

Molecular phylogenetic analysis among species of Paridae, Remizidae and *Aegithalos* based on mtDNA sequences of *COI* and *cyt b*

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Abstract The phylogeny of Paridae and allies has been studied intensively during past decades. However, the phylogenetic relationship among species tends to become increasingly controversial as different genetic markers emerge. In our study, the partial mitochondrial genes cytochrome *b* (*cyt b*) and cytochrome *c* oxidase subunit I (*COI*) were obtained from 15 species that included 10 tits, 4 long-tailed tits and a Chinese penduline tit. Analyses were conducted on the combined *cyt b* and *COI* sequences with maximum likelihood and Bayesian algorithms. Based on strong, congruent support among the different temporal partitions and models of sequence evolution, a highly resolved consensus of the relationships among Parids and their allies has been formed. The monophyly of Paridae and Remizidae is strongly supported. However, the monophyly of Paridae and *Aegithalos* is rejected. This agrees with previous studies using other molecular markers. Our results suggest the promotion of the subgenus *Machlolophus* from genus *Parus* to a separate genus. The phylogeny of *Aegithalos* is robust in the current study. However, by considering differences of both morphological and molecular characters within species, we conclude that more data are needed to define their phylogeny. Based on the patterns of taxonomic diversity and endemism, we suggest the southwestern mountain ranges of China might be the center of origin of the *Aegithalos* species. Divergence time estimates for the long-tailed tits range from the late Miocene to the Pleistocene (from 5.5 to 0.1 Mya) using a calibration of 2% divergence per million years. In a comparative sense, we found a congruent genetic differentiation among sympatric distribution taxa.

Keywords phylogeny, tit, monophyly, center of origin, separation, genetic differentiation

Introduction

Tits and titmice are small, familiar cavity-nesting songbirds forming the family Paridae which contains

4 genera, 55 to 65 species, depending on different classifications (Harrap and Quinn, 1996; Salzburger et al., 2002; Dickinson, 2003). They are distributed in the entire Holarctic, Oriental and Afrotropical regions with a hot spot of diversity in the Himalayan and Chinese mountain ranges (Päckert et al., 2007). Because of their overall morphological similarity, all but three species have been classified into the genus *Parus* and the genus is divided into 10 subgenera

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(Harrap and Quinn, 1996) or 12 species groups (Eck, 1988). The three other genera, *Melanochlora*, *Sylviparus* and *Pseudopodoces* are monotypic. A well-supported, corroborated and presumably accurate phylogeny is essential for an understanding of ecological and behavioral variation within the group. Therefore, in recent years, a plethora of studies using molecular markers, such as allozyme comparisons (Gill et al., 1989), mitochondrial restriction fragment-length polymorphism (RFLP) data (Gill and Slikas, 1992; Gill et al., 1993), DNA-DNA hybridization distances (Sheldon et al., 1992; Slikas et al., 1996) and cytochrome-*b* sequences (Kvist et al., 1996; Gill et al., 2005; Martens et al., 2006) have been applied to the phylogeny of Paridae. However, the phylogenetic relationship among species tends to become increasingly controversial as different genetic markers emerge. For instance, based on nuclear DNA-DNA hybridization, *Parus caeruleus* and *P. major* formed a clade which is the sister group of all other Parids (Sheldon et al., 1992; Slikas et al., 1996), but the phylogeny inferred from *cyt b* sequences did not support that result (Gill et al., 2005).

Aegithalos, at one time, was classified as a genus in the family Paridae (Gadow, 1883; Hellmayr, 1903; Mayr and Amandon, 1951). However, Snow (1967) considered *Aegithalos* and a few related genera as a separate family, the Aegithalidae. Phylogenetic analyses based on DNA-DNA hybridization (Sibley and Ahlquist, 1990; Sheldon and Gill, 1996), protein allozyme comparisons (Ohta et al., 2000) and nuclear gene sequences (Barker et al., 2002; Spicer and Dunipace, 2004) suggested that *Aegithalos* was indeed not Parids and the closest sister group to Parids was the penduline tits, species of Remizidae. However, genera closely related to *Aegithalos* are still under dispute, as well as the phylogenetic relationships within *Aegithalos* (Päckert et al., 2010).

In this study, we sampled part of representative taxa of tits, long-tailed tits and penduline tits in an attempt to resolve the relationships among these three groups and also to infer the phylogenetic relationships within tits and long-tailed tits based on two mitochondrial gene sequences, *COI* and *cyt b*.

Materials and methods

Taxon selection and choice of outgroups

Altogether, twenty-nine individuals of ten species from Paridae, nine individuals of four species from

Aegithalos and two Chinese penduline tits were included in this study. Six species had more than one subspecies included in the analysis (Table 1). According to Sibley and Monroe (1990), Sheldon and Gill (1996), Barker et al. (2002), Spicer and Dunipace (2004), an individual of *Pica pica*, *Uragus sibiricus*, *Garrulax lunulatus* and *G. ocellatus* were selected as outgroups.

Extraction, amplification and sequencing

Genomic DNA was extracted from blood, feathers or tissue specimens using the QIAamp™ DNA Mini Kit as per manufacturer's instructions. Nucleotide sequence data were obtained from the two mitochondrial, cytochrome *c* oxidase I (*COI*) and cytochrome *b* (*cyt b*) genes. PCR amplification and sequencing of cytochrome *c* oxidase I followed the method suggested by Sorenson et al. (1999), while Gill et al. (2005) described protocols for cytochrome *b*.

For the sequencing reactions, the same primers were used. Both strands of each PCR product were sequenced. For each gene and sample, multiple sequence fragments were obtained by sequencing with different primers. Complete sequences were assembled using Seqman II (DNASTAR®). Sequences were compared visually to the original chromatograms to avoid reading errors. Assembled sequences were aligned by eye. All sequences were deposited in GenBank.

Phylogenetic analysis

Based on *a priori* assumption and partition homogeneity test ($p = 0.44$), the two mitochondrial genes were analyzed as one data set with a total nucleotide length of 2149 base pairs (bp). The data were analyzed using maximum likelihood (ML, Felsenstein, 1981) and Bayesian inference methods (BI, Rannala and Yang, 1996; Yang and Rannala, 1997; Larget and Simon, 1999). Statistics for nucleotide variation and genetic distance were computed with MEGA 4 (Tamura et al., 2007). A Jukes-Cantor estimate of the number of nucleotide substitutions per site was computed for the *cyt b* gene (Jukes and Cantor, 1969). Following the suggestion of Nei and Kumar (2000), if the Jukes-Cantor distance is less than 0.05, the *p*-distance would be used. Otherwise, a more complicated distance model would be employed.

Following alignment, we partitioned the data by genes in order to allow different rates for the various

Table 1 List of taxonomic samples and sequences used in the study

Family	Genus	Species and subspecies	Museum No.	Collection locality	GenBank Accession Nos.		
					<i>cyt b</i>	<i>COI</i>	
Paridae	<i>Parus</i>	<i>Parus major commixtus</i>	IOZ1254	Guangxi	HM185345	HM185334	
		<i>Parus major subtibetanus</i>	IOZ5236	Yunnan	HM185346	HM185333	
		<i>Parus major subtibetanus</i>	IOZ3918	Sichuan	HM185347	HM185332	
		<i>Parus major artatus</i>	IOZ5661	Jilin	HM185348	HM185331	
		<i>Parus monticolus yunnanensis</i>	IOZ3633	Sichuan	HM185349	HM185330	
		<i>Parus monticolus yunnanensis</i>	IOZ2465	Shaanxi	HM185350	HM185329	
		<i>Parus spilonotus rex</i>	IOZ3026	Fujian	HM185351	HM185328	
		<i>Parus cyanus</i>	IOZ 781	Xinjiang	HM185352	HM185327	
		<i>Parus venustulus</i>	IOZ2806	Hubei	HM185353	HM185326	
		<i>Parus venustulus</i>	IOZ1945	Shaanxi	HM185354	HM185325	
		<i>Parus ater aemodius</i>	IOZ1094	Gansu	HM185355	HM185324	
		<i>Parus ater</i>	IOZ2331	Zvolen	HM185356	HM185323	
		<i>Parus ater ater</i>	IOZ9029	Heilongjiang	HM185357	HM185322	
		<i>Parus palustris hypermelas</i>	IOZ1239	Gansu	HM185358	HM185321	
		<i>Parus palustris hypermelas</i>	IOZ7991	Hubei	HM185359	HM185320	
		<i>Parus palustris brevirostris</i>	IOZ5675	Jilin	HM185360	HM185319	
		<i>Parus palustris hellmayri</i>	IOZ8680	Shanxi	HM185361	HM185318	
		<i>Parus palustris hellmayri</i>	IOZ2458	Shaanxi	HM185362	HM185343	
		<i>Parus montanus baicalensis</i>	IOZ5944	Heilongjiang	HM185363	HM185317	
		<i>Parus montanus baicalensis</i>	IOZ5945	Heilongjiang	HM185364	HM185316	
		<i>Parus montanus</i>	IOZ2237	Zvolen	HM185365	HM185315	
		<i>Parus montanus affinis</i>	IOZ2178	Shaanxi	HM185366	HM185342	
		<i>Parus dichrous dichrous</i>	IOZ2489	Shaanxi	HM185367	HM185314	
	<i>Parus dichrous dichrous</i>	IOZ6117	Shaanxi	HM185368	HM185313		
	<i>Parus cristatus</i>	IOZ2333	Zvolen	HM185369	HM185312		
	<i>Sylviparus</i>	<i>Sylviparus modestus modestus</i>	KIZgls1240	Yunnan	HM185370	HM185311	
		<i>Sylviparus modestus modestus</i>	KIZgls1241	Yunnan	HM185371	HM185310	
	<i>Pseudopodoces</i>	<i>Pseudopodoces humilis</i>	IOZ4783	Qinghai	HM185372	HM185309	
		<i>Pseudopodoces humilis</i>	IOZ4785	Qinghai	HM185373	HM185308	
	Remizidae	<i>Remiz</i>	<i>Remiz consobrinus</i>	IOZ10640	Liaoning	HM185374	HM185298
			<i>Remiz consobrinus</i>	IOZ10652	Liaoning	HM185375	HM185297
	Aegithalidae	<i>Aegithalos</i>	<i>Aegithalos caudatus caudatus</i>	IOZ5677	Jilin	HM185376	HM185307
<i>Aegithalos caudatus glaucogularis</i>			IOZ2769	Hubei	HM185377	HM185306	
<i>Aegithalos caudatus glaucogularis</i>			IOZ3322	Shaanxi	HM185378	HM185305	
<i>Aegithalos concinnus concinnus</i>			IOZ1152	Gansu	HM185379	HM185304	
<i>Aegithalos concinnus concinnus</i>			IOZ1499	Hunan	HM185380	HM185303	
<i>Aegithalos concinnus concinnus</i>			IOZ3661	Sichuan	HM185381	HM185302	
<i>Aegithalos concinnus talifuensis</i>			IOZ5344	Yunnan	HM185382	HM185301	
<i>Aegithalos bonvaloti</i>			IOZ3766	Sichuan	HM185383	HM185300	
<i>Aegithalos fuliginosus</i>			IOZ2476	Shaanxi	HM185384	HM185337	
Timaliidae	<i>Garrulax</i>	<i>Garrulax lunulatus</i>	IOZ2594	Shaanxi	HM185385	HM185341	
		<i>Garrulax ocellatus</i>	IOZ5083	Yunnan	HM185386	HM185340	
Fringillidae	<i>Uragus</i>	<i>Uragus sibiricus</i>	IOZ5870	Heilongjiang	HM185387	HM185344	
Corvidae	<i>Pica</i>	<i>Pica pica</i>	IOZ3931	Xinjiang	HM185388	HM185339	

Note: IOZ, Institute of Zoology, Chinese Academy of Sciences; KIZ, Kunming Institute of Zoology, Chinese Academy of Sciences.

partitions for ML and BI analyses. Nucleotide substitution models were selected separately by genes and then used for different data partitions in reconstruction. However, the TVM + I + G model ($-\ln L = 15397.6758$, $K = 9$, $AIC = 30813.3156$) was identified as the best fit for these two genes, using both the likelihood-ratio test (LRT) and Akaike Information Criterion (AIC) implemented in MODELTEST 3.7 (Posada and Crandall, 1998). The parameter values for the model include a symmetric rate matrix specifying relative probabilities for all possible nucleotide changes (Rmatrix = 0.6396 [A-C], 8.0280 [A-G], 1.1611 [A-T], 0.0498 [C-G], 8.0280 [C-T], 1.0000 [G-T]), the proportion of invariant sites (pinvar = 0.6183) and the shape parameter for the gamma distribution of rate variation (shape = 1.2128). The base frequencies were set as follows: A = 0.3263, C = 0.4100, G = 0.1009, T = 0.1628.

ML reconstruction (1000 replicates) was performed in TREEFINDER (Jobb, 2007) and BI performed in MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003). In the BI analysis, we ran two analyses of two million generations and trees sampled every 100 generations. One cold and three heated Markov chains were used in our analysis. The trees saved during the “burn-in” phase (the first 200000 generations in our analysis) were discarded. The remaining trees from two runs were used to create a 50% majority rule consensus tree.

Results

Sequence characteristics

We obtained a total of 88 sequences, with their GenBank accession numbers listed in Table 1. No stop codons were identified in a contiguous 1201 bp stretch of the *COI* gene and 948 bp of *cyt b*. The overlapping sequences from different PCR products and a single peak in the electropherograms suggest that these sequences do not come from “numts” (nuclear sequences of mitochondrial origin). The average base composition of sequence was skewed,

which is similar to that found in previous avian studies (Barhoum and Burns, 2002; Webb and Moore, 2005). The characteristics of these sequences are summarized in Table 2.

Pairwise genetic distances

Because the mean Jukes-Cantor distance was 0.112 ± 0.006 SE (> 0.05) for the *cyt b* sequence data set, the Tamura-Nei model was chosen to compute the genetic distance (Tamura and Nei, 1993). Sequence divergences within species range from 0% (*Parus dichrous*) to 2.8% (*Aegithalos concinnus*), with an average divergence of 1.15%. The mean *cyt b* sequence distance of *Aegithalos* was 0.081 ± 0.007 SE, with the lowest 0.002 between *A. bonvaloti* and *A. fuliginosus* and the highest 0.118 between *A. concinnus* and *A. caudatus*. The mean distance of genus *Parus* was 0.077 ± 0.005 SE, with the lowest 0.053 between *P. major* and *P. monticolus*. Between taxa in *Parus* and the other two species in the Paridae, average pairwise divergences are as follows: 0.115 ± 0.009 SE to *Sylviparus modestus* and 0.097 ± 0.007 SE to *Pseudopodoces humilis*. The distances were 0.16 ± 0.01 SE between *Aegithalos* and Paridae, 0.18 ± 0.013 SE between *Aegithalos* and Remizidae, and 0.126 ± 0.01 SE between Paridae and Remizidae.

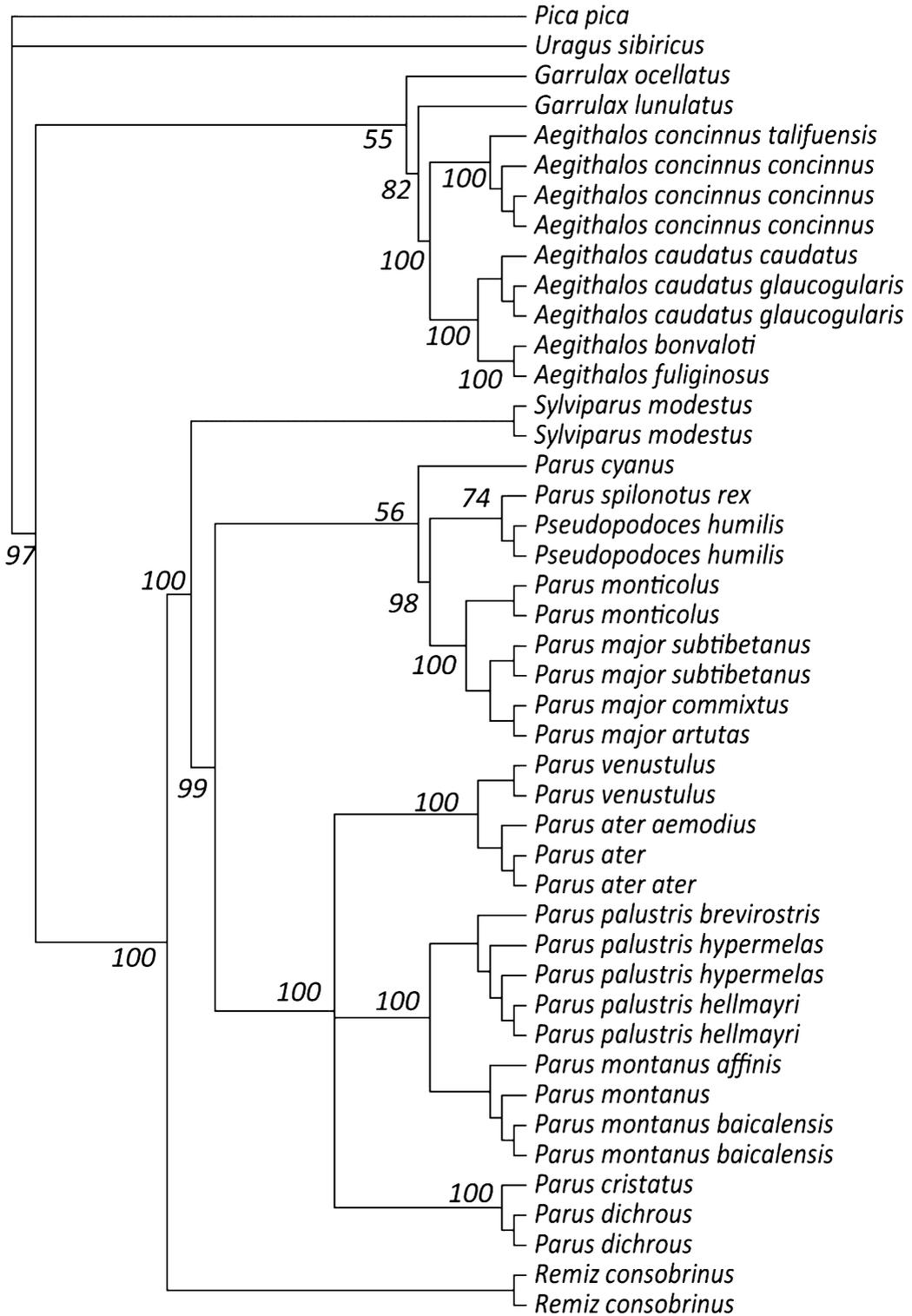
Phylogenetic relationships

The consensus tree from the Bayesian analysis is identical to the ML tree, except for a few weakly supported nodes. A few nodes that are resolved in the ML tree are polytomies in the Bayesian consensus tree (Fig. 1). In all optimal trees, no species which includes several subspecies were found to be paraphyletic in our study. Paridae, Remizidae, *Aegithalos* and *Garrulax* species formed a monophyletic group with high support (bootstrap support = 70%, Bayesian posterior probability = 97%) and branched off to two monophyletic clades. The clade, representing individuals from species of Paridae and Remidae, was highly supported (bootstrap = 98%, BPP = 100%).

Table 2 Molecular characterization of the sequences

Genes	No. of sequences	Total sites	Variable sites (%)	Avg R (si/sv)	Nucleotide frequencies			
					A (%)	T (%)	G (%)	C (%)
<i>COI</i>	40	1201	370	1.7	25.8	23.8	17.3	33.1
<i>cyt b</i>	40	948	326	1.5	26.6	23.8	13.2	36.3

a



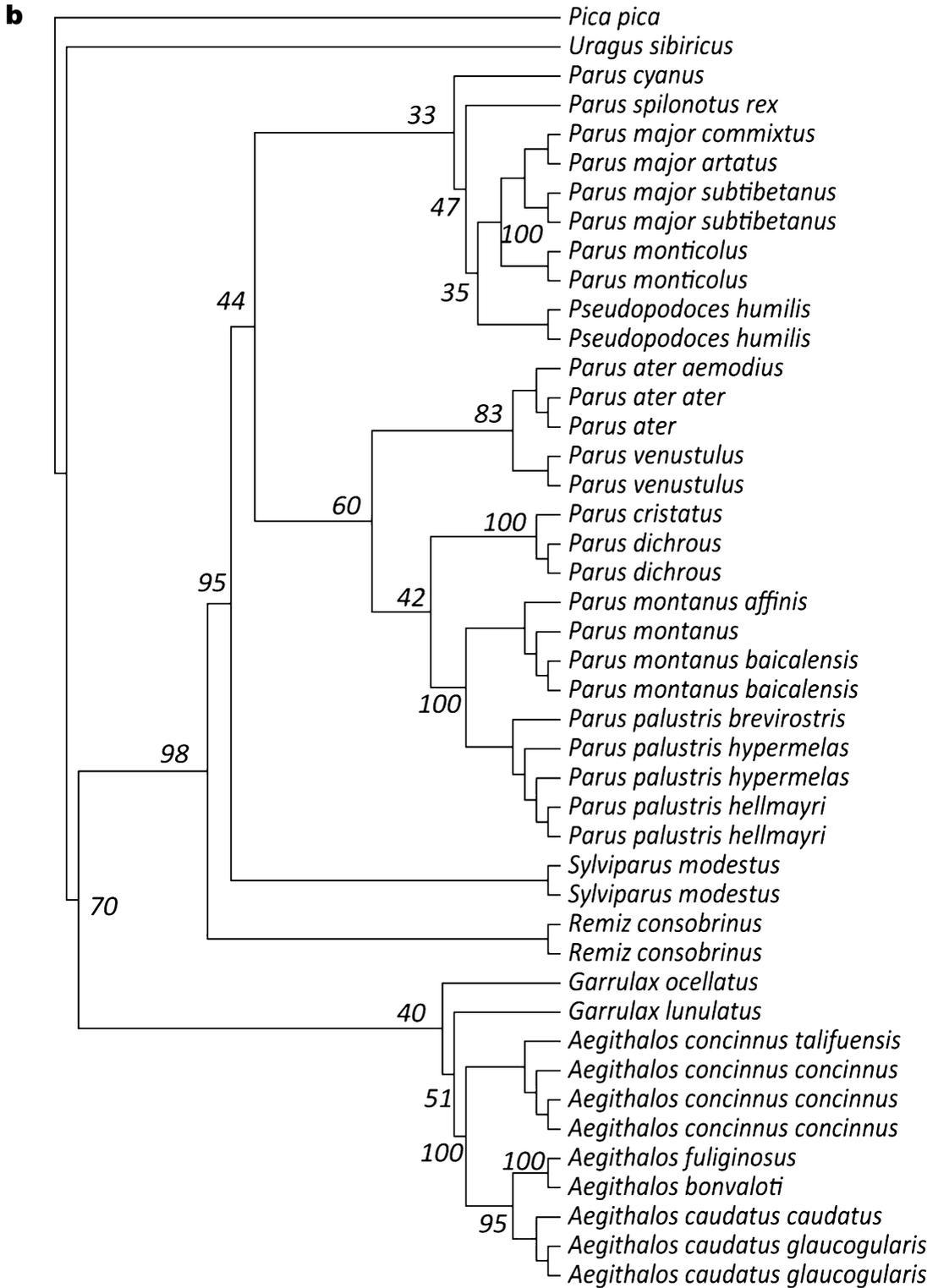


Fig. 1 Trees obtained from the analysis of the combined *COI* and *cyt b* data: (a) Bayesian and (b) maximum likelihood. The optimal model as estimated by Modeltest following the AIC and LRT was estimated to be TVM + I + G. Support values are indicated to the left of the nodes.

Remiz consobrinus positions as a sister species to the Paridae, including *Sylviparus*, *Pseudopodoces* and the species in *Parus*. *Aegithalos* is monophyletic, grouped with two *Garrulax* species and, with weak support, formed the other clade.

Within *Aegithalos*, four species are monophyletic and grouped in all optimal trees (Bayesian posterior probability = 100%, bootstrap = 100%). Within the clade, *Aegithalos bonvaloti* is sister to *A. fuliginosus* and that pair is sister to *A. caudatus*. *Aegithalos concinnus* is sister to these three species. Phylogenetic relationships have strong support (Bayesian posterior probability = 100%, bootstrap > 95%) and are identical in the two phylogenetic trees.

Within the family Paridae, *Sylviparus modestus* positions as a sister species to the remaining species of Paridae from the two trees. These remaining species form two high-support monophyletic clades in the BI tree (BPP = 99%), but the bootstrap value is rather low (bootstrap = 44%) in the ML tree. One clade includes *Parus cyanus*, *P. monticolus*, *P. major*, *P. sibilotus* and *Ps. humilis*. The relationships among these five species are congruent in the two trees except for the position of *Ps. humilis*. In the two trees, *Parus cyanus* is a sister species to the remaining species, although the nodal support value is low. *Parus major* and *P. monticolus* pair as sister taxa with high support. In the BI tree, *P. sibilotus* and *Ps. humilis* pair as sister taxa but in the ML tree, *Pseudopodoces humilis* is a sister species to *P. sibilotus*, *P. major* and *P. monticolus*. However, the nodal supports are low. Another clade includes *P. ater*, *P. venustus*, *P. palustris*, *P. montanus* and two Eurasian crested tits, *P. dichous* and *P. cristatus*. *Parus palustris* and *P. montanus*, *P. ater* and *P. venustus*, *P. dichous* and *P. cristatus* pair as sister taxa with high support. Relationships among these three pairs are unclear because of polytomies in the BI tree and low nodal support value in the ML tree.

Discussion

Phylogenetic relationships among Paridae, Remizidae and *Aegithalos*

Our data support the classification for the family Paridae proposed by Sibley and Ahlquist (1990). Based on DNA-DNA hybridization, they combined family Remizidae with Paridae, hence the family Paridae includes two subfamilies, Remizinae and Parinae. Sheldon and Gill (1996) studied the phy-

logeny of song birds using DNA-DNA hybridization and concluded that the sister group of Paridae is the Remizidae. Based on combined myoglobin intron II and cytochrome *b* sequences, Alström et al. (2006) revealed the close relationships between *P. major* and *R. pendulinus*. In our study, the Chinese penduline tit (Remizidae) positioned as sister group to the Parids (Paridae) with high support. This may indicate very close relationships between Paridae and Remizidae. Although, in the present study, only one species of Remizidae was tested, the high nodal support (> 95%) signifies that the designated branch is most possibly unaffected by sampling among variation existing in the data (Slikas et al., 1996).

Long-tailed tits, species of *Aegithalos*, because of their morphological similarity with tits, were earlier believed to be Parids by several authors (Gadow, 1883; Hellmayr, 1903; Mayr and Amandon, 1951). However, Stresemann (1923) described several characteristics of *Aegithalos* that differ from those of the Parids, such as the presence of a complete juvenile-molt, nest structure and naked hatching. Since then, *Aegithalos* and two other monotypic or small genera are usually placed in the family Aegithalidae (e.g., Paynter, 1967; Morony et al., 1975; Sibley and Ahlquist, 1990; Sibley and Monroe, 1990). Sturmbauer et al. (1998) suggested a close relationship between *Leptopoeile* and *Aegithalos* based on the mitochondrial 16S sequence. Alström et al. (2006) confirmed the close relationship between *Aegithalos* and *Leptopoeile* based on the combined myoglobin intron II and cytochrome *b* sequences. Using protein allozyme comparison, Ohta et al. (2000) found the genetic distance between *Aegithalos* and *Parus* appears to be at a familial level. In our study, two distinct clades and *Aegithalos* grouped with two out-group *Garrulax* species, in spite of weak nodal support, show distant relationships between *Aegithalos* and Paridae. In addition, the genetic divergence of *cyt b* between *Aegithalos* and Paridae is larger than that between Remizidae and Paridae. Hence, the morphological similarity between tits and long-tailed tits may have resulted from evolutionary convergence.

Phylogenetic relationships within Paridae

Based on plumage pattern, Hellmayr (1903) had recognized eleven subgenera for the genus *Parus*. However, the delimitation of these subgenera is in dispute. For instance, Wolters (1982) classified the

species of *Pardaliparus* into *Pariparus* and this classification was supported by molecular analyses (Slikas et al., 1996; Gill et al., 2005). In our study, *Parus ater* and *P. venustulus* also formed a closely related group, but the divergence of *cyt b* is ~7% (uncorrected *p*-distance). According to Hellmayr's classification, our samples belong to seven subgenera: *Cyanistes* (*P. cyanus*), *Baeolophus* (*P. dichous*, *P. cristatus*), *Poecile* (*P. montanus*, *P. palustris*), *Periparus* (*P. ater*), *Pardaliparus* (*P. venustulus*), *Machlolophus* (*P. spilonotus*) and *Parus* (*P. major*, *P. monticolus*). Similar to previous studies, our data did not reveal phylogenetic relationships among these subgenera. Therefore, the *Parus* phylogeny needs to be studied further. We intend to add more species and subspecies and new markers, including nuclear loci to classify this challenge in the future.

Although our results came to quite similar conclusions as previous studies, different phylogenetic relationships among species were detected as well. The question of greatest interest is: what is the phylogenetically most closely related species to *Ps. humilis* in Parids? This aberrant and enigmatic Tibetan species, earlier thought to be a corvid, turned out to be a Parid (James et al., 2003). Past phylogenetic analyses based on *cyt b* gene sequences positioned *Ps. humilis* as sister species to *P. major* (James et al., 2003; Gill et al., 2005). However, in optimal trees based on the *cyt b* and *COI* gene sequences in our study, *Ps. humilis* is sister to *P. spilonotus*, although the nodal support is weak. This result may indicate that more data of the species and different markers are needed to determine the phylogenetic status of Parids.

Gill et al. (2005) recommended upgrading six subgenera of *Parus* into genera for facilitating future evolutionary analyses among Parids. The retained genus *Parus* only included *P. major*, *P. monticolus*, *P. xanthogenys*, *P. spilonotus*, *P. holsti* and the African tits (*P. leucomelas*, *P. niger*, *P. carpi*, *P. albiventris*, *P. leuconotus*, *P. rufiventris*, *P. funereus*, *P. fringillinus*, *P. fasciiventer*, *P. thruppi*, *P. griseiventris*, *P. cinerascens* and *P. afer*). However, in our present study, we also believe that the genus *Machlolophus* should be recognized in consideration of the phylogenetic relationships between *P. spilonotus* and *Ps. humilis* and distinct plumage pattern between *P. major* and *P. spilonotus* (Ohta et al., 2000). Furthermore, in our data, the divergence of the *cyt b* gene between *P. major*–*P. monticolus* group and *P. spilonotus* (8.4%, uncorrected *p*-distance) is no less than the divergence among recommended genera (ranging from 7.3% between

Pariparus and *Baeolophus* to 9.7% between *Poecile* and *Machlolophus*, uncorrected *p*-distance).

Intra- and interspecies relationships and history of *Aegithalos* taxa

On the one hand, intraspecific divergence is significant. The Black-throated Tit (*Aegithalos concinnus*) includes six highly distinct subspecies. These subspecies are remarkably different in their plumage pattern. Divergence differences of the *cyt b* gene among these subspecies are also significant. In our data, the pairwise *p*-distance between specimens from Bengal (India, ssp. *iredalei*, sequence obtained from GenBank) and Gaoligong (Yunnan, ssp. *talifuensis*) was 4.5%. *p*-distance between specimens from Gaoligong and Pingjiang (Hunan, ssp. *concinus*) was 6.1%. *p*-distance between specimens from Bengal and Pingjiang was 5.1%. Eck and Martens (2006) sequenced three specimens, including subspecies ssp. *iredalei*, ssp. *talifuensis* and ssp. *manipurensis*, and they found the pairwise *p*-distance was 5.1% between ssp. *iredalei* and ssp. *talifuensis*, 5.3% between ssp. *talifuensis* and ssp. *manipurensis* and 6% between ssp. *iredalei* and ssp. *manipurensis*. These four subspecies are reddish-crowned. The other two subspecies *A. c. pulchellus* and *A. c. annamensis* are grey-headed. They are obviously different in presence of reddish breast band, the latter of which is absent. Hence, it seems possible that *A. concinnus* represents an unresolved species swarm (Eck and Martens, 2006).

As for the Long-tailed Tit (*Aegithalos caudatus*), based on comparative morphology, up to 19 subspecies are currently recognized, divided by Harrap and Quinn (1996) into four groups. Zink et al. (2005) studied the mitochondrial phylogeography, including five subspecies or representatives of *A. caudatus* group and *A. alpinus* group of the long-tailed tit. Within their samples, no correspondence between five subspecies and mitochondrial subdivision has been discovered. However, two geographically unsorted lineages were displayed and differentiation seems to exist between the two main groups. In this study, the *cyt b* divergence of two specimens belonging to the subspecies *A. c. caudatus* and *A. c. glaucogularis*, is 1.3%. Thus, phylogeographic structures would be developed if additional subspecies were taken into consideration.

On the other hand, interspecies molecular divergence is small. The morphologically distinct sister

species *A. fuliginosus* and *A. bonvaloti* are separated by unexpectedly small *cyt b* divergences (0.2%, uncorrected *p*-distance) and associated very short branch lengths in the *cyt b* tree. The pairwise *cyt b* divergence between the species is comparable to that within populations of the same species in other passerine birds. This could indicate recent separation and divergence from their common ancestor. However, surprisingly, the more slowly evolving nuclear locus β -fibrinogen intron 7 shows relatively greater divergence (2.5%, uncorrected *p*-distance) than the faster-evolving *cyt b* (unpublished data). This might be the result of amplification of nuclear pseudogenes instead of mitochondrial DNA (Zhang and Hewitt, 1996; Sorensen and Quinn, 1998). However, our sequences show no evidence of being of nuclear origin. Introgression of mitochondrial DNA seems to be a more likely explanation. *A. fuliginosus* and *A. bonvaloti* are not known to hybridize, but their current distributions partly overlap. Hybridization or past hybridization leading to introgression is nevertheless a possibility. Weckstein et al. (2001) argued that introgressive hybridization is the cause of discordant patterns of mitochondrial and allozyme data in the North American sparrows *Zonotrichia leucophrys* and *Z. atricapilla*.

Furthermore, species limits within the *A. niveogularis* are not, as yet, reliably defined. Harrap and Quinn (1996) proposed the species to be split in two species while Dickinson (2003) proposed a three way split. Therefore, currently, five (Martens and Eck, 1995) or six (Harrap and Quinn, 1996) or seven (Dickinson, 2003) species have been recognized within the genus *Aegithalos*. Our study includes only a few species and subspecies from *Aegithalos* because of insufficient or unavailable samples. Recently, Päckert et al. (2010) studied the phylogeny of long-tailed tits and allies based on mitochondrial and nuclear markers and resolved the status of some species on the basis of phylogenetic relationships. However, by considering the long evolutionary time of species and differences within species, both morphological and molecular characters, a study which includes more species and subspecies is necessary to define the phylogeny of *Aegithalos*.

We suggest that the southwestern mountain ranges of China might be the center of origin of *Aegithalos* species and that the taxa of this genus colonized new habitats from west to east across China. Supporting this hypothesis are the patterns of taxonomic diversity and endemism. For example, members of all

long-tailed tits are found in the areas of southwestern China except *A. niveogularis*. The oldest lineage in the genus in our study is the *A. concinnus talifuensis* which is distributed in Yunnan Province of China. The historical biogeography can be deduced by the relationships among species and the areas of species distribution (Gill et al., 2005). For instance, the closely related phylogenetic relationships between *Aegithalos fuliginosus* and *A. bonvaloti* is accompanied by a partly overlapped distribution. *Aegithalos caudatus caudatus* is older than *A. c. glaucogularis* in our phylogenetic analysis and is thus distributed farther from the original areas than *A. c. glaucogularis*. Given a rough calibration of 2% divergence per million years, we hypothesize that the separation of *A. concinnus* may occur ~5.5 Mya, on the basis of the estimate of 11% sequence divergence between *A. concinnus* and other three species. Then, about 4.5 Mya ago, *A. caudatus* was divided and colonized the palearctic region. More recently, separation between *Aegithalos fuliginosus* and *A. bonvaloti* is hypothesized to have taken place in the late Pleistocene, i.e., ~100000 years ago. However, that break may be earlier than late Pleistocene, for the possible introgression between two species may obscure the real separation time.

Congruent genetic differentiation among co-distributed species

Comparative phylogeography helped elucidate the relative effect of shared historical earth events on current patterns of biodiversity by comparing historical patterns of gene flow and divergence among species that overlap in time and space (Hickerson et al., 2010). In other words, co-distribution species may have similar population genetic differentiation or congruent phylogeographical patterns as a result of sharing common environmental and geological changes. In our study, the Great Tit (*Parus major*) and the Black-throated Tit (*A. concinnus*) overlap in southern and southwestern China, the Coat Tit (*P. ater*), the Willow Tit (*P. montanus*), the Marsh Tit (*P. palustris*) and the Long-tailed Tit are distributed in the northeast, center and west of China. Although our sample size was small, we found congruent genetic differentiation among these sympatric distribution taxa. The Great Tit and the Black-throated Tit have similar phylogeographical patterns. They differentiated into two clades, one including the samples of Yunnan Province and the other one belonging to the

other sample locations in China. Similarly, the Coat Tit, the Marsh Tit, the Willow Tit and the Long-tailed Tit also divided into two branches, one covering the areas of northeastern China and Europe and the other including the species distribution in central and western China. These phylogeographical patterns or population structures of sympatric species may indicate that the historical earth events they experienced, including climatic and geological, had almost the same effect on these closely related taxa.

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基于线粒体基因 *COI* 和 *cyt b* 序列的山雀科、攀雀科及长尾山雀属鸟类的分子系统发育分析

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摘要: 过去的几十年来对山雀科鸟类及其亲缘类群的系统发育关系的研究很多, 然而, 由于各研究采用不同的遗传标记, 关于这些物种间系统发育关系的争议越来越大。在本研究中, 我们采用最大似然法和贝叶斯法分析所获得的15个物种(10个山雀科物种, 4个长尾山雀属物种和中华攀雀)的线粒体基因*cyt b*和*COI*片段的联合序列。基于不同片段和分子进化模型间的一致性且强烈的支持, 山雀科及其亲缘类群物种间的系统发育关系得到了很好的解决。和以往用不同的遗传标记研究结果一样, 山雀科和攀雀科的单系性得到支持, 而山雀科和长尾山雀属的单系性则被拒绝。我们的结果建议将山雀属亚属*Machlolophus*提升为属。虽然在我们的研究中, 长尾山雀属的系统发生关系获得很高的支持, 然而, 考虑到该属物种内的分子和形态性状的差异, 还需要更多的数据来解决该属的系统发育。基于该属物种的多样性和特有化格局, 我们认为中国西南山地很可能就是该属的演化中心。以每百万年2%的进化速率来估算物种的分化时间, 结果表明该属物种的分化时间为从晚第三纪到第四纪(从5.5至0.1百万年)。经比较分析, 我们发现同域分布的物种存在着一致的遗传分化格局。

关键词: 系统发育, 山雀, 单系, 演化中心, 分离, 遗传分化