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Phylogenetic relationships of some Sylviidae species based on complete mtDNA *cyt b* and partial *COI* sequence data

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Abstract Members of the passerine family Sylviidae are distributed widely around the Old World and the phylogenetic relationships of many species still remain controversial. In this study, we investigated the phylogeny and relationships among 37 species of 11 genera by analyzing DNA sequences obtained from the complete mitochondrial cytochrome *b* (*cyt b*) and partial cytochrome oxidase I (*COI*) genes. The data were analyzed by maximum-likelihood analysis and Bayesian inference. The results demonstrate that the current genus *Phylloscopus* is non-monophyletic, while *Seicercus* is synonymized with *Phylloscopus* or is a group within the genus *Phylloscopus.* We may conclude that within *Phylloscopus* there are close relations between *P. magnirostris* and *P. borealis*; among *P. proregulus*, *P. yunnanensis*, *P. pulcher* and *P. maculipennis*, as well as among *P. occisinensis*, *P. schwarzi*, *P. armandii*, *P. collybita* and *P. fuscatus*. Monophyly of the genus *Cettia* could not be corroborated; it is closely related to the genera *Abroscopus* and *Tesia*. However monophyly of *Acrocephalus* is supported. Furthermore, the close relationships between *Sylvia* and *Zosterops* and among *Prinia*, *Orthotomus* and *Cisticola* are also supported.

Keywords molecular phylogeny, Sylviidae, *Phylloscopus*, cytochrome *b* gene, cytochrome oxidase I gene, China

Introduction

Sylviidae, a family of small Old World warblers, has proven to be a controversial group and, for a long time, problematic in taxonomy, owing to subtle morphological distinctions in many species and subspecies (Zheng, 2005). Traditionally, taxonomists considered that these warblers should be grouped in the subfamily Sylviinae, consisting of 60 genera and 348 species (Mayr and Cottrell, 1986). Many previous studies on the relations among the Sylviidae species and their taxonomic status are mostly based on morphological and ecological characteristics (La Touche, 1925–1934; Vaurie, 1965; Mayr and Cottrell, 1986). However, cryptic species are quite common in Sylviidae and sonogram analysis and molecular genetic approaches have been frequently used to solve issues of species delimitation and taxonomic relationships (Drovetski et al., 2004; Alström et al., 2006). Since the first analyses of DNA-DNA hybridization, Sylviinae has been elevated as the family Sylviidae and

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divided into four subfamilies: Acrocephalinae, Megalurinae, Garrulacinae and Sylviinae, while some genera have been modulated (Sibley and Monroe, 1990). After this, most species were studied using molecular approaches and their relationships were revised repeatedly, especially in Europe (Helbig and Seibold, 1999; Drovetski et al., 2004; Alström et al., 2006).

In China, based on traditional morphological taxonomic approaches, Cheng (1994, 2000) recognized 98 or 95 species in 18 genera and listed them in the subfamily Sylviinae under the family Muscicapidae, including the genera *Tesia*, *Cettia*, *Bradypterus*, *Megalurus*, *Locustella*, *Acrocephalus*, *Hippolais*, *Sylvia*, *Phylloscopus*, *Regulus*, *Seicercus*, *Abroscopus*, *Tickellis*, *Leptopoecile*, *Orthotomus*, *Cisticola*, *Graminicola* and *Prinia*. Recently, Cheng's classification was revised by Zheng (2005) mostly based on Sibley and Monroe's taxonomic treatment and sequences, in which Sylviinae has been elevated to the family Sylviidae consisting of 16 genera, while *Regulus* was promoted as the family Regulidae, while *Cisticola* and *Prinia* were placed (similar as in Dickinson (2003)) in the family Cisticolidae. Although a few studies on the phylogenetic relations of the species are available from Asia, there are still many taxonomic problems about the relationships among some genera, especially from China which harbors abundant warbler diversity and the classification and phylogenetic relationships of many more putative species and genera in Sylviidae still remain unsolved (Alström et al., 2007, 2008; Martens et al., 2008; Päckert et al., 2009). In our study we investigated the phylogenetic relationships among genera and some species of Sylviidae, based on sequence data of mitochondrial DNA, in an attempt to reconstruct a phylogenetic topology for the constituents of this group and to assess the validity of the taxonomic status of some controversial genera and species.

The mitochondrial cytochrome *b* (*cyt b*) gene is the most widely used genetic marker for phylogenetic studies and has been the most readily available source of sequence data in avian studies (Johnson, 2001; Klicka et al., 2001; Thomassen et al., 2003; Sheldon et al., 2005). Cytochrome oxidase I (*COI*) gene is also a very useful tool for DNA-barcoding, allowing studies of avian species delimitation and their phylogenies (DeFilippis, 1995; Weibel and Moore, 2002; Hebert et al., 2004; Webb and Moore, 2005; Aliabadian et al., 2009). In this study, we selected species as in-group following Zheng's classification of Sylviidae (Zheng, 2005) and then investigated the phylogeny and relations among some species and genera by DNA sequencing of the complete *cyt b* and partial *COI* genes.

Materials and methods

Selection of in-group taxon and out-groups

We included 36 Sylviidae species in the study (Table 1). In attempting to enhance viewing the phylogenetic relationships of Sylviidae species, we also included *Zosterops japonica* from Zosteropidae. We used *Lanius isabellinus* and *Dicrurus hottentottus* as outgroups. Samples were collected mostly from China. Only the *Locustella fluviatilis*, *Sylvia communis* and *Phylloscopus collybita* species are from Slovakia (Europe). All birds were collected complying with the current laws in China and Slovakia.

Extraction, amplification and sequencing

Total genomic DNA was extracted from blood or muscle specimens using the TIANamp Genomic DNA Kit (TIANGEN) as per instructions of the manufacturer. Nucleotide sequence data were obtained from the mitochondrial *cyt b* gene and *COI*.

The primers used to amplify the *cyt b* gene were L14827 and H16065 (Pasquet et al., 2002), L14731 and H16067 (Saetre et al., 2001), L14851 and H16058 (Groth 1998), L14863 and H16058 (Groth, 1998). The primers L6615 and H7956 (Sorenson et al., 1999) were used for the *COI* gene. Amplification products were sequenced with the same primers as used for PCR amplification.

PCR reactions were carried out under the following conditions: an initial denaturation at 94°C for 8 min; 36 cycles at 94°C for 30 s, 45–48°C for 1 min and 72°C for 2 min, followed by a final extension of 10 min at 72°C. For all taxa, both strands of DNA were sequenced using an ABI3730 automated sequencer. The DNA sequences are deposited at GenBank (accession number from HQ608821 to HQ608894).

Alignment and sequence properties

All DNA sequence datasets were edited using the DNASTAR package (SeqMan), and the sequences of the two gene regions were aligned using Clustal W1.83 (Thompson et al., 1997). No gaps, insertions,

Table 1 Species list, samples used, mitochondrial DNA *cyt b* and *COI* gene sequences

Species	Museum No.	Sample type	Collection sites	GenBank Accession No.	
				cyt b	*COI*
Locustella certhiola	IOZ571	Muscle	Qinhuangdao, Hebei, China	HQ608848	HQ608859
Locustella fluviatilis	IOZ2264	Blood	Slovakia	HQ608847	HQ608858
Locustella lanceolata	IOZ576	Muscle	Qinhuangdao, Hebei, China	HQ608849	HQ608860
Sylvia curruca	IOZ2282	Muscle	Xinjiang, China	HQ608839	HQ608880
Sylvia communis	IOZ2272	Blood	Slovakia	HQ608840	HQ608881
Phylloscopus fuscatus	IOZ4369	Muscle	Suifenhe, Heilongjiang, China	HQ608823	HQ608868
Phylloscopus coronatus	IOZ10776	Blood	Qinhuangdao, Liaoning, China,	HQ608834	HQ608874
Phylloscopus collybita	IOZ2304	Blood	Slovakia	HQ608821	HQ608872
Phylloscopus schwarzi	IOZ432	Blood	Tianjin, China	HQ608825	HQ608863
Phyllosopus armandii	IOZ581	Blood	Qinhuangdao, Hebei, China	HQ608831	HQ608871
Phylloscopus occisinensis	IOZ1104	Muscle	Wenxian, Gansu, China	HQ608829	HQ608869
Phylloscopus pulcher	IOZ3960	Muscle	Yanbian, Sichuan, China	HQ608826	HQ608864
Phylloscopus maculipennis	IOZ4081	Muscle	Miyi, Sichuan, China	HQ608828	HQ608866
Phylloscopus proregulus	IOZ1766	Muscle	Wuxue, Hubei, China	HQ608830	HQ608861
Phylloscopus yunnanensis	IOZ8273	Muscle	Shennongjia, Hubei, China	HQ608833	HQ608873
Phylloscopus reguloides	IOZ3746	Muscle	Panzhihua, Sichuan, China	HQ608827	HQ608865
Phylloscopus magnirostris	IOZ4204	Muscle	Meigu, Sichuan, China	HQ608822	HQ608867
Phylloscopus borealis	IOZ429	Muscle	Foping, Shaanxi, China	HQ608832	HQ608870
Phylloscopus trochiloides	IOZ430	Muscle	Tianjin, China	HQ608824	HQ608862
Cettia fortipes	IOZ4583	Muscle	Taibai, Shaanxi, China	HQ608836	HQ608878
Cettia robustipes	IOZ2563	Muscle	Foping, Shaanxi, China	HQ608835	HQ608876
Cettia diphone	IOZ4648	Muscle	Taibai, Shaanxi, China	HQ608838	HQ608879
Cettia flavolivaceus	IOZ4241	Muscle	Meigu, Sichuan, China	HQ608837	HQ608877
Seicercus burkii	IOZ1125	Muscle	Wenxian, Gansu, China	HQ608856	HQ608892
Seicercus castaniceps	IOZ3586	Muscle	Foping, Shaanxi, China	HQ608857	HQ608893
Tesia castaneocoronata	IOZ4261	Muscle	Meigu, Sichuan, China	HQ608852	HQ608888
Abroscopus albogularis	IOZ3126	Muscle	Wuyi mountain, Fujian, China	HQ608846	HQ608894
Acrocephalus orientalis	IOZ4331	Muscle	Dunhua, Liaoning, China	HQ608853	HQ608889
Acrocephalus aedon	IOZ418	Muscle	Suifenhe, Heilongjiang, China	HQ608854	HQ608890
Acrocephalus bistrigiceps	IOZ578	Blood	Qinhuangdao, Hebei, China	HQ608855	HQ608891
Prinia criniger	IOZ4183	Muscle	Yanyuan, Sichuan, China	HQ608842	HQ608884
Prinia atrogularis	IOZ4703	Muscle	Guilin, Guangxi, China	HQ608845	HQ608886
Prinia flaviventris	IOZ1432	Muscle	Jiedong, Guangdong, China	HQ608843	HQ608883
Prinia subflava	IOZ1250	Muscle	Nanning, Guangxi, China	HQ608844	HQ608885
Orthotomus sutorius	IOZ82	Muscle	Chaoan, Guangdong, China	HQ608841	HQ608882
Cisticola juncidis	IOZ1310	Muscle	Haifeng, Guangdong, China	HQ608851	HQ608887
Zosterops japonica	IOZ2538	Muscle	Foping, Shaanxi, China	HQ608850	HQ608875
Dicrurus hottentottus	IOZ2049	Muscle	Foping, Shaanxi, China	EF113121	EF422250
Lanius isabellinus	IOZ717	Muscle	Xinjiang, China	EF113120	EF422251

or deletions were found in the aligned sequences and all sequences were translated into amino acid sequences to verify the alignments. Both separated and combined datasets were analyzed. The final sequences included complete *cyt b* gene (1143 bp) and part of *COI* gene (1176 bp). Statistics for nucleotide variation and pairwise genetic distances were computed with MEGA 3.1 (Kumar et al., 2004).

Phylogenetic analyses

Phylogenetic analyses were performed on the combined sequences from the *cyt b* and *COI* genes. In addition, phylogenetic signals in the two datasets were compared by analyzing each gene region separately.

Maximum-likelihood (ML) analyses and incongruence length difference (ILD or partition homogeneity) tests were performed using Paup* 4.0b10 (Swofford, 2002). For ML, the optimal model of evolution was determined by hierarchical likelihood ratio tests (hLRTs) in Modeltest 3.06 (Posada and Crandall, 1998). Parameters for the ML analyses were estimated from the data (Table 2). Furthermore, the GTR + I + G model was identified as the best fit for our data using hLRTs criteria in Modeltest. Bootstrap support values were based on 100 replicate, maximum-likelihood analyses.

The datasets were also analyzed by Bayesian inference. The models for nucleotide substitutions were selected for the two genes individually using the Akaike Information Criterion (Akaike, 1973). We ran four Markov chains for 5 million generations each with trees sampled every 100 generations. The trees saved during the “burn-in phase” (the first 100000 generations in each analysis) were discarded. The posterior probabilities were then calculated from the remaining 49000 saved trees. The remaining trees from both analyses (produced automatically in MrBayes v3.1b) were used to create a majority rule consensus tree. Posterior probabilities greater or equal to 95% were considered significant (Leache and Reeder, 2002).

Results

Sequence characteristics

In *cyt b*, 523 of 1143 sites varied among taxa and 448 sites (39%) were parsimony-informative. The *COI* gene was less variable than *cyt b*: 424 of 1176 sites varied among taxa and 386 sites (33%) were parsimony-informative. The combined sequences of the two gene segments had 2319 sites, of which 834 (36%) were parsimony-informative.

Pairwise distances among the 37 in-group species and 2 out-group species are summarized in Table 2. In *cyt b* gene, the observed intra-generic sequence divergence ranged from 0.001 (*Acrocephalus orientalis* and *A. aedon*, *Phylloscopus proregulus* and *P. yunnanensis*) to 0.138 (*Phylloscopus collybita* and *P. trochiloides*). Inter-generic *cyt b* comparisons ranged from 0.118 (*Phylloscopus coronatus* and *Cettia diphone*, *Prinia criniger* and *Orthotomus sutorius*) to 0.204 (*Sylvia curruca* and *Acrocephalus aedon*). The smallest divergence in *cyt b* between the in-group and out-group was 0.182 (*Locustella lanceolata* and *Lanius isabellinus*), and the largest 0.232 (*Sylvia curruca* and *Dicrurus hottentottus*). In the *COI* gene, the smallest intra-generic sequence divergence within the in-group was 0.001 (*Phylloscopus proregulus* and *P. yunnanensis*, *Phylloscopus schwarzi* and *P. armandii*, *Acrocephalus orientalis* and *A. aedon*) and the largest 0.144 (*Phylloscopus collybita* and *P. maculipennis*). Inter-generic *COI* comparisons ranged from 0.111 (*Cettia fortipes* and *Abroscopus albogularis*) to 0.176 (*Prinia criniger* and *Seicercus castaniceps*, *Cisticola juncidis* and *Phylloscopus trochiloides*). The smallest divergence observed between the in-group and the two out-groups was 0.150 (*Orthotomus sutorius* and *Dicrurus hottentottus*, *Sylvia communis* and *Dicrurus hottentottus*) while the largest divergence was 0.187 (*Acrocephalus bistrigiceps* and *Dicrurus hottentottus*).

cyt b and *COI* had very similar nucleotide compositions, so the two genes, when combined, had a more uniform nucleotide composition than any individual gene. Nucleotide bias of the two genes was similar to that observed in birds in previous studies (Weibel and Moore, 2002; Webb and Moore, 2005). At the first codon position, the four bases were equally distributed. At the second position, the amount of G was decreased and that of T increased. The strong bias for an excess of C and paucity of G was shown at the third codon positions.

Phylogenetic analysis

We analyzed the topologies of ML and Bayesian trees produced by the combined sequences of the two gene segments. The trees, resulting from the maximum-likelihood analysis and Bayesian infer-

Table 2 Observed pairwise genetic distances for the *cyt b* gene (below diagonal) and the *COI* gene (above diagonal)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1		0.130	0.101	0.156	0.154	0.152	0.170	0.149	0.158	0.160	0.147	0.164	0.163	0.162	0.149	0.149	0.155	0.156	0.156	0.153
2	0.115		0.107	0.156	0.156	0.163	0.165	0.140	0.145	0.146	0.144	0.157	0.157	0.150	0.155	0.143	0.147	0.153	0.156	0.155
3	0.108	0.117		0.149	0.144	0.152	0.147	0.136	0.145	0.146	0.138	0.143	0.143	0.158	0.156	0.133	0.149	0.143	0.153	0.150
4	0.178	0.192	0.183		0.121	0.128	0.150	0.145	0.130	0.131	0.130	0.145	0.145	0.154	0.144	0.146	0.151	0.144	0.131	0.150
5	0.173	0.183	0.169	0.134		0.145	0.154	0.151	0.127	0.126	0.136	0.153	0.154	0.156	0.148	0.152	0.146	0.164	0.141	0.157
6	0.183	0.188	0.174	0.187	0.170		0.153	0.149	0.150	0.148	0.137	0.145	0.144	0.144	0.126	0.141	0.137	0.139	0.130	0.144
7	0.159	0.158	0.150	0.156	0.172	0.167		0.128	0.127	0.128	0.124	0.128	0.127	0.144	0.129	0.132	0.136	0.136	0.134	0.137
8	0.161	0.174	0.159	0.166	0.162	0.168	0.117		0.117	0.118	0.111	0.114	0.113	0.116	0.127	0.119	0.114	0.128	0.114	0.141
9	0.168	0.168	0.153	0.174	0.167	0.174	0.122	0.123		0.001	0.097	0.114	0.113	0.120	0.114	0.131	0.120	0.127	0.113	0.131
10	0.166	0.164	0.148	0.168	0.163	0.171	0.117	0.121	0.008		0.098	0.113	0.112	0.121	0.113	0.132	0.121	0.126	0.114	0.132
11	0.169	0.178	0.161	0.165	0.166	0.160	0.128	0.118	0.111	0.106		0.120	0.122	0.130	0.121	0.135	0.126	0.126	0.127	0.130
12	0.163	0.150	0.156	0.155	0.155	0.155	0.119	0.119	0.115	0.109	0.113		0.001	0.117	0.106	0.115	0.117	0.121	0.127	0.137
13	0.164	0.149	0.155	0.154	0.157	0.156	0.118	0.119	0.116	0.110	0.114	0.001		0.116	0.105	0.115	0.117	0.121	0.127	0.137
14	0.175	0.160	0.156	0.172	0.156	0.163	0.124	0.126	0.126	0.121	0.116	0.086	0.087		0.109	0.134	0.133	0.127	0.122	0.143
15	0.166	0.166	0.173	0.166	0.165	0.172	0.114	0.126	0.122	0.118	0.115	0.091	0.092	0.095		0.130	0.125	0.127	0.125	0.136
16	0.153	0.164	0.169	0.167	0.152	0.164	0.131	0.117	0.112	0.112	0.116	0.101	0.102	0.104	0.107		0.076	0.103	0.105	0.100
17	0.170	0.176	0.164	0.178	0.172	0.165	0.134	0.140	0.126	0.122	0.122	0.120	0.121	0.113	0.125	0.085		0.112	0.094	0.111
18	0.161	0.167	0.158	0.163	0.168	0.151	0.127	0.132	0.142	0.139	0.132	0.114	0.113	0.125	0.125	0.101	0.108		0.104	0.112
19	0.163	0.164	0.148	0.160	0.159	0.159	0.135	0.127	0.134	0.134	0.130	0.117	0.118	0.126	0.124	0.095	0.109	0.091		0.126
20	0.158	0.166	0.159	0.170	0.162	0.171	0.138	0.132	0.129	0.125	0.122	0.113	0.114	0.119	0.129	0.101	0.106	0.110	0.110	
21	0.149	0.153	0.154	0.178	0.160	0.158	0.148	0.134	0.143	0.136	0.123	0.123	0.124	0.129	0.118	0.109	0.120	0.101	0.117	0.106
22	0.161	0.149	0.145	0.164	0.158	0.145	0.134	0.123	0.140	0.136	0.115	0.104	0.105	0.117	0.133	0.101	0.113	0.097	0.110	0.108
23	0.187	0.188	0.181	0.203	0.198	0.190	0.178	0.172	0.171	0.167	0.172	0.161	0.162	0.164	0.176	0.156	0.171	0.183	0.160	0.176
24	0.188	0.190	0.182	0.204	0.200	0.191	0.179	0.171	0.172	0.168	0.173	0.162	0.163	0.165	0.177	0.158	0.172	0.184	0.161	0.177
25	0.169	0.181	0.167	0.182	0.186	0.182	0.151	0.151	0.156	0.152	0.159	0.150	0.149	0.155	0.163	0.149	0.156	0.162	0.156	0.155
26	0.168	0.170	0.164	0.186	0.169	0.177	0.159	0.146	0.153	0.146	0.158	0.122	0.123	0.147	0.143	0.129	0.143	0.145	0.141	0.145
27	0.169	0.171	0.165	0.187	0.170	0.178	0.160	0.147	0.154	0.147	0.159	0.123	0.125	0.148	0.145	0.128	0.144	0.146	0.142	0.146
28	0.162	0.169	0.157	0.185	0.155	0.163	0.155	0.141	0.146	0.140	0.137	0.132	0.133	0.139	0.153	0.119	0.127	0.136	0.134	0.126
29	0.168	0.167	0.167	0.191	0.168	0.167	0.152	0.145	0.158	0.152	0.156	0.133	0.132	0.154	0.147	0.127	0.146	0.139	0.137	0.138
30	0.199	0.201	0.188	0.189	0.191	0.204	0.168	0.149	0.182	0.177	0.157	0.160	0.161	0.173	0.168	0.149	0.162	0.169	0.156	0.181
31	0.191	0.181	0.174	0.200	0.198	0.187	0.164	0.161	0.166	0.160	0.173	0.157	0.156	0.170	0.161	0.157	0.167	0.158	0.156	0.173
32	0.158	0.182	0.168	0.177	0.150	0.176	0.145	0.148	0.149	0.144	0.160	0.156	0.157	0.156	0.157	0.139	0.156	0.168	0.157	0.146
33	0.168	0.181	0.172	0.184	0.160	0.192	0.162	0.160	0.159	0.156	0.164	0.151	0.152	0.158	0.172	0.146	0.160	0.174	0.164	0.155
34	0.171	0.192	0.171	0.186	0.171	0.184	0.166	0.161	0.162	0.155	0.169	0.151	0.152	0.166	0.162	0.157	0.160	0.174	0.156	0.151
35	0.169	0.191	0.167	0.181	0.168	0.184	0.164	0.159	0.165	0.157	0.167	0.149	0.150	0.164	0.163	0.155	0.160	0.171	0.154	0.149
36	0.163	0.169	0.164	0.171	0.173	0.173	0.150	0.152	0.156	0.152	0.161	0.142	0.143	0.154	0.152	0.149	0.158	0.165	0.149	0.155
37	0.165	0.168	0.166	0.191	0.165	0.209	0.165	0.156	0.176	0.171	0.170	0.149	0.150	0.163	0.155	0.160	0.165	0.168	0.161	0.160
38	0.190	0.193	0.182	0.218	0.203	0.218	0.198	0.186	0.199	0.196	0.194	0.195	0.197	0.196	0.199	0.192	0.200	0.208	0.183	0.196
39	0.203	0.211	0.194	0.232	0.214	0.200	0.212	0.205	0.210	0.203	0.203	0.193	0.194	0.214	0.207	0.202	0.208	0.203	0.213	0.198

Table 2 (Continued)

	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
1	0.158	0.162	0.161	0.159	0.146	0.146	0.146	0.155	0.167	0.162	0.161	0.148	0.154	0.141	0.142	0.146	0.148	0.172	0.171
2	0.138	0.159	0.153	0.154	0.144	0.152	0.153	0.150	0.169	0.144	0.163	0.162	0.163	0.160	0.161	0.150	0.147	0.177	0.167
3	0.148	0.148	0.149	0.148	0.145	0.137	0.138	0.142	0.149	0.143	0.150	0.152	0.159	0.156	0.155	0.133	0.140	0.160	0.163
4	0.144	0.144	0.135	0.136	0.138	0.136	0.136	0.154	0.140	0.131	0.141	0.157	0.148	0.156	0.159	0.146	0.142	0.168	0.158
5	0.147	0.155	0.129	0.130	0.145	0.154	0.153	0.154	0.153	0.144	0.154	0.165	0.158	0.170	0.171	0.144	0.148	0.171	0.150
6	0.130	0.147	0.141	0.14	0.133	0.137	0.138	0.144	0.144	0.144	0.150	0.166	0.151	0.158	0.160	0.159	0.148	0.179	0.162
7	0.132	0.141	0.156	0.155	0.162	0.149	0.146	0.150	0.157	0.148	0.154	0.163	0.164	0.163	0.165	0.160	0.151	0.179	0.171
8	0.121	0.131	0.147	0.148	0.141	0.131	0.130	0.139	0.143	0.146	0.136	0.169	0.153	0.156	0.159	0.145	0.168	0.179	0.158
9	0.127	0.119	0.132	0.131	0.129	0.126	0.125	0.139	0.153	0.133	0.141	0.161	0.145	0.157	0.158	0.141	0.148	0.164	0.158
10	0.128	0.118	0.133	0.132	0.131	0.127	0.126	0.138	0.154	0.134	0.142	0.160	0.146	0.158	0.160	0.142	0.149	0.163	0.159
11	0.128	0.128	0.140	0.141	0.130	0.125	0.122	0.140	0.140	0.149	0.146	0.156	0.151	0.154	0.154	0.133	0.152	0.154	0.165
12	0.114	0.128	0.139	0.138	0.143	0.124	0.123	0.134	0.141	0.134	0.138	0.174	0.164	0.161	0.163	0.147	0.155	0.155	0.160
13	0.113	0.128	0.138	0.137	0.142	0.125	0.124	0.133	0.140	0.133	0.137	0.174	0.163	0.161	0.163	0.147	0.154	0.155	0.161
14	0.116	0.137	0.136	0.135	0.141	0.153	0.151	0.150	0.154	0.140	0.157	0.174	0.169	0.165	0.165	0.164	0.166	0.172	0.172
15	0.118	0.122	0.135	0.134	0.127	0.133	0.132	0.148	0.148	0.146	0.153	0.155	0.166	0.161	0.166	0.159	0.142	0.174	0.177
16	0.100	0.115	0.149	0.150	0.138	0.134	0.133	0.144	0.145	0.147	0.149	0.164	0.163	0.155	0.156	0.141	0.169	0.169	0.172
17	0.106	0.114	0.145	0.146	0.132	0.133	0.134	0.138	0.150	0.137	0.142	0.158	0.152	0.156	0.155	0.140	0.164	0.175	0.174
18	0.103	0.106	0.143	0.142	0.133	0.134	0.134	0.152	0.161	0.144	0.155	0.161	0.161	0.161	0.161	0.160	0.166	0.167	0.162
19	0.100	0.112	0.131	0.130	0.128	0.133	0.131	0.148	0.144	0.136	0.133	0.154	0.139	0.154	0.153	0.135	0.157	0.175	0.175
20	0.112	0.116	0.148	0.149	0.148	0.149	0.147	0.149	0.150	0.152	0.150	0.154	0.160	0.156	0.157	0.144	0.176	0.164	0.165
21		0.117	0.151	0.152	0.139	0.130	0.129	0.151	0.143	0.150	0.146	0.176	0.158	0.150	0.154	0.139	0.155	0.179	0.168
22	0.113		0.146	0.145	0.135	0.128	0.127	0.146	0.144	0.154	0.151	0.151	0.157	0.152	0.155	0.140	0.158	0.182	0.192
23	0.165	0.157		0.001	0.113	0.130	0.131	0.136	0.151	0.138	0.151	0.164	0.162	0.163	0.160	0.151	0.143	0.166	0.156
24	0.166	0.158	0.001		0.112	0.129	0.130	0.135	0.152	0.137	0.150	0.163	0.160	0.162	0.159	0.152	0.142	0.168	0.157
25	0.155	0.144	0.125	0.126		0.128	0.130	0.147	0.142	0.148	0.144	0.162	0.157	0.148	0.147	0.148	0.139	0.163	0.187
26	0.137	0.125	0.172	0.173	0.160		0.003	0.113	0.088	0.112	0.137	0.154	0.153	0.159	0.161	0.143	0.144	0.166	0.172
27	0.138	0.126	0.173	0.175	0.161	0.001		0.114	0.087	0.111	0.136	0.156	0.151	0.159	0.161	0.143	0.145	0.164	0.171
28	0.135	0.118	0.169	0.170	0.151	0.086	0.087		0.127	0.120	0.129	0.169	0.167	0.162	0.162	0.155	0.163	0.173	0.181
29	0.131	0.132	0.178	0.18	0.159	0.101	0.102	0.090		0.127	0.148	0.171	0.158	0.167	0.169	0.146	0.165	0.162	0.180
30	0.165	0.156	0.175	0.176	0.170	0.134	0.135	0.121	0.151		0.134	0.161	0.160	0.175	0.172	0.147	0.150	0.176	0.178
31	0.165	0.148	0.179	0.181	0.173	0.127	0.128	0.122	0.128	0.135		0.170	0.150	0.161	0.160	0.144	0.162	0.179	0.184
32	0.161	0.146	0.181	0.182	0.157	0.152	0.153	0.153	0.169	0.175	0.170		0.096	0.123	0.124	0.130	0.137	0.166	0.182
33	0.166	0.149	0.181	0.182	0.161	0.166	0.167	0.145	0.176	0.183	0.183	0.089		0.127	0.124	0.118	0.147	0.174	0.167
34	0.161	0.152	0.192	0.193	0.187	0.159	0.161	0.160	0.173	0.172	0.172	0.119	0.114		0.004	0.136	0.146	0.166	0.175
35	0.158	0.147	0.190	0.191	0.185	0.157	0.158	0.160	0.171	0.170	0.170	0.117	0.114	0.004		0.137	0.147	0.165	0.179
36	0.148	0.144	0.177	0.178	0.171	0.152	0.153	0.141	0.156	0.165	0.172	0.118	0.129	0.124	0.124		0.127	0.160	0.150
37	0.148	0.154	0.179	0.180	0.177	0.181	0.182	0.158	0.170	0.192	0.183	0.138	0.142	0.129	0.127	0.127		0.165	0.166
38	0.196	0.184	0.209	0.211	0.200	0.197	0.198	0.185	0.212	0.192	0.204	0.205	0.213	0.203	0.206	0.198	0.196		0.163
39	0.201	0.199	0.228	0.229	0.215	0.210	0.209	0.188	0.211	0.222	0.214	0.211	0.225	0.217	0.219	0.204	0.219	0.177	

1, *Locustella fluviatilis*; 2, *Locustella certhiola*; 3, *Locustella lanceolata*; 4, *Sylvia curruca*; 5, *Sylvia communis*; 6, *Zosterops japonica*; 7, *Phylloscopus collybita*; 8, *Phylloscopus fuscatus*; 9, *Phylloscopus schwarzi*; 10, *Phyllosopus armandii*; 11, *Phylloscopus occisinensis*; 12, *Phylloscopus proregulus*; 13, *Phylloscopus yunnanensis*; 14, *Phylloscopus maculipennis*; 15, *Phylloscopus pulcher*; 16, *Phylloscopus magnirostris*; 17, *Phylloscopus borealis*; 18, *Phylloscopus reguloides*; 19, *Seicercus burkii*; 20, *Phylloscopus trochiloides*; 21, *Seicercus castaniceps*; 22, *Phylloscopus coronatus*; 23, *Acrocephalus orientalis*; 24, *Acrocephalus aedon*; 25, *Acrocephalus bistrigiceps*; 26, *Cettia robustipes*; 27, *Cettia fortipes*; 28, *Cettia diphone*; 29, *Cettia flavolivaceus*; 30, *Abroscopus albogularis*; 31, *Tesia castaneocoronata*; 32, *Prinia criniger*; 33, *Prinia atrogularis*; 34, *Prinia flaviventris*; 35, *Prinia subflava*; 36, *Orthotomus sutorius*; 37, *Cisticola juncidis*; 38, *Lanius isabellinus*; 39, *Dicrurus hottentott*

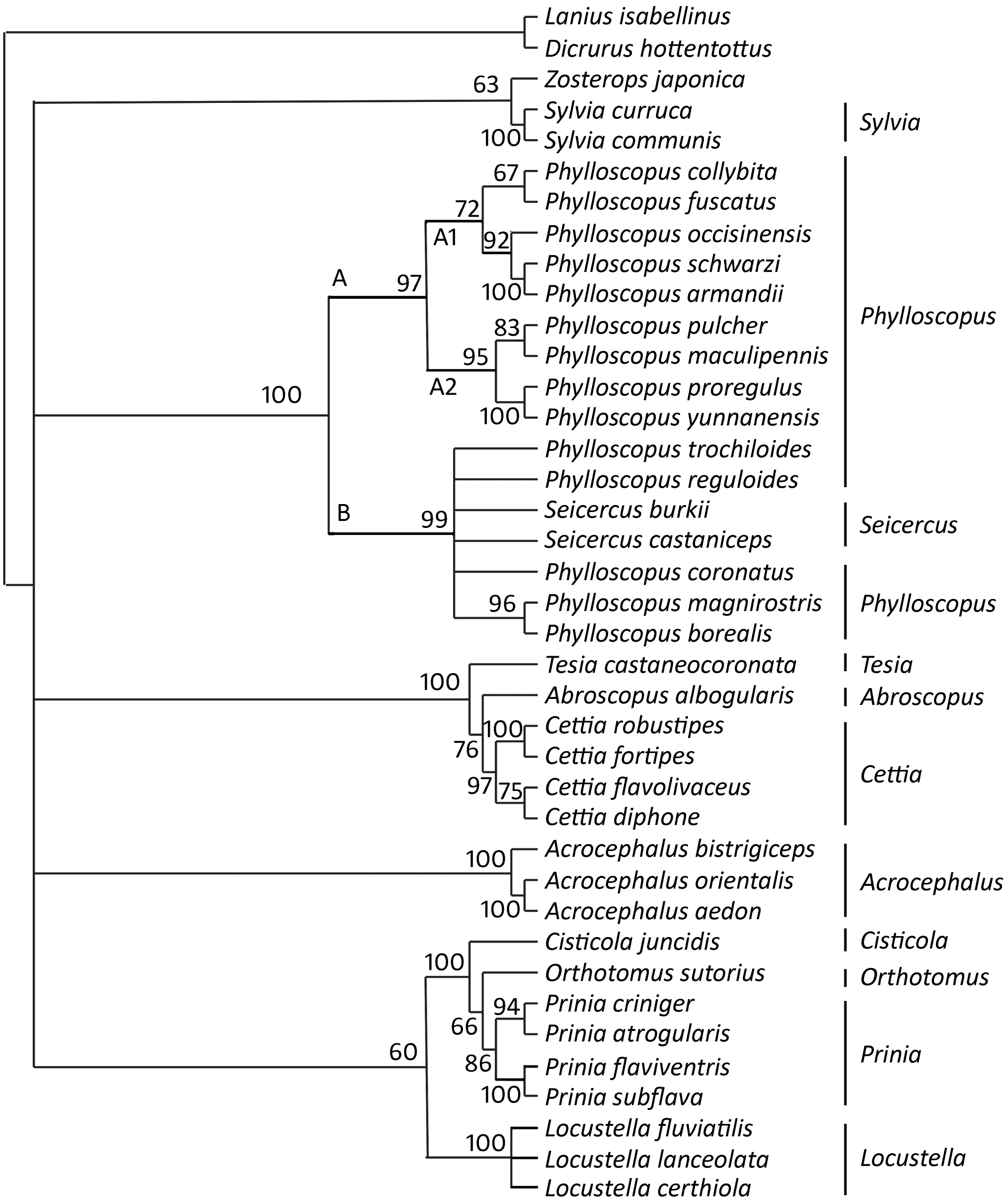

Fig. 1 The maximum likelihood tree (Bootstrap values are shown at nodes on the maximum likelihood trees.) from analysis of the *cyt b* and *COI* sequences

ence have practically identical topologies when the frequency of occurrence is set to 50% (Figs. 1 and 2).

The taxa fall into five major clades. *Sylvia* and *Zosterops* are clustered within Clade 1 (ML: 63%; Bayesian: 100%). In Clade 2, *Seicercus* is nested within *Phylloscopus* (ML: 99%; Bayesian: 94%) and the latter genus is divided into three clades in ML tree: Clade A1 with *P. collybita*, *P. fuscatus*, *P. occisinensis*, *P. schwarzi* and *P. armandii* (ML: 72%; Bayesian: 100%), Clade A2 with *P. pulcher*, *P. maculipennis*, *P. proregulus* and *P. yunnanensis* (ML: 95%; Bayesian: 100%) and Clade B with *P. trochiloides*, *P. reguloides*, *P. coronatus*, *P. magnirostris*, *P. borealis*, *Seicercus burkii* and *S. castaniceps* (ML: 99%, Bayesian: 100%). However, in the Bayesian tree, Clade B is divided into two small clades: Clade B1 with *P. reguloides*, *P. coronatus*, *Seicercus burkii*

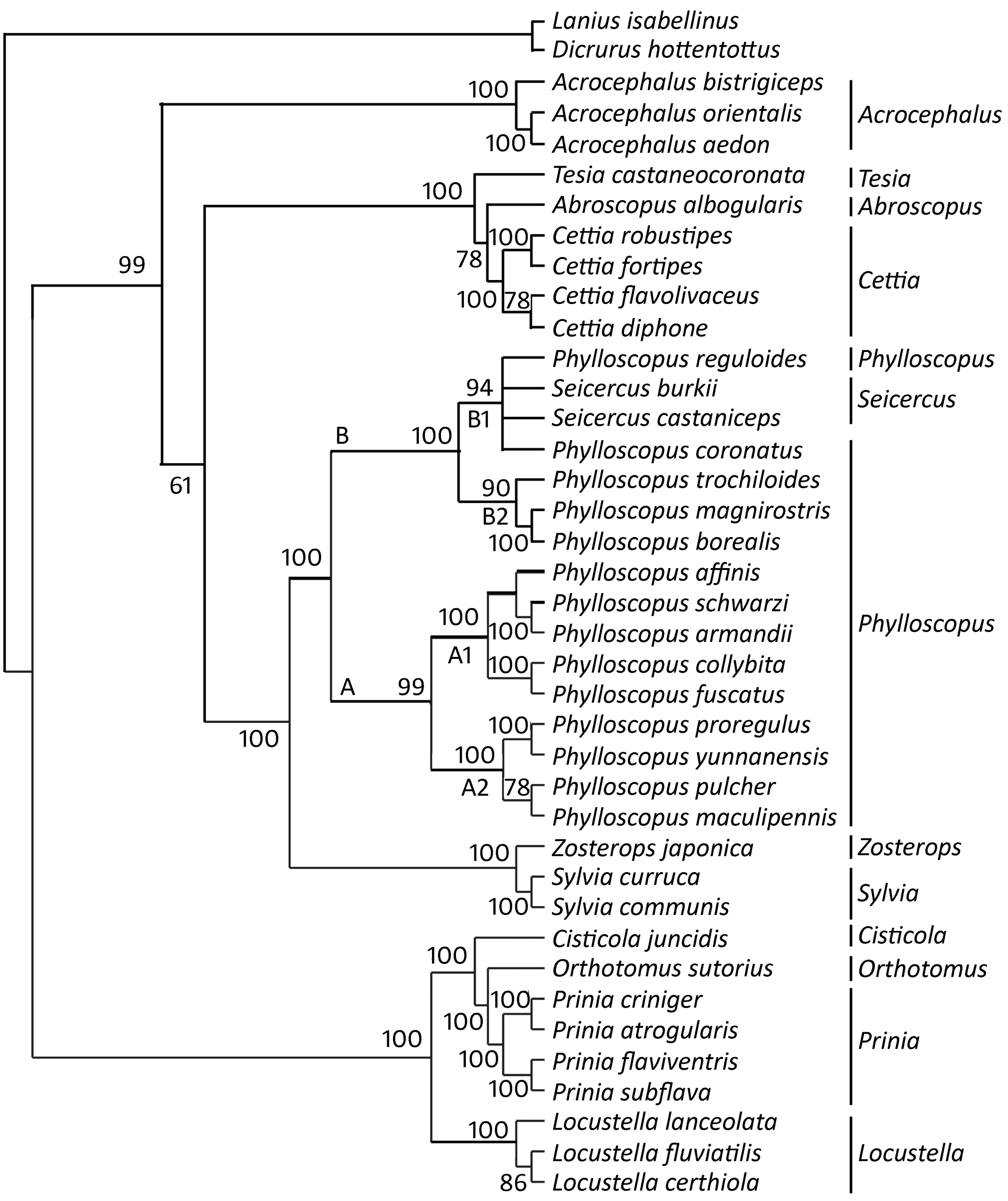

Fig. 2 The Bayesian tree (The mean posterior probabilities on the Bayesian tree are given only where they were 50% or higher) from analysis of the *cyt b* and *COI* sequences

and *S. castaniceps* (94%) and Clade B2 with *P. trochiloides*, *P. magnirostris* and *borealis* (90%). The close relationship among *Tesia*, *Abroscopus* and *Cettia* receives good bootstrap and posterior probability support (ML: 100% and 76%; Bayesian: 100% and 78%). Clade 4 comprises only three members of *Acrocephalus*. *Locustella*, *Prinia*, *Orthotomus* and *Cisticola* are clustered within Clade 5 (ML: 60%; Bayesian: 100%). Our results here show a close relationship among *Cisticola*, *Orthotomus* and *Prinia* with good nodal support (ML: 100% and 66%; Bayesian: 100% and 100%).

Discussion

Relationships between *Phylloscopus* and *Seicercus*

The genera *Seicercus* and *Phylloscopus* have been traditionally believed to be closely related (Ticehurst, 1938; Watson et al., 1986; Sibley and Ahlquist, 1990). Sibley and Ahlquist (1990) and Sibley and Monroe (1990) placed *Phylloscopus* and *Seicercus* in Acrocephalinae of Sylvioidea, while Dickinson (2003) erected the subfamily Phylloscopinae including *Phylloscopus*, *Seicercus* and *Abroscopus*. Olsson et al. (2004, 2005) suggested that both *Phylloscopus* and *Seicercus* are paraphyletic. Alström et al. (2006) strongly corroborated the viewpoints of Olsson et al. (2004, 2005) that *Seicercus* is nested within *Phylloscopus* and thus the latter genus is non-monophyletic. However, *Seicercus* and *Phylloscopus* were still, for twenty years, widely believed to be two distinct genera in the China checklist (Cheng, 1994, 2000; Zheng, 2005).

In our study, the maximum-likelihood and Bayesian analyses both suggest that *Seicercus* is a close relative of *Phylloscopus*, especially of *P. reguloides*, *P. coronatus*, *P. trochiloides*, *P. magnirostris* and *P. borealis.* Although only two species of *Seicercus* (*S. burkii* and *S. castaniceps*) were studied, we strongly support the idea that the monophyly of *Phylloscopus* is invalid (Olsson et al., 2004, 2005). Two species of *Seicercus* were grouped with five species of *Phylloscopus* (Clade B) and the largest genetic distance among them (0.117) was lower than the largest distance among 14 species of *Phylloscopus* (0.138). Furthermore, *Phylloscopus* and *Seicercus* species have many similar morphological characters, such as incompact feathers on forehead, prolonged shaft propers, many supplementaries before rectal bristles and twelve tail feathers. In view of this evidence, we support the viewpoint that *Phylloscopus* is non-monophyletic, which should include *Phylloscopus* and *Seicercus*, and suggest that *Phylloscopus* and *Seicercus* could be combined into one genus and that the complete species of these two former genera are necessarily involved in further review.

Relationships within *Phylloscopus*

The genus *Phylloscopus* has the most taxonomic problems. Little is known about this genus in China, except for the morphological review by Jia et al. (2003). As well, new species in *Phylloscopus* have frequently been found (Olsson et al., 2005; Martens et al., 2008; Päckert et al., 2009), e.g. twelve new species were found in China over a period of ten years during the last century (Irwin et al., 2001). Therefore, a taxonomic revision of some species and subspecies is still needed. However, taxonomic arrangements have traditionally relied on similarities in morphology and ecology (Cheng, 1994, 2000; Zheng, 2005). Based on DNA sequence data from our current study with strong support from some closely related species, allow us to cast new insights into the evolution of these birds.

We found two deeply distinct divergent clades (Clades A and B) of *Phylloscopus* in both maximum-likelihood and Bayesian trees (Figs. 1 and 2). Clade A includes two small clades: Clade A1 (including *P. collybita*, *P. fuscatus*, *P. occisinensis*, *P. schwarzi* and *P. armandii*) and Clade A2 (including *P. pulcher*, *P. maculipennis*, *P. proregulus* and *P. yunnanensis*). Clade B includes five species of *Phylloscopus* in a ML tree and there is a close relationship between *P. magnirostris* and *P. boreali*. Clade B is divided into two sister groups in a Bayesian tree: Clade B1 (including *P. reguloides*, *P. coronatus*, *Seicercus burkii* and *S. castaniceps*) and Clade B2 (including *P. trochiloides*, *P. magnirostris* and *P. borealis*). The positions of these species were stable and strongly supported by the trees. Our molecular results are also corroborated by some morphological and ecological characters. There are some morphological similarities of *P. magnirostris* and *P. boreali* among these three species, except for the sixth primary remiges, for example the olive green body and a pair of brown wings. In Clade A1, species *P. occisinensis*, *P. collybita*, *P. fuscatus*, *P. schwarzi* and *P. armandii* share the same morphological character (no stripes on the wings). Species *P. proregulus*, *P. pulcher*, *P. maculipennis* and *P. yunnanensis* in Clade A2 have some distinct morphological characters (one yellow caestus and two yellow stripes on the wings) and inhabit elevations above 1500 m a.s.l. Olsson et al. (2005) also supported the two close relations between *P. proregulus* and *P. maculipennis* and between *P. collybita* and *P. schwarzi* on the basis of DNA analysis (*cyt b*, 12S and myoglobin intron II). We may conclude then that there are close relationships between *P. magnirostris* and *P. borealis*, among *P. proregulus*, *P. yunnanensis*, *P. pulcher* and *P. maculipennis*, among the following five species, *P. occisinensis*, *P. collybita*, *P. fuscatus*, *P. schwarzi* and *P. armandii*. Because the Clade B1 and Clade B2 were not supported on the ML trees, the relationships among these species cannot be resolved in this study.

However, there are currently over 30 *Phylloscopus* species recognized in China and over 50 across the world (Monroe and Sibley, 1993; Zheng, 2005). Unfortunately, because only 16 representatives from *Phylloscopus* and *Seicercus* were included in this study, the validity of *Phylloscopus* is premature for a revision by us and the suggestion needs to be proven in future studies.

Taxonomic status of *Sylvia* and *Zosterops*

Monroe and Sibley (1993) considered the *Sylvia* genus within Sylviidae and *Zosterops* genus in Zosteropidae (both families in the superfamily Sylvioidea). Cheng (2000) placed *Sylvia* into Sylviinae under the family Muscicapidae and *Zosterops* into Zosteropidae. Mackinnon and Phillipps (2000) and Zheng (2005) also considered that *Sylvia* and *Zosterops* fell into two separate families, Sylviidae and Zosteropidae. Recently, a close association of *Zosterops* and *Sylvia* has been suggested by several studies on the basis of mitochondrial and nuclear DNA sequences (Barker et al., 2002, 2004; Cibois, 2003; Ericson and Johansson, 2003). Furthermore, Alström et al. (2006) showed that *Sylvia*, *Zosterops*, *Garrulax* and *Timaliini* are clustered within the same clade and suggested the name *Timaliidae* for this clade. Although few morphological similarities exist between *Zosterops* and *Sylvia*, their close relationship is strongly supported in our study, based on mitochondrial gene sequences. However, we have only one sample of *Zosterops* and two samples of *Sylvia.* A phylogenetic study of these two genera should be considered, at best, as uncertain, but needs to be undertaken in the future.

Relationships among *Cettia*, *Abroscopus* and *Tesia*

The genera *Cettia*, *Abroscopus* and *Tesia* were placed in Acrocephalinae by Sibley and Monroe (1990). Some taxonomists considered that *Cettia*, *Tesia* and *Urosphena* were near relatives, as were *Tickellia* and *Abroscopus*, but *Abroscopus* has not previously been considered to be closely related with *Cettia* and *Tesia* (Wolters, 1975–1982; Mayr and Cottrell, 1986; Sibley and Monroe, 1990; Inskipp et al., 1996; Dickinson, 2003). The study by Alström et al. (2006) of myoglobin intron II and mt-cytochrome *b* gene confirmed that *Cettia* was non-monophyletic and that there were near relationships among *Cettia*, *Tesia*, *Urosphena*, *Abroscopus* and *Tickellia*.

In this study, the *Tesia* and *Abroscopus* species grouped with four species from *Cettia*, forming a strongly supported clade (Figs. 1 and 2). The sequence divergence in *cyt b* between *Abroscopus albogularis* and *Cettia* species is from 0.121 (*A. albogularis* and *C. diphone*) to 0.151 (*A. albogularis* and *C. flavolivaceus*). However, the sequence divergence in *cyt b* between *A. albogularis* and the taxa of other genera in Sylviidae is from 0.131 to 0.192. Hence, the sequence divergences in *cyt b* between *A. albogularis* and *Cettia* species are smaller than those between *A. albogularis* and other generic species in Sylviidae. Similarly, the sequence divergences in *cyt b* between *T. castaneocoronata* and *Cettia* species are also smaller than those between *T. castaneocoronata* and other Sylviid genera. Although both trees depict *Cettia* as a monophyletic group, we await more study samples to clarify it as a monophyletic or non-monophyletic group.

Relations among other genera

Sibley and Monroe (1990) suggested that the Cisticolidae family included *Prinia* and *Cisticola* and that both *Orthotomus* and *Locustella* were placed into Acrocephalinae of Sylviidae. All the same, in some molecular studies, *Prinia*, *Orthotomus* and *Cisticola* have been found to be closely related, based on mitochondrial *cyt b* and 16S RNA (Cibois et al., 1999), mitochondrial ND2 and 12S RNA (Sefc et al., 2003) and nuclear RAG-1 and RAG-2 (Beresford et al., 2005). Nguembock et al. (2007) supported the placement of two *Orthotomus* species within the Cisticolidae. Alström et al. (2006) also supported this and suggested that *Prinia*, *Orthotomus*, *Cisticola* and other genera not studied here could be placed into Cisticolidae. Our results revealed a close relationship among *Prinia*, *Orthotomus* and *Cisticola* with good nodal support. Although *Locustella* species are clustered with a sister group comprising *Prinia*, *Orthotomus* and *Cisticola* in a terminal branch, the nodal support value derived by bootstrap of this clade is low in the maximum-likelihood analysis. Accordingly, more species from four genera *Locustella*, *Prinia*, *Orthotomus* and *Cisticola* are needed to resolve their evolutionary and phylogenetic relationships decisively.

Haffer (1991) suggested that *Acrocephalus* and *Locustella* are closely related, but that was disputed by Helbig and Seibold (1999). Leisler et al. (1997) and Helbig and Seibold (1999) proposed that *Acrocephalus* is non-monophyletic. In the present study, three members of *Acrocephalus* are clustered within the same clade with very high bootstrap and posterior probability. However, we have only a limited supply of samples and further extensive studies are therefore needed to review the taxonomic status and phylogeny of these two genera.

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基于线粒体基因*cyt b*和*COI*的莺科部分鸟类系统发育

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摘要：雀形目（Passeriformes）莺科（Sylviidae）鸟类广泛分布于旧大陆，该科许多种类的系统发育关系一直存在争议。本研究采用分子系统学方法，对莺科11属37种鸟类的*cyt b*全基因序列和*COI*部分基因序列进行系统发育分析，构建了ML和Bayesian系统发育树。结果显示，柳莺属（*Phylloscopus*）并非单系发生，鹟莺属（*Seicercus*）可能是其同类或其属下的一个类群；在柳莺属内，乌嘴柳莺（*P. magnirostris*）与极北柳莺（*P. borealis*）亲缘关系较近；黄腰柳莺（*P. proregulus*）、云南柳莺（*P. yunnanensis*）、橙斑翅柳莺（*P. pulcher*）及灰喉柳莺（*P. maculipennis*）亲缘关系较近；黄腹柳莺（*P. occisinensis*）、巨嘴柳莺（*P. schwarzi*）、棕眉柳莺（*P. armandii*）、叽喳柳莺（*P. collybita*）及褐柳莺（*P. fuscatus*）亲缘关系较近；树莺属（*Cettia*）并非单系发生，与拟鹟莺属（*Abroscopus*）和地莺属（*Tesia*）聚在一起；大苇莺属（*Acrocephalus*）为单性系；此外，林莺属（*Sylvia*）与绣眼鸟属（*Zosterops*）的亲缘关系、以及鹪莺属（*Prinia*）、缝叶莺属（*Orthotomus*）及扇尾莺属（*Cisticola*）三者间的亲缘关系也被支持。

关键词：系统发育，莺科，柳莺属，*cyt b*，*COI*，中国