INTRODUCTION

The Blackthroat Calliope obscura and Firethroat C. pectardens are two of the world’s rarest and least known ‘chats’ (Muscicapidae). They have been considered colour morphs of the same species (Firethroat, by priority), although they are nowadays usually treated as separate species. The taxonomic status of these two taxa is here investigated based on analyses of mitochondrial and nuclear DNA, vocalisations and reassessed distributions. Phylogenetic analysis confirms that they are sisters. Their genetic divergence (cytochrome b 6.4%, GTR+T+I corrected) is comparable to several other species pairs of ‘chats’. Discriminant function analysis of songs correctly classified 88% of the recordings. The breeding ranges appear to be mainly parapatric. Based on congruent differences in morphology, songs and molecular markers, it is concluded that Blackthroat and Firethroat are appropriately treated as separate species.

MATERIAL AND METHODS

Sequencing and phylogenetic analyses

Total genomic DNA was extracted from an adult male Blackthroat collected in Foping, Shaanxi province (33.693°N 107.849°E), in June 2011 (Institute of Zoology, Chinese Academy of Sciences, Beijing No. IOZ 62531) using the QIAamp DNA Mini Kit (Qiagen) following the manufacturer’s protocol.

Partial sequences of the mitochondrial cytochrome b and flanking RNA-Thr (hereafter cyt b) were obtained through PCR amplification. The primer pair L14851 and H16058 (Grotz 1998) was used for cyt b, with annealing temperature 46–48°C. We also amplified two nuclear markers, myoglobin intron 2 (Myo) and ornithine decarboxylase (ODC). Primer pair myo2 and myo3F (Kimball et al. 2009) were used for Myo, and primer pair OD6 and ODS8 for ODC (Allen & Omland 2003), with annealing temperatures 55°C and 59°C, respectively. PCR products were purified using QIAquick PCR purification Kit (Qiagen). Sequencing was carried out using an ABI 3730 automatic sequencer following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. Both strands were sequenced using the same primers as for PCR. All sequences have been submitted to GeneBank (Table S1). For the phylogenetic analyses, sequences of Firethroat and 10 other chats, all of which have been placed in the genus Luscinia (Dickinson 2003), and two more distant outgroup species (choice based on Sangster et al. 2010), were obtained from GeneBank (Table S1).

Sequences were aligned and checked manually in MEGA4 (Tamura et al. 2007). The phylogeny was estimated by Bayesian inference using MrBayes 3.2 (Huelsenbeck & Ronquist 2001, 2010; Ronquist et al. 2011). All loci were analysed separately, as well as concatenated in the multi-locus analyses, the data were partitioned by locus, using rate multipliers to allow different rates for the different partitions (Ronquist & Huelsenbeck 2003, Nylander et al. 2004). Appropriate substitution models were determined based on the AIC (Akaike information criterion: Akaike 1973) as calculated in MrModeltest2 (Nylander 2004). For cyt b, the general time-reversible (GTR) model (Laneave et al. 1984, Tavaré 1986, Rodríguez et al. 1990), assuming rate variation across sites according to a discrete gamma distribution with four rate categories (Γ; Yang 1994) and an estimated proportion of invariant sites (I; Gu et al. 1995), was selected. For Myo, the HKY model (Hasegawa
et al. 1985) + $\Gamma$ and for ODC the GTR + $\Gamma$ model were selected. Two simultaneous runs of four incrementally heated Metropolis-coupled MCMC (Markov Chain Monte Carlo) chains were run for 5 million generations and sampled every 1,000 generations in MrBayes. Convergence to the stationary distribution of the single chains was inspected using a minimum threshold for the effective sample size. Joint likelihood and other parameter values were inspected in Tracer 1.5.0 (Rambaut & Drummond 2009) and indicated large effective sample sizes ($>1,000$). Good mixing of the MCMC and search reproducibility were established by running each analysis at least twice, and topological convergence was examined by eye and by the average standard deviation of split frequencies ($<0.01$). The first 25% of the generations was discarded as "burn-in", well after stationarity of chain likelihood values had been established, and the posterior probabilities were calculated from the remaining samples.

Pairwise sequence divergences among all 12 chats were calculated in PAUP* (Swofford 2002) for all individual loci, following the recommendations of Fregin et al. (2012), i.e. by comparing homologous parts of the genes (same parts, same length), deleting all positions with any uncertain base pairs from the matrix, and using the best-fit model (same as the model used in phylogenetic analyses; choice of model determined in MrModeltest2 [Nylander 2004]) with the two distant outgroup species, Oriental Magpie Robin Copsychus malabaricus and Spotted Flycatcher Muscicapa striata, excluded). The shape parameter alpha and estimated proportion of invariable sites were obtained from a database of the birds of China (http://www.cnbird.org.cn/first.htm), Chinese bird gallery birdtalker/report/index.asp?lan=0), a database of the birds of China (http://www.wwfchina.org/birdgallery), Oriental Bird Images (http://orientalbirdimages.org), the Internet Bird Collection (http://ibc.lynxeds.com) and museum collections that we thought might hold specimens of Blackthroat or Firethroat (museums in the USA searched through ORNIS: http://ornis2.ornisnet.org).

RESULTS

Molecular analyses

We obtained 1,076 bp of cyt$\beta$, 664 bp (674 bp aligned) of Myo and 705 bp (737 bp aligned) of ODC from Blackthroat. The tree based on the concatenated sequences (Figure 1) showed Blackthroat and Firethroat to be sisters with strong support. These were, in turn, inferred to be sisters to Siberian Rubythroat Calliope calliope and White-tailed Rubythroat C. pectoralis, with high support. The sister relationship between Blackthroat and Firethroat was strongly supported in single-locus analyses of all three markers (not shown).

Figure 1. Blackthroat Calliope obscura and Firethroat C. pectardens are sister species, as shown in this phylogenetic tree of all Calliope species sensu Sangster et al. (2010) (grey shade) and a selection of outgroup species. The tree is based on concatenated mitochondrial cyt$\beta$ b and nuclear myoglobin intron 2 and ODC introns 6–7 sequences (see Sangster et al. 2010 and Zuccon & Ericson 2010 for a broader taxon sampling within Muscicapidae). Values at nodes represent Bayesian posterior probabilities; * indicates posterior probability 1.00.

The genetic divergences between Blackthroat and Firethroat (cyt$\beta$ 6.4%, Myo 0.32%, ODC 0.35%) were considerably lower in all three loci than in the majority of pairwise comparisons between the chats in the present dataset (Figure 2). However, they were comparable to the divergences between the well-accepted species pairs Larvivora cyaniceps/L. brunniceps, L. akahige/L. komadai, Luscinia luscinia/L. megarhynchos and Tarsiger rufilatus/T. hyperythrus (Figure 2).

Vocalisations

The song of Blackthroat consists of rather short, rapidly delivered, varied strophes that include both whistles and harsh notes, and masterful mimicry of other species (Song et al. in press; Figure 3). The song of Firethroat (Figure 3) is very similar, and due to the masterful mimicry of other species (Song et al. in press; Figure 3).

Distributions

Records of Blackthroat were taken from Song et al. (in press). To revise the distribution of Firethroat we reviewed the literature, as well as the BirdLife International species database (http://www.birdlife.org/), China Bird Report (http://birdtalker.net/birdtalker/report/index.asp?lan=0), a database of the birds of China (http://www.cnbird.org.cn/first.htm), Chinese bird gallery (http://www.wwfchina.org/birdgallery), Oriental Bird Images (http://orientalbirdimages.org), the Internet Bird Collection (http://ibc.lynxeds.com) and museum collections that we thought might hold specimens of Blackthroat or Firethroat (museums in the USA searched through ORNIS: http://ornis2.ornisnet.org).
them by ear or by sonograms. However, DFA correctly classified 88% of the recordings (Wilks’s Lambda = 0.379, Chi-square = 20.364, P = 0.000), and identified the top frequency and number of elements as the variables most important in the discrimination (Table 1).

Distributions
The breeding areas of Blackthroat and Firethroat appear to be mainly non-overlapping (Figure 4, Table S2). Blackthroat has been recorded at presumed or proven breeding localities in southern Shaanxi (Qinling Mountains), southern Gansu and northern Sichuan, whereas records of Firethroat at presumed breeding localities are from central Sichuan, north-western Yunnan, south-east Xizang (Tibet) and Arunachal Pradesh (a single record from latter area). One old and one recent record of Firethroat were made in Shaanxi, at localities where Blackthroat has been found to breed (Figure 4, Table S2). Records from wintering areas are completely segregated, with Blackthroat only observed in Thailand and Firethroat reported from north-east India, Bangladesh and northern Myanmar (Figure 4, Table S2). Observations of birds assumed to be migrating (at places unsuitable for breeding, during August to October and March to May) were made of Blackthroat in Thailand.

Figure 2. The genetic distances between Blackthroat Calliope obscura and Firethroat C. pectardens are comparable to the divergences between other chat sister species (highlighted by blue lines). The symbols represent genetic distances for all pairwise comparisons among the 12 chats in Figure 1; distances corrected (cyt b GTR + I, MyoHKY + I, ODC GTR + I). As expected, the mitochondrial cyt b is overall more divergent than the two nuclear introns Myo and ODC.

Table 1. Standardised canonical discriminant function coefficients for song variables in Blackthroat and Firethroat.

<table>
<thead>
<tr>
<th>Function 1</th>
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<tbody>
<tr>
<td>Mean low frequency</td>
<td>0.201</td>
</tr>
<tr>
<td>Mean top frequency</td>
<td>0.844</td>
</tr>
<tr>
<td>Mean delta time</td>
<td>-0.972</td>
</tr>
<tr>
<td>Mean number of elements</td>
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<tr>
<td>Eigenvalue</td>
<td>1.637</td>
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<tr>
<td>Variance explained</td>
<td>100%</td>
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</tbody>
</table>
DISCUSSION AND CONCLUSION

The close relationship between Blackthroat and Firethroat, which has been assumed by all previous workers (e.g. Ripley 1964, Dickinson 2003, Collar 2005, Sangster et al. 2010), is confirmed by the molecular data and further supported by the similarity in song between these two taxa. Their mainly parapatric distributions (see below) might call into question their status as separate species, and support earlier suggestions that they are conspecific (Goodwin & Vaurie 1956, Cheng 1958, Vaurie 1959, Etchécopar & Hüe 1983). However, the cyt$b$ divergence is actually greater than in the two sympatrically breeding species pairs *Luscinia luscinia*/*L. megarhynchos* and *Tarsiger hyperythrus*/*T. rufilatus*, and only marginally lower than the allopatric *Larvivora cyane*/*L. brunnea* and parapatric *L. akabige*/*L. komadori*. The cyt$b$ divergence also agrees well with pairwise comparisons between 69 parapatric, non-hybridising species (mean 6.17% Kimura 2-parameter [K2P] corrected distances: Aliabadian et al. 2009) (however, as different correction methods and different datasets have been used in these two studies, the genetic distances are not directly comparable; see Fregin et al. 2012). Although the present genetic analyses are based on only one individual per taxon, the cyt$b$ divergence between Blackthroat and Firethroat is far greater than normal intraspecific variation in birds (mean 0.74% K2P divergence in 656 species investigated by Aliabadian et al. 2009).

The plumage differences between Blackthroat and Firethroat are of a similar magnitude to the differences between *L. cyane* and *L. brunnea* and between *L. akabige* and *L. komadori*, and much more pronounced than the difference between *L. luscinia* and *L. megarhynchos*. The songs of Blackthroat and Firethroat may seem surprisingly similar for different species (see Alström & Ranft 2003). However, 88% of the analysed recordings were correctly identified by the DFA, and it should also be noted that especially *L. cyane* and *L. brunnea* have closely similar songs (Rasmussen & Anderton, 2012, pers. obs.; recordings on www.xeno-canto.org).

Based on present knowledge, the breeding distributions of Blackthroat and Firethroat appear to be mainly parapatric. There are two undocumented records of Blackthroat from potential breeding sites in Sichuan province, a female in June 1931 and a male in May 1991 (Song et al. in press). The latter is from Wolong, which is a stronghold for Firethroat, indicating potential sympatry. However, as both are undocumented, and the earliest record is the first report ever of a female Blackthroat, they should be considered uncertain. Moreover, a ‘May’ record could represent a bird on migration (Song et al. in press). According to Goodwin & Vaurie (1956), on 12 July 1905 a Firethroat and four male Blackthroats were collected at the same locality and by the same collectors on Taibai Shan, Shaanxi province. On 8 and 24 May 2013, a singing male Firethroat was observed in Changqing, Shaanxi province, in close proximity to singing Blackthroats (P. Morris and T. Townshend in litt.). These reports indicate sympatric breeding of the two taxa, although both Firethroat records could have concerned individuals that had strayed north of their usual breeding
range, and nothing is known of the interactions between Blackthroat and Firethroat in these places. Although hybridisation is a possibility, it seems unlikely in view of the large genetic distances between them.

In conclusion, the congruence between morphological, vocal, genetic and distributional data show that Blackthroat and Firethroat represent independently evolving lineages, and it is reasonable to treat them as separate species under both the ‘phylogenetic’ (Cracraft 1983, 1989) and ‘biological’ (Mayr 1942, 1963) species concepts. More research is needed on their distributions and possible geographical overlap, as well as on their numerical status and potential threats.

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REFERENCES


SUPPLEMENTARY ONLINE MATERIAL

Table S1. Sequences used in the present study
Table S2. Records of Firethroat

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