mainland records were downloaded from http://birdtalker.net/ and Taiwanese records were provided by the Chinese Wild Bird Federation). Only those localities with georeferenced data specific enough for the longitude and latitude to be estimated with confidence using GOOGLE EARTH (http://earth.google.com/) were used. We removed sites separated from each other by less than 0.1° to reduce the effect of spatial autocorrelation. A total of 149 localities were used in the final analysis. Eighty per cent of the occurrence data were randomly selected to construct models and the remaining 20% were used to test the models. We used the default convergence threshold (10^{-5}) and set the number of maximum iterations to 2000 and the number of replicates to 10. The output format was set to logistic. This output ranges from 0 to 1 and quantifies the probability of suitable environmental conditions for the species in each grid cell. Model performances were evaluated by averaging areas under the receiver operating characteristic curves (AUC) and the binomial probabilities over ten replicate runs. To investigate the degree of elevational shift in the green-backed tit, we selected a threshold of 10%, which ejected only the lowest 10% of possible predicted values and classified others as 'present' (Pearson *et al.*, 2007). We used ARCGIS 9.3 (ESRI, Redlands, CA) to calculate the range of suitable distributions and average elevations during the three periods.

RESULTS

Genetic diversity

In the entire 3555-bp mtDNA alignment, 181 sites were variable and 111 were parsimony informative. There was one insertion/deletion (indel) in the control region and it was excluded in all analyses. Polymorphic sites defined 99 haplotypes, 78 of which were found in single individuals. The most abundant haplotype was shared by 19 individuals and distributed widely across central China. Within sampling locations, haplotype diversity (h) ranged from 0.70 to 1.00 and nucleotide diversity (π) from 0.00022 to 0.00347 (Table 1). Haplotype diversity for nDNA loci ranged from 0.51 to 0.96 and nucleotide diversity ranged from 0.00132 to 0.01292 (Table 2). The McDonald–Kreitman test showed no significant deviation from neutrality for the three mtDNA coding segments ($P > 0.05$, with Fisher's exact test). Neither Chakraborty's amalgamation test nor the Ewens–Watterson test was significant for any nDNA locus. There was no evidence for recombination in any nuclear locus after applying Bonferroni correction. The molecular clock was rejected for the mtDNA alignment, but it could not be rejected for any of the nDNA alignments.

Phylogenetic analyses

The mtDNA haplotype network resulted in three phylogroups (Fig. 2a): a south-western phylogroup including samples from the Himalayas, south-western China and north-eastern India; a central phylogroup containing samples from central China; and a Taiwanese phylogroup restricted to Taiwan Island and Vietnam. The network analysis grouped the central and Taiwanese phylogroups together. The central/Taiwanese clade was separated from the south-western clade by approximately 30 mtDNA mutational changes (0.9%). The mtDNA gene tree produced from the *BEAST analysis had the central clade sister to the Taiwanese clade and the south-western clade sister to the central/Taiwanese clade (Fig. 2b). This tree topology was consistent with the relationships uncovered by the network analysis. Although the mtDNA did not resolve the monophyly of Vietnamese population, there were fixed base pairs differences between Vietnamese and other populations.

In all nDNA haplotype networks (Fig. 3) except *TGFB2*, there was no haplotype sharing between the Taiwanese and the south-western/central samples. Although the

Table 1 Summary of genetic diversity in the green-backed tit (*Parus monticolus*) in East Asia based on mitochondrial DNA (mtDNA). Sample size (n), number of haplotypes (nh), nucleotide diversity (π), haplotype diversity (h), Fu's F_S and Tajima's D are shown. Only sampling sites of fresh tissue samples are included in this table. Genetic diversity was only calculated for locations with more than three samples.

Population code	Location	Longitude	Latitude	n	nh	π	h	Fu's F_S	Tajima's D
Central	—	—	—	86	52	0.00126	0.94	−25.603***	−2.525***
ZQ	Zhouqu	104.171	33.697	2	2	—	—	—	—
ZZ	Zhouzhi	108.221	34.160	9	7	0.00131	0.94	−1.129	−1.513
FP	Foping	107.768	33.596	10	7	0.00094	0.91	−1.510	−1.595*
NS	Ningshan	108.318	33.313	3	3	—	—	—	—
HP	Haoping	107.711	34.088	12	10	0.00134	0.96	−3.454*	−1.789*
MG	Meigu	103.308	28.518	7	7	0.00152	1.00	−2.621*	−1.543*
DX	Dingxi	110.195	31.566	8	8	0.00100	1.00	−2.898*	−1.121
BQ	Banqiao	108.638	29.933	4	4	0.00140	1.00	−2.084	−1.285
LJS	Laojunshan	110.918	31.880	4	4	0.00095	1.00	−0.825	−0.817
LD	Luding	102.277	29.920	5	5	0.00109	1.00	−1.554	−0.526
SM	Shimian	102.338	29.100	4	4	0.00163	1.00	1.655	0.837
LL	Longlin	104.871	24.666	1	1	—	—	—	—
GD	Guiding	107.135	26.582	3	3	—	—	—	—
KKS	Kuankuoshui	107.190	27.947	12	8	0.00090	0.91	−1.948	−0.544
LS	Leishan	108.078	26.384	2	2	—	—	—	—
South-western	—	—	—	45	34	0.00315	0.99	−16.044***	−1.133
MY	Miyi	101.899	27.127	5	5	0.00347	1.00	−0.080	0.439
YB	Yanbian	101.535	27.106	13	10	0.00271	0.95	−0.869	−0.615
YL	Yulong	100.261	27.097	2	2	—	—	—	—
BS	Baoshan	98.783	24.820	11	9	0.00332	0.96	−0.602	−0.013
BM	Bomi	95.759	29.873	2	2	—	—	—	—
CY	Chayu	97.084	28.562	5	5	0.00222	1.00	−0.608	−0.375
ZM	Zhangmu	85.988	27.989	5	3	0.00022	0.70	−0.829	−0.973
NE	Rasuwa District	28.173	85.314	2	2	—	—	—	—
Taiwanese	—	—	—	22	13	0.00178	0.92	−2.113	−0.787
NT	Nantou	121.182	23.977	14	7	0.00154	0.86	0.809	−0.081
HL	Hualian	121.388	24.192	8	7	0.00214	0.96	−0.992	−0.072

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Table 2 Summary of nuclear DNA (nDNA) genetic diversity in the green-backed tit (*Parus monticolus*) in East Asia. n, sample size; s, polymorphic sites; nh, number of haplotypes; π, nucleotide diversity; h, haplotype diversity; P(recombination), recombination test using the Φ_w-statistic (the P for *ARNTL* is below 0.05, but it is not statistically significant when Bonferroni correction is applied); rate, nuclear substitution rates scaled to the mitochondrial DNA (mtDNA) alignment (0.01365 per site per million years).

Locus	n	Length	s	nh	π	h	P(recombination)	Rate
FGB	40	460	8	9	0.00268	0.68	0.78	0.00097
MB	84	654	13	14	0.00214	0.54	1.00	0.00116
TGFB2	39	471	4	4	0.00269	0.69	1.00	0.00155
ARNTL	35	426	19	29	0.01292	0.96	0.04	0.00824
ACO1	95	929	33	27	0.00695	0.83	0.50	0.00301
Z-185	48	801	8	7	0.00132	0.51	1.00	0.00084
MUSK	96	526	20	22	0.00816	0.81	0.08	0.00427

south-western and central samples shared haplotypes with each other, they also each contained private haplotypes in most of the nDNA loci. This suggested that the south-western and central phylogroups were also divergent to some extent in nDNA, though the separation between them was statistical and did not include fixed differences. The *ACO1* and *MUSK* haplotype networks showed fixed differences in the Vietnamese samples.

The species tree analysis in *BEAST, which included the mtDNA and all seven nuclear loci, produced a well-resolved phylogeny (Fig. 4a). The species tree topology placed the Taiwanese OTU basal to the south-western/central clade and the south-western OTU sister to the central OTU. The topology was consistent with a species tree analysis that included only the nuclear data. The species tree constructed with mtDNA, *ACO1* and *MUSK* but adding Vietnamese toepad

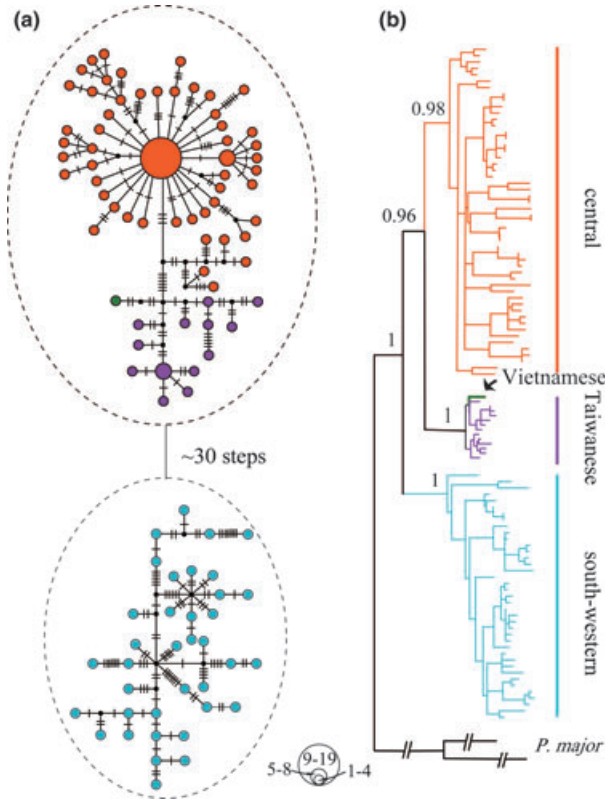

Figure 2 (a) Mitochondrial DNA (mtDNA) median-joining network of the green-backed tit (*Parus monticolus*) in East Asia. Each circle represents a haplotype, and the size of the circle is proportional to that haplotype's frequency. Dots represent unsampled haplotypes and dashes represent the corresponding mutation steps. Colours denote phylogroup membership and are the same as in Fig. 1. (b) MtDNA gene tree. Posterior probabilities for major clades are shown above nodes. Sequences from the great tit (*Parus major*) were used to root the tree.

samples (Fig. 4b) indicated that the Vietnamese OTU was sister to the Taiwanese OTU.

Divergence time dating of the species tree (Fig. 4) in *BEAST indicated that divergence within the green-backed tit was relatively recent. The species tree constructed with all eight loci revealed that the Taiwanese OTU split from the south-western/central clade approximately 0.57 Ma (95% highest posterior density, HPD: 0.30–0.96 Ma). The central OTU split from the south-western OTU approximately 0.07 (0.04–0.10 HPD) Ma. The divergence dating of the species tree with Vietnamese toepad samples showed the basal split within the green-backed tit was approximately 0.28 (0.14–0.51 HPD) Ma. The divergence time between the Taiwanese and Vietnamese OTUs was about 0.18 (0.08–0.33 HPD) Ma.

Demographic reconstruction

Fu's F_S (Table 1) was negative for most populations and significantly negative for the central and south-western phylogroups. Tajima's D (Table 1) was significantly negative for the central phylogroup, negative but not significant for the south-western and Taiwanese phylogroups. Mismatch distributions for the south-western and central phylogroups were unimodal, whereas mismatch distribution for the Taiwanese phylogroup was multimodal. The EBSP (Fig. 5) rejected population stability in the south-western and central phylogroups, but it could not reject population stability in the Taiwanese phylogroup. EBSP estimates of the south-western phylogroup suggested that the population has expanded approximately fivefold, from about 0.4 to about 2.1, beginning around 0.06 Ma, with no evidence of contraction afterwards. EBSP estimates of the central phylogroup suggested that the population expanded approximately 30-fold, from about 0.1 to about 3.0, beginning around 0.04 Ma. Both population expansions were coincident with the transition from the LIG to the LGM.

Ecological niche modelling

The ecological niche models for the green-backed tit (Fig. 6) had excellent predictive power, with an average training AUC of 0.968. The binomial probabilities ($P \ll 0.0001$) for the eleven common thresholds also showed that predictions were substantially better than that of a random model. The average elevation (Table 3) was highest during the LIG (1727 $\pm$ 699 m) and lowest during the LGM (1262 $\pm$ 770 m). The present-day average elevation was in between these values, at 1496 $\pm$ 847 m. The predicted distribution of the south-western and central phylogroups was restricted to south-western China during the LIG. It expanded eastwards and northwards after the LIG. The potential present-day distribution was more similar to the potential distribution at the LGM than it was to that in the LIG. The predicted distributions for the Taiwanese phylogroup were similar during the three periods.

DISCUSSION

Phylogeographical structure

Phylogeographical structure within the green-backed tit indicated a history of isolation within East Asia. Three well-supported reciprocally monophyletic mtDNA phylogroups were distributed without geographical overlap (Figs 1 & 2). Individuals from central China formed a clade (central clade) that was sister to a clade made up of individuals from Taiwan and Vietnam (Taiwanese clade). Individuals from the Himalayas, south-western China and north-eastern India formed a third well-supported, basal clade (south-western clade). The mtDNA gene tree topology was different from the species tree topology (Fig. 4). A species tree analysis that included mtDNA, *ACO1* and *MUSK* (Fig. 4b) revealed that the Taiwanese and Vietnamese OTUs were sister and that the central and south-western OTUs were sister. The fact that individual gene trees can differ from the species tree is well known (Maddison, 1997). This incongruence between the mtDNA gene tree and species tree illustrates the potential

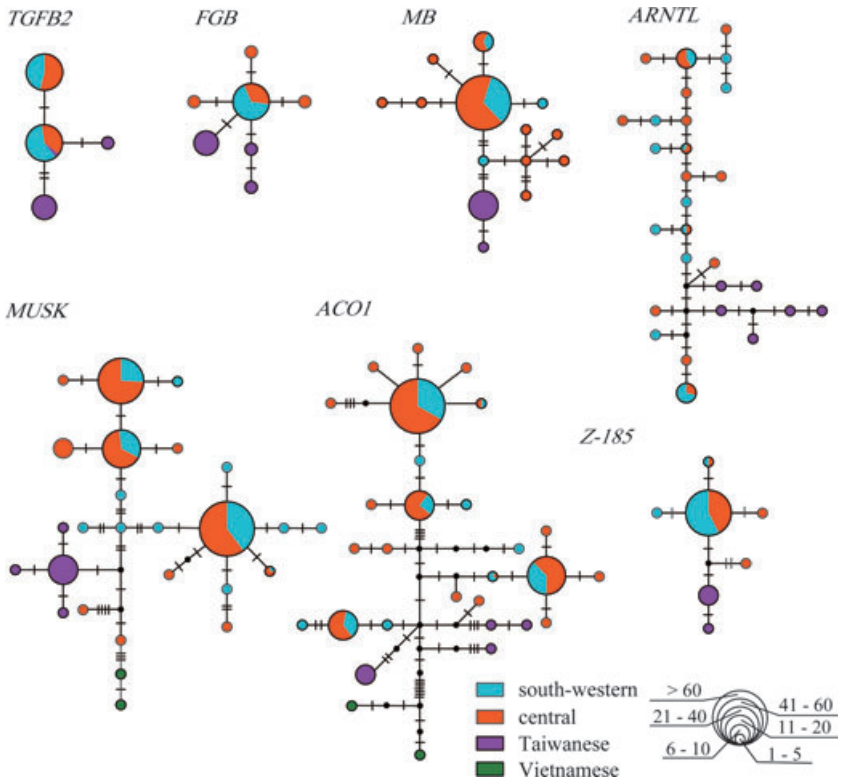

Figure 3 Nuclear DNA median-joining networks of the green-backed tit (*Parus monticolus*) in East Asia. Each circle represents a haplotype, and the size of the circle is proportional to that haplotype's frequency. Dots represent unsampled haplotypes and dashes represent the corresponding mutation steps. Colour coding is as in Fig. 1. The four loci above (*TGFB2*, *FGB*, *MB* and *ARNTL*) are autosomal markers. The three loci below (*MUSK*, *ACO1* and *Z-185*) are Z-linked markers.

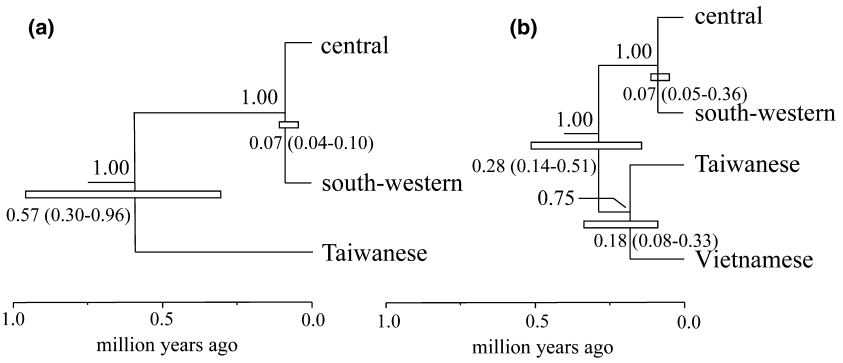

Figure 4 (a) Species tree of the green-backed tit (*Parus monticolus*) in East Asia, constructed with the mitochondrial DNA (mtDNA) and all seven nuclear DNA loci. (b) Species tree constructed with mtDNA, *ACO1* and *MUSK* but adding Vietnamese (toepad) samples. Posterior probabilities of clade support are shown at nodes above branches. Estimates of divergence time with 95% highest posterior densities are shown at nodes as white bars and numbers below branches.

dangers of making inferences about phylogeny from mtDNA alone. It also reinforces the importance of incorporating multilocus nuclear data to reconstruct the historical relationships of species and populations.

There are two disjunct subspecies within the green-backed tit: *P. m. insperatus* and *P. m. legendrei*. *Parus m. insperatus* is endemic to Taiwan Island and *P. m. legendrei* is restricted to the Da Lat Plateau in southern Vietnam. Both subspecies exhibit significant morphological differentiation from other subspecies (Harrap & Quinn, 1995). This suggests that the two subspecies may have evolved independently for a long time. We also found evidence of genetic distinctiveness in both subspecies. Taiwanese individuals had fixed differences in mtDNA, as well as several nDNA loci. Both mtDNA and nDNA haplotype networks with Vietnamese toepad samples showed that no haplotype was shared between Vietnamese and other populations. The species tree that included Vietnamese toepad samples suggested that these two disjunct

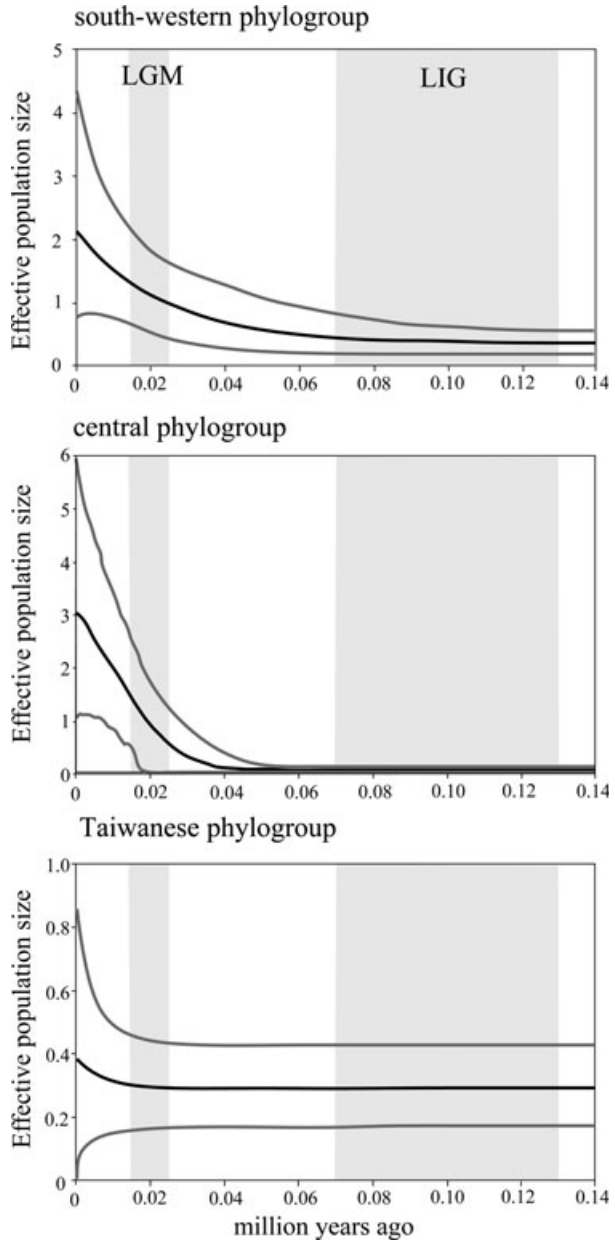

Figure 5 Extended Bayesian skyline plots representing historical demographic trends of the south-western, central and Taiwanese phylogroups of the green-backed tit (*Parus monticolus*) in East Asia. Time (in million years ago) is shown along the *x*-axis, and the scaled effective population size is shown along the *y*-axis. The mean estimate is enclosed within the 95% highest posterior densities. LGM represents the Last Glacial Maximum, and LIG represents the Last Interglacial.

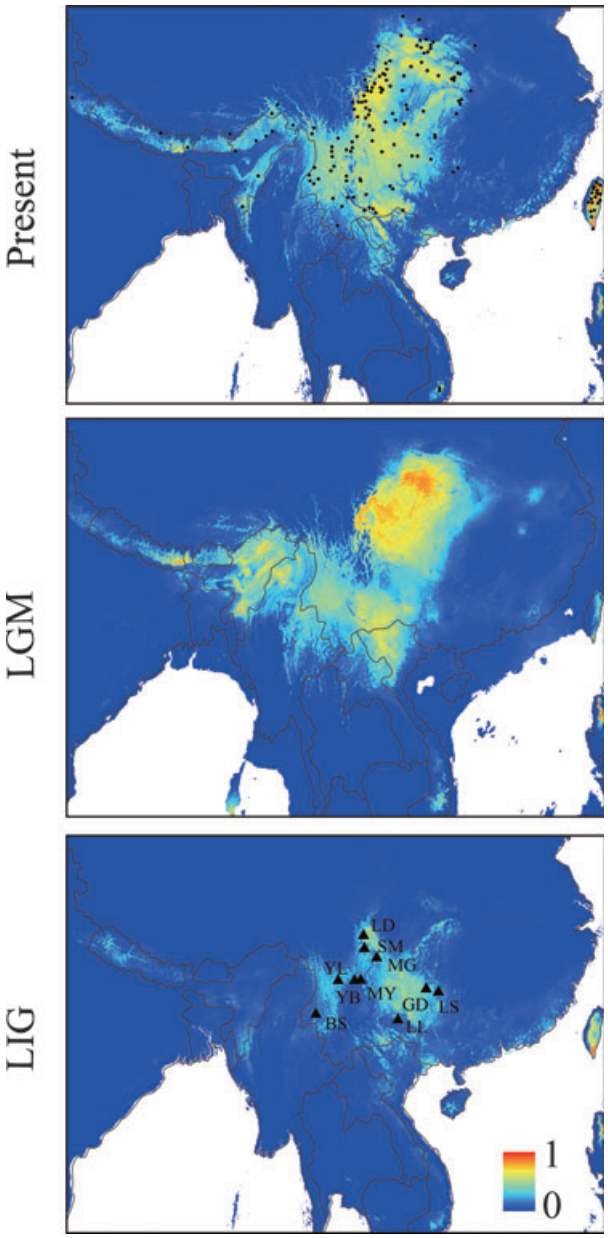

Figure 6 Ecological niche models for the green-backed tit (*Parus monticolus*) in East Asia in the present day, at the Last Glacial Maximum (LGM), and during the Last Interglacial (LIG). Circles represent the localities used to build the ecological niche models. Triangles denote sampling sites within the refugial area. Different colours correspond to different fitting indices, with low in blue and high in red.

populations were sister taxa. This close relationship was also evident in the mtDNA and nDNA haplotype networks. The genetic peculiarity of southern Vietnamese populations has been reported elsewhere (Fuchs *et al.*, 2008). Morgan *et al.* (2011) suggested that the southern Vietnamese populations of several mosquito species were derived from recent colonization from populations in northern Vietnam and south-western China. Zou *et al.* (2007) revealed that the southern Vietnam population of the bird species, the grey-cheeked fulvetta (*Alcippe morrisonia*), was more closely related to the south-western China population than to northern Vietnamese and Taiwanese populations. Fuchs *et al.* (2008) found that the southern Vietnamese population of another bird species, the white-browed piculet (*Sasia ochracea*), was genetically distinct. Interestingly, to our best knowledge, we found a novel pattern wherein a genetically distinct southern Vietnamese population is closely related to an endemic

Table 3 The numbers of grid cells and average elevations of predicted distributions of the green-backed tit (*Parus monticolus*) under the threshold of 10% during the three periods: the present day, the Last Glacial Maximum, and the Last Interglacial.

	Suitable area	Average elevation (m)	SD elevation
Present day	140,628	1,496	847
Last Glacial Maximum	152,799	1,262	770
Last Interglacial	80,879	1,727	699

Taiwanese population. A possible reason for this unexpected pattern is the Vietnamese and Taiwanese populations were derived from long-distance dispersal from an ancestral taxon, which may have lived in mountainous areas in or around the Hengduan Mountains. It dispersed to Taiwan and southern Vietnam when disjunct areas were covered by suitable habitat. In agreement with this hypothesis, previous phylogeographical studies have suggested that most montane fauna in Taiwan were derived from dispersal of continental populations when a dried-out land bridge connected montane habitats in Taiwan and the Hengduan Mountains (Kano, 1940; Päckert *et al.*, 2012). Besides, several species experienced a southward expansion from northern refugia (mountainous areas in or around the Hengduan Mountains) to southern Vietnam (Morgan *et al.*, 2011) or southern Thailand (Pramual *et al.*, 2005; O'Loughlin *et al.*, 2008). We note, however, that the current Vietnam and Taiwan populations may also be relicts of an older, more widespread taxon that connected Taiwan to southern Vietnam. Extinction within the ancestral taxon would have left relict populations on Taiwan and Vietnam, and this would also have resulted in the phylogenetic pattern that we uncovered within the green-backed tit.

Two widespread mtDNA phylogroups, the south-western and central phylogroups, were uncovered in the central and south-west portions of the Asia continent. The geographical division between them coincides with the Daxue Mountains. It has been suggested that the Daxue Mountains, which run north to south, played an important role in shaping the genetic structure within the Chinese white-bellied rat (*Niviventer confucianus*; Chen *et al.*, 2011). Furthermore, another bird species, the grey-cheeked fulvetta (*Alcippe morrisonia*; Song *et al.*, 2009), showed a similar phylogeographical division at the Daxue Mountains. It seems that the Daxue Mountains have also contributed to the genetic divergence within the green-backed tit. We note that this phylogeographical break differs from the Mekong–Salween Divide (Ward, 1921) found in some plants (Qiu *et al.*, 2011), mammals (Geissmann *et al.*, 2011) and birds (Päckert *et al.*, 2012). Individuals of the green-backed tit from west and east of the Mekong–Salween Divide showed no evidence of genetic divergence. A sampling site (BS) west of the Mekong–Salween Divide shared haplotypes with a sampling site (MY) from the eastern part of the divide. Further comparative phylogeographical studies with dense sampling are needed to explore if the various phylogeographical breaks related to different habitat requirements, history processes or some other factors.

Divergence times derived from the application of the molecular clock to our species tree analysis indicated that diversification within the green-backed tit was relatively recent (Fig. 4). All diversification probably occurred within the last 0.9 million years. It has been suggested that our planet experienced a dramatic climate shift around 0.9 Ma, termed the 'mid-Pleistocene revolution' (Ruddiman *et al.*, 1989). Around this time, climate oscillations shifted from the 41,000-year cycles of the earlier Pleistocene to the 100,000-year cycles of the later Pleistocene (Huang *et al.*, 2005). The later cycles were characterized by increasing severity and duration of cold stages, which are thought to have had profound impacts on the biota, especially in the Northern Hemisphere (Head & Gibbard, 2005). A major faunal turnover has been detected in Europe (Palombo *et al.*, 2005), Siberia (Foronova, 2005) and East Asia (Wang *et al.*, 2000) around the time of the climate transition. Therefore, it is possible that the drier and colder environment in the later Pleistocene induced the population divisions within the green-backed tit.

Climate-driven spatial and demographic histories

Several studies have suggested that climate conditions during the LIG supported vegetation similar to that observed today (Li *et al.*, 2006; Qu *et al.*, 2011). Thus, we expected the predicted distribution for the green-backed tit during the LIG to be similar to its present-day distribution. Surprisingly, however, our ecological niche models suggested a restricted geographical distribution of the green-backed tit in East Asia during the LIG (Fig. 6). The predicted refugial area was consistent with our genetic data. We found higher nucleotide diversity (π) south-west of the Sichuan Basin and in the Hengduan Mountains (sampling sites YL, BS, YB, MY, LD, SM, MG, GD, LS and LL; π ranged from 0.00109 to 0.00347, with an average of 0.00229) than in other parts of the Asian continent (π ranged from 0.00022 to 0.00140, with an average of 0.00114). We suggest that the restricted distribution during the LIG may be due to higher temperatures during the LIG compared to the present day. It has been suggested that temperatures during the LIG were about 2 °C warmer than present, and even up to 5 °C in the Arctic during summer (Otto-Bliesner *et al.*, 2006). The warmer temperature led to the sea level reaching approximately 6 m above the present sea surface in the Pacific Ocean (Camoin *et al.*, 2001). A warming climate is thought to push montane species to higher elevations (Davis & Shaw, 2001; Colwell *et al.*, 2008), and if the climate warms too much then montane species may be completely extirpated, even from mountains. We suggest that the climate might have warmed enough during the LIG to extirpate the green-backed tit from some of the mountains where it occurs today. This would have effectively trapped the green-backed tit in high-mountain refugia

in the Himalayan foothills in south-western China (Fig. 6, Table 3). If present-day climates continue to warm, we can predict a similar distribution shift in the future.

As the climate cooled following the LIG and leading up to the LGM, both the south-western and central phylogroups underwent population expansions (Fig. 5). The expansion for the south-western phylogroup was more gradual and began at approximately 0.06 Ma. In contrast, population expansion for the central phylogroup was more sudden and began approximately 0.04 Ma. This is consistent with the ecological niche models and the scenario of range contraction and expansion we described above. As climate cooled following the LIG and leading up to the LGM, the green-backed tit moved down in elevation to occupy a larger geographical area that included dispersion into lower mountain ranges with newly available suitable habitat.

Our ecological niche modelling results indicated that the current distribution of the green-backed tit was more similar to its LGM distribution than it was to its LIG distribution. This is obviously different from the post-LGM expansion scenario detected in many organisms (Hewitt, 1999). Nevertheless, a significant impact of the LIG is not unique to the green-backed tit, and has also been detected in another East Asian bird, the black-throated tit (*Aegithalos concinnus*; Dai *et al.*, 2011). However, Dai *et al.* briefly commented on this unusual scenario, and our study is the first report attempting to explore the possible reasons. Further, Dai *et al.* evaluated demographic history with a single mitochondrial locus, and it is now apparent that data from an array of independent loci are required to recover complex demographic histories (Eytan & Hellberg, 2010; Lascoux & Petit, 2010). We integrated mitochondrial and seven nuclear loci in this study and then utilized a coalescent-based method, extended Bayesian skyline plots, to incorporate the demographic signals of all loci. Thus, our study should provide more robust conclusions about the effects of the LIG.

Previous phylogeographical studies have suggested that responses to Pleistocene climate fluctuations are expected to vary among species and regions (Hewitt, 2000; Bisconti *et al.*, 2011). For example, glacial cycles affected several cold-tolerant species in a way that was the opposite of their effect on temperate species, with interglacials promoting restricted distributions in northern refugia (Provan & Bennett, 2008). Furthermore, glacial expansions were hypothesized for the Tyrrhenian tree frog (*Hyla sarda*; Bisconti *et al.*, 2011) and the pool frog (*Rana lessonae*; Canestrelli & Nascetti, 2008) due to a vast widening of lowland flood-plain habitats following falling sea levels, which could counterbalance the negative demographic consequences of climate changes. Finally, several studies have indicated that species' population sizes have not been adversely affected by glacial cycles because of mild climate in East Asia (Ding *et al.*, 2011) and southern Patagonia (Jakob *et al.*, 2009). Our results implicate a significant role of the LIG in shaping the demographic history of the green-backed tit; this study thus contributes to the growing idea that species' responses to Pleistocene climate fluctuations have been more varied than previously believed.

ACKNOWLEDGEMENTS

We thank Zuohua Yin, Wulin Liu, Huatao Liu and Bin Gao for field assistance, and Congtian Lin, Jianhai Chen, Chunlan Zhang, Atul Kathait and Feng Jiang for assistance with data analyses. We also thank Scott Edwards, Jon Fjeldså and Per Alström for giving some valuable comments on the research. We are very grateful to Michael Patten and three anonymous referees for their helpful comments and suggestions. Invaluable tissue and toepad samples from Tibet, Nepal, India and Vietnam were provided by the National Zoological Museum of China, American Museum of Natural History and Muséum National d'Histoire Naturelle. This work was supported by the National Science Fund for Distinguished Young Scientists (grant number 30925008), Major International (Regional) Joint Research Project (grant number 31010103901), CAS-IOZ Innovation Program (KSCX2-EW-J-2), NSFC (30870270) and a grant (number O529YX5105) from the Key Laboratory of the Zoological Systematics and Evolution of the Chinese Academy of Sciences to F.M.

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Editor: Michael Patten