Deep phylogeographic divergence of a migratory passerine in Sino-Himalayan and Siberian forests: the Red-flanked Bluetail (*Tarsiger cyanurus*) complex

Site Luo1,2,*, Yuchun Wu1,*, Qing Chang2, Yang Liu3, Xiaojun Yang4, Zhengwang Zhang5, Min Zhang1, Qiang Zhang1 & Fasheng Zou1

1Guangdong Entomological Institute, South China Institute of Endangered Animals, Guangzhou 510260, China
2Institute of Genetic Resources, College of Life Sciences, Nanjing Normal University, Nanjing 210046, China
3State Key Laboratory of Biocontrol and School of Life Sciences, SunYat-sen University, Guangzhou 510275, China
4State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming 650223, China
5Ministry of Education Key Laboratory for Biodiversity Sciences and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing 100875, China

**Keywords**
Sex chromosome, species delimitation, *Tarsiger cyanurus* complex, the phylogenetic species concept.

**Correspondence**
Fasheng Zou, Guangdong Entomological Institute, South China Institute of Endangered Animals, No. 105 Xingang West Road, 510260 Guangzhou, China.
Tel: +8620 84182827; Fax: +8620 84183704;
E-mail: zoufs@gdei.gd.cn

**Funding Information**
The National Natural Science Foundation of China-Guangdong Joint Fund (U0833005) to Fasheng Zou, the National Natural Science Foundation of China (31172076, 31201709 and 31200327) to Fasheng Zou, Yuchun Wu, and Qiang Zhang, Science and Technology Planning Project of Guangdong Provinc, China to Fasheng Zou, Natural Science Foundation of Guangdong Province, China (52013010 012976) to Yuchun Wu, “Hundred Talent Program (Bai Ren Ji Hua)” from Sun Yat-sen University, China to Yang Liu.

Received: 25 July 2013; Revised: 13 December 2013; Accepted: 31 December 2013

**Abstract**
Enormous mountaneous forests in Sino-Himalayas and Siberia harbor important avian biodiversity in the Northern Hemisphere. Numerous studies in last two decades have been contributed to systematics and taxonomy of passerines birds in these regions and have revealed various and complex phylogeographic patterns. A passerine species Red-flanked Bluetail *Tarsiger cyanurus* provided a good system to manifest such evolutionary complexity. The subspecies *T. c. cyanurus* and *T. c. rufilatus* (*or/and T. c. pallidior*), divergent in morphology, acoustics, and migratory strategies are allopatric in Siberia and Sino-Himalayan forests, respectively. The two taxa most likely deserve full species status but rigorous genetic analysis is missing. In this study, multilocus phylogeography based on mitochondrial DNA and Z-linked DNA reveals that *T. c. cyanurus* and *T. c. rufilatus* are reciprocally monophyletic with significant statistical support and differ with a large number of diagnostic nucleotide sites resulting substantial genetic divergence. Our finding supports the proposed split of *Tarsiger cyanurus s.l.* that *T. cyanurus* and *T. rufilatus* should be treated as two full species. Whether “pallidior” is a subspecies or geographical form of *T. rufilatus* is still uncertain. Additionally, these two forest passerine species may have diverged 1.88 (3.25–1.30) Mya, which might be shaped by geographical vicariance due to grassland and desert steppe on the central Loess Plateau during the Pliocene. Taken together, this study and further suggests another independent example of North Palearctic–Sino-Himalayan phylogeographic pattern in Palearctic birds.
**Introduction**

The uplifts of Tibetan Plateau, especially the most recent and abrupt uplift in 3.4 million years ago (Mya), have substantially influenced the phylogeographic patterns of the component fauna of the plateau as well as the adjacent areas including the Sino-Himalayan, the Siberia, and tropical Asia (Päckert et al. 2009, 2012; Yang et al. 2009; Price 2010; Martens et al. 2011). These large Eurasian mountain ranges in this area are considered an important hotspot of avian biodiversity in the Northern Hemisphere (Roselaar et al. 2007). Extensive ornithological studies on the systematics and taxonomy have been focused on many taxa distributed in this region, especially passerines (Päckert et al. 2009, 2012; Price 2010; Martens et al. 2011). Most of species distributed on/around the Tibetan Plateau and in North Palearctic regions demonstrated various degrees of differentiation in morphology, acoustics, ecology (i.e., migratory strategies), and genetics, which can been assigned to different geographical affinities, long been known as “subspecies”. Most of these divergences can be dated to the Pliocene–Pleistocene associated with geographical vicariance (Johansson et al. 2007; Päckert et al. 2009, 2012; Martens et al. 2011). Considering their long and independent evolution in geographical separation, however, some of them may merit treatment as “species” (Martens et al. 2011). Recent findings mainly based on the evidence of genetic or/and acoustic characters have revealed discordance between conventional taxonomy and phylogenetic relationships in many taxa (Cibois et al. 1999; Luo et al. 2009; Sangster et al. 2010; Zuccon and Ericson 2010; Martens et al. 2011). This has led to many taxonomic changes, especially the increase in species by splitting of long-established polyploid species or species complexes (Luo et al. 2009; Sangster et al. 2010; Zuccon and Ericson 2010; Martens et al. 2011), leading to the inflation the species-level diversity in Old World passerine birds.

One example of a passerine species complex distributed in the North Palearctic region and Sino-Himalayas is the Red-flanked Bluetail *Tarsiger cyanurus* Pallas (1773), which is placed in Old World flycatcher family Muscicapidae (Zhao 2001; Dickinson 2003; del Hoyo et al. 2005; Sangster et al. 2010). This species is a small migratory insectivorous bird that occupies a large breeding range in Eurasia, from northern Finland to Kamchatka and Japan in the Northern Palearctic, the northern, western, and southern Himalayas, and southwestern China. The Red-flanked Bluetail occurs in Southern China, Myanmar, Indochina, and the Himalayan foothills during nonbreeding period. It is sexually dimorphic and shows a habitat preference for both coniferous and broad-leaf mixed forests, subalpine forests and up to treeline in the Himalayas (del Hoyo et al. 2005).

The Red-flanked Bluetail is currently split into two or three subspecies based on morphological differentiation and breeding range: *T. c. cyanurus* in Northern Eurasia including N Japan; *T. c. rufilatus* in C and E Himalayas and southwest China, and the proposed *T. c. pallidior* exclusively in northwest Himalayas that is sometimes lumped into *T. c. rufilatus* (Dickinson 2003; del Hoyo et al. 2005). Most recently the nominate race and *T. c. rufilatus* have been regarded as two distinct species based on their plumage, song-types, and distribution (del Hoyo et al. 2005; Rasmussen and Anderton 2005; Robson 2008). The two taxa are morphologically prominent in plumages, that is, more dark blue pigmentation of the adult males and the greyer pigmentation of the females and immatures in *T. c. rufilatus* than in *T. c. cyanurus* (Fig. 1). More *T. c. rufilatus* has bright blue in brows rather than white in the latter taxon (Fig. 1). Ecologically, the former taxon is a short-distance altitudinal migrant, whereas *T. c. cyanurus* is a long-distance migrant traveling between Siberia and south China. No overlap breeding zone is found between *T. c. cyanurus* and *T. c. rufilatus* (including *T. c. pallidior*). Though substantial differentiation in several aspects (including plumage, song-types, and allopatric distribution) indicates the two subspecies may merit separate species status, no genetic evidence has been presented (del Hoyo et al. 2005; Rasmussen and Anderton 2005; Robson 2008).

The allopatric distribution of the Red-flanked Bluetail resembles the pattern of North Palearctic and Sino-Himalayas within several passerine species complex, attributed to

**Figure 1.** (A) Male *Tarsiger cyanurus* cyanurus. (B) Male *T. c. rufilatus*. (B) can be morphologically distinguished from (A) by darker blue tone and light blue supercilium (photographs by Menxiu Tong/China Wild Tour).
climatic and vegetation changes since the Pliocene (Päckert et al. 2009, 2012; Price 2010; Martens et al. 2011). We assume that similar biogeographic patterns may reflect a consensus evolutionary history, and thus rationally hypothesized that the general phylogeographic pattern of the Bluetail complex possibly followed the divide between Northern Palearctic and Sino-Himalayan taxa given the distributional and ecological evidence mentioned above. However, species-specific phylogeographic patterns have to be carefully investigated using rigorous genetic approaches because random nongenetic variations in morphology and migratory strategies within a species (Rolshausen et al. 2009; Liu et al. 2012) can evolve very fast than neutral genetic markers (Zink and Barrowclough 2008).

Moreover, another main goal of the present study is to investigate taxonomic status and species delimitation of the Red-flanked Bluetail. Species delimitation also strongly relies on the application of species concepts (Mayr 1942; Cracraft 1989; Andersson 1990). In fact, the intent of most species concepts shares a fundamental idea that a species in nature is an evolutionary distinct lineage (de Queiroz 2007). So to define a species, we identify separately evolving lineages based on monophyly and diagnosability under the phylogenetic species concept (Cracraft 1989) as well as the well-known biological species concept (Mayr 1942). We applied these criteria to elucidate the species limits in the Red-flanked Bluetail complex in case of genetic analysis based on two Z-linked fragments and two mitochondrial genes. The Z-linked loci are considered to be involved in species recognition traits and may be important for genes involved in reproductive isolation in birds (Sæther et al. 2007). Meanwhile, compared with nuclear genes, mtDNA can provide similar but more sensitive genetic information about the divergence of closely related taxa (Zink and Barrowclough 2008). Furthermore, considering similar phylogeographic pattern of North Palearctic region and Sino-Himalayas within several passerine birds in previous studies (Päckert et al. 2009, 2012; Price 2010; Martens et al. 2011), molecular dating between the two taxa of Red-flanked Bluetail was also approximately estimated based on a common mitochondrial clock (cytochrome b). All genetic markers and analysis methods employed in this study have been proved to be effective and enough to species delimitation and phylogenetic analysis (Backström et al. 2006; Zink and Barrowclough 2008; Päckert et al. 2009, 2012; Martens et al. 2011; Wu et al. 2011).

**Materials and Methods**

**DNA sampling and sequencing**

Tissue samples were collected from our field sampling trips and museum tissue collection. Because both *T. c. cyanurus* and *T. c. rufilatus* are migrants, we included samples of these two taxa from both the breeding and nonbreeding ranges (Fig. 2 and Table 1). They were two breeding and three nonbreeding localities of *T. c. cyanurus* (32 individuals, including 17 females, nine males, and six sex unidentified individuals), one breeding and four nonbreeding localities of *T. c. rufilatus* (28 individuals, including 11 female, nine males, and eight sex unidentified individuals), and two sex unidentified individuals from two nonbreeding localities of *T. c. pallidior*. The homologs sequences of congenus species, the Golden Bush-robin (*Tarsiger chrysaeus*), and related genus species, the Plumbeous Water-redstart (*Rhyacornis fuliginosus*) were sequenced as outgroups to root the phylogenetic tree (Sangster et al. 2010).

Genomic DNA was extracted from tissues using the method described by Zhang and Hewitt (1998). Two mitochondrial genes (cytb and the second subunit of

![Figure 2. Sampling of Tarsiger cyanurus complex (For the abbreviation of populations see Table 1). Solid symbols are breeding regions and hollow are wintering regions for T. c. cyanurus (red) and T. c. rufilatus (dark blue), T. c. pallidior are light blue and samples from nonbreeding seasons.](image-url)
nicotinamide adenine dinucleotide dehydrogenase (ND2) and two nuclear Z-linked fragments (GC-rich promoter binding protein 1 gene intron [GPBP1] and nicotinamide nucleotide transhydrogenase intron [NNT]) were amplified and sequenced in both directions with published primers and PCR protocols (Hackett 1996; Johnson and Sorenson 1998; Backström et al. 2006; Li et al. 2006; Table 2). The resulting sequences of *T. cyanurus* and *T. rufilatus* have been deposited in GenBank (Accession No. cytB, KJ024109–KJ024114; ND2, KJ024147–KJ024153; GPBP1, KJ024218–KJ024225; NNT, KJ024456–KJ024462).

**Table 1.** *Tarsiger cyanurus* samples used in this study.

| Subspecies | Population | Locality | Sample sizes | GenBank Accession Numbers |
|------------|------------|----------|--------------|---------------------------|
|            |            |          |              | cytB  | ND2     | GPBP1   | NNT     |
| T. c. cyanurus | RQLD      | S. Kurile Is., Iturup I., Russia | 6 | KJ024109–KJ024114 | KJ024173–KJ024178 | KJ024237–KJ024242 | KJ024298–KJ024304 |
|             | RKYD      | Sakhalin I., Dolinsk & Nogicky District, Russia | 7 | KJ024115–KJ024121 | KJ024179–KJ024185 | KJ024243–KJ024249 | KJ024305–KJ024311 |
| HNDZ       | Dongzhai, Henan, China | 10 | KJ024122–KJ024128 | KJ024186–KJ024195 | KJ024250–KJ024259 | KJ024312–KJ024315 |
| GXXIX      | Jinxiu, Guangxi, China | 4 | KJ024132–KJ024135 | KJ024196–KJ024199 | KJ024260–KJ024263 | KJ024322–KJ024325 |
| SCLZ       | Luzhou, Sichuan, China | 5 | KJ024140–KJ024145 | KJ024200–KJ024204 | KJ024264–KJ024268 | KJ024324–KJ024328 |
| T. c. rufilatus | YNPW | Pingbian, Yunnan, China | 6 | KJ024141–KJ024146 | KJ024205–KJ024210 | KJ024269–KJ024274 | KJ024331–KJ024336 |
|             | YNNJ      | Nuijiang, Yunnan, China | 7 | KJ024147–KJ024153 | KJ024211–KJ024217 | KJ024275–KJ024281 | KJ024337–KJ024343 |
|             | YNLC      | Lincang, Yunnan, China | 8 | KJ024154–KJ024160 | KJ024218–KJ024225 | KJ024282–KJ024289 | KJ024344–KJ024351 |
|             | SCBC      | Beichuan, Sichuan, China | 3 | KJ024162–KJ024164 | KJ024226–KJ024228 | KJ024290–KJ024292 | KJ024352–KJ024354 |
|             | GSLH      | Lianhua Mountain, Gansu, China | 4 | KJ024165–KJ024168 | KJ024229–KJ024232 | KJ024293–KJ024296 | KJ024355–KJ024358 |
| T. c. pallidior | MALI | Manalind, India | 1 | KJ024169 | KJ024233 | KJ024297 | KJ024359 |
|             | PAKI      | Pakistan | 1 | KJ024170 | KJ024234 | KJ024298 | KJ024360 |

**Table 2.** Primer pairs used for the amplification and sequencing of *Tarsiger cyanurus*.

| Locus | Primer | Primer sequence (5’–3’) | References |
|-------|--------|-------------------------|------------|
| cytB  | L13653 | TAGGACTTCGCCCCTATC       | Li et al. (2006) |
|       | H14296 | TTGGTAGATCCTGTTCTGTG      | Li et al. (2006) |
|       | L14192 | CCTAGTGAAGACTAGTAGAGG      | Li et al. (2006) |
|       | H14853 | TTACCAAGCAAATGTCTTTATA     | Li et al. (2006) |
| ND2   | L5215  | TATCGGGCCATACCCCGAAAAAT   | Hackett (1996) |
|       | H5766  | CTCTATTTAAGCCTGTGAGG      | Johnson and Sorenson (1998) |
|       | L5758  | GGCTGAAATGGCTNAACTCARAC   | Johnson and Sorenson (1998) |
|       | H6313  | GGATGAGAAGGCTAGGATTKCG     | Johnson and Sorenson (1998) |
| GPBP1 | Forward | CTTTTGTGAGACGGAGATCG | Backström et al. (2006) |
|       | Reverse | ATCTCTTCTGTTGAGACGCC   | Backström et al. (2006) |
| NNT   | Forward | GCTGAAATGCAAATCTTTGCC | Backström et al. (2006) |
|       | Reverse | TCCACACAAACTGACCTTC | Backström et al. (2006) |
Genetic diversity

The number of segregating sites, haplotypes, and base substitutions of cytb, ND2 and their combined sequences (mitochondrial DNA, mtDNA) were estimated on T. c. cyanurus, T. c. rufilatus, and/or T. c. pallidior by DnaSP 5 (Librado and Rozas 2009) and MEGA 5 (Tamura et al. 2011). The estimation of standard genetic diversity indices, including the haplotype diversity (h) and nucleotide diversity (π) and the neutrality tests, is, that is, Tajima’s D and Fu’s Fs were carried out in DnaSP 5. We also calculated the number of polymorphic sites and the number of genotypes of the unphased sequences of Z-linked loci, GPBP1, NNT, and their combined sequences (zDNA) by MEGA 5.

Lineage divergence analysis

The TPM2uf+I+G and TPM3uf+I+G model for the mtDNA and zDNA were selected, respectively, by the Akaike information criterion in jModelTest 0.1.1 (Posada 2008). Using these models, phylogenetic reconstruction based on the mitochondrial and Z-linked (excluding the indels) data set was carried out using maximum-likelihood (ML) trees and Bayesian inference (BI) approaches. The ML trees were constructed with PhyML 3.0 (Guindon et al. 2010) using approximate likelihood-ratio test (aLRT) branch supports (Anisimova and Gascuel 2006) to estimate the reliability of each node. For BI analysis, two independent runs of Markov chain Monte Carlo (MCMC) were launched for 1.0 × 10^7 generations and sampled every 1000 generations by MrBayes 3.12 (Ronquist and Huelsenbeck 2003), and the rest parameters were kept as default settings, respectively. The stability of two runs and convergence of the MCMC were checked with Tracer 1.5 (Rambaut and Drummond 2007). The first 25% of samples were discarded as burnin, and the remaining saved samples were used to estimate posterior probabilities (PP) of each bipartition. These phylogenetic trees were viewed by FigTree V. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Further, we depicted the haplotype relationships among populations of the Bluetail using the median-joining (MJ) networks in Network 4.5.1.0 (http://www.fluxus-engineering.com/sharenet.htm).

The average P-distance within T. c. cyanurus, T. c. rufilatus, and/or T. c. pallidior and the net distance among/between them were also computed based on mitochondrial DNA with MEGA 5. Due to lack of fossil calibrations for passerine birds, the commonly assumed average mutation rate of cytb (1.035 × 10^{-8} substitutions/site/year, s/s/y) was applied (Weir and Schluter 2008). Its conservative confidence interval was also estimated by an extremely “slow” rate (0.6 × 10^{-8} s/s/y) and an extremely “fast” rate (1.505 × 10^{-8} s/s/y) (Weir and Schluter 2008). Divergent timings of T. c. cyanurus, T. c. rufilatus, and/or T. c. pallidior were estimated based on the net genetic distance of cytb between them.

Results

Genetic diversity and lineage diagnosability

A total of 62 cytb (1143 bp) and ND2 (1041 bp) sequences were obtained, and they generated 32 and 28 haplotypes, respectively. However, although cytb complete sequences were longer than ND2 complete sequences and also provided more haplotypes, ND2 yielded more variable sites (88) than that of cytb (76). With respect to T. c. rufilatus and/or T. c. pallidior, T. c. cyanurus has 85 diagnostic sites (fixed base sites, 33 in cytb and 52 in ND2) including five nonsynonymous sites (one in cytb and four in ND2). However, for T. c. rufilatus and T. c. pallidior, only five (including two nonsynonymous sites) and four diagnostic sites (including one nonsynonymous site) in ND2 sequences were detected, respectively. The number of haplotype, haplotype diversity (h), and nucleotide diversities (π) was higher in T. c. cyanurus than in T. c. rufilatus (Table 3). Furthermore, 62 combined sequences of two mitochondrial genes (cytb and ND2) including five nonsynonymous sites (one in cytb and four in ND2) were launched for 1.0 × 10^7 generations and sampled every 1000 generations by MrBayes 3.12 (Ronquist and Huelsenbeck 2003), and the rest parameters were kept as default settings, respectively. The stability of two runs and convergence of the MCMC were checked with Tracer 1.5 (Rambaut and Drummond 2007). The first 25% of samples were discarded as burnin, and the remaining saved samples were used to estimate posterior probabilities (PP) of each bipartition. These phylogenetic trees were viewed by FigTree V. 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Further, we depicted the haplotype relationships among populations of the Bluetail using the median-joining (MJ) networks in Network 4.5.1.0 (http://www.fluxus-engineering.com/sharenet.htm).

The average P-distance within T. c. cyanurus, T. c. rufilatus, and/or T. c. pallidior and the net distance among/between them were also computed based on mitochondrial DNA with MEGA 5. Due to lack of fossil calibrations for passerine birds, the commonly assumed average mutation rate of cytb (1.035 × 10^{-8} substitutions/site/year, s/s/y) was applied (Weir and Schluter 2008). Divergent timings of T. c. cyanurus, T. c. rufilatus, and/or T. c. pallidior were estimated based on the net genetic distance of cytb between them.

Significantly negative values of Tajima’s D and Fu’s Fs tests were attributed to population expansion rather than a deviation from neutrality by selection (Table 3). No associated statistical analysis was executed for T. c. pallidior due to a small sample size (N = 2).

Also, 60 GPBP1 (534/ 528 bp) and NNT (504 bp) sequences were obtained from 32 T. c. cyanurus and 28 T. c. rufilatus. Unlike the two mtDNA genes, the two zDNA genes showed more number of genotypes in T. c. rufilatus (27 for GPBP1 and 13 for NNT) than in T. c. cyanurus (14 for GPBP1 and 12 for NNT), as well as the number of polymorphic sites for GPBP1 did (33 in T. c. rufilatus and 11 in T. c. cyanurus) (Table 3). However, the number of polymorphic sites for NNT was larger in T. c. cyanurus (16) than in T. c. rufilatus (13) (Table 3). A total of 6 bp indels from three loci were found between T. c. cyanurus (534 bp) and T. c. rufilatus (528 bp) for GPBP1 in addition to two diagnostic base variation sites between them. Sixty concatenated Z-linked sequences without gaps (zDNA, 1032 bp) identified 56 haplotypes, 28 for each subspecies.
Lineage divergence and population structure analysis

Both ML and BP trees based on the mtDNA (2184 bp) revealed two major clades with strong statistical support: 
*T. c. cyanurus* clade (aLRT = 1.0, PP = 1.0) and 
*T. c. rufilatus* and 
*T. c. pallidior* clade (aLRT = 0.98, PP = 0.99) (Fig. 4, A mtDNA). Furthermore, the second clade also included a substantial subclade including haplotypes from 
*T. c. rufilatus* (aLRT = 1.0, PP = 0.63).

The phylogenetic analysis based on zDNA (1032 bp) executed with two outgroups indicated a weakly supported paraphyletic group of 
*T. c. cyanurus*. Therefore, an unrooted ingroup phylogenetic tree was provided (Fig. 4, B zDNA). As with the mtDNA phylogenetic tree, both ML and BI tree of the zDNA data set also suggested two reciprocal monophyletic groups corresponding to two subspecies with significant statistic supports (aLRT = 0.99, PP = 1.0). The MJ network showed the similar topology as the phylogenetic trees (Fig. 3). The haplotypes of the mtDNA from 
*T. c. pallidior* was distinguished from groups of haplotypes from 
*T. c. cyanurus* and 
*T. c. rufilatus* by 98 and 11 mutation steps, respectively (Fig. 3). Further, a few frequent haplotypes were shared by individuals from different breeding or nonbreeding regions in both 
*T. c. cyanurus* and 
*T. c. rufilatus*, probably suggesting that no prominent genetic subdivision within these two taxa.

### Table 3. Summary of nucleotide variation and demographic parameters.

| Subspecies     | Locus   | L   | n   | s   | N   | h   | π   | Tajima’s D | Fu’s Fs |
|----------------|---------|-----|-----|-----|-----|-----|-----|------------|---------|
|                | cytb    | 1143 | 32  | 21  | 18  | 0.925 | 0.003 | -1.242     | -9.722***|
| T. c. cyanurus | ND2     | 1041 | 32  | 15  | 15  | 0.917 | 0.002 | -1.171     | -8.021***|
|                | GPBP1   | 534  | 32  | 11  | 14  | NA   | NA   | NA         | NA      |
|                | NNT     | 504  | 32  | 16  | 12  | NA   | NA   | NA         | NA      |
| T. c. rufilatus| cytb    | 1143 | 28  | 15  | 13  | 0.783 | 0.001 | -2.104**   | -9.612***|
|                | ND2     | 1041 | 28  | 13  | 11  | 0.831 | 0.001 | -1.807*    | -6.047***|
|                | GPBP1   | 528  | 28  | 33  | 27  | NA   | NA   | NA         | NA      |
|                | NNT     | 504  | 28  | 13  | 13  | NA   | NA   | NA         | NA      |
| T. c. pallidior| cytb    | 1143 | 2   | 9   | 2   | 1.0  | 0.008 | NA         | NA      |
|                | ND2     | 1401 | 2   | 1   | 2   | 1.0  | 0.001 | NA         | NA      |

n, sample size; L, sequence length (bp); s, number of polymorphic sites; N, number of haplotypes for mitochondrial DNA or number of genotypes for Z-linked fragments; h, genetic diversity; π, nucleotide diversity; *P < 0.05; **P < 0.01; ***P < 0.001; NA: not available.
The average genetic distances for mtDNA within each clade were small: 0.003 for *T. c. cyanurus* clade and 0.002 for *T. c. rufilatus* and *T. c. pallidior* clade. However, the net genetic distance between the two clades was much more substantial: 0.039 for cyt b, 0.061 for ND2, and 0.049 for mtDNA. The estimated divergent time of two clades was about 1.88 (3.25–1.30) million years ago (Mya), approximately falling into the Pliocene–Pleistocene boundary (Päckert et al. 2009).

**Discussion**

Our study demonstrates the deep phylogeographic divergence between the two subspecies *cyanurus* and *rufilatus* of Red-tailed Bluetail based on both mitochondrial and Z-linked genetic markers. Such a divergence can be predicated to Pliocene–Pleistocene vicariance and indicates the both subspecies merit a full species each. It is noted that our samples of *cyanurus* and *rufilatus* does not well represent their breeding range with only a limited proportion of samples from breeding grounds in Russia and Gansu and the remaining from nonbreeding range in China. This is because of logistic difficulties to visit vast and remote Siberia. However, we assume this issue could not influence our estimation of the level and tempo of divergence between *cyanurus* and *rufilatus* as field identification between the two are very obvious. But the detailed demographics and spatial substructures with these two taxa require a comprehensive coverage of the range. Nevertheless, this first phylogeographic analysis has a few implications discussed below:

**Phylogeographic pattern of Tarsiger cyanurus complex**

The phylogeographic pattern of *T. cyanurus* complex is similar to some other passerine birds with Sino-Himalayan and North Palearctic split, such as *Phylloscopus fusca-tus* and *P. fuligiventer*, complex and *Certhia familiaris* superspecies (Martens et al. 2011), and *Bradypterus thoracicus* and *B. david* (Alström et al. 2008). This pattern has a large divergence between taxa on/around the Tibetan Plateau and the Northern Palearctic related taxa dating back to the Pliocene–Pleistocene boundary (Johansson et al. 2007; Päckert et al. 2009, 2012; Martens et al. 2011). During this period, major vegetation-type shifts occurred on the central Loess Plateau: from dry steppe first to a temperate more humid forest ecosystem and successively to dry grassland and desert steppe until 3.7 Mya (Wang et al. 2006; Päckert et al. 2012). As small passerine birds inhabiting in forests, the deep genetic divergence between *T. cyanurus* and *T. rufilatus* might have been shaped by geographical vicariance due to grassland and desert steppe on the central Loess Plateau following the uplift of the Tibetan Plateau during the Pliocene (Wang et al. 2006; Päckert et al. 2012; Gu et al. 2013). As a result of geographical isolation and possible adaptation to different biotic environments, the two taxa began to evolve separately and diverged in several morphological and life-history traits, such as plumage, vocalizations, and migration (del Hoyo et al. 2005; Rasmussen and Anderton 2005; Robson 2008). With the augment of these divergences, the two taxa were qualified as distinct evolutionary entities.

*Tarsiger cyanurus* winters in Southeast Asia several thousand kilometers from their breeding ranges, whereas *T. rufilatus* are mostly residents or seasonally altitudinal migrants from E Himalaya to southwest China (del Hoyo et al. 2005; Rasmussen and Anderton 2005; Robson 2008). The wintering grounds of two taxa are overlapped in a small extent in Yunnan and Sichuan province, China (Zhao 2001; Y. C. Wu and F. S. Zou, unpubl. data). The breeding ranges of the two taxa are regarded as completely separated (del Hoyo et al. 2005). However, a few males of *T. cyanurus* form were noticed in Huzhui Mountains near Xining, the provincial capital of Qinghai on July 2008 (Y. Liu, pers. obs.), where the northern limit of *T. rufilatus* also lies in southeast Qinghai and Gansu, China (Zhao 2001). This can indicate that *T. cyanurus* and *T. rufilatus* may be slightly sympatric in this region. If so, distinct song-types and male plumages can present strong behavioral pre-mating reproductive barriers under sexual selection (Coyne and Orr 2004; Price 2010).

**Taxonomic recommendations**

The present study provides the first molecular genetic analysis to delimit the taxonomic status of *T. cyanurus* complex. Our results suggest that three currently recognized *T. cyanurus* subspecies were identified to be at least two significant distinct lineages in case of mtDNA, *cyanurus*, and *rufilatus* (including *pallidior*). The two distinct genetic groups were reciprocal monophyly with substantial statistical support in the mtDNA and zDNA phylogenies. The *cyanurus* had a large number fixed base variation in mtDNA and zDNA data set with respect to *rufilatus*. Although no zDNA data of *pallidior* were available to allow genetic analysis, this may not alter the conclusions of the general pattern of major split between *cyanurus* and *rufilatus*. Further, *cyanurus* and *rufilatus* were separated from each other by large (98) mutation steps in mtDNA haplotype network. Under the phylogenetic species concept (Cracraft 1989), *cyanurus* and *rufilatus* (including *pallidior*) are reciprocal monophyly, and both have diagnosable criterion, which all show that both of them merit the level of separate species. Substantial
zDNA differentiation between the two lineages is sufficient to demonstrate isolation here. These genetic characteristics are also congruent with their widely separate breeding distribution and substantial difference in male plumage, vocalizations, and migration routes (Martens and Eck 1995; del Hoyo et al. 2005; Robson 2008). Under the general lineage concept of species (de Queiroz 2007), species are delimitated based on different criteria, including morphology, ecology, behaviors, and genetic characteristics. Traits such as plumage, allopatry, vocalizations, migration routes, and reciprocal monophyly could be grouped together to define the three taxa of the Red-flanked Bluetail complex. In conclusion, this study identifies genetic distinctness of cyanurus and rufilatus based on both mtDNA and nuclear DNA. Thus, this study confirms the pervious taxonomic treatment that T. cyanurus and T. rufilatus should be two full species (del Hoyo et al. 2005; Rasmussen and Anderton 2005; Robson 2008) under both the phylogenetic and general lineage concept of species, possibly as well as biological species concept (Mayr 1942).

While T. cyanurus is monotypic species, T. rufilatus is a probably polytypic species, which includes the subspecies rufilatus from E Himalayas to southwest China and pallidior exclusively in northwest Himalayas. The pallidior generally resembles rufilatus but upper part paler upper than the later taxa, sometimes treated as a subspecies (Rasmussen and Anderton 2005) or geographical form (Dickinson 2003; del Hoyo et al. 2005). The two haplotypes of pallidior were grouped into the rufilatus clade with high statistical supports in phylogenetic-tree-based analysis. Interestingly, we found a larger average genetic distance within pallidior (0.008) than that within cyanurus (0.003) and rufilatus (0.001), two also group into rufilatus with substantial support. Several passerine birds, that is, Certhia [familiaris] superspecies and Coal Tit Parus ater complex, are diversified with W-E Himalayan limits along this region (Martens et al. 2011). Despite the Himalayan range plays as a hotspot for bird diversification, most of these intraspecific variations along this mountain chain were immigration rather than in situ speciation suggested by several phylogeographic studies (Johansson et al. 2007; Päckert et al. 2009, 2012). Genetic differentiation among Himalayan populations of most species is relatively low and apparent lack of Pleistocene divergences (Johansson et al. 2007; Päckert et al. 2009, 2012). In line with this assumption, pallidior may represent westward colonization of rufilatus given its close affiliation to the latest taxa. However, whether pallidior deserves a species status and its evolutionary history needs further validation through a larger data set genotyped at nuclear loci.

In conclusion, our genetic analysis corroborates the previous split of the Red-flanked Bluetail Tarsiger cyanurus s.l. into T. cyanurus and T. rufilatus. The divergence of the two taxa likely started around 1.88 Mya, approximately corresponding to the Pliocene–Pleistocene epoch, and such a divergence was probably associated with geographical vicariance in the central Loess Plateau. This study further suggests another independent example of the phylogeographic pattern of North Palearctic and Sino-Himalayan split in Palearctic passerine birds in which congruent patterns of divergence emerged between morphological, acoustic, distributional, and genetic evidence.

Acknowledgments

We appreciate Yongmi Hong and Xincai Wang for collecting samples. We sincerely thank Prof. Trevor Price, Yanhua Qu, Jinping Chen, Chengmin Shi, Liang Leng, Junfeng Liu, De Ma, and Feng Dong who provided valuable comments on data analysis and early drafts of this manuscript. We are indebted to Prof. Trevor D. Price for providing samples of India and Pakistan, to Ornithology Department of Zoological Museum, Moscow State University for Russian samples. The research was supported by the National Natural Science Foundation of China-Guangdong Joint Fund (U0833005) to Fasheng Kou, the National Natural Science Foundation of China (31172067, 31201709 & 31200327) to Fasheng Kou, Yuchuan Wu and Qiang Zhang, Science and Technology Planning Project of Guangdong Province, China (2010B060200034) to Fasheng Kou, Natural Science Foundation of Guangdong Province, China (S201301012976) to Yuchuan Wu, “Hundred Talent Program (Bai Ren Ji Hua)” from Sun Yat-sen University, China to Yang Liu.

Conflict of Interest

None declared.

References

Alström, P., P. C. Rasmussen, U. Olsson, and P. Sundberg. 2008. Species delimitation based on multiple criteria: the Spotted Bush Warbler Bradypterus thoracicus complex (Aves: Megaluridae). Zool. J. Linn. Soc. 154: 291–307.
Andersson, L. 1990. The driving force: species concepts and ecology. Taxon 39:375–382.
Anisimova, M., and O. Gascuel. 2006. Approximate likelihood ratio test for branches: a fast, accurate, and powerful alternative. Syst. Biol. 55:539–552.
Backström, N., M. Brandström, L. Gustafsson, A. Qvarnström, H. Cheng, and H. Ellegren. 2006. Genetic mapping in a natural population of collared flycatchers (Ficedula albicollis): conserved synteny but gene order rearrangements on the avian Z chromosome. Genetics 174:377–386.
Cibois, A., E. Pasqueta, and T. S. Schulenberg. 1999. Molecular systematics of the Malagasy Babblers (Passeriformes: Timaliidae) and Warblers (Passeriformes: Sylviidae), based on Cytochrome b and 16S rRNA sequences. Mol. Phyl. Evol. 13:581–595.

Coyne, J. A., and H. A. Orr. 2004. Speciation. Pp. 545. Sinauer Associates, Sunderland, MA.

Cracraft, J. 1989. Speciation and its ontology: the empirical consequences of alternative species concepts for understanding patterns and processes of differentiation. Pp. 28–59 in D. Otte and J. A. Endler, eds. Speciation and its consequences. Sinauer Associates, Sunderland, MA.

Dickinson, E. C. 2003. The Howard and Moore Complete checklist of the birds of the world, 3rd edn. Pp. 677. Princeton Univ. Press, London.

Gu, L. Y., Y. Liu, P. J. Que, and Z. W. Zhang. 2013. Quaternary climate and environmental changes shape genetic differentiation of a Chinese endemic pheasant at the eastern edge of the Tibetan Plateau. Mol. Phyl. Evol. 67:129–139.

Guindon, S., J. F. Dufayard, V. Lefort, M. Anisimova, W. Hordijk, and O. Gascuel. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. Syst. Biol. 59:307–321.

Hackett, S. J. 1996. Molecular phylogenetics and biogeography of tanagers in the genus Ramphocelus (Aves). Mol. Phyl. Evol. 5:368–382.

del Hoyo, J., A. Elliott, and J. Sargatal, eds. 2005. Handbook of the birds of the world. Vol. 10. Cuckoo-shrikes to thrushes. Pp. 754. Lynx Edicions, Barcelona.

Johansson, U. S., P. Alström, U. Olsson, P. G. R. Ericson, P. Sundberg, and T. D. Price. 2007. Build-up of the Himalayan avifauna through immigration: a biogeographical analysis of the Phylloscopus and Seicercus warblers. Evolution 61:324–333.

Johnson, K. P., and M. D. Sorenson. 1998. Comparing molecular evolution in two mitochondrial protein coding genes (cytochrome b and ND2) in the dabbling ducks (Tribe: Anatini). Mol. Phyl. Evol. 10:82–94.

Li, S. H., J. W. Li, L. X. Han, C. T. Yao, H. T. Shi, F. M. Lei, et al. 2006. Species delimitation in the Hwamei Garrulax canorus. Ibis 148:698–706. 

Librado, P., and J. Rozas. 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25:1451–1452.

Liu, Y., I. Keller, and G. Heckel. 2012. Breeding site fidelity and winter admixture in a long-distance migrant, the tufted duck (Aythya fuligula). Heredity 109:108–116.

Luo, X., Y. H. Qu, L. X. Han, S. H. Li, and F. M. Lei. 2009. A phylogenetic analysis of laughing thrushes (Timaliidae: Garrulax) and allies based on mitochondrial and nuclear DNA sequences. Zool. Scr. 33:9–22.

Martens, J., and S. Eck. 1995. Towards an ornithology of the Himalayas: systematics, Ecology and Vocalizations of Nepal Birds. Bonner Zool. Monogr. 38:1–445.

Martens, J., D. T. Tietze, and M. Päckert. 2011. Phylogeny, biodiversity, and species limits of passerine bird in the Sino-Himalayan region- a critical review. Ornithol. Monogr. 70:64–94.

Mayr, E. 1942. Systematics and the origin of species. Columbia Univ. Press, New York.

Päckert, M., J. Martens, Y. H. Sun, and D. T. Tietze. 2009. Phylogeography and the evolutionary time-scale of passerine radiations in the Sino-Himalaya. Pp. 71–80 in M. Hartmann, J. Weipert, eds. Biodiversity and natural heritage of the Himalaya, Vol. III. Verein der Freunde und Förderer des Naturkundemuseums Erfurt, Erfurt.

Päckert, M., J. Martens, Y. H. Sun, L. L. Severinghaus, A. A. Nazarenko, J. Ting, et al. 2012. Horizontal and elevational phylogeographic patterns of Himalayan and Southeast Asian forest passerines (Aves: Passeriformes). J. Biogeogr. 39: 556–573.

Posada, D. 2008. jModelTest: phylogenetic model averaging. Mol. Biol. Evol. 25:1253–1256.

Price, T. D. 2010. The roles of time and ecology in the continental radiation of the Old World leaf warblers (Phylloscopus and Seicercus). Philos. Trans. R. Soc. Lond. B Biol. Sci. 365:1749–1762.

de Queiroz, K. 2007. Species concepts and species delimitation. Syst. Biol. 56:879–886.

Rambaut, A., and A. J. Drummond. 2007. Tracer v1.5. Available from http://beast.bio.ed.ac.uk/Tracer (accessed 25 June 2011).

Rasmussen, P. C., and J. C. Anderton. 2005. Birds of South Asia: the Ripley Guide. Lynx Edicions, Barcelona.

Robson, C. 2008. A field guide to the birds of South-east Asia. Pp. 218–461. 2nd edn. New Holland, London.

Rolshausen, G., G. Segelbacher, A. K. Hobson, and M. Schaefer. 2009. Contemporary evolution of reproductive isolation and phenotypic divergence in sympathy along a migratory divide. Curr. Biol. 19:2097–2101.

Ronquist, F., and J. P. Huelsenbeck. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19:1572–1574.

Roselaar, C. S., R. Sluys, M. Aliabadian, and P. G. M. Mekenkamp. 2007. Geographic patterns in the distribution of Palaearctic songbirds. J. Ornithol. 148:271–280.

Sæther, S. A., G. P. Sætre, T. Borge, C. Wiley, N. Svedin, G. Sæther, and T. D. Price. 2007. Build-up of the Himalayan east edge of the Tibetan Plateau. Mol. Phyl. Evol. 45:1452.

Sangster, G., P. Alström, E. Forsmark, and U. Olsson. 2010. Multi-locus phylogenetic analysis of Old World chats and flycatchers reveals extensive paraphyly at family, subfamily and genus level (Aves: Muscicapidae). Mol. Phyl. Evol. 57:380–392.
Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28:2731–2739.

Wang, L., H. Y. Lv, N. Q. Wu, J. Li, Y. P. Pei, G. B. Tong, et al. 2006. Palynological evidence for late Miocene-Pliocene vegetation evolution recorded in the red clay sequence of the central Chinese Loess Plateau and implication for palaeoenvironmental change. Palaeogeogr. Palaeoclimatol. Palaeoecol. 241:118–128.

Weir, J. T., and D. Schluter. 2008. Calibrating the avian molecular clock. Mol. Ecol. 17:2321–2328.

Wu, H. C., R. C. Lin, H. Y. Hung, C. F. Yeh, J. H. Chu, X. J. Yang, et al. 2011. Molecular and morphological evidences reveal a cryptic species in the Vinaceous Rosefinch Carpodacus vinaceus (Fringillidae; Aves). Zool. Scr. 40:468–478.

Yang, S. J., H. L. Dong, and F. M. Lei. 2009. Phylogeography of regional fauna on the Tibetan Plateau: a review. Prog. Nat. Sci. 19:789–799.

Zhang, D. X., and G. M. Hewitt. 1998. Isolation of animal cellular total DNA. Pp. 5–9 in A. Karp, P. G. Isaac and D. S. Ingram, eds. Molecular tools for screening biodiversity: plants and animals. Chapman and Hall, London.

Zhao, Z. J. 2001. A handbook of the birds of China. Passeriformes Vol. II. Pp. 256–258. Jilin Scientific and Technology Press, Changchun.

Zink, R. M., and G. F. Barrowclough. 2008. Mitochondrial DNA under siege in avian phylogeography. Mol. Ecol. 17:2107–2121.

Zuccon, D., and P. G. P. Ericson. 2010. A multi-gene phylogeny disentangles the chat-flycatcher complex (Aves: Muscicapidae). Zool. Scr. 39:213–224.