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Phylogeography of ground tit (*Pseudopodoces humilis*) based on mtDNA: Evidence of past fragmentation on the Tibetan Plateau

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Abstract

Pseudopodoces humilis, a long misclassified terrestrial tit, is the only species of parid whose distribution is limited to treeless terrain and endemic to the Tibetan Plateau. We revealed the phylogeographic structure of the species by using mitochondrial control region, as well as comparing morphological characters. The distinct geographic distributions of two major clades suggest spatial and temporal separations that coincide with important climatic and paleogeographic changes following the uplift of the Tibetan Plateau. Population expansion was inferred for the population at the platform of the Plateau 0.17 million years before present (Ma B.P.), and restricted gene flow with isolation by distance was detected within this region, congruent with expansion occurring after the extensive glacial period. A significant decrease in body size with decreasing altitude was found, possibly indicating selection for larger-sized birds at higher altitude.

Keywords: *Pseudopodoces humilis*; Phylogeography; Population expansion; Past fragmentation; Tibetan Plateau

1. Introduction

Many studies have focused on speciation events and genetic structure associated with the Pleistocene Refugia Hypothesis, wherein repeated glaciations followed by glacial retreat and habitat recovery are proposed to account for patterns of avian species diversity across Eurasia and North America (Baker and Marshall, 1997; Klicka and Zink, 1997; Merilä et al., 1997; Avise, 2000; Mila et al., 2000; Peck and Congdon, 2004). Phylogeography, the study of the distribution of genealogical lineages to understand factors involved in the origin and maintenance of present-day spatial genetic structure (Avise, 2000), has proved very useful in tests of this hypothesis. These factors include geographical barriers resulting in recurrent but restricted gene flow and historical events operating at the population level (e.g. past fragmentation, colonization, or range expansion events) (Hewitt, 1999, 2004; Avise, 2000). The uplift of the Tibetan Plateau (Qinghai-Xizang Plateau) beginning in the Pliocene and continuing through the Quaternary glaciations has considerably influenced the evolution and distributions of many species (Zheng et al., 1981; Shi et al., 1998; Liu et al., 2002; Pang et al., 2003). Three stages of the uplift have been hypothesized to potentially account for speciation of some plant, fish and pika species (Liu et al., 2002; He et al., 2001; Yu et al., 2000). Much intraspecific differentiation occurred in mammals and birds during Quaternary (Avise et al., 1998; Klicka and Zink, 1997), therefore the phylogeographic studies within some species endemic to the Tibetan Plateau can help us to understand the influence of the topographic and environmental changes on the speciation process. Nevertheless, it is poorly understood below species level since there are few intraspecific phylogeographic studies about the species distributed on the Tibetan Plateau due to inaccessibility of samples. Two recent studies of the phylogeography of the red-necked snow finch

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(*Pyrgilauda ruficollis*) and the white-rumped snow finch (*Onychostruthus taczanowskii*), both endemic to the Tibetan Plateau, found no distinct phylogeographic structure. These two related species show some difference in overall nucleotide diversity, but their phylogeographic patterns are congruent, suggesting rapid range expansion following a population bottleneck and high gene flow due to strong mobility (Qu et al., 2005; Yang et al., 2006).

The Hume's Ground Tit (*Pseudopodoces humilis*) is another species endemic to the Tibetan Plateau (Fig. 1). Previously known as Hume's Ground Jay, it had long been misclassified as a member of crow and jay family (Corvidae) according to its initial description (Hume, 1871) and was considered to be close to the genus *Podoces* until 1978 because of several traits shared in common with it. A recent study (James et al., 2003) removed it from the Corvidae and suggested that it should be the most aberrant member of the tits and chickadees (family Paridae) based on three independent datasets drawn from comparative osteology, the nuclear *c-myc* gene, and the mitochondrial cytochrome *b* gene, although *Pseudopodoces* shows little superficial resemblance to tits and chickadees (Chin, 2003; Lei et al., 2003). *Pseudopodoces* is the only species of parid with a distribution limited to treeless terrain (3000 to perhaps 5480 m) and one of the few species of parid with weak flight. This explanation is consistent with a hypothesis that the uplift of the Tibetan Plateau provided a tectonically active geomorphological region in which *Pseudopodoces* has a limit to its distribution, that a species of parid invaded the emergent high steppes and became the ancestor of *Pseudopodoces*; finally, that morphological evolution in a novel adaptive zone altered its appearance.

In this paper, we analyze variation in both mitochondrial control region DNA sequence and morphology of *P. humilis* from seven populations covering most of its distribution. We address three questions: (1) Is *P. humilis* genetically structured over its distribution or does it exhibit the weak structure evident in other sympatric species? (2) How have potential geological barriers (e.g. mountains, deserts, habitats) affected contemporary patterns of genetic variation in this species? (3) What is the importance of historic-geographical versus contemporary processes in generating intraspecific phylogeographic pattern?

2. Materials and methods

2.1. Sampling and laboratory methods

Samples of 67 *P. humilis* individuals from 17 localities (Fig. 1 and Table 1) covering almost the entire distribution, were collected at elevations from 3200 to 4550 m during the years 2000–2003.

DNA was extracted from muscle, feather and blood with EZ Spin Column Genomic DNA Isolation Kit (BBI) following the manufacturer's protocols. Polymerase chain reactions (PCRs) were performed with primers L16700 and H636 which cover the first domain and part of the second domain of the mitochondrial control region as described in Kvist et al. (2002). DNA samples were isolated from toe pads of three individuals in Northern Tibet and two in Central Tibet collected from the years 1960 to 1980 following the protocols as described in Nishiguchi et al. (2002), and were amplified in two fragments using primers: (L16700 + HP328: 5′-CTGTGACATTATTCGTATTCG

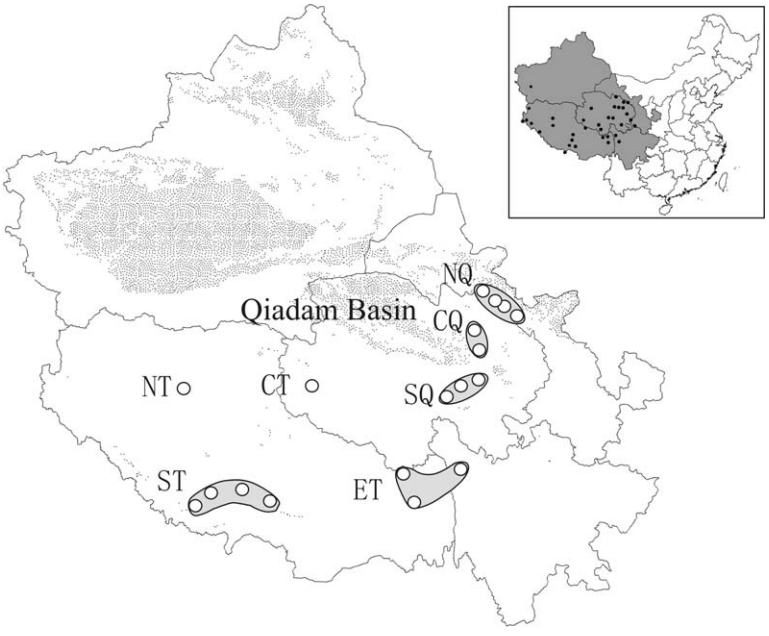

Fig. 1. Distributions and sampling localities of *P. humilis*. Filled circles represent distributions; unfilled circles represent sampling localities; sands represent desert area. (Note: NT, Northern Tibet; CT, Central Tibet; ST, Southern Tibet; ET, Eastern Tibet; SQ, Southern Qinghai; CQ, Central Qinghai; NQ, Northern Qinghai.)

Table 1
Polymorphic sites within mtDNA control region of *P. humilis*

Haplotype	1111222222223333333355	NT	CT	ST	ET	SQ	CQ	NQ
	46745792225779922347911	$n = 5$	$n = 5$	$n = 12$	$n = 7$	$n = 10$	$n = 8$	$n = 20$
	75180630179068565763433 4	$\pi = 0.392\%$	$\pi = 0.285\%$	$\pi = 0.505\%$	$\pi = 0.136\%$	$\pi = 0.400\%$	$\pi = 0.000\%$	$\pi = 0.060\%$
		Hd = 0.700	Hd = 0.700	Hd = 0.742	Hd = 0.667	Hd = 0.778	Hd = 0	Hd = 0.337
Hap_1	CAATTTGGTTCTGACGTCAGACAC						8	16
Hap_2	. . . . . G.							4
Hap_3	. . GC. . . . CCT. . GTAC. G. G. GT			2				
Hap_4	. . GC. . . . CCT. . GTA. . GAGTGT					1		
Hap_5	. . GC. . . . CCT. . GTA. . G. GT. T					1		
Hap_6	. . GC. . . . CCT. . GTA. . G. G. . T			1				
Hap_7	T. GC. . . . CCT. . GTA. . G. GT. .			2				
Hap_8	. . GC. . . . CCT. . GTA. . G. G. . .		1		1	1		
Hap_9	. . GC. . . ACCT. . GTA. . G. G. . .		3	6	4	5		
Hap_10	. . GCC. . ACCTC. GTA. . G. G. . .					1		
Hap_11	. . GCC. . ACCTCAGTA. . G. G. . .		1					
Hap_12	. . . C. . . ACCT. . GTA. . G. G. . .				2			
Hap_13	. . GC. . . . CCT. . GT. . . G. G. . .					1		
Hap_14	. . GC. CA. CC. . . GTA. TG. G. . .			1				
Hap_15	. . GC. . . ACCT. AGTA. . G. G. . .	2						
Hap_16	. GGC. . . . CCT. AGTA. . G. G. . .	1						
Hap_17	. . GC. . . . CCT. AGTA. . G. G. GT	2						

π, nucleotide diversity; Hd, haplotype diversity; n, sample size.

A-3′ and LP288: 5′-AAACATCTTGCACTCGAATACG A-3′ + H636) modified from Kvist et al. (2003).

The amplification profile was 94 °C for 5 min followed by 35 cycles of 94 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min and a final extension in 72 °C for 5 min. Sequencing reactions were performed with the primers H636, HP328 and HP451: 5′-AGTTTAAGTCCCTGAAGC-3′ (modified from H450 in Kvist et al., 2002) with Big Dye Terminator Cycle Sequencing Kit v.2.0 and run with ABI 377 automatic sequencer. Sequences have been deposited in GenBank under Accession Nos. DQ267830–DQ267895.

2.2. Genetic data analysis

The DNA sequences were aligned using the program ClustalX1.83. Nucleotide diversity (π, Nei, 1987; Eq. 10.5), and haplotype diversity (Hd; Nei, 1987; Eqs. 8.4 and 8.12) were calculated with DnaSP v. 4.00 (Rozas et al., 2003) for each of the seven groups (see Fig. 1). Haplotypes were connected in a network obtained using the 95% parsimony criterion implemented in the program TCS (Clement et al., 2000). We calculated long term effective female population size using the equation: $N_e = 10^8 \times 0.5 \times p/g$ (Avise et al., 1988), where p is the mean pairwise sequence divergence, and g is the generation time. The generation time was reported to be two years for the *Parus major* (Garant et al., 2005). We assumed that the generation time of *P. humilis* was two years also, because the study (James et al., 2003) clearly suggested a close relationship between the two species despite morphological distinction.

Analysis of molecular variance (AMOVA) and population differentiation was performed using pairwise differences and haplotype frequencies (Excoffier et al., 1992), measure of the extent of DNA divergence between populations (F_{ST}; Excoffier et al., 1992) was calculated, and the significance was tested using 10,000 permutations with Arlequin (version 2.0, Schneider et al., 2000). Mismatch distributions (SSD) and Harpending's raggedness index (*Hri*; Harpending, 1994) were estimated to test whether the sequence data deviate significantly from the expectation of a population expansion model. The parameters θ, tau (τ) and Fu's *F*s were also calculated with Arlequin. The θ estimates (θ_0 and θ_1) are the product of $2\mu N$, where N is the effective population size, and μ is the mutation rate; here, we assumed the widely accepted average mutation rate of 2% Ma^{-1} per site of mtDNA control region for passerine birds, despite the controversies concerning the 2% rule (Klicka and Zink, 1997; Avise and Walker, 1998; Randi et al., 2001; Lovette, 2004). Tau (τ) is equal to $2\mu kT$, where k is the number of nucleotides assayed, and T is the population expansion time (Rogers and Harpending, 1992; Gaggiotti and Excoffier, 2000). Fu's *F*s tests whether mutations are neutral or under influence of selection, and significantly negative values indicate population expansion (Fu, 1997).

The haplotype genealogy was converted into a network by grouping haplotypes separated by one mutational change into one-step clades, and continuing until all subclades were nested into a single clade using the procedure described by Templeton et al. (1987). Test for geographical association was carried out by performing an exact permutational contingency test for each clade at each nesting level using Geodis (Posada et al., 2000). Clade distance (D_c—a measure of the geographical range of a particular clade) and nested clade distance (D_n—a measure of how a particular

clade is geographically distributed relative to other clades in the same higher-level nesting category) were used to calculate average interior distance minus the average tip distances [$(I-T)_c$ and $(I-T)_n$]. The null hypothesis was tested to make spatio-temperal inferences about the evolutionary processes (fragmentation, range expansion or restricted gene flow) likely to contribute to the observed intraspecific pattern of genetic diversity.

2.3. Multivariate morphometrics

We measured 12 morphological characters from 108 museum-preserved adult individuals, spanning the range of the whole distribution. All specimens were measured by the same observer to avoid observer bias. We tested for character differences between males and females using *t*-test in SPSS (14.0). For the characters which were not significantly different, we treated males and females together when performing principle components analysis (using correlation matrix) and discriminant function analysis, otherwise we treated males and females separately.

3. Results

3.1. Genetic diversity

Sequence analysis of 561 base pairs of the first domain and part of the second domain of the mitochondrial control region revealed 24 polymorphic sites, 19 of which were parsimony informative, representing 17 unique haplotypes. No evidence of heteroplasmy or nuclear-mitochondrial (numt) copies was found in the sequence analysis (Sorenson and Quinn, 1998), because: (i) the electropherograms present only one specific band without length mutations; (ii) amplification of DNA from muscle gave a product with the same sequence as that obtained from blood; (iii) no significant disagreement for any particular individuals in genetic distances or phylogeographic relationships. Haplotype distributions among the 67 individuals that were divided into seven geographical groups (populations) are shown in Table 1. The distribution of individuals in the minimum spanning network of 17 haplotypes is shown in Fig. 2. Total haplotype diversity was estimated to be 0.800 and varied from 0 in CQ to 0.778 in SQ. Overall nucleotide diversity was 1.139% and ranged from 0 in CQ to 0.505% in ST (Table 1).

Neither test for neutrality on the total datasets was significant (Tajima's $D=0.8119$, $P>0.10$, Fu and Li's $D=0.0421$, $P>0.10$), providing no evidence for selection acting on this part of the mtDNA control region.

3.2. Genetic structure

For the analysis of molecular variance, the seven groups were best recognized, because the grouping maximized values of among-group variance (Φ_{CT}; Excoffier et al., 1992). The analysis showed that 14.82% of the variance resulted from differences among individuals and 80.81% from differences among the groups ($P<0.001$) (Table 2).

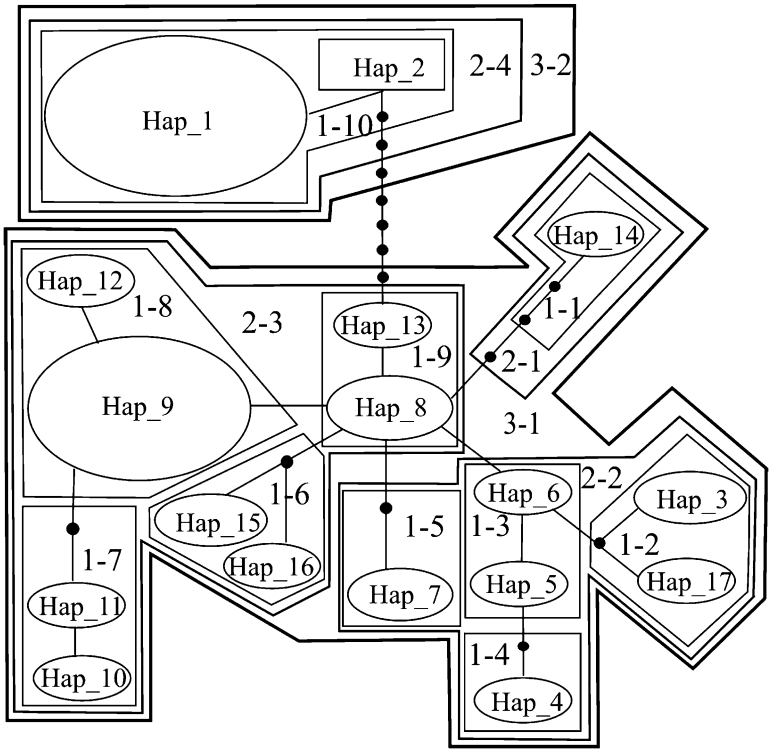

Fig. 2. Minimum spanning network and nested clades of *P. humilis*. Black dots represent missing (or unsampled) haplotypes. The area of the circle and rectangle is proportional to the frequency of the haplotype. Each segment connecting haplotypes represents one mutation. One-step clades are indicated by '1-#', two-step clades by '2-#', three-step clades by '3-#', where # is the number assigned to the clades within each level.

Table 2
AMOVA analysis of *P. humilis*

Source of variation	Sum of squares	Variance components	Percentage of variation	Fixation indices
Among groups	168.805	2.9865 Va	80.81	Φ_{CT}: 0.8081
Among samples/within groups	10.236	0.1616 Vb	4.37	Φ_{SC}: 0.2278
Within samples	27.383	0.5477 Vc	14.82	Φ_{ST}: 0.8518

Table 3
Pairwise F_{ST} values between the seven populations of *P. humilis*

	Central Tibet	Southern Tibet	Eastern Tibet	Central Qinghai	Northern Qinghai	Southern Qinghai
Southern Tibet	0.0567					
Eastern Tibet	−0.0081	0.1170				
Central Qinghai	**0.9298**	**0.8777**	**0.9640**			
Northern Qinghai	**0.9135**	**0.8608**	**0.9470**	0.1579		
Southern Qinghai	−0.0335	−0.0363	0.0688	**0.9007**	**0.8837**	
Northern Tibet	**0.3116**	**0.2634**	**0.4817**	**0.9113**	**0.8960**	**0.3012**

Significant values ($P < 0.05$) are shown in bold.

The minimum spanning network demonstrated two distinct haplotype clades connected by seven mutations, one clade (haplotypes 1 and 2) was comprised of individuals from NQ and CQ populations (referred as NCQ region) and the other clade (haplotypes from 3 to 14) including all other populations (referred as QT region) including SQ, CT, ST, ET and NT populations (Table 1). There are no common haplotypes shared between NCQ and QT regions. Overall nucleotide diversity was much higher in QT region ($\pi = 0.402\%$) than that in NCQ region ($\pi = 0.045\%$).

The pairwise F_{ST} values between the groups are shown in Table 3, the highest values are between NCQ and QT regions and very close to value one, indicating substantial isolation between the populations of NCQ and QT regions. For the substitutions in our datasets are all transition mutations, the net *p*-distance was calculated between NCQ and QT to be 0.017 (implemented in software Mega 3.0; Kumar et al., 2004), which implies isolation age of 0.85 Ma B.P. based on the mutation rate of 2% Ma^{-1} for birds.

The nested clade network of haplotypes of *P. humilis* contains three loops, indicating ambiguous connections. These were resolved using coalescent theory according to the rules outlined in Crandall and Templeton (1993) (Fig. 2). Some phylogeographical structure within clades was detected (Table 4). Table 5 presents the results of nested contingency analysis in which sampling localities are treated as categorical variables. It revealed significant associations between clades 1–8, 2–2, 2–3, 3–1 and their

Table 4
The nested cladistic analysis of geographical distance for the mtDNA haplotypes of *P. humilis*

Haplotypes			One-step clades			Two-step clades			Three-step clades		
Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n	Clade	D_c	D_n
14			1–1			2–1	0	522.63	3–1		
3	0	179.58	1–2	238.36	322.73	2–2	474.50	573.28			
17	0	355.92									
5	0	455.14	*1–3*	*525.56*	*589.66*						
6	0	619.45									
4			1–4	0	970.61						
7			1–5	0	426.06						
			I − T	406.38	124.5						
15			1–6	0^S	796.09^L	2–3	532.54	536.98			
16											
10	0	428.22	1–7	358.15	459.80						
11	0	308.10									
9	522.76^L	529.90^L	1–8	505.56	511.34						
12	0	338.91^S									
I − T	522.76^L	190.99^L									
8	331.18	328.73	*1–9*	*295.04*	*481.04*						
13	0	207.44									
			I − T	−138.06	−60.34						
1	99.09	99.46	1–10			2–4			3–2		
2	*0*	*97.76*									
I − T	−99.09	−1.70									

Tests determine whether the within-clade (D_c) or nested clade (D_n) geographical distances are significantly large (^{L}) or significantly small (^{S}). Where interior and tip clades are present, significance is also tested for the average difference between these two types of clade ($I - T$). Tip clades are in italics. Note that the number of steps indicated refers to the clades within the nested clades given in Fig. 2.

Table 5
Nested contingency analysis of geographical associations based upon 1000 iterations

Clades	Permutational χ^2 statistic	probability
1–2	3	0.346
1–3	2	1
1–7	2	1
1–8	20	0.026[a]
1–9	1.3333	1
1–10	2.5926	0.569
2–2	19.5556	0.045[a]
2–3	48.7683	0.007[a]
3–1	31.9592	0.017[a]

Clades are the same as in Table 4.
[a] Significant geographical structuring ($P < 0.05$).

Table 6
Inference chains based on results of geographical dispersion analysis (GEODIS)

Clades	Inference chain	Inferred pattern
1–8	1–2–3–4–No	Restricted gene flow with isolation by distance
2–3	1–2–3–5–6–7–8–No	Sampling design inadequate to discriminate between isolation by distance versus long distance dispersal
Total	1–19–No	Allopatric fragmentation

geographical locations. The null hypothesis of no association between geographical distribution of haplotypes and mtDNA genealogy was rejected for 1–8, 2–3 and the total clades (Table 6). Use of the inference key led us to reject the null hypothesis in favor of isolation by distance or long distance dispersal for 1–8 and 2–3, and for the total clade, implied allopatric fragmentation.

3.3. Population expansion and effective population size

The Arlequin analyses showed large differences in θ_0 and θ_1 within the two isolated regions NCQ and QT, suggesting rapid population expansion (Table 7). Mismatch distributions did not differ significantly from those distributions expected under population expansion in these two regions (Fig. 3 and Table 7) (Slatkin and Hudson, 1991; Rogers and Harpending, 1992), but did in the whole sample. Fu's *F*s-value was negative and significantly different from zero only for the QT region, also consistent with population expansion. The τ-values (Table 7) were incongruent between NCQ and QT, indicating that, even if the population in NCQ region experienced expansion event, it happened much later than that in QT. The τ-value for the whole sample may imply a date of the isolation between NCQ and QT (0.561 Ma B.P., which is calculated from τ-value), much earlier than the date of population expansion in QT (0.169 Ma B.P.) and NCQ (0.015 Ma B.P.) (Table 7).

The long term effective female population size was estimated to be 363,000 (with p calculated to be 0.0115). Because $\theta(=2N_e\mu)$ is biased upwards, it was not used to calculate N_e (Alexandrino et al., 2002).

Table 7
Mismatch distribution analysis of *P. humilis*

	QT	NCQ	Total
n	39	28	67
S	17	1	24
Θ_0	0.003	0	0.002
Θ_1	4.196	4.701	10.229
τ	3.800	0.331	12.584
T(Ma)	0.169	0.015	0.561
SSD	0.0047	0.0009	0.0531*
Hri	0.0160	0.3066	0.0566
Fu's *F*s	−7.1045***	0.4480	−0.2604

NCQ, containing NQ and CQ groups; QT, containing SQ, NT, CT, ET and ST groups. * and *** significantly at $P < 0.05$ and $P < 0.001$, respectively.
Number of individuals (n), number of polymorphic sites (S), theta at time 0 and 1 (θ_0 and θ_1), tau (τ), the estimates of expansion time calculated from τ (T), sum of squared deviations from mismatch analyses (SSD), Harpending's Raggedness index (*Hri*) and Fu's *F*s.

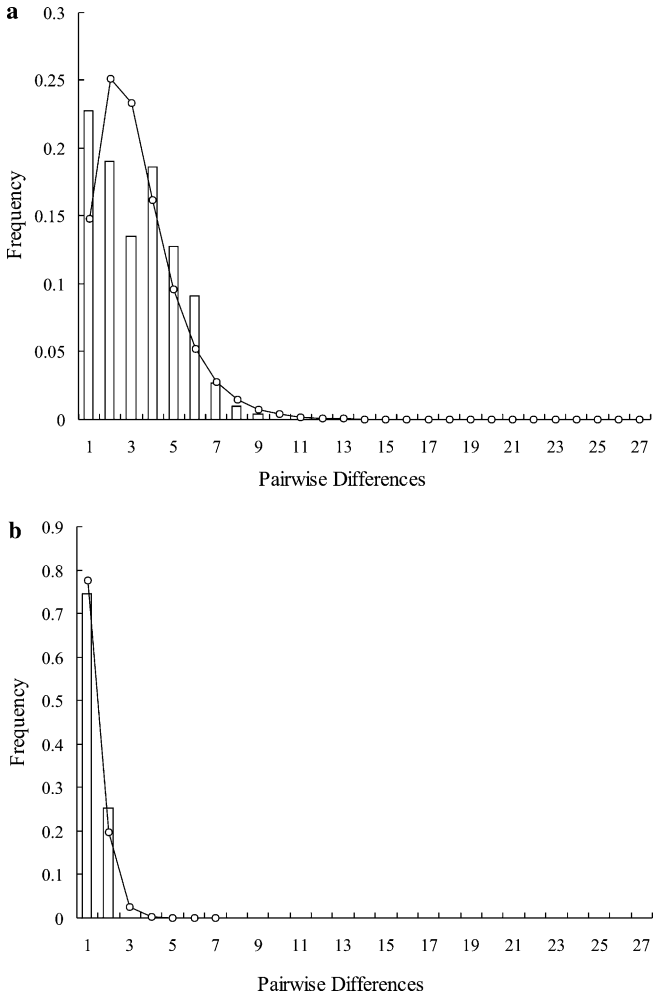

Fig. 3. Mismatch distributions in the two regions. The histograms represent the observed frequencies of pairwise differences among haplotypes and the line refers to the expectation under the model of population expansion. (a) Mismatch distribution for the QT region. (b) Mismatch distribution for the NCQ region.

3.4. Spatial patterns of morphological variation

Among the 12 characters, significant differences between males and females were only found in wing length, bill height, tarsus and third claw length. The first two axes from the principal component (PC) analysis of characters that did not differ between sexes explained 46.196% and 15.552% of the total original variation, respectively. PC1 corresponded mainly to variation in beak dimensions and claw length of the first toe while PC2 reflected variation in weight, and body and tail length. There was a significant decrease in the PC2 score with increasing longitude and latitude ($F_{(1,68)} = -1.423$, $P = 0.016$; $F_{(1,68)} = -0.891$, $P = 0.001$, respectively), and PC2 score was significantly different between QT and NCQ regions ($P = 0$). Among the four sexually different characters, female tarsus and male wing length were significantly different between QT and NCQ regions. The discriminant function analysis results show that 83%, 75% and 73% of the whole sample, males and females, respectively, could be correctly classified to QT and NCQ regions.

4. Discussion

Overall nucleotide diversity was moderately high for *P. humilis* compared to other avian species (Baker and Marshall, 1997; Avise et al., 2000; Moum and Arnason, 2001; Godoy et al., 2004; Tiedemann et al., 2004). Our analysis identified two distinct geographically non-overlaping clades in NCQ and QT regions. Although CQ is immediately adjacent to SQ spatially, these two populations had no common haplotypes and belonged to NCQ and QT regions, respectively. Geographically, NCQ and QT are separated by Qiadam Basin located at the northern border of the Tibetan Plateau in Qinghai Province, China (see Fig. 1). Nested clade analysis showed that restricted gene flow with isolation by distance is likely to be widespread across QT region without deep divergence.

Phenotypic quantitative studies on *P. humilis* revealed significant decrease in body size measures including weight, body length and tail length with increasing longitude and latitude; that is to say that the individuals from NCQ region, on average, were smaller than those in QT region. The discriminant function analysis results show that 73–83% individuals could be correctly classified to the two regions. However, no evident difference was found among other morphological characters like beak size and shape, claw, as well as plumage coloration between the two genetically distinct clades (Chen et al., 1998). The ecological environments flanking Qiadam Basin are similar except for altitude, the collection altitude in NCQ region ranged from 3000 to 3600 m and was much lower than that in QT region (3900–4800 m). Therefore, the significant decrease in body size of *P. humilis* was possibly associated with the decreasing altitude, consistent with Bergman's rule, for the more extreme environment on the Plateau may select for larger-size organism (Li, 1981). Genetic divergence in putatively neutral genes, reflects history of isolation, through mutation and genetic drift, whereas phenotypic divergence may reflect recent divergent ecological selection (Moritz et al., 2000).

The altitude of Qiadam Basin ranges from 2500 to 3000 m, whereas the surrounding Mt., Kunlun, Altun and Qilian Mt. have elevations exceeding 5000 m. Furthermore, the habitat is markedly different from its flanking areas, with both saline swamp and desert habitats. Because *P. humilis* is only distributed in treeless terrain (above 3000 m), we speculate that the low latitude and poorer habitat in Qiadam Basin form an effective barrier to movement. Based on our knowledge from field trips undertaken from the years 2000 to 2004, *P. humilis* was not found in this area, but was encountered frequently in the adjacent areas. The event inferred for the total two deeply divergent clades from Nested clade analysis, was past fragmentation, occurring between NCQ and QT regions. *P. humilis* populations appear to have been subject to past fragmentation caused by the original formation and desertification of Qiadam Basin. There would appear to be no current gene flow across the uninhabitable belt because of the weak flying ability of this species. According to geological data, the first glaciations occurred 1.21–4.43 Ma B.P. on the Tibetan Plateau, and with the ongoing uplift, the summer and winter monsoon circulations were altered immensely (Zheng and Rutter, 1998). New geological events caused the elevation of mountains surrounding the Qiadam Basin, subsequently, the arid climate and abundant sand source from lake deposits lead to the desertification in Qiadam Basin (Wang et al., 1996; Zheng et al., 2002; Lu et al., 2004). *P. humilis* possibly was isolated by the Qiadam Basin at about 0.85 Ma B.P. according to our divergence estimates based on the molecular clock. The time T calculated from τ-value by equation $\tau = 2\,\mu k T$, may also indicate the date of isolation between populations in QT and NCQ (roughly 0.561 Ma B.P.). Therefore, we deduce that desertification in Qiadam Basin might have separated the ancestral population into QT and NCQ regions gradually. The subsequent largest glaciations which occurred 0.7–0.5 Ma B.P. might have made an additional contribution to the geographical isolation.

Analysis of cytochrome *b* sequences of *P. humilis* and most species of the genus *Parus* suggested that the divergence of *P. humilis* from its closest sister taxa *Parus major* occurred at roughly 4 Ma B.P. based on the average genetic distance 8.02% and the assumed rate of 2% Ma^{-1} (Lu, 2004). James et al. (2003) suggested that the parid ancestor of *P. humilis* invaded the emergent high steppes that formed in the Plio-Pleistocene (Sun and Liu, 2000). Subsequent morphological evolution in this novel adaptive zone altered the species' appearance, obscuring its relationship with the Paridae. It is likely that *P. humilis* evolved with the ongoing uplift of the Plateau and the multiple glaciations. Divergent ecological selection of *P. humilis* in this environment atypical of other parid species undoubtedly produced its current unusual suit of morphological and behavioral

attributes and led to its original misclassification as a ground jay in the genus *Podoces*.

Very different phylogeographic patterns were previously described in two snow finch species, both of which are largely co-distributed with *P. humilis* on the Plateau (Qu et al., 2005; Yang et al., 2006). No distinct geographical divergence was detected in these two species across their entire ranges, presumably because of contemporary gene flow during a spatial expansion of the population and a relatively homogeneous habitat available throughout the Tibetan Plateau for that snow finches have strong ability of flight and a tendency to seasonal locomotion. The high level of haplotypic diversity coupled with low nucleotide diversity indicated a rapid range expansion following a population bottleneck for these two species, and time estimates derived from the mismatch distributions suggest that the present postglacial colonization occurred at 0.07–0.19 Ma B.P. (Qu et al., 2005; Yang et al., 2006). In this study, all statistical analyses suggested population expansion for *P. humilis* in the QT region, estimated to have occurred at 0.169 Ma B.P. Both are consistent with expansion occurring after the extensive glacial period (Late Mid Pleistocene, 0.2 Ma B.P.) (Zheng et al., 2002). Although mismatch distributions analysis indicates population expansion in the NCQ region as well, the expansion time estimated was much later, 0.015 Ma B.P. Furthermore, Fu's *Fs* did not suggest a population expansion for this region. Only two different haplotypes with one substitution were recognized in the 28 individuals in NCQ region. The weak evidence of population expansion and low genetic diversity for *P. humilis* in the NCQ region may be a result of a very small population size and the small area of suitable Plateau habitat to the north of Qiadam Basin, which might have lead to strong genetic drift after separation from the larger population in QT.

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