Records of Paddyfield Warbler *Acrocephalus agricola* in Turkey and evidence for a monotypic taxon

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The Paddyfield Warbler *Acrocephalus agricola* has a patchy distribution in eastern Europe and eastern/central Asia. The nominate subspecies *agricola* is thought to breed from the Caspian sea and Aral lake eastwards through Kazakhstan to western Mongolia and northwest China and southwards to Tajikistan, eastern Iran, northern Afghanistan and western Pakistan. The subspecies *septimus* should breed on the coast of the Black sea in Bulgaria, Romania and eastwards through southern Ukraine until the Ural mountains and river Ilek (Kennerley & Pearson 2010). Some authors have even introduced a third subspecies *A. a. capistratus* (Severtsov, 1873), which is however often included in *A. a. agricola*.

Paddyfield Warblers have been recorded from Turkey but their taxonomic status has not been elucidated (Kirwan *et al* 2008a). It is uncertain which of the two putative subspecies breed there *ie agricola* or *septimus* (Kennerley & Pearson 2010). Based on photographs of Paddyfield Warblers at lake Van 8 and 9 May 1987 (van den Berg & Bosman 1988), there was speculation that Turkish birds belong to subspecies *agricola* (Kirwan *et al* 2008a). However, a bird caught and photographed 22 September 1991 in the Göksü delta showed greyish-white underparts and not the typical reddish-orange colouration of the nominate subspecies (van der Have & van den Berk 1995, Kirwan *et al* 2008a). In addition, a number of quite good photos taken in Turkey are at www.surfbirds.com/gallery, most however without mentioning a subspecies.

Studying birds at lake Van (marshes east side of the lake, 38° 29′ 58.25″ N, 43° 19′ 13.94″ E, 1648 m ASL, Plate 1), JH caught four Paddyfield Warblers 26 June 2012. Measurements, photos and blood samples were taken. These data were evaluated to help unravel the

Plate 1. Reeds at the eastern shore of lake Van, east Anatolia, Turkey, 26 June 2012. © *Jens Hering*

taxonomic status of Turkish Paddyfield Warblers. The DNA analysis included Paddyfield Warblers of both subspecies coming from different parts of the distributional range. Previous phylogenetic analyses in the Heidelberg laboratory had suggested that both subspecies can be distinguished by distinctive sequence differences in the mitochondrial cytochrome *b* gene (Leisler *et al* 1997). We expected that allocation of these Turkish birds to one of the subspecies would be straightforward. However, we obtained the surprising result that the two subspecies cannot be distinguished by their mtDNA sequence data nor morphology indicating that the taxon is monotypic.

MATERIALS AND METHODS

Birds were caught using mist nets/tape recordings (recordings from JH and those of Schulze 2003). Biometric data were collected according to the recommendations of the German Ornithologists Union (DO-G 2011). In the vicinity, several other reed warbler species were seen or heard (*Acrocephalus scirpaceus*, *A. melanopogon*, *A. arundinaceus*).

DNA analysis: Samples from *A. agricola* came from different populations located in eastern Europe and Asia (Table 1). The DNA was obtained from blood samples stored in a modified EDTA buffer at −20°C until processing in the Heidelberg laboratory. Total DNA was isolated using standard proteinase K (Merck, Darmstadt) and phenol/chloroform procedures.

We amplified a fragment of the mitochondrial cytochrome *b* gene (cyt *b*) (> 940 nt) as an informative marker gene which has been used by MW before for phylogeny reconstruction of reed warblers (Leisler *et al* 1997, Arbadi *et al* 2014a, b). The PCR amplifications of cyt *b* and COI were performed in 50 µl reaction volumes containing 1 × PCR buffer (Bioron, Ludwigshafen), 100 µM dNTPs, 0.2 units of *Taq* DNA polymerase (Bioron, Ludwigshafen), 200 ng of DNA and 5 pmol of primers (as in Arbadi *et al* 2014a, b). Thermal cycling was carried out under the following conditions: 5 min at 94°C, followed by 35 cycles of 40 s at 94°C, 40 s at 52.0°C, 1 min at 72°C and a final extension at 72°C for 10 min. PCR products were precipitated with 4 M NH_4Ac and ethanol (1:1:6) and a centrifugation for 15 min (13 000 rpm). Sequencing was performed using the ABI 3730 automated capillary sequencer (Applied Biosystems, CA, USA) with the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit 3.1 (carried out by STARSEQ GmbH, Mainz, Germany). For sequencing, the same primers were used as for the initial PCR amplifications.

The nucleotide sequences were aligned manually with BioEdit version 7.0.9.0. No internal stop codons or frame-shifts were observed in the sequences that were translated entirely by using the chicken mitochondrial code. Phylogenetic trees were reconstructed using Maximum likelihood (ML) in MEGA version 5.2 (Tamura *et al* 2011) with related *Acrocephalus* species as outgroups.

Table 1. Origin of Paddyfield Warblers *Acrocephalus agricola* used for DNA analysis. Song types: A = partly rather similar to *A. schoenobaenus*; B = most similar to *A. palustris*; C = partly rather similar to a fast singing *A. scirpaceus*; D = different to A–C.

Taxon	Song type	Number of samples	Origin	Collector
Acrocephalus agricola				
A. a. septimus	B	9	Charkiv region, NE Ukraine	A Poluda
	A	10	Odessa, SW Ukraine	A Poluda
A. a. agricola	D	5	W Mongolia	A Bräunlich
	C	10	Omsk region, W Siberia	Y Redkin, M Kalyakin
	B	2	Lake Alakol, Kazakhstan	G Nikolaus
?		3	Lake Van, Turkey	J Hering

Plates 2–4. Paddyfield Warblers *Acrocephalus agricola* caught at lake Van 26 June 2012 and used for DNA studies. © *Jens Hering*

Robustness of nodes was assessed by 1000 bootstrap replications. Sequence data have been submitted to GenBank (accession numbers KR181847–KR181885).

RESULTS

The four Paddyfield Warblers caught at lake Van were two males and two females with pronounced brood patches (Plates 2–5). Therefore, we consider that the birds were breeders. The plumage of all Paddyfield Warblers was worn out, which made a biometric analysis problematic.

DNA analysis: We had samples from the two putative subspecies *A. a. septimus* (n = 22) and *A. a. agricola* (n = 17). As expected *A. agricola* clusters as a sister with *A. concinens* (Leisler *et al* 1997) (Figure 1). To our surprise, there was no consistent difference between

Figure 1. Phylogeny and phylogeography of Paddyfield Warblers based on a ML analysis of the mitochondrial cytochrome *b* gene. Outgroup sequences (identified by an appropriate accession number) were retrieved from GenBank: *Acrocephalus concinens* (FJ88027, Fregin *et al* 2009), *A. scirpaceus* (NC0100227, Singh *et al* 2008), *A. palustris* (AJ004774, Helbig & Seibold 1999), *A. paludicola* (AJ004292, Leisler *et al* 1997) and *A. dumetorum* (AJ004773, Helbig & Seibold 1999). Number at the nodes are Bootstrap values from 1000 replications.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (–2708.3223) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2849)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 14.7058% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 44 nucleotide sequences. Codon positions included were 1st+2nd+3rd position. All positions with less than 95% site coverage were eliminated. There were a total of 940 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.2 (Tamura *et al* 2011).

Table 2. Haplotype variation in Paddyfield Warblers. Documentation of variable sites in the cyt *b* dataset. Numbers refer to IPMB accession numbers. '.'= identical base to the sequence in the top lane. ? = unresolved nucleotide.

#A. a. _agricola_6579_Alakol_Kazakhstan	C C T C T C A G A C	C G G G G T G G A T	T
#A. a. _agricola_60253_Russia_Omsk	. . . . C	. . . .	.
#A. a. _agricola_60250_Russia_Omsk	. . . . C	. . . .	.
#A. a. _agricola_60251_Russia_Omsk	. . . . C	. . . .	.
#A. a. _agricola_60256_Russia_Omsk	. . . . C	. . . .	.
#A. a. _agricola_60027_W_Mongolia	. . . . C	. . . .	.
#A. a. _septimus_60019_Odessa_Ukraine	. . . . C	. . . .	?
#A. a. _septimus_60019_Odessa_Ukraine	. . . . C	. . . .	?
#A. a. _agricola_60030_W_Mongolia	. . . . C	. . . .	.
#A. a. _septimus_60013_Odessa_Ukraine	. . . . C	. . . .	.
#A. a. _septimus_60014_Odessa_Ukraine	. . . . C	. . . .	.
#A. a. _septimus_60016_Odessa_Ukraine	. . . . C	. . . .	.
#A. a. _septimus_60236_Ukraine_Charkiv	. . . . C	. . . .	.
#A. a. _septimus_60238_Ukraine_Charkiv	. . . . C	. . . .	?
#A. a. _septimus_60239_Ukraine_Charkiv	. . . . C	. . . .	.
#A. a. _septimus_60240_Ukraine_Charkiv	. . . . C	. . . .	.
#A. a. _septimus_60243_Ukraine_Charkiv	. . . . C	. . . .	?
#A. a. _septimus_60244_Ukraine_Charkiv	. . . . C	. . . .	.
#A. a. _septimus_60019_Odessa_Ukraine	. . . . C	. . . .	?
#A. a. _Turkey_65875	. . . . C	. . . .	.
#A. a. _agricola_6601_Alakol_Kazakhstan	. . . . C	. . . .	G
#A. a. _septimus_60015_Odessa_Ukraine	. . . . C	. . . .	G
#A. a. _agricola_60252_Russia_Omsk	. . . . C	. . . A	.
#A. a. _agricola_60254_Russia_Omsk	. . . . C	. . . A	.
#A. a. _agricola_60247_Russia_Omsk	. . . . C	. . . . A . C	?
#A. a. _agricola_60028_W_Mongolia	. . . . C	. . . . . C	.
#A. a. _agricola_60032_W_Mongolia	. . . . C	. . . . . C	.
#A. a. _agricola_60249_Russia_Omsk	. . C . C	. A . . . C	.
#A. a. _agricola_60248_Russia_Omsk	. . . . C	. . A	.
#A. a. _agricola_60255_Russia_Omsk	. . . . C	T	.
#A. a. _agricola_60031_W_Mongolia	. . . . C	. . . . C .	.
#A. a. _septimus_60010_Odessa_Ukraine	. . . . C	. . . A	.
#A. a. _septimus_60011_Odessa_Ukraine	. . . . C	. . . . . . A . . .	.
#A. a. _septimus_60012_Odessa_Ukraine	. . . . C T	. . . .	?
#A. a. _septimus_60017_Odessa_Ukraine	. . . . C T	. . . .	.
#A. a. _septimus_60020_Odessa_Ukraine	. . . . C . . A . .	. . . . A . . A . C	?
#A. a. _septimus_60235_Ukraine_Charkiv	. T . . C	. . . .	.
#A. a. _septimus_60237_Ukraine_Charkiv	. . . . C . G . . .	. . . .	.
#A. a. _septimus_60242_Ukraine_Charkiv	. . . . C . G . . .	. . . .	?
#A. a. _Turkey_65873	T . . . C	. . . .	.
#A. a. _Turkey_65874	. . . T C . . . G T	. . . A	.

both subspecies as can be seen from a haplotype analysis (Table 2) and the phylogeny reconstruction by maximum likelihood (ML) (Figure 1). An identical result was obtained from an analysis using the mitochondrial COI gene (data not shown). Twenty-one variable sites were found in the cytochrome *b* data set (Table 2). Individuals differed by one or a

few single nucleotide exchanges indicating a high degree of gene flow within this species. Twenty birds, which include both subspecies and one of JH's birds from Turkey show an identical haplotype, which would be quite atypical if two distinct subspecies exist (Arbadi *et al* 2014a, b).

As can be seen from Table 2 and Figure 1, the genetic analysis does not give evidence for two distinct subspecies nor allows subspecies allocation of the Turkish birds (only three of the four birds caught by JH produced DNA sequences; in one the DNA had apparently been degraded).

DISCUSSION

Paddyfield Warblers were seen the first time in Turkey in May 1986 at lake Van and observed almost yearly there during the breeding season (van den Berg & Bosman 1988, van den Berk *et al* 1993, Kirwan *et al* 2008a, b). However, a breeding record (nests, feeding adults) has not been obtained there yet or elsewhere in Turkey. A fledgling was recorded at lake Van 23 June 1994 (Kirwan *et al* 2008b). JH's record of two females with brood patches is a strong indication that the Paddyfield Warbler breeds in Turkey.

Plate 5. Female *Acrocephalus agricola* caught at lake Van 26 June 2012 and showing an obvious brood patch. © *Jens Hering*

We were surprised that the genetic evaluation of a larger set of Paddyfield Warblers coming from areas inhabited by both subspecies, did not show any significant or distinctive differentiation. In the initial work (Leisler *et al* 1997) only a few specimens were available, which came from Crimea (presumably *septimus*) and eastern Kazakhstan (presumably *agricola*). Because the sequence divergence between the samples was quite high (4.5%) it was concluded that both subspecies deserved distinct taxonomic status, although it is assumed that they cannot be distinguished by morphological data. As a consequence of the DNA analyses, which had been summarized by Sangster (1997), Kennerley & Pearson (2010) treated *A. agricola* as a polytypic species. Our new analyses, based on a large data set, clearly show that the analyses reported in Leisler *et al* (1997) must have been wrong. It is difficult to identify the source of error almost 18 years later: it could have been a wrongly labelled sample, but more likely a DNA sequence which came from a nuclear copy of the mitochondrial cyt *b* gene (such nuclear copies are sometimes amplified by PCR and can give misleading results).

The low number of haplotypes within the species indicates recent speciation and range expansion, similar to the situation in *A. palustris* (Arbadi *et al* 2014b). It should be mentioned that an unambiguous identification of both subspecies using morphology has not yet been achieved (Kennerley & Pearson 2010). Combining the arguments from morphology and genetics it is likely that the Paddyfield Warbler is a monotypic species and that the patchy distribution is not indicative of differentiation into two distinct subspecies.

Paddyfield Warblers from the Ukrainian Black sea coast, from Kazakhstan, from the west Siberian lowlands (Irtysh marshes, Omsk oblast) and from west Mongolia show quite different song types (see Table 1). Song recordings have been collected by Hans-Heiner Bergmann (Kazakhstan), Anatoly Poluda (southwest Ukraine and eastern central Ukraine), Martin Flade, Yaroslav Redkin and Mikhail Kalyakin (west Siberia) and Axel Bräunlich (west Mongolia), together with blood samples of the singing males (Ukraine, west Siberia, Mongolia), which were analysed in this study. However, although the warblers produced very different song types their mtDNA sequences did not reveal a similar differentiation.

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