Molecular Phylogenetics and Evolution 58 (2011) 513–526

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Multilocus analysis of a taxonomically densely sampled dataset reveal extensive non-monophyly in the avian family Locustellidae

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ARTICLE INFO

Article history:
Received 12 October 2010
Revised 17 December 2010
Accepted 20 December 2010
Available online 15 January 2011

Keywords:
Species trees
Gene trees
Phylogeny
Concatenation
BEST
MrBayes
Classification

ABSTRACT

The phylogeny of most of the species in the avian passerine family Locustellidae is inferred using a Bayesian species tree approach (Bayesian Estimation of Species Trees, BEST), as well as a traditional Bayesian gene tree method (MrBayes), based on a dataset comprising one mitochondrial and four nuclear loci. The trees inferred by the different methods agree fairly well in topology, although in a few cases there are marked differences. Some of these discrepancies might be due to convergence problems for BEST (despite up to 1×10^9 iterations). The phylogeny strongly disagrees with the current taxonomy at the generic level, and we propose a revised classification that recognizes four instead of seven genera. These results emphasize the well known but still often neglected problem of basing classifications on non-cladistic evaluations of morphological characters. An analysis of an extended mitochondrial dataset with multiple individuals from most species, including many subspecies, suggest that several taxa presently treated as subspecies or as monotypic species as well as a few taxa recognized as separate species are in need of further taxonomic work.

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1. Introduction

The avian family Sylviidae (“Old World warblers”) has long been recognized as one of the main passerine families, although the composition has varied among authors. Traditionally, a large number of taxa were included, e.g. 60 genera and 358 species in the classification of Watson et al. (1986). Sibley and Monroe (1990), based on the DNA–DNA hybridization work by Sibley and Ahlquist (1990), split off Cisticolidae from Sylviidae, and further divided Sylviidae into the subfamilies Megalurinae, Acrocephalinae and Sylviinae. This was followed by Dickinson (2003) and Bairlein et al. (2006). Later studies, based on DNA sequence data, revised this classification. Alström et al. (2006) and Johansson et al. (2008) proposed recognition of a number of well supported major clades at family level. These authors synonymized Sylviidae with the family Timaliidae (“babblers”). Gelang et al. (2009), again based on DNA sequence data, resurrected Sylviidae, but restricted it to a clade containing mainly traditional Timaliidae species.

The subfamily Megalurinae sensu Sibley and Monroe (1990) contained the genera *Megalurus*, *Cincloramphus*, *Eremiornis*, *Amphilais*, *Megalurulus*, *Buettikoferella*, *Chaetornis*, *Graminicola* and *Schoenicola*. In contrast, the family Megaluridae sensu Alström et al. (2006) and Johansson et al. (2008) comprised the genera *Megalurus*, *Bradypterus*, *Locustella* and *Dromaeocercus*, i.e. including three of the genera placed in Acrocephalinae by Sibley and Monroe (1990). Other DNA sequence studies have shown that *Cincloramphus* and *Schoenicola* form a clade with *Bradypterus* and *Megalurus* (Beresford et al., 2005), while *Graminicola* belongs to the babbler family Timaliidae (Alström et al., 2006; Gelang et al., 2009). Beresford et al. (2005) also revealed that the aberrant *Bradypterus victorini* is not related to Megaluridae/Megalurinae.

The name Locustellinae Bonaparte, 1854, has priority over Megalurinae Blyth, 1875 (Bock, 1994: p. 152), and thus the family name Locustellidae Bonaparte, 1854 is applied in the present paper for Megaluridae sensu Alström et al. (2006) and Johansson et al. (2008). The relationships within this family are poorly known. Drovetski et al. (2004) used mitochondrial ND2 to study the relationships of all *Locustella*, two Asian and three African *Bradypterus*, and two *Megalurus*. They found the Asian *Bradypterus* and *Megalurus pryeri* nested within *Locustella*, the African *Bradypterus* in a separate clade, and *M. gramineus* on a branch on its own.

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1055-7903/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.ympev.2010.12.012

The species in Locustellidae are distributed across Africa, Eurasia and Australasia, frequenting mostly bushy, but sometimes also marshy, habitats from sea level up to above the tree limit (c. 4500 m in the Himalayas) (Bairlein et al., 2006). Most species are notoriously secretive and difficult to observe. All are non-descript, mostly various shades of brown above and at least slightly paler below; *Megalurus*, *Cincloramphus* and some *Locustella* are streaked above, some of these and some *Bradypterus* also on the underparts (Bairlein et al., 2006). *Cincloramphus cruralis* is exceptional in that the male is uniformly dark sooty brown below (Bairlein et al., 2006). Most species are fairly small, with an overall length of 13–16 cm, but some are considerably larger (22–28 cm in *Megalurus palustris*) (Bairlein et al., 2006). The songs are mostly simple but distinctive, and in general differ more than morphology among closely related species (Bairlein et al., 2006). Due to the generally cryptic plumages, there has been much confusion regarding species level taxonomy (e.g. Dickinson et al., 2000), and recent studies involving vocalizations and/or DNA have led to suggestions that some taxa currently treated as subspecies should be raised to the rank of species (e.g. Drovetski et al., 2004; Alström et al., 2008) as well as to the identification of a new cryptic species (Rasmussen et al., 2000).

In the present study, we infer the relationships of nearly all species in the family Locustellidae using one mitochondrial gene and four nuclear introns. We use traditional gene tree methods (Bayesian inference, maximum likelihood bootstrapping, parsimony bootstrapping) as well as a Bayesian species tree approach (Bayesian Estimation of Species Trees, BEST; Liu and Pearl, 2007; Liu, 2008) that accounts for lineage sorting processes that might produce discordance between gene trees. We also analyse mitochondrial DNA for a larger sample, comprising multiple individuals and several subspecies of polytypic species. A revised taxonomy is proposed based on our results.

2. Materials and methods

2.1. Study group

In total, we include 37 species from seven genera considered to belong to Locustellidae (=Megaluridae sensu Alström et al., 2006 and Johansson et al., 2008). Our sample comprises 16 species of *Bradypterus* plus cytochrome *b* (cyt*b*) sequences for three additional species (two from GenBank and one provided by Trevor Price and Udayan Borthakur; only two African and three Asian species are missing); all eight *Locustella* species; four *Megalurus* species plus cyt*b* for one more species (two species are lacking); both species of *Cincloramphus*; one of the two species of *Schoenicola*; and the monotypic genera *Dromaeocercus* and *Eremiornis*. For cyt*b*, we have in total 82 unique haplotypes, including 24 sequences from GenBank, comprising several taxa treated as subspecies of polytypic species. Sequences from four nuclear markers (ODC, myo, GAPDH, LDH) were obtained for most taxa (see Appendix A for details regarding loci coverage across the taxa).

Species level taxonomy follows Dickinson (2003) and Bairlein et al. (2006), with the exception of the recognition of *Bradypterus thoracicus kashmirensis* as a distinct species, based on a study of morphology, vocalizations and mitochondrial DNA (Alström et al., 2008).

2.2. DNA extraction and sequencing

DNA was extracted from blood, feathers, or muscle, using QIA Quick DNEasy Kit (Qiagen, Inc.) according to the manufacturer's instruction, but with 30 µl 0.1% DTT added to the initial incubation step of the extraction of feathers. We sequenced five loci: the main part of the mitochondrial cytochrome *b* gene and part of the flanking tRNA-Thr (cyt*b*); the nuclear ornithine decarboxylase exon 6 (partial), intron 6, exon 7, intron 7 and exon 8 (partial) (ODC); the entire nuclear myoglobin intron 2 (myo), the nuclear glyceraldehyde-3-phosphodehydrogenase intron 11 (GAPDH), and the complete nuclear lactate dehydrogenase intron 3 (LDH). Amplification and sequencing of cyt*b* and myo followed the protocols described in Olsson et al. (2005), of ODC Allen and Omland (2003), of GAPDH Fjeldså et al. (2003), and of LDH Fregin et al. (2009). Cyt*b* was amplified as one fragment to decrease the risk of amplifying nuclear pseudocopies (e.g. Sorensen and Quinn, 1998). DNA was also extracted from one museum specimen (*Schoenicola brevirostris*). For extraction, PCR-amplification, and sequencing procedures from this one, the procedures described in Irestedt et al. (2006) were followed, with specially designed primers obtainable from the authors upon request. All new sequences have been deposited in GenBank (Appendix A).

2.3. Phylogenetic analyses

Sequences were aligned using MegAlign 4.03 in the DNASTAR package (DNAstar Inc.); some manual adjustment was necessary for the non-coding sequences. For the nuclear loci, haplotypes were not separated, but coded as ambiguous bases.

Gene trees were estimated by Bayesian inference (BI) using MrBayes 3.1.2 (Huelsenbeck and Ronquist, 2001, 2005) according to the following: (1) All loci were analysed separately (single-locus analyses, SLAs). (2) Sequences were also concatenated, either all nuclear loci, or all loci together. In the multilocus analyses, the data were either (a) partitioned by locus, using rate multipliers to allow different rates for the different partitions (Nylander et al., 2004; Ronquist and Huelsenbeck, 2003), or (b) unpartitioned, using a homogeneous model for the entire dataset. In the analyses of all loci, species with missing data were included or excluded in various constellations. Ambiguous base pairs and indels were treated as missing data, but indels were plotted on the trees a posteriori. As outgroups, two species belonging to the family Bernieridae (*Hartertula flavoviridis* and *Thamnornis chloropetoides*) were chosen, as this family has been suggested to be sister to Locustellidae (Beresford et al., 2005; Johansson et al., 2008). Analyses were also run with 28 outgroup species, representing all families in the superfamily Sylvioidea (Alström et al., 2006; Johansson et al., 2008).

Appropriate substitution models were determined based on the Akaike Information Criterion (Akaike, 1974) and a hierarchical likelihood ratio test (Posada and Crandall, 1998), both calculated using MrModeltest2 (Nylander, 2004) in conjunction with PAUP* (Swofford, 2002). For all loci, posterior probabilities (PPs) were calculated under 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, for the cyt*b* data, also an estimated proportion of invariant sites (I; Gu et al., 1995). Default priors in MrBayes were used. Four Metropolis-coupled MCMC chains with incremental heating temperature 0.1 or 0.2 were run for 10–30 $\times$ 10^6 generations and sampled every 1000 generations. Chain likelihood and other parameter values and effective sample sizes (>200, generally >1000) were inspected in Tracer 1.5.0 (Rambaut and Drummond, 2009). The first 25% of the generations were discarded as "burn-in", well after stationarity of chain likelihood values had been established, and the posterior probability was estimated for the remaining generations. Every analysis was run at least twice, and the topologies and posterior probabilities compared by eye.

Species tree analysis was performed using Bayesian Estimation of Species Trees (BEST) 2.3 (Liu and Pearl, 2007; Liu, 2008). Only

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