

# Comparative phylogeography of five avian species: implications for Pleistocene evolutionary history in the Qinghai-Tibetan plateau

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## Abstract

Pleistocene climate fluctuations have shaped the patterns of genetic diversity observed in extant species. In contrast to Europe and North America where the effects of recent glacial cycles on genetic diversity have been well studied, the genetic legacy of the Pleistocene for the Qinghai-Tibetan (Tibetan) plateau, a region where glaciation was not synchronous with the North Hemisphere ice sheet maxima, remains poorly understood. Here, we compared the phylogeographical patterns of five avian species on the Qinghai-Tibetan plateau by three mitochondrial DNA fragments: the Tibetan snow finch (*Montifringilla adamsi*), the Blanford's snow finch (*Pyrgilauda blanfordi*), the horned lark (*Eremophila alpestris*), the twite (*Carduelis flavirostris*) and the black redstart (*Phoenicurus ochruros*). Our results revealed the three species mostly distributed on the platform region of the plateau that experienced population expansion following the retreat of the extensive glaciation period (0.5–0.175 Ma). These results are at odds with the results from avian species of Europe and North America, where population expansions occurred after Last Glacial Maximum (LGM, 0.023–0.018 Ma). A single refugium was identified in a restricted semi-continuous area around the eastern margin of the plateau, instead of multiple independent refugia for European and North American species. For the other two species distributed on the edges of the plateau (the twite and black redstart), populations were maintained at stable levels. Edge areas are located on the eastern margin, which might have had little or no ice cover during the glaciation period. Thus, milder climate may have mitigated demographic stresses for edge species relative to the extremes experienced by platform counterparts, the present-day ranges of which were heavily ice covered during the glaciation period. Finally, various behavioural and ecological characteristics, including dispersal capacities, habitat preference and altitude specificity along with evolutionary history might have helped to shape different phylogeographical structures appearing in these five species.

**Keywords:** comparative phylogeography, edge and platform species, glaciation refugia, postglacial expansion, Qinghai-Tibetan plateau

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## Introduction

The Pleistocene was characterized by worldwide climatic perturbations that resulted in the development of large ice sheets and changes in the landscape and ecol-

ogy of high-latitude continental areas of the northern hemisphere (Rising & Avise 1993; Hewitt 1996; Merila *et al.* 1997). For regions that were glaciated, such as Europe and North America, climate cycles are considered to be critical in shaping the distribution and genetic attributes of species (e.g. reviewed by Avise 2008; Hewitt 2000, 2004). For example, phylogeographical studies have shown that European and North

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American species expanded from southern refugia after the Last Glacial Maximum (LGM, Hewitt 1996, 2000, 2004). For these species, genetic diversity is usually geographically structured and can be partially attributed to the admixture of divergent lineages originating from separate refugia.

Interestingly, the glaciations of montane regions such as the Qinghai-Tibetan (Tibetan) plateau appear to have occurred asynchronously relative to Northern Hemisphere glaciation events (Zhang *et al.* 2006). Therefore, species demographic histories remain poorly understood in this region (Beheregaray 2008). The Qinghai-Tibetan plateau is the highest plateau in the world. The uplift of mountain ranges between 1.1 and 0.6 Ma (Kunlun-Huanghe Tectonic Movement) initiated widespread mountain glaciations during ice ages. The maximum extent of glacier development occurred during the late Pleistocene (0.5–0.175 Ma, according to marine isotope stages, MIS 12–6). This differs from European and North American ice sheet that generally had their maximum extent in the global LGM (0.023–0.018 Ma, MIS2) (Zhang *et al.* 2006; Zhou *et al.* 2006). Consistent with this, Gillespie & Molnar (1995) revealed differences in frequency and types between mountain and continental glaciations. The glaciations developed only around high mountains, not on the whole plateau surface (Zhou *et al.* 2006), instead of heavy ice cover throughout high-latitude regions in Europe and North America. Milder Pleistocene climate may have mitigated stresses for cold-tolerant alpine birds relative to the extremes experienced by European and North American counterparts. Thus, we may expect species on the Qinghai-Tibetan plateau to bear different genetic imprints of demographic expansion.

Here, we used five species distributed on the Qinghai-Tibetan plateau: the Tibetan snow finch (*Montifringilla adamsi*), the Blanford's snow finch (*Pyrgilauda blanfordi*), the horned lark (*Eremophila alpestris*), the twite (*Carduelis flavirostris*) and the black redstart (*Phoenicurus ochruros*), to investigate the historical demographic and genetic effects of recent ice ages on the plateau species. These five species differ in dispersal ability. The Blanford's snow finch has a relatively high dispersal potential, and often flocks in autumn and winter with altitudinal movements (Cramp & Perrins 1994). By contrast, the other four birds have weak dispersal potential (Voous 1962; Cramp & Perrins 1994; Gebauer & Kaiser 1994; Fu 1998). Variations in ecological adaptations, such as these, seem probably to affect geographical patterns of genetic variation (Endler 1977; Gavrilets 2003). We thus assume that these behavioural differences may have contributed to different phylogeographical structures of these birds.

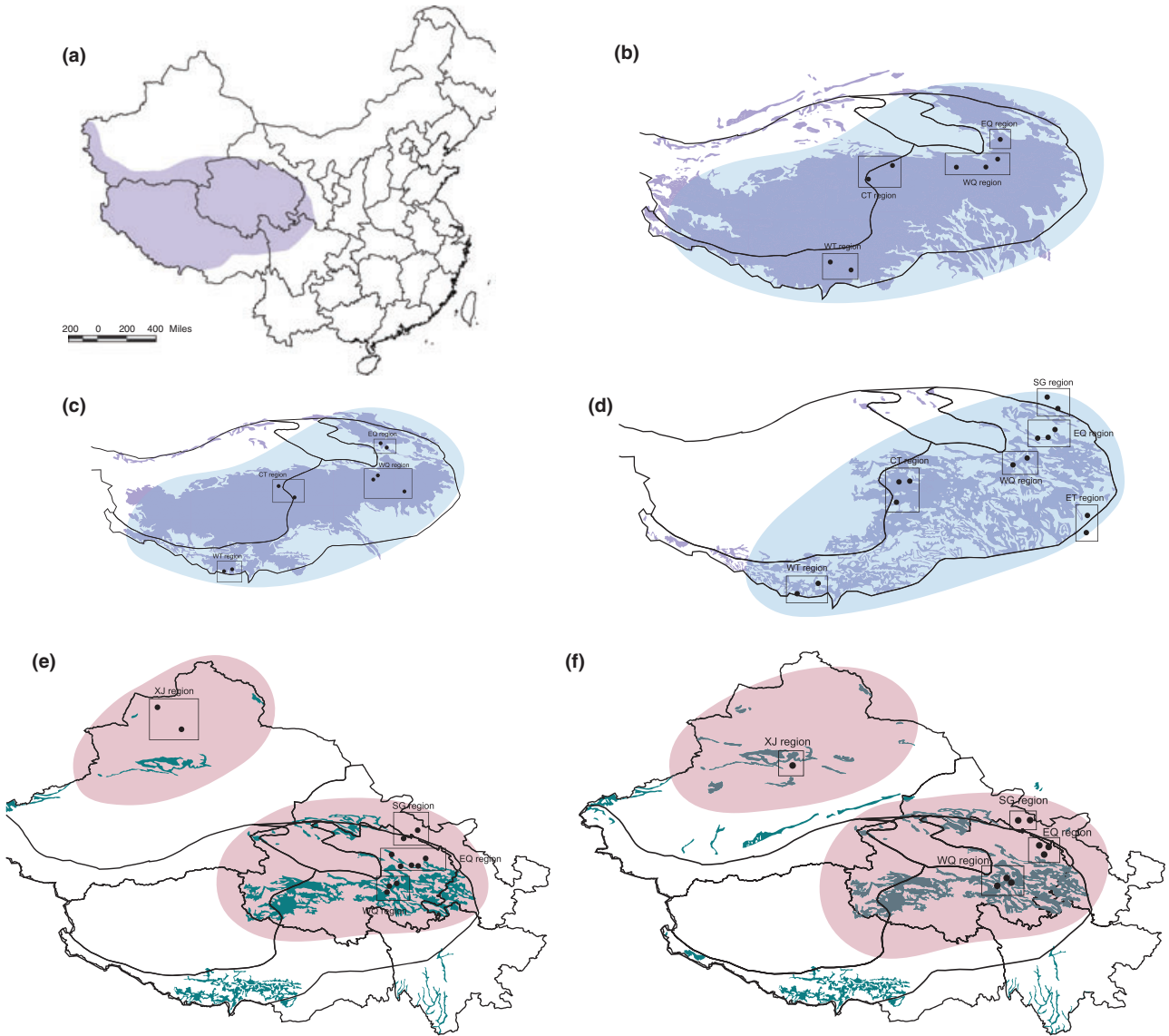
On the other hand, although these species have sympatric or partially sympatric distributions, they are different in the altitudinal patterns. The Tibetan snow finch, Blanford's snow finch and horned lark are mostly distributed in the platform region with a high altitudinal distribution (3500–5000 m), whereas the twite and black redstart dwell at lower altitudes and mostly in the edge areas (2000–3500 m). As montane glaciations predominated on the Qinghai-Tibetan plateau, we hypothesized that these only affected the habitats of species with high altitude distributions. Therefore, we expect the three species with high altitude distributions to show evidence of population expansion concurrent with the retreat of the glaciers, whereas the two lower altitude species should not have this expansion evidence. Some congruent results have been observed in previous studies (Yang *et al.* 2006; Qu & Lei 2009). While the platform populations experienced postglacial expansions and showed weak phylogeographical structures, significant isolation by distance patterns and no trace of population size fluctuations were detected in the edge populations. In this study, the 'platform' and 'edge' species were thus selected to test the hypothesis that these plateau species have responded differentially to glacial cycles as a result of variations in habitats and altitude distributions.

By analysing three gene fragments of the mitochondrial genome, we have been able to capture the genetic imprints of the matriarchal demographic dynamics of these plateau birds during glacial period. Although results were extracted from a limited scope, details of the historical demography of these species revealed in this study could provide insights into how these plateau birds and possible other plateau species respond to the climatic fluctuations of the late Pleistocene.

## Materials and methods

### *Sampling, DNA extraction, polymerase chain reaction and DNA sequencing*

The sampling of these species is shown in Fig. 1. Birds were caught in mist nets, and each bird within a site was taken from a different part of the colony to avoid sampling close relatives (Hansen *et al.* 1997). Groups with adequate sample sizes were created by pooling the sampling sites into three edge and four platform regional groups. The three edge regional groups are SG (South Gansu region, average altitude 3000 m), XJ (Xinjiang region, average altitude 2000 m) and EQ (East Qinghai region, average altitude 3400 m); the four platform regional groups are WQ (West Qinghai region, average altitude 4200 m), CT (Central Tibet region,



**Fig. 1** The study areas and sampling sites for the five species on the Qinghai-Tibetan plateau. The sampling sites were further pooled into the three 'edge' and four 'platform' regional groups. Blue and green areas represent suitable habitats for the platform and edge species, whereas their distribution ranges were highlighted in light blue and red respectively, (a) The study area; (b) Tibetan snow finch; (c) Blanford's snow finch; (d) Horned lark; (e) Twite; and (f) Black redstart.

average altitude 5500 m), WT (West Tibet region, average altitude 4800 m) and ET (East Tibet region, average altitude 4500 m).

DNA was extracted from blood or tissue samples using the DNeasy Tissue kit (QIAGEN). We amplified the cytochrome *b*, cytochrome *c* oxidase subunit I (COI) and NADH dehydrogenase subunit 2 (ND<sub>2</sub>) genes via polymerase chain reaction (PCR). The primers OSCL1 and OSCH2 were used for the amplification of the cytochrome *b* gene (Qu & Lei 2009). PCR reactions were run using the following parameters: dena-

turation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 40 s, 49 °C for 40 s, and 72 °C for 1 min, and a final 5 min at 72 °C. The COI gene was amplified using the primers BirdF1 and BirdR1 (Hebert *et al.* 2004). The parameters used for amplification were 2 min at 94 °C followed by five cycles of 1 min at 94 °C, 1.5 min at 47 °C, and 1.5 min at 72 °C, followed in turn by 35 cycles of 1 min at 94 °C, 1.5 min at 52 °C, and 1.5 min at 72 °C, and a final 5 min at 72 °C. The primers and amplification conditions used for ND<sub>2</sub> were as described in Sorenson *et al.* (1999).

Each round of PCR reactions also included one negative control to check for contamination.

The PCR products were purified using QIAquick PCR purification kit (QIAGEN), and sequenced on a Perkin-Elmer 377 semiautomated DNA sequencer (Applied BioSystems), using a Perkin-Elmer Prism terminator cycle sequencing kit (Applied BioSystems) with Ampli Taq FS polymerase and BigDye terminators. Both strands of each PCR product were sequenced. The sequencing program consisted of 25 cycles of denaturation at 96 °C for 30 s, annealing at 50 °C for 15 s and extension at 60 °C for 4 min. Complete sequences were assembled using Seqman II (DNASTAR). Sequences were compared visually to the original chromatograms to avoid reading errors. Complete sequences were aligned by eyes. All sequences are accessible at GenBank (Accession numbers: FJ624113 – FJ624146, FJ952386 – FJ952506).

#### *Nucleotide polymorphism*

We calculated the number of segregating sites, haplotype diversity and nucleotide diversity for each regional group and all populations combined using DnaSP 4.0 (Rozas *et al.* 2003). McDonald & Kreitman (1991) test was used to examine the selective neutrality of the mitochondrial protein-coding fragments (for outgroups see Table 1). Two additional neutrality tests, Fu's  $F_S$  (Fu 1997) and Fu & Li's  $D$ , (Fu & Li 1993) were used to detect departures from the mutation-drift equilibrium that would be indicative of changes in historical demography and natural selection. All three tests were implemented in DnaSP.

#### *Historical demography*

The dynamics of population size fluctuations were estimated using the Bayesian skyline plot (BSP) method implemented in BEAST 1.4.6 (Drummond & Rambaut 2006). This approach incorporates uncertainty in the genealogy by using Markov Chain Monte Carlo (MCMC) integration under a coalescent model, where the timing of dates provides information about effective population sizes through time. Chains were run for 200–500 million lengths, and the first 10% was discarded as 'burn-in'. The model HKY + invariable sites was selected for the Tibetan snow finch, Blanford's snow finch and twite, whereas the model HKY + invariable sites + Gamma distribution was selected for the horned lark according to MRMODELTEST 2.2 (Nylander 2002). For black redstart, the model GTR + invariable sites was used. The linear growth was selected for skyline model. As no fossil data were available for calibration mutation rates, we assumed a conventional

molecular clock for the avian mitochondrial cytochrome *b* gene ( $1 \times 10^{-8}$  per site per year, Klicka & Zink 1997; Weir & Schluter 2008). The mutation rate was modulated by multiplying the ratio of the average distance for the combined sequence vs. that for *cyt b* alone to deduce the substitution rate for all fragments combined. Pilot analyses showed that the *ucl.d.stdev* parameter was close to zero for the horned lark, Tibetan snow finch and Blanford's snow finch, suggesting these data are clocklike; thus a strict clock model was selected. For the twite and black redstart, the *ucl.d.stdev* parameter was much greater than 1.0, thus the uncorrelated log-normal model was used to account for rate variation among lineages (Drummond *et al.* 2006). Demographic history through time was reconstructed using Tracer version 1.4 (Rambaut & Drummond 2005). Time and effective population size were defined as year and  $\theta$  ( $N_{fe}\tau$ ;  $\tau$ , generation for time) for the BSP, although the authors cautioned that this method cannot estimate effective population size precisely. For the historical demography analyses, we did not include samples from XJ region for the two edge species because of a geographical break between XJ and other three regions.

The exponential growth rate was also estimated for each regional group and species by FLUCTUATE (version 1.4, Kuhner *et al.* 1998). FLUCTUATE was initiated with a Watterson (1975) estimate of theta ( $\Theta$ ), and a random topology, performing 10 short chains, sampling every 20 genealogies for 200 steps, and two long chains, sampling every 20 genealogies for 20 000 steps. FLUCTUATE analyses were repeated five times, and the mean and standard deviation of  $\Theta$  and ' $g$ ' were calculated from the results of these separate runs. However, this genealogical method was known to yield estimates of  $g$  with an upward bias (Kuhner *et al.* 1998). Thus, we corrected  $g$  values following the conservative approach of Lessa *et al.* (2003) and only considered the  $g$  value indicative of population growth when  $g > 3SD$  ( $g$ ).

#### *Phylogeographical structures*

We used unrooted networks to evaluate haplotype relationships for each species. The network was constructed with TCS version 1.23 (Clement *et al.* 2000) with 95% parsimoniously plausible branch connections. An analysis of molecular variance (AMOVA) was performed to test for regional structures in the partitioning of haplotypes using ARLEQUIN version 3 (Excoffier *et al.* 2005) (For regional subdivisions see Fig. 1 and Table 2). Significance levels were determined by conducting non-parametric procedures 1000 times. Isolation by distance was tested based on pairwise geographical and genetic distances ( $F_{ST}$ ) with the Isolation by Distance Web

**Table 1** Nucleotide polymorphism and results of neutrality tests at each gene and all genes combined

Taxa	Gene	Cyt <i>b</i>	CoI	ND <sub>2</sub>	Combined
Tibetan snow finch	S	9	14	8	31
<i>Montifringilla adamsi</i>	Nhap	8	12	8	17
	Hd	0.87	0.9	0.86	0.97
	$\pi$	0.0027	0.0035	0.0018	0.0025
	Fu's $F_S$	-1.32	-6.37**	-2.39	-6.87*
	Fu and Li's <i>D</i>	-0.22	-1.66	-0.41	-1.06
	MK test	1.00	1.00	1.00	
Blanford's snow finch <i>Pyrgilauda blanfordi</i>	S	17	7	14	38
	Nhap	13	9	12	24
	Hd	0.823	0.749	0.698	0.989
	$\pi$	0.0028	0.0019	0.0021	0.0023
	Fu's $F_S$	-6.05**	-4.58*	-5.66**	-18.52***
	Fu and Li's <i>D</i>	-2.20	-1.43	-1.30	-2.02
Horned lark <i>Eremophila alpestris</i>	S	47	20	22	89
	Nhap	36	20	20	43
	Hd	0.975	0.878	0.81	0.993
	$\pi$	0.0041	0.0026	0.0014	0.0027
	Fu's $F_S$	-39.6***	-17.27***	-19.32***	-42.50***
	Fu and Li's <i>D</i>	-4.51**	-2.02	-4.23*	-4.38
Twite <i>Carduelis flavirostris</i>	S	11	14	15	37
	Nhap	6	4	4	13
	Hd	0.588	0.233	0.645	0.873
	$\pi$	0.0017	0.0016	0.0021	0.0019
	Fu's $F_S$	-0.03	-0.72	3.20	-0.93
	Fu and Li's <i>D</i>	1.44	0.90	1.56	1.63
Black redstart <i>Phoenicurus ochruros</i>	S	75	33	80	188
	Nhap	23	11	15	25
	Hd	0.975	0.828	0.844	0.992
	$\pi$	0.0305	0.0117	0.0278	0.0244
	Fu's $F_S$	-1.978	1.11	4.283	-0.988
	Fu and Li's <i>D</i>	1.136	0.157	1.376	1.112
	MK test	0.146	1	1	

S, number of segregating sites; Nhap, number of haplotypes; Hd, haplotype diversity;  $\pi$ , nucleotide diversity; Fu's  $F_S$ , statistics of Fu's  $F_S$  test (Fu 1997) (\* $P < 0.02$ ; \*\*\* $P < 0.001$ ); Fu and Li's *D*, statistics of Fu & Li's *D*-test (Fu and Li 1993) (\* $P < 0.05$ ; \*\* $P < 0.01$ ); MK test, probability to reject the neutrality by Fisher's exact test in the McDonald & Kreitman (1991). For MK test, the Tibetan snow finch and Blanford's snow finch were selected as outgroup for each other, whereas the skylark was selected as outgroup for the horned lark. The Daurian redstart was selected as outgroup for the black redstart, and the redpoll and siskin were selected as outgroups for the twite.

Service (Jensen *et al.* 2005) (<http://ibdws.sdsu.edu/~ibdws/>).

#### Gene flow among regional groups

To examine the levels of gene flow among defined regional groups, maximum-likelihood migration rates were estimated by MIGRATE version 2.3.2 (Beerli 1997). This approach, based on coalescence using MCMC searches, takes into account unequally effective population sizes and asymmetrical gene flow (Beerli & Felsenstein 1999). To reduce the numbers of parameters and

degrees of freedom in our gene flow analyses, we used a stepping-stone model of population structure (Kimura & Weiss 1964). Thus, gene flow was only estimated between regional groups that were adjacent to one another. Effective population sizes and gene flow rates were estimated from  $F_{ST}$  values and set as initial values. We performed 10 short chains (500 trees used out of 10 000 sampled) and three long chains (5000 trees used out of 100 000 sampled). Adaptive heating with four to five chains of different temperatures was used. We ran the program four times with different random seed numbers using the same condition.



**Table 2** The nucleotide diversity and haplotype diversity in regional groups

Taxa	Regional groups	Sampling size	S	Haplotype sizes	Nucleotide diversity	Haplotype diversity	Theta ( $\Theta$ )	Corrected $g$ ( $g-3SD$ )
Tibetan snow finch	Whole set	22	31	17	0.0025	0.974	0.0285	1331.28
	EQ	2	3	2	0.00128	1	*	*
	WQ	6	14	5	0.0025	0.933	0.0245	1109.88
	CT	7	15	5	0.0023	0.0857	0.0117	199.68
	WT	7	14	6	0.0021	0.952	0.0142	471.85
Blanford's snow finch	Whole set	27	38	24	0.0023	0.989	0.1816	4570.96
	EQ	6	14	5	0.0024	0.933	0.1945	665.63
	WQ	11	22	11	0.0023	1	0.2624	5104.38
	CT	7	17	6	0.0021	0.952	0.0133	897.47
	WT	3	8	3	0.0021	1	0.0151	819.42
Horned lark	Whole set	50	89	43	0.0027	0.993	0.2863	2619.16
	SG	11	34	11	0.0032	1	1.9651	3220.41
	EQ	11	24	10	0.0023	0.982	0.0418	1366.99
	WQ	9	26	9	0.0028	1	2.48	2816.9
	ET	8	16	6	0.0023	0.929	0.0163	854.58
	CT	6	11	4	0.0019	0.8	0.0205	1277.01
	WT	5	20	4	0.0031	0.9	0.0309	608.45
	Whole set	40	37	13	0.0019	0.873	0.0024	-532.26
Twite	Whole set	38	8	11	0.0008	0.859	0.0023	-120.33
	without XJ							
	EQ	14	6	6	0.0008	0.802	0.0020	-92.5
	WQ	11	5	6	0.0007	0.891	0.0024	-72.05
	SG	13	5	7	0.0007	0.885	0.0038	-51.74
	XJ	2	1	2	0.0004	1	*	*
	Whole set	28	188	25	0.0244	0.992	0.0577	-0.18
	Whole set	24	186	22	0.0242	0.993	0.061731	0.89
Black redstart	without XJ							
	EQ	10	128	10	0.0235	1	0.0435	-8.99
	WQ	8	148	8	0.0239	1	0.0500	-3.71
	SG	6	82	5	0.0109	0.933	0.0050	-263.7
	XJ	4	77	4	0.0198	1	0.0098	-184.4
	Whole set	4	77	4	0.0198	1	0.0098	-184.4

S, number of segregating sites. ML estimates of effective population size (theta) and population growth coefficient ( $g$ ) estimated by FLUCTUATE.

\*Blanks indicated that ML estimates of effective population size and population growth coefficient could not be performed because of limited sample sizes.

### Glaciation refugia modelling

Estimates of the potential glaciation refugia for species having undergone postglacial expansions were made using ArcGIS (ESRI). The refugia were constructed using the known distribution range, the paleovegetation layer and the range of ice sheet during the extensive glaciation period. The paleovegetation layer was created from paleoclimatic simulation for the Qinghai-Tibetan plateau during the maximum glaciation period using two models: the AGCM (McAvaney *et al.* 1978; Simmonds 1985) coupled with a simplified simple biosphere model (SSiB, Xue *et al.* 1991). Both models generated the vegetation layer with eleven vegetation types, which were derived from a variety of sources: geological data, calibrations of field measurements, the

biometrics and physiological data for representative species and wide-range survey of the ecological and geographical literature (Peterson 1993; Edwards *et al.* 2000; Elenga *et al.* 2000; Takahara *et al.* 2000; Tarasov *et al.* 2000; Thompson & Anderson 2000; Williams *et al.* 2000; Yu *et al.* 2000) (for details, see Liu *et al.* 2002).

The prediction of glaciation refugia was created by generating suitable vegetation types from the paleovegetation layer; the newly constructed suitable vegetation types were then projected onto the ice sheet layer to obtain the ice-free suitable habitats. The predicted areas were overlapped with the current distribution of predicted species; areas predicted to have been suitable at the glaciation period, but for which no current distribution was observed were logically excluded.

## Results

### Genetic variation

We obtained 2546 bp of the mitochondrial DNA, including partial *cyt b* (880 bp), *CoI* (652 bp) and *ND2* (1014 bp) genes. In the horned lark, 43 haplotypes were identified from 50 individuals, whereas 13 haplotypes were detected from 40 individuals in the twite. Twenty-five and 24 haplotypes were defined from the black redstart and Blanford's snow finch respectively. In the Tibetan snow finch, 17 haplotypes were observed from 22 individuals. Within the three platform species, haplotype diversities ranged from 0.974 to 0.993 and nucleotide diversities ranged from 0.0023 to 0.0027. The haplotype diversities of the edge species were 0.873 and 0.992; whereas the nucleotide diversities were 0.0019 and 0.0244 for the twite and black redstart respectively (Tables 1 and 2).

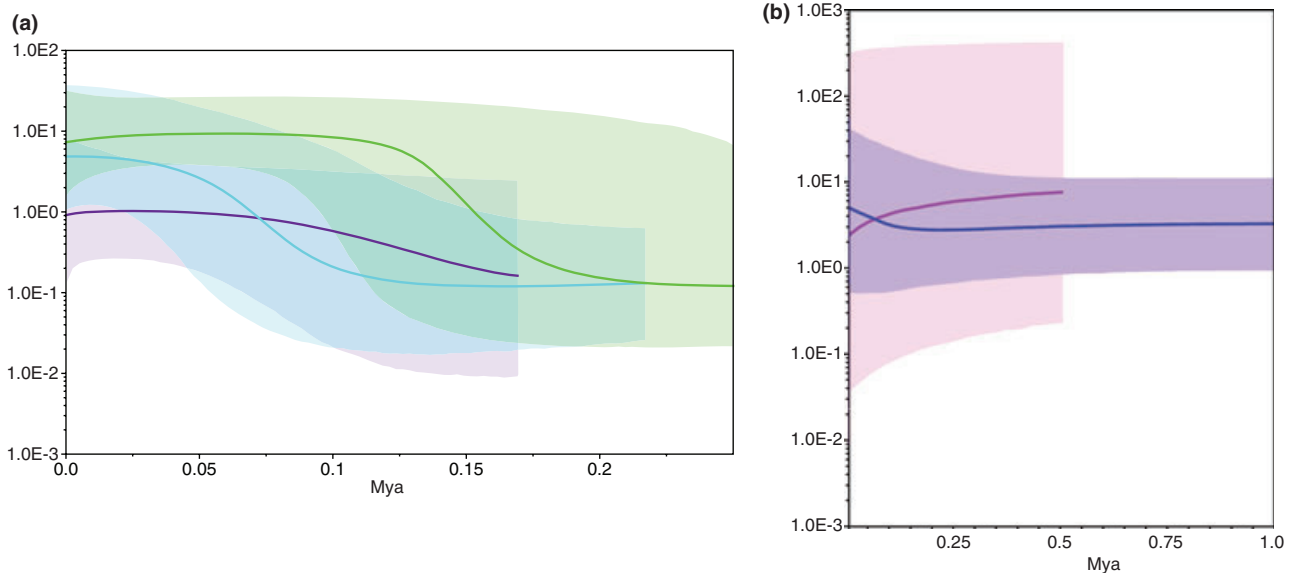
None of the coding regions we sequenced significantly deviated from neutrality (McDonald & Kreitman's test,  $P > 0.05$ , Table 1). In the three platform species, results of the Fu's  $F_S$  test and Fu and Li's  $D$ -test indicated an over-abundance of singleton mutations and rare alleles for most of the fragments (negative  $D$  or  $F_S$  values with  $P < 0.02$ ). Contradictory results between two neutrality tests and the McDonald and Kreitman's test implied that intraspecific polymorphism of these regions might have been mainly influenced by historical demography and not selection. In the two

edge species, results of the Fu's  $F_S$  test and Fu and Li's  $D$ -test did not deviate from neutrality (positive  $D$  or  $F_S$  values with  $P > 0.02$ ), suggesting that the observed nucleotide polymorphism was selectively neutral.

### Historical demography

The historical population trends of the three platform species inferred by the BSP seemed to fit the climate trend relatively well since the extensive glaciation period (Fig. 2). Past population dynamics of the three species indicated rapid population growth for the last 150 000 years. The age of TMRCA (root age) was estimated to be 0.249 Myr (0.17–0.348), 0.22 Myr (0.11–0.34) and 0.16 Myr (0.089–0.25) for the horned lark, Blanford's snow finch and Tibetan snow finch respectively. Recent population growths of the three platform species were also supported by Maximum Likelihood estimates of the exponential growth rate for each regional population and species (positive corrected  $g$  values in Table 2).

For the two edge species, we did not include samples from XJ region in the historical demography analyses because of a geographical break between XJ and other three regions. The population sizes of the other three regions of the two edge species appeared to have remained relatively stable during the whole glaciation period (Fig. 2). The age of TMRCA was estimated to be 0.5 Myr (0.08–18.48) for the twite and 4.08 Myr (1.59–7.03) for the black redstart. FLUCTUATE also did not



**Fig. 2** Bayesian skyline plots of past population demographic trends in mitochondrial lineages: *x*-axis: time in  $10^6$  ya; *y*-axis: estimated population size [units =  $N_e t$ , the product of effective population size and generation length in years (log-transformed)]. The mean estimate and both 95% HPD limits are indicated. (a) Three platform species. Green, horned lark; blue, Blanford's snow finch; red, Tibetan snow finch; (b) Two edge species. Blue, black redstart; red, twite.

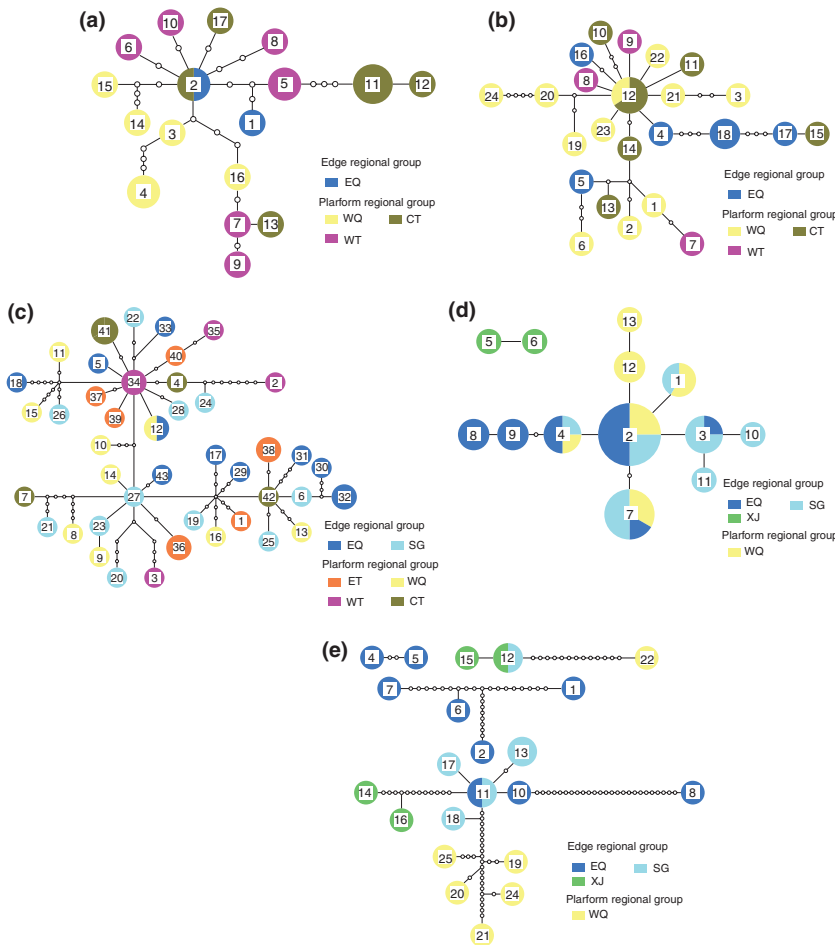
detect positive population growth rates for the twite or black redstart (Table 2).

*Phylogeographical structures*

The phylogenetic relationships of species to geographical areas were assessed by application of the maximum parsimony criterion (Fig. 3). In the black redstart, we observed four separate networks and three unconnected haplotypes (3, 9 and 23). However, the separated networks were not associated with geographical divisions, with the haplotypes from different regional groups intermixed together. In the twite, two haplotypes observed in XJ region were separated with those haplotypes in other three regions. For the three platform species, all haplotypes were connected together in a star-like topology, without distinct geographically separated clades. The ancestral haplotype of the horned lark was assigned to EQ region. Many of the other haplotypes were derived from it by one or two mutations, and the network contained several extinct or unsampled haplotypes. The basal haplotypes of the Tibetan and Blanford's snow finch were assigned to EQ, WQ and

CT regions, whereas the other haplotypes either directly attached to it or originated from it by one or several missing haplotypes.

AMOVA analyses for the Blanford's snow finch and black redstart showed weak population differentiations across regional groups and sampling sites. The total genetic variance was located at the smallest geographical scale, among individuals within sampling sites. Results of AMOVA in the Tibetan snow finch and horned lark indicated that most of the variations were found among individuals within sampling sites, 86.98% and 88.67% of all genetic variation; the components located among sampling sites were 13.9% and 14.25% respectively. No distinct population differentiation was detected among either regional groups or sampling sites in the twite (Table 3). However, when we divided twite populations into two regional groups according to network result (XJ and other three regions), a 9.91% of variance was found between regional groups (Table S1). IBD tests revealed significant isolation by distance for the twite ( $r = 0.986, P = 0.012$ ), nevertheless, the distance pattern was not detected when we removed the samples from XJ region (twite,  $r = -0.2469, P = 0.814$ ).



**Fig. 3** Maximum parsimony networks for five species on the Qinghai-Tibetan plateau. Numbers in the networks represent haplotype designations and the areas of the circles are proportional to the frequencies of those haplotypes. Small circles represent 'missing' haplotypes. (a) Tibetan snow finch; (b) Blanford's snow finch; (c) Horned lark; (d) Twite; and (e) Black redstart.



**Table 3** Hierarchical analyses of molecular variance for five species on the Tibetan plateau

Taxa	Source of variation	Variance component of $\Phi$ statistics	Variance explained (%)	P-value	Fixation index
Tibetan snow finch	Among groups	-0.0043	-0.88	0.6158	-0.0088
	Among populations within groups	0.0685	13.9	0.0811	0.1378
	Within population	0.4286	86.98	0.0078	0.1302
Blanford's snow finch	Among groups	0.0000	0.00	0.4184	0.0000
	Among populations within groups	0.0156	3.13	0.1877	0.0313
	Within population	0.4813	96.86	0.0782	0.0314
Horned lark	Among groups	-0.0147	-2.93	0.6433	-0.0293
	Among populations within groups	0.0715	14.25	0.0469	0.1385
	Within population	0.4455	88.67	0.0000	0.1133
Twite	Among groups	0.0140	3.18	0.1945	0.0318
	Among populations within groups	-0.0022	-0.49	0.5748	-0.0051
	Within population	0.4271	97.31	0.2669	0.0269
Black redstart	Among groups	0.0019	0.39	0.3900	0.0039
	Among populations within groups	0.0140	2.8	0.1554	0.0281
	Within population	0.4825	96.81	0.0684	0.0319

### Gene flow among regional groups

The orientations and levels of gene flow were estimated only for species that had undergone postglacial expansions (i.e. the three platform species). The results from four runs are summarized in Fig. 4. In most cases, MIGRATE gave asymmetrical estimates of gene flow between group pairs. The directions were more or less similar: westward migration from east to west regions. The estimates of effective female population sizes, scaled by mutation rate, were larger for EQ, ET and WQ than for other regions.

### Glaciation refugia modelling

The three platform species have similar habitat requirements; the predicted glaciation refugia layers were thus integrated to generate a final consensus model. The potentially suitable areas for the three platform species during the glaciation are shown in Fig. 5. In terms of current distributions (Fig. 1), the predicted suitable habitats were severely restricted to the eastern margin of the Qinghai-Tibetan plateau, which were defined as contiguous areas that included Gansu, Qinghai, Tibet and Sichuan. Notably, the EQ, WQ and ET regions, which were identified as the source of gene flow by MIGRATE, fell entirely within the predicted refugia.

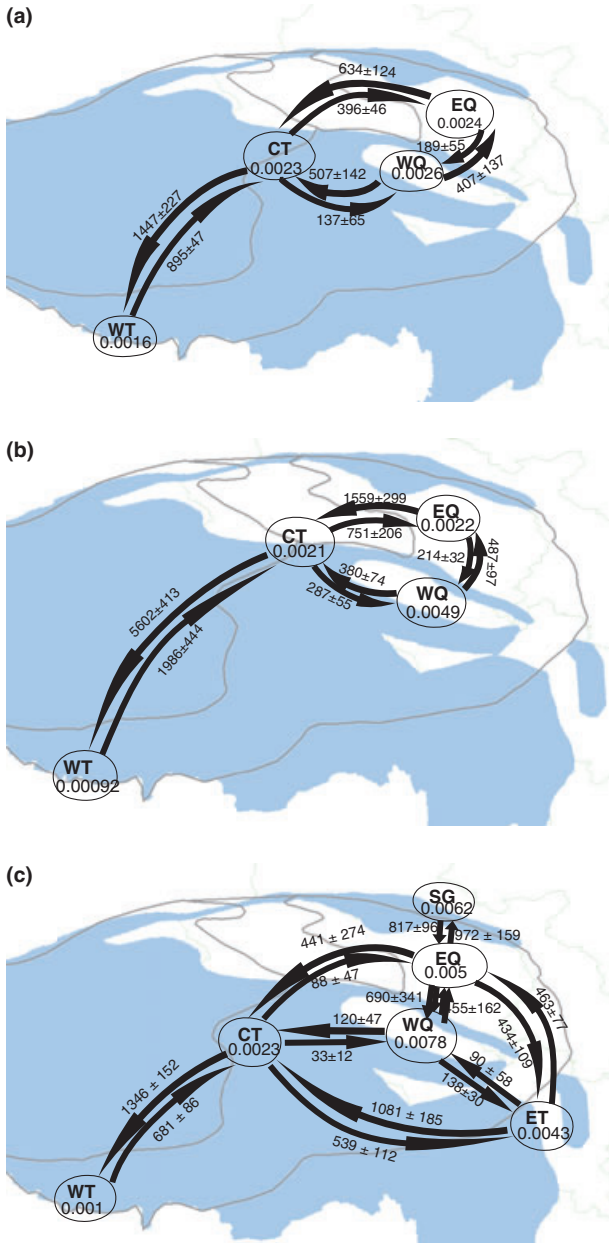
### Discussion

The comparative phylogeography of the five plateau birds revealed that the three platform species experienced postglacial expansion following the retreat of the extensive glaciation period (0.5–0.175 Ma), whereas the

two edge species maintained stable population sizes through glacial cycles. Various phylogeographical structures were observed in these species. While the molecular genetic results presented here are of limited scope, and data on larger samples and additional species are necessary to fully evaluate the evolutionary history of the Qinghai-Tibetan plateau avian fauna, we nevertheless offer a cautious interpretation of the current results.

### Historical demography, Pleistocene refugia of the Qinghai-Tibetan plateau species and comparison with those of avian species from Europe and North America

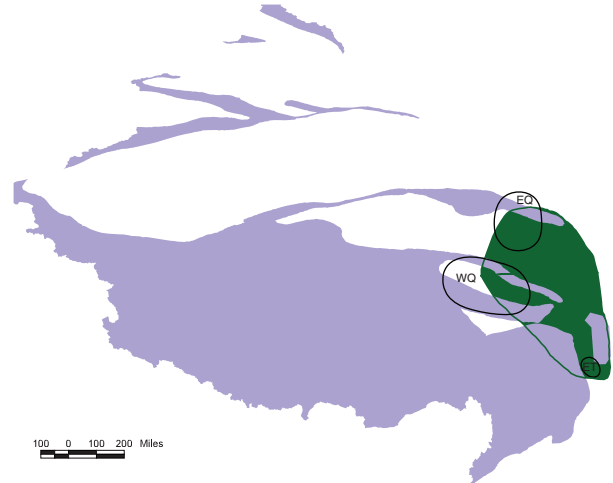
All analyses we conducted unambiguously retrieved signals of recent population expansions in the three species mostly distributed on the platform region of the Qinghai-Tibetan plateau. Unlike species in temperate North America and Europe that commonly expanded in post LGM era (0.023–0.018 Ma, Table S2), the estimated expansion dates of the plateau birds appeared much earlier, ~0.15–0.17 Ma, following the retreat of the extensive glaciation period (Qu *et al.* 2005; Yang *et al.* 2006; Qu & Lei 2009). In the Quaternary Period, the Qinghai-Tibetan plateau was uplifted to 4000–4500 m. Patterns of high rainfall moved eastward and away from the plateau centre. The development of plateau glaciers shrank and their sizes were greatly decreased (Sharma & Owen 1996; Zheng *et al.* 2002; Zhang *et al.* 2006). The largest glacier development in the Qinghai-Tibetan plateau occurred during the late Pleistocene (0.5 Ma, MIS 12-6). Glacial retreat has occurred since 0.175 Ma (Zhang *et al.* 2000; Shi 2002; Zheng *et al.* 2002). Our results, along with those from previous studies on the postglacial expansions of the



**Fig. 4** Gene flow estimations among regional groups for the three platform species. Arrows represent the direction of gene flow. The results from four independent runs were averaged. (a) Tibetan snow finch; (b) Blanford's snow finch; and (c) Horned lark. The shadowed areas represent the range of ice cover during the extensive glaciation period (data from 1: 4 000 000 digital data developed by the Institute of Geography, Chinese Academy of Sciences).

plateau avian species (Table S2), suggest that these plateau bird populations expanded after the retreat of the extensive glaciation period.

According to gene flow patterns estimated from coalescent analyses, expansions by the three platform species seem to have taken similar paths: via the westward



**Fig. 5** Predicted glaciation refugia for the three platform species. Areas predicted to be suitable habitats during the extensive glaciation period were shaded in green; blue indicated the approximate locations of ice sheets. EQ, WQ and ET regions, which were identified as the sources of gene flow by MIGRATE, were indicated by black outlines.

approach from the eastern margin of the plateau to the platform region. These westward patterns might suggest population expansions from eastern refugia. Previous studies have revealed similar expansion routes and Pleistocene refugia, which suggest that the eastern margin of the Qinghai-Tibetan plateau served as the major refugium during the Pleistocene glaciations (Qu *et al.* 2005; Zhang *et al.* 2005; Meng *et al.* 2007; Qu & Lei 2009). In contrast with the multiple separated refugia for species in Europe and North America (Table S2), the glacial refugia of the plateau birds appeared to have been restricted to the semi-continuous areas along the eastern margin of the plateau. A potential explanation for the difference might be that the plateau species are distributed in a smaller geographical and regional scale, whereas most European and North American birds were distributed on a broader, more geographically complex area that may have provided potential refugia (Hewitt 1996, 2000, 2004).

In contrast to the deep phylogeographical partitions commonly found in European and North American species, we did not find noticeable phylogeographical divisions within most plateau species (Table S2). Geography and climate might determine the distinctive phylogeography of species in different regions (Hewitt 2000). Glaciation events most probably had long-term influences in Europe and North America (Hewitt 1996, 2004). Phylogeographical analyses reveal the survival of deep lineages, often in several glacial refugia, indicating survival of populations in different refugia over many ice ages (Table S2). With repeated range changes,

surviving populations may pass through many adaptations and reorganizations, allowing their lineages to diverge and accumulate genetic differences (Hewitt 1996, 2000, 2004). By contrast, the Qinghai-Tibetan plateau has been less affected by ice sheets than highly glaciated European and North American regions during the last two glacial cycles (MIS 4 and MIS 2) (Sharma & Owen 1996; Zheng *et al.* 2002; Zhang *et al.* 2006), and the glacial period might have been too short for genetic divergences to arise. Moreover, the semi-continuous refugium of the plateau birds was potentially continuous, or at least less fragmented than refugia in Europe and North America. It is plausible that the retreat and advance of glaciations might have had less influence on the populations in such habitats. If so, haplotype composition would have remained relatively homogeneous during range shifts and demographic fluctuations.

Although our comparisons revealed some interesting results, many factors probably influence expansion timing estimates and patterns, for instance, mutation rates, generation time and idiosyncrasies of genetic loci. Increased sampling may clarify our interpretations.

#### *Different demographic history between platform and edge species*

Three of the five species mostly distributed on the platform region of the Qinghai-Tibetan plateau showed recent population expansion. Conversely, the two edge species did not exhibit these signatures of demographic expansion. This discordance could be explained by the differentiation of habitat distribution. During the extensive glaciation period, most platform regions might have been heavily covered with ice, but glaciers were probably less frequent in the east than in the west (Zhang *et al.* 2000). Consistently, the edge areas are located on the eastern margin of the plateau, which was free or less ice covered (Li 1986; Shi *et al.* 1990; Shi 1996). Comprehensive pollen analyses and our glaciation refugia model indicated that both alpine meadow and steppe might move to the eastern part of the plateau during the ice ages (Kong *et al.* 1981; Ke & Sun 1992; Liu *et al.* 2002). Following eastward retreat of suitable habitats, the platform species might have had to migrate to these ice-free refugia. From there, they colonized platform regions after glacial retreat.

However, edge areas might have remained relatively ice-free during the ice ages. The suitable habitats of the two edge species (alpine grassland and bush) could still present in this region (Kong *et al.* 1981; Ke & Sun 1992; Liu *et al.* 2002). Without pressure from lack of habitats, it is possible that the edge species might remain in the stable niche and did not experience drastic demographic fluctuations such as bottlenecks and expan-

sions. Similar examples were found from other avian species in North America. While some species showed population expansions related to recolonization of formerly glaciated areas, others that inhabited the ice-free areas during the glacial maximum did not appear to exhibit any signature of recent demographic expansions (Drovetski *et al.* 2005; Soltis *et al.* 2006).

#### *Phylogeographical structures*

No distinct phylogeographical structure was found in the Blandford's snow finch, presumably because of contemporary gene flow during postglacial expansion and the relatively homogeneous refugia. The Blanford's snow finch is characterized by strong flight and a tendency to altitudinal migration (Cramp & Perrins 1994; Gebauer & Kaiser 1994; Fu 1998). Its high dispersal capacity usually promotes gene flow from neighbouring populations and thus erodes genetic divergence (Endler 1977; Gavrillets 2003). The minimal phylogeographical divisions were observed among sampling sites for the Tibetan snow finch and horned lark. The behavioural traits of both species may explain this pattern, to a certain extent. These birds are habitat specific and have restricted dispersal ability (Cramp & Perrins 1994; Gebauer & Kaiser 1997; Fu 1998), thus gene flow might be limited after postglacial expansions. Relative isolation could have allowed populations to persist in separated habitats and promote to genetic drift.

A distinct genealogical subdivision was observed in the black redstart, and possible coalescence time dated to the late Pliocene. The most recent uplift of the Qinghai-Tibetan plateau, also known as the 'Tibetan movement', occurred between 3.6 and 1.7 Ma, which caused large geomorphologic and tectonic reconfigurations (Li *et al.* 1996; Li & Fang 1999). Our data suggest that the genealogical divergence of the black redstart occurred during this period. Potential dispersal limits, geological and climatic changes would have facilitated the genetic divergence of this species. In the twite, a geographical break occurred between populations from XJ region and other three regions, which may result from a gene cline across the large gap in our sampling between the two areas. Although isolation by distance was observed, the distance pattern could be attributed to the long distance between XJ and other three regions. Various phylogeographical structures appearing in these five species could be related to differentiation of species-specific dispersal abilities, habitat preferences, altitude specificities and evolutionary histories. The genetic diversity of these plateau species in response to climatic oscillation in the Pleistocene has most probably been controlled by many complex factors, reflecting species-specific responses to common historical events.

## Conclusion

By comparing the demographic histories and phylogeographical structures of the five species on the Qinghai-Tibetan plateau, we demonstrated that the species distributed on the platform region of the plateau experienced rapid population expansion after the retreat of extensive glaciers (0.5–0.175 Ma). These results are at odds with those of the avian species of Europe and North America, population expansion of which occurred after LGM (0.023–0.018 Ma). The potential refugium was identified in a restricted semi-continuous area around the eastern margin of the plateau, instead of multiple independent refugia for European and North American species. Population sizes of the two species distributed on the edges of the plateau, however, were maintained at stable levels. The ice-free habitats during glaciation period might provide milder climate and allow populations to stay in the stable niche. Finally, different phylogeographical structures were identified for these species, which inferred the hypothesis that various behavioural and ecological characteristics, including dispersal capacities, habitat preference and altitude specificity along with evolutionary history might have contributed to species-specific responses to the Pleistocene climatic fluctuations.

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### Supporting information

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

**Table S1** Hierarchical analyses of molecular variance for two regions of the twite and black redstart

**Table S2** Comparison of postglacial colonization patterns of the Qinghai-Tibetan birds with other birds from North American and European (see References for details)

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This research is part of Yanhua Qu's long-term study on the comparative phylogeography of the Tibetan avian species. Yan-