

Taxonomic status of Blackthroat *Calliope obscura* and Firethroat *C. pectardens*

PER ALSTRÖM, GANG SONG, RUIYING ZHANG, XUEBIN GAO, PAUL I. HOLT, URBAN OLSSON & FUMIN LEI

The Chinese endemic breeders 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+ Γ +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.

INTRODUCTION

The Blackthroat *Calliope obscura* and Firethroat *C. pectardens* are two of the world's rarest and least known 'chats'. Males are uniformly blue-grey above, with blackish tail with white sides basally. As the names imply, male Blackthroat has a black throat, breast and side of the head, whereas male Firethroat has a 'shining' orange throat and breast with black sides, and black sides of the head. Male Firethroat also has a small white patch on the side of the neck. Females are, as usual in chats, much more cryptically coloured (Meyer de Schauensee 1984, MacKinnon & Phillipps 2000, Collar 2005, Rasmussen & Anderton 2012, Song *et al.* in press). Both breed in the mountainous regions of central China, Firethroat also in south-east Tibet and perhaps Arunachal Pradesh (Meyer de Schauensee 1984, Cheng 1987, MacKinnon & Phillipps 2000, Collar 2005, Rasmussen & Anderton 2012, BirdLife International 2013a,b). The non-breeding ranges are poorly known, but there are records of Blackthroat from north-west Thailand and of Firethroat from north-east India, northern Myanmar and Bangladesh (Cheng 1987, Collar 2005, Rasmussen & Anderton 2012, BirdLife International 2013a,b). Both species were described in the late nineteenth century, but there have been rather few records since then, especially of the Blackthroat, whose breeding grounds were only rediscovered in 2011 (Song *et al.* in press). The Blackthroat is classified as Vulnerable and Firethroat as Near Threatened (BirdLife International 2013 a,b).

Blackthroat and Firethroat are usually placed in either *Luscinia* (Sibley & Monroe 1990, Dickinson 2003, Collar 2005, Gill & Donsker 2013) or *Erithacus* (Ripley 1964). However, *Luscinia sensu* Dickinson (2003) was recently shown to be non-monophyletic and proposed to be divided into the genera *Luscinia (sensu stricto)*, *Larvivora*, *Tarsiger* and *Calliope*, with Firethroat in the *Calliope* clade (Sangster *et al.* 2010). Blackthroat was not included in that study, but it was placed in *Calliope* due to its assumed close relationship with Firethroat (Sangster *et al.* 2010). The species status of Blackthroat has been questioned, and it has been considered a colour morph of Firethroat (Goodwin & Vaurie 1956, Cheng 1958, Vaurie 1959, Etchécopar & Hüe 1983). This view was rebutted by Ripley (1958) based on morphological differences, and later authors have treated it as a distinct species (e.g. Ripley 1964, Sibley & Monroe 1990, Dickinson 2003, Collar 2005, Gill & Donsker 2013). However, the relationship between the Blackthroat and Firethroat has not yet been properly studied. As both are very rare (BirdLife International 2013a,b, Song *et al.* in press), it would be helpful for conservation purposes if their taxonomic status could be clarified.

Here the taxonomic status of the Blackthroat and Firethroat is discussed, based on analyses of mitochondrial and nuclear DNA

and songs, and the distributions of the two species are reviewed. It is concluded that Blackthroat and Firethroat are best 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 tRNA-Thr (hereafter *cytb*) were obtained through PCR amplification. The primer pair L14851 and H16058 (Groth 1998) was used for *cytb*, 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 *OD8R* 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 GenBank (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 GenBank (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 *cytb*, the general time-reversible (GTR) model (Lanave *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) + Γ and for ODC the GTR + Γ 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 lengths), 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 Bayesian Inference, since PAUP* cannot estimate these parameters under the distance criterion.

Vocalisations

Songs of a total of 18 male Blackthroats (17 from Foping, one from Changqing) were recorded in June 2011 and May 2012 using a Marantz PMD661 solid state recorder and a Telinga Pro Twin Science microphone (five individuals, by PA), a Marantz PMD661 solid state recorder and Sennheiser MKH416 (five individuals, by XG), and a Sound Devices 722 hard drive recorder with a Telinga Pro 7 parabolic microphone (eight individuals, by PIH). These recordings were compared by ear and by inspection of sonograms to 11 recordings of songs of Firethroat, all from Sichuan, China (seven from Wolong, one from Longcangguo, two from Jiuding Shan and one from Hailuogou—six by PA, three by PIH and two from www.xeno-canto.org). Sonograms were produced in Raven Pro 1.4 (Cornell Laboratory of Ornithology 2012). The following measurements were taken on entire song strophes of 14 Blackthroats and eight Firethroats: Δ time (s) = duration; Δ frequency (Hz) = frequency range; minimum frequency (Hz); maximum frequency (Hz); and number of elements. Discriminant function analysis (DFA) of the song variables was carried out in SPSS Statistics version 20 (IBM Corp.); mean values for each male were used as input in these analyses, as there were few individuals but several different strophes recorded per individual. Three of PA's recordings of Blackthroat are available on-line at www.xeno-canto.org (XC91803, XC1804, XC143220) as well as www.slu.se/per-alstrom-research; two of PA's recordings of Firethroat are available on Xeno-canto (XC143218, XC143219); and all of PIH's recordings have been deposited at the National Sound Archive, London.

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

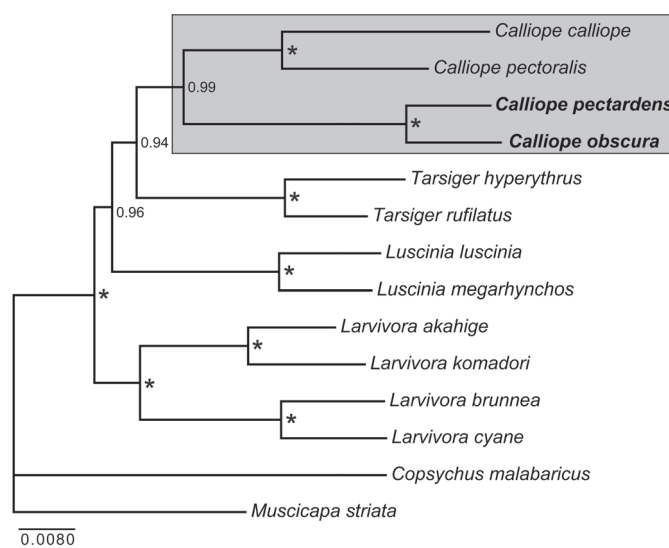
(<http://www.wwfchina.org/birdgallery>), Oriental Bird Images (<http://orientalbirdimages.org>), the Internet Bird Collection (<http://ibc.lynxeds.com>) and museum collections that we thought might hold specimens of Blackthroat or Firethroat (museums in the USA searched through ORNIS: <http://ornis2.ornisnet.org>).

RESULTS

Molecular analyses

We obtained 1,076 bp of *cytb*, 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 cytochrome *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 Muscipidae). Values at nodes represent Bayesian posterior probabilities; * indicates posterior probability 1.00.



The genetic divergences between Blackthroat and Firethroat (*cytb* 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 cyane*/*L. brunnea*, *L. akahige*/*L. komadori*, *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 complexity of the songs (including mimicry), fairly large repertoire sizes of individual males (Blackthroat 5–22 strophes recorded per male, mean 10.1 ± 4.2 ; Firethroat 4–30, mean 13.6 ± 7.9) and pronounced variation among males in both species, as well as limited sample sizes, we were unable to differentiate safely between

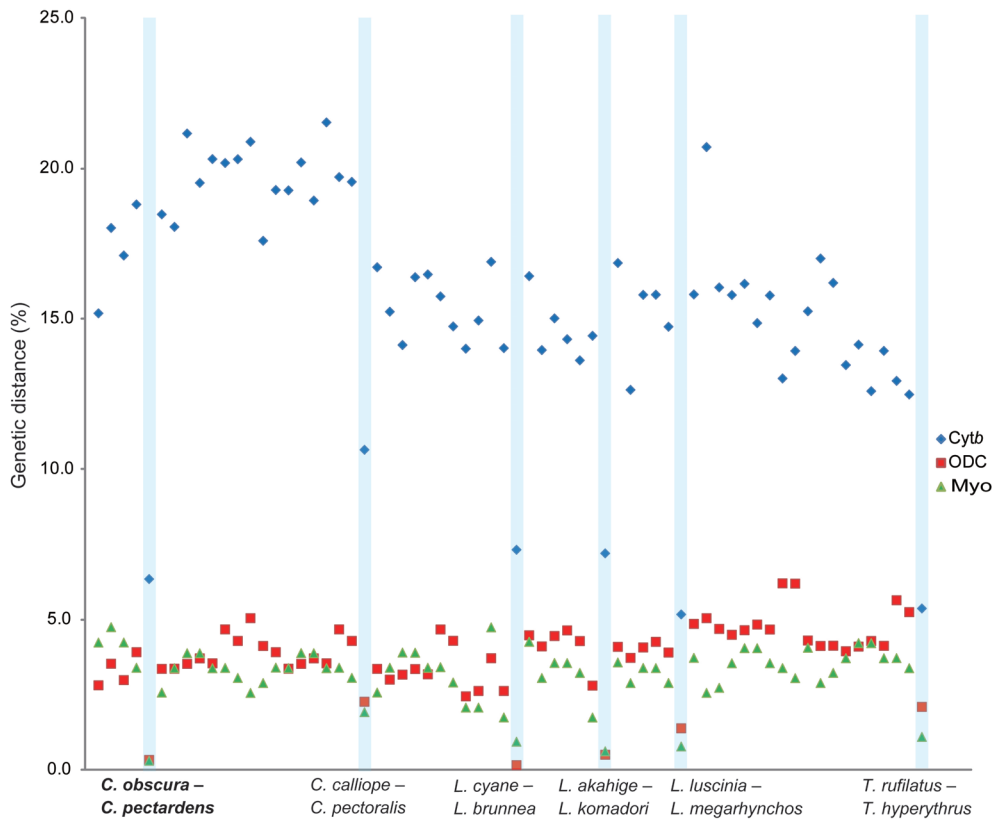
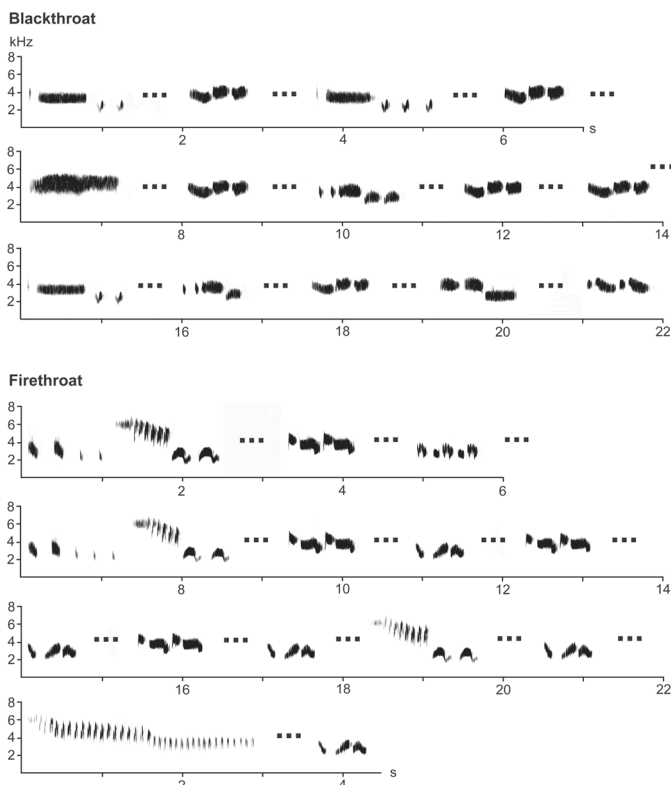


Figure 2. The genetic distances between Blackthroat *Calliope obscura* and Firethroat *C. pectardens* are comparable to the divergences between other chat sister species (highlighted by blue lines). The symbols represent genetic distances for all pairwise comparisons among the 12 chats in Figure 1; distances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

Figure 3. Songs of Blackthroat (top) and Firethroat are hard to distinguish owing to the fairly large repertoire sizes of individual males and extensive variation among males of both species (differences hence exaggerated in sonograms; a sonogram of another male Blackthroat appears in Song *et al.* in press). However, statistical analyses of songs reveal differences (see Table 1). Blackthroat recorded at Foping, Shaanxi province, China (June 2011) and Firethroat at Wolong, Sichuan province, China (May 1990), both by Per Alström (recordings available at www.xeno-canto.org, XC143220 and XC143219, respectively). Pauses between strophes have been shortened (indicated by dots).



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).

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

Function 1	
Mean low frequency	0.201
Mean top frequency	0.844
Mean delta time	-0.972
Mean number of elements	0.855
Eigenvalue	1.637
Variance explained	100%

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

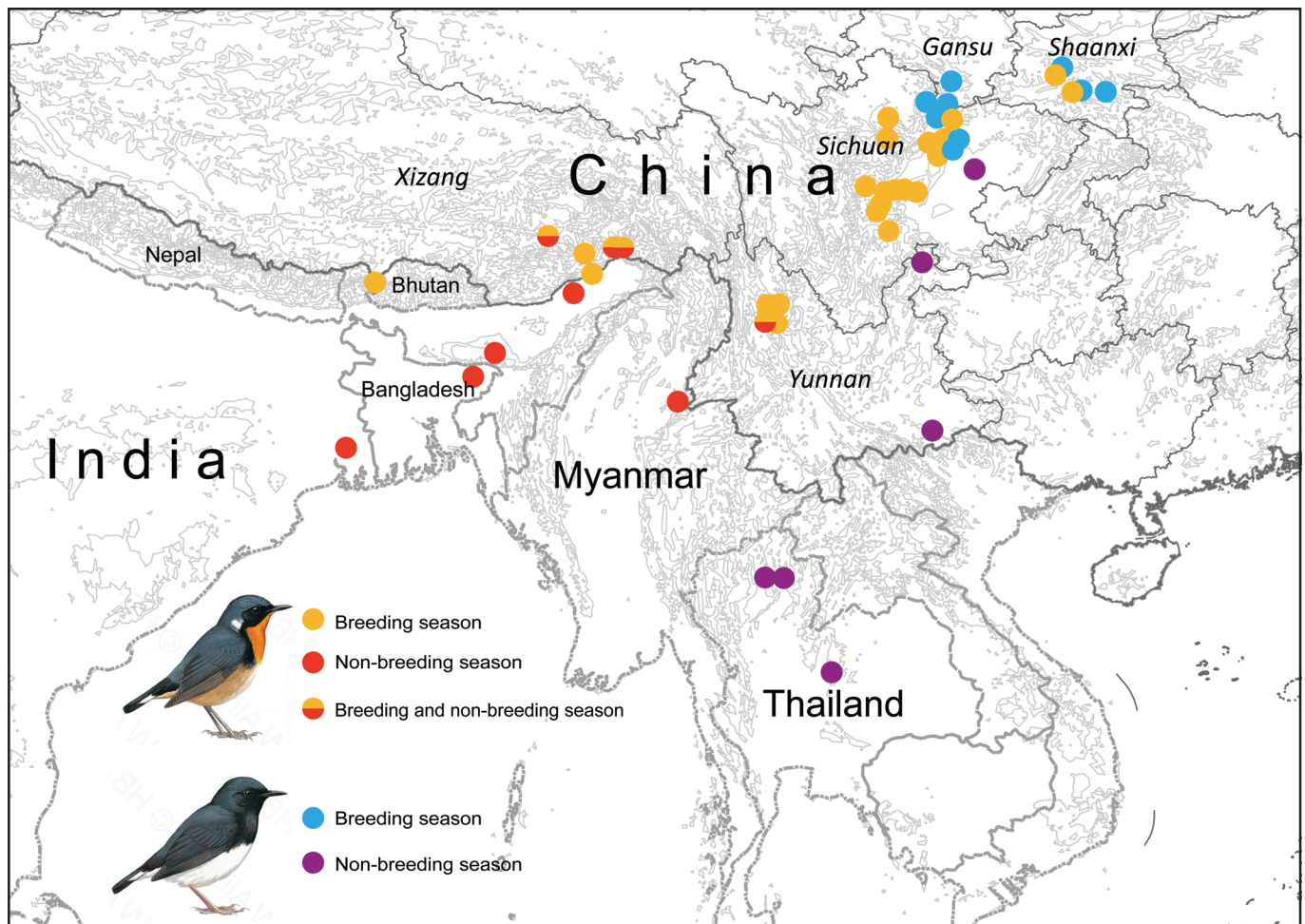


Figure 4. Blackthroat and Firethroat are mainly parapatric, with only limited evidence of sympatric breeding. Breeding season is defined as May–August, but records during this period of birds obviously on migration have been placed in the ‘non-breeding season’ category; for further details, see Table S2. Paintings by Hilary Burn from Collar (2005), reproduced with permission from the publishers.

and Yunnan, China, and of Firethroat in north-east India and Sichuan, China (Figure 4, Table S2).

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 & Hübner 1983). However, the *cytb* 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. akahige*/*L. komadori*. The *cytb* 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 *cytb* 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. akahige* 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. Townsend *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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- Per ALSTRÖM**, Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China; and Swedish Species Information Centre, Swedish University of Agricultural Sciences, Box 7007, SE-750 07 Uppsala, Sweden. Email: per.alstrom@slu.se (corresponding author)
- Gang SONG and Ruiying ZHANG**, Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China. Email: songgang@ioz.ac.cn, zhangry@ioz.ac.cn
- Fumin LEI**, Key Laboratory of Zoological Systematics and Evolution, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China; and School of Life Sciences, Shaanxi Normal University, Xi'an 710062, China. Email: leifm@ioz.ac.cn (corresponding author)
- Xuebin GAO**, Shaanxi Institute of Zoology, No. 88, Xingqing Road, Xi'an 710032, China. Email: gaodb63@163.com
- Paul I. HOLT**, Bracken Dean, Pendleton, Clitheroe, Lancashire, BB7 1PT, England. Email: piholt@hotmail.com
- Urban OLSSON**, Systematics and Biodiversity, Department of Biology and Environmental Sciences, University of Gothenburg, Box 463, SE-405 30 Goteborg, Sweden. Email: urban.olsson@bioenv.gu.se

SUPPLEMENTARY ONLINE MATERIAL

Available on Oriental Bird Club website, links at <http://www.orientalbirdclub.org/publications/forktail29>

Table S1. Sequences used in the present study

Table S2. Records of Firethroat