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S17-5 The evolutionary history of Eurasian redstarts, *Phoenicurus*

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Abstract The bird genus *Phoenicurus* comprises 10 to 11 species that occur from Europe through Asia. To resolve their phylogenetic relationships, their phylogeography and the tempo of their evolution, the mitochondrial cytochrome-*b* gene was sequenced for 16 taxa. The Bayesian maximum likelihood tree and neighbor-joining dendrogram computed from the resulting data are nearly identical. The genus seems to have diverged from turdine stock approximately 5.25 ± 0.25 MYA in central Asia, and then underwent a rapid radiation during the recent orogenesis of the Himalayas. This radiation took place in two waves, at 4.9 ± 0.4 and 3.05 ± 0.35 MYA, producing a core-group represented by *P. auroreus*, *P. erythrogaster*, *P. hodgsoni*, *P. ochruros*, *P. phoenicurus* and *P. schisticeps*, and three more distantly related species: *P. caeruleocephalus*, *P. erythronotus* and *P. frontalis*. The latter species are about equally divergent from one another as well as from the core group.

Key words Cytochrome *b*, Molecular clock, *Phoenicurus*, Phylogeny, Central Asia

1 Introduction

The passerine genus *Phoenicurus* consists of 10 to 11 small to medium-sized species ranging throughout Europe and Asia, with foci in the Caucasus and the Himalayas/China where up to 6 species are sympatric. The phylogenetic relationships of this highly diverse, predominantly Sino-himalayan genus have not yet been worked out. This seems to be particularly important for the systematics of the group as hybridization in *Phoenicurus* seems to be a regular event and was observed quite often in *P. phoenicurus* and *P. ochruros*.

The traditional view that hybrids are a rare and irrelevant phenomenon in nature has changed in recent decades; and it is estimated today that approximately 10% of all bird species hybridize regularly (Grant and Grant, 1992). Within *Phoenicurus*, moreover, it does not seem to be a trivially infrequent event. Similarities in natural hybrids between the Redstart (*P. phoenicurus*) and Black Redstart (*P. ochruros*) in Europe to several subspecies of the Black Redstart in the Middle East and east Asia (Kleinschmidt, 1908; Landmann, 1987) suggest that historic hybridization may be involved in the origin of those forms. These morphological similarities have been reflected in breeding experiments (Berthold et al., 1996), where hybrids and backcrosses have been studied for several years.

Additional breeding experiments (Görl, unpublished) and results from the following microsatellite-analysis further indicate that hybridization is also found occasionally between *P. hodgsoni*/*P. auroreus* and *P. hodgsoni*/*P. ochruros phoenicuroides* or *P. o. rufiventris*. Because of many intermediate forms, especially in the subspecies of *P. ochruros* in Turkey, the Middle East and Asia, it is often difficult to identify single individuals from morphology alone. The sequence data presented here now permit estimation of phylogenetic relationships among the taxa and determination of whether those that hybridize are distinct species.

2 Materials and methods

2.1 Sampling

Blood samples where collected from across Europe and on two expeditions to Asia in 1996 and 1997. Asian locations were east Turkey where populations of the Common Redstart (*P. phoenicurus*) and Black Redstart (*P. ochruros*) were the focus of sampling, and Sichuan and Gansu Provinces in China, where many other members of the genus were collected. Birds were caught with mist nets, scored for morphological characters and sampled for blood by extraction from the ulnar (wing) vein. Additionally, diverse tissue samples (feathers, skin, sole-pads, tarsus, tongue) were taken from specimens in museum collections in Berlin, Bonn, Frankfurt, Munich and Vienna.

2.2 Laboratory procedures

PCR was used on 75 blood samples to amplify the cytochrome *b* gene in mitochondrial DNA (mtDNA). Depending on the nature of the sample, total cellular mtDNA was extracted from over 500 samples with either QIAGEN-extraction-kits or with phenol/chloroform-, ethanol/isopropanol (with and without guanidine)-, or chelex- extraction-protocols. Seventy five samples were then chosen for sequencing reactions, representing all available taxa and populations. To minimize the risk of sequencing fragmented chromosomal-copies of the mitochondrial genome (Quinn,

1992; Smith et al., 1992; Kornegay et al., 1993), the whole gene was amplified first with the amplification-primers A and F (Helbig et al., 1995), yielding a PCR product of approximately 1 000 bp for the cytochrome *b* gene. After isolation and purification of the PCR-product, sequencing followed using an ABI 377 cycle-sequencer with internal primers B, D and G, which guarantee further control because of overlapping regions.

2.3 Phylogenetic analysis

To identify pseudogene PCR products, the sequences were edited and inspected for stop-codons or unusual transition/transversion ratios. Phylogenetic analysis was performed using the programs MEGA 2.0 for the neighbor-joining dendrogram (Kimura-2-Parameter and pairwise distance method; Kumar et al., 2001) and MrBayes 2.01 (Huelsenbeck et al., 2001) for the maximum likelihood tree (Monte-Carlo-Markov-Chain algorithm; MCMC). Outgroups were *Acrocephalus scirpaceus* and *Sylvia atricapilla*.

3 Results

3.1 Variability in cytochrome b gene sequences

280 out of 398 variable nucleotide positions were phylogenetically informative and usable for tree construction. The analysis yielded distance values of up to 4.0% at subspecies level, with *rufiventris* in *P. ochruros* the most divergent subspecies at 2.3%–4.0%. *P. phoenicurus samamisicus* from East Turkey differed by 2.3 % from European nominate *phoenicurus*; and central Asian *P. erythrogaster grandis* also differed from nominate *erythrogaster* in the Caucasus by the same value. Interspecific distance values ranged from 4.9% to 11.3%, and generally matched expectations from morphological studies. Most surprising was the unexpectedly high divergence value of 10.5% between the two populations of *P. frontalis* in Gansu (China) and Nepal.

3.2 Variability in amino acid sequences

In contrast to synonymous substitutions, the distribution of non-synonymous substitutions shows a bias, revealing highly variable areas in the transmembrane domains, the inner regions of the cytochrome *b* protein, and more conservative redox-centers of the molecule (Howell, 1989). Within the genus *Phoenicurus*, the total number of non-synonymous substitutions ranges from 0–3 at subspecies level and 0–14 at species level. *P. schisticeps* (4–11), *P. caeruleocephalus* (5–14), and the two *P. frontalis* populations (7–14) show particularly high numbers of amino-acid replacements. The distantly related outgroup taxa, *Acrocephalus scirpaceus* and *Sylvia atricapilla*, showed

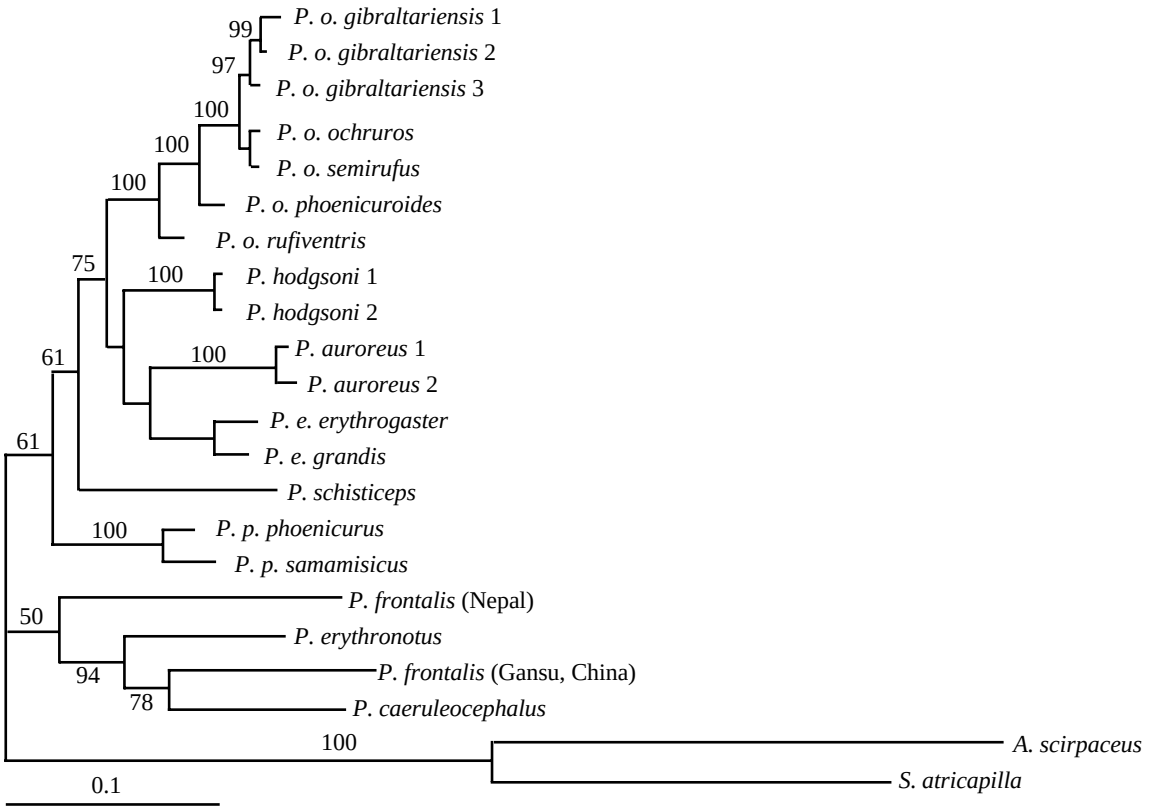

Fig. 1 Bayesian maximum likelihood tree of phylogentic relationships of taxa in the genus *Phoenicurus* (P.), based on mtDNA sequence data from the cytochrome-b gene

Program MrBayes 2.01 here uses the Monte-Carlo-Markov-Chain algorithm (MCMC) to calculate the statistical support (indicated in numerals against branches). o = *P. ochruros*, e = *P. erythrogaster*, p = *P. phoenicurus*. For identity of outgroups *A. scirpaceus* and *S. atricapilla*, see text.

the highest number of substitutions (24–37), as expected.

4 Discussion

The topology of the resulting Bayesian maximum likelihood tree and neighbor-joining dendrogram (Figs. 1, 2) are nearly identical, with most branches supported by high statistical values. In the dendrogram, branches with bootstrap values under 60 were collapsed, which resulted in improved concordance with the Bayesian tree. Each species represented by 2 or more unique sequences was resolved as a monophyletic group, except *P. frontalis*. The cytochrome-*b* sequences also confirmed that the hybridizing taxa (Ertan, 2000) are distinct species. *P. ochruros*, *P. phoenicurus*, *P. hodgsoni*, *P. auroreus*, *P. erythrogaster* and *P. schisticeps* form a core group in the genus, with uncorrected distance values of 4.9% to 7.9%. *P. caeruleocephalus*, *P. erythronotus* and the two populations of *P. frontalis* are about equally divergent from both one another and the core-group (7.3%–11.1%). This result is concordant with morphological studies, which also separate these three (four) species from the rest of the genus (Alí and Ripley, 1987; Glutz von Blotzheim and Bauer, 1988). The significance of the unexpectedly high divergence (10.5%) between the two populations of *P. frontalis* from Gansu and Nepal, which are not monophyletic on either the Bayesian tree or neighbor-joining dendrogram, still needs elucidation.

Early research on a number of mammalian lineages revealed corrected mitochondrial third-position divergence rates of approximately 10% per million years (Irwin et al., 1991; Thomas and Martin, 1993). Other studies, however, have shown lower rates, as in whales (Martin and Palumbi, 1993), consistent with considerable evidence suggesting that molecular evolutionary rates vary among taxonomic lineages (Britten, 1986; Li et al., 1987) and are much lower in birds (Martin and Palumbi, 1993). Therefore it is not surprising that studies calibrating molecular clocks for mitochondrial DNA have resulted in different evolutionary rates (of 0.4% – 2%, uncorrected and 1.6% – 2.9%, corrected per million years) for several bird orders (Arnnaiz-Villena et al., 1999; Helm-Bychowski and Wilson, 1986; Klicka and Zink, 1997; Krajewski and King, 1996; Nunn et al., 1996; Tarr and Fleischer, 1993). Most convincing and applicable here are those studies that calibrated long fragments of the cytochrome-*b* gene against the fossil record. These record average evolutionary rates of 0.4% to 1.7% per million years for the Galliformes (chicken and pheasants), Gruiformes (cranes) and Procellariformes (albatrosses and petrels).

According to such rates, the genus *Phoenicurus* would have diverged from other turdine stock 6–25 million years ago (MYA). Because most redstarts are centered in the high mountain regions of central Asia, it is more likely that their radiation occurred more recently, driven by isola-

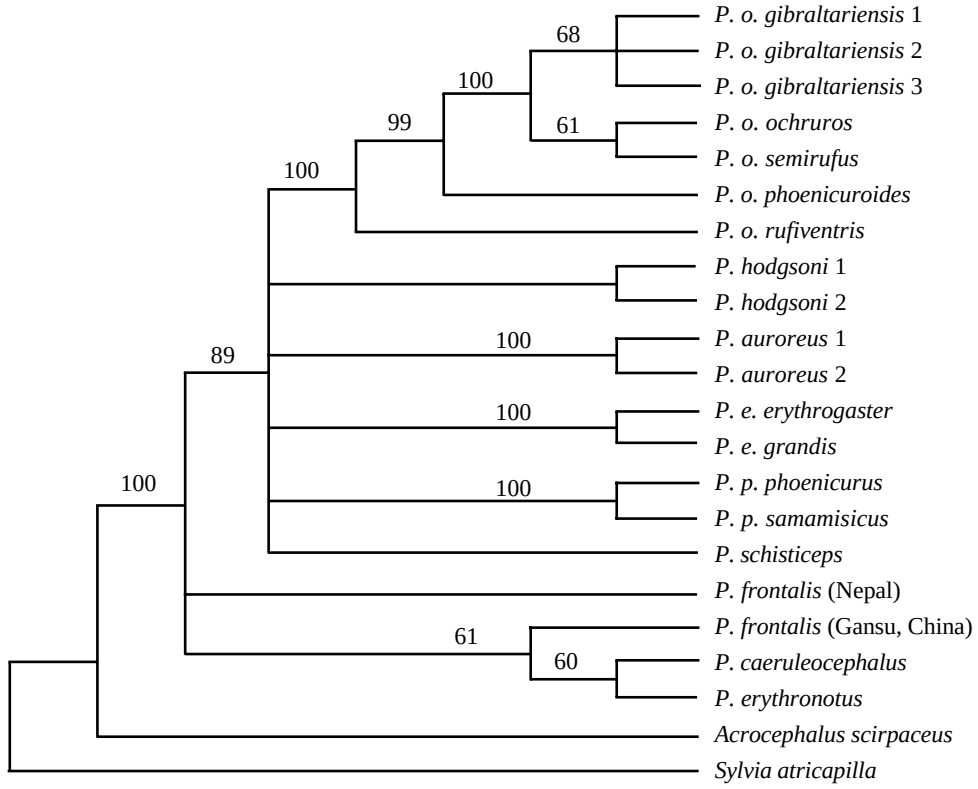

Fig. 2 Neighbour-joining dendrogram of taxa in the genus *Phoenicurus* (P.), based on mtDNA sequence data of the cytochrome-*b* gene

Tree generated with MEGA 2.0 (Kimura-2-parameter and pairwise distance method). Branches with bootstrap values lower than 60 are collapsed.

tion resulting from the rapid Himalayan orogenesis around 5 MYA. Calibrating their highest mtDNA distance values with this geological event would result in average evolutionary rates of approximately 2% per million years, a value was also recorded in passerines using restriction enzymes (Tarr and Fleischer, 1993). This would shift the second wave of radiation within the genus, that of the core group, into a time frame of 3.05 ± 0.35 MYA.

Judged from mtDNA distance values, proto western subspecies of the Black Redstart (proto *semirufus*, *ochruros*, *gibraltariensis* and *aterrimus*) were separated from central Asian stock (proto *phoenicuroides* and *rufiventris*) 1.5 ± 0.25 MYA, and began to expand westwards in distribution. In the course of this expansion, subsequent populations were cut off in the Middle East (proto *semirufus*) and East Turkey (proto *ochruros* and *semirufus*) around 1.15 ± 0.58 and 0.53 ± 0.16 MYA respectively, until finally central Europe was reached (Ertan, 2002). The central European population (*gibraltariensis*) and morphologically quite different Iberian population (*aterrimus*) probably diverged from one another when separated during the last ice age. The central European stock probably withdrew to ice-free parts of south and southeast Europe then, until the onset of warmer postglacial periods allowed expansion once more. Reports of historical shifts in the European avifauna support this reconstruction. Indeed, general northwards expansion is still in progress, the Black Redstart itself gradually occupying northwest Europe as a breeding bird over the last 180 years (Glutz von Blotzheim and Bauer, 1988).

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