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The Large-billed Reed Warbler *Acrocephalus orinus* revisited

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The Large-billed Reed Warbler *Acrocephalus orinus* is known only from the type specimen, collected in Himachal Pradesh, India, in 1867. The specimen is poorly prepared, and it has been suggested that it could represent an isolated form of the Clamorous Reed Warbler *A. stentoreus* or an aberrant Blyth's Reed Warbler *A. dumetorum*. We tested the affinity of *A. orinus* by (1) re-examining the morphology of the type specimen and (2) amplifying and sequencing a portion of its mitochondrial cytochrome *b* gene. Both the morphological and the mitochondrial analyses showed the specimen to be similar to *dumetorum*, but distinct enough to qualify as a species of its own. Relative to *dumetorum*, it has a more rounded wing, longer bill, longer and more graduated tail with more pointed tail feathers, and larger claws. The divergence in mitochondrial DNA between *orinus* and *dumetorum* was 7.8%, well above the value expected between subspecies. *A. orinus* is smaller than any of the forms of *A. stentoreus* or the related Australian Reed Warbler *A. australis*. It has a somewhat longer first primary, more pointed tail feathers and paler, less robust feet and claws. DNA comparison places it in the clade of small unstreaked *Acrocephalus* warblers, and apart from the clade of large unstreaked warblers that contains *stentoreus* and *australis*.

In recent decades, there have been more discoveries of new bird species (Vuilleumier *et al.* 1992) than anyone would have dared to anticipate in the middle of the last century (Mayr 1957). This is partly because of the increasing skill of the many bird-watchers nowadays travelling to remote areas, and also due to technical improvements, such as audio recording systems and sonagram analyses (Whitney & Alvarez Alonso 1998) and PCR-based DNA analyses (Smith *et al.* 1991, Helbig *et al.* 1995).

Several species of warblers have recently been discovered in southern Asia (e.g. Alström *et al.* 1992, Olsson *et al.* 1993, Alström & Olsson 1999) where many cryptic species perhaps remain to be found (Price 1996). In this paper we re-examine a (possible) cryptic species from the Himalayas known only from the type specimen, the Large-billed Reed Warbler *Acrocephalus orinus*. This specimen (BMNH registration no. 1886.7.8. 1742) was collected on 13 November 1867 in the Sutlej Valley near Rampoor (31°26′N, 77°37′E), Himachal Pradesh, by Allan Hume (Hume 1869). It remained in his collection until 1885 when this came in its entirety to the British Museum (BMNH). The specimen was first provisionally described as *Phyllopneuste macrorhyncha* (Hume 1869) but the name was changed two years later to *Acrocephalus macrorhynchus* Hume, 1871 when its generic affinity was established. However, Oberholser (1905) pointed out that this latter name was untenable because a specimen from Egypt, described by von Müller in 1853 as *Calamoherpe macrorhyncha*, appeared to be a synonym of Clamorous Reed Warbler *Acrocephalus stentoreus*. Hence, *Acrocephalus macrorhynchus* was abandoned in favour of the new name *Acrocephalus orinus* Oberholser, 1905.

Describing a new species from just one different-looking individual might be questionable (LeCroy & Vuilleumier 1992), since it might be an aberrant example of an already known species, or a hybrid. This concern has followed *A. orinus* ever since it was first described. Most handbooks and lists do recognize it as a species, but usually add a question mark to this treatment. Details and measurements of the species were given by Vaurie (1955) and Williamson (1968). Vaurie concluded that it was closely related to the Blunt-winged Warbler *A. concinens* and the Paddyfield Warbler *A. agricola*, but remarked on the

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long, broad bill, larger and less attenuated than in those species, or indeed the larger Blyth's Reed Warbler *A. dumetorum*. He described the rounded wing structure, with a short second primary as in *concinens*, but with the third and fourth primaries also short of the fifth. Williamson considered this unusual wing formula to be due to incomplete feather growth. He pointed out that the bird was still in moult and noted traces of waxy sheaths on the outer primaries. He concluded that the wing formula details were therefore unhelpful, and that wing and tail were probably short of their fully grown length. He suggested that the bird 'might represent a rare and isolated form of the widely but patchily distributed *A. stentoreus*'.

The present study took shape when one of us (D.P.) briefly inspected the type of *A. orinus* at the Natural History Museum, Tring, and noted its close resemblance to a moulting Blyth's Reed Warbler *A. dumetorum*. Other warbler specialists have also apparently been struck by this similarity (Per Alström, Urban Olsson and Lars Svensson pers. comm.). We decided to re-evaluate the species status of the specimen by (1) remeasuring it carefully together with a representative number of *A. dumetorum*, *A. concinens* and appropriate forms of the *A. stentoreus/australis* complex, and (2) identifying a portion of its mitochondrial cytochrome *b* sequence. We expected to find that both morphological characters and the cytochrome *b* sequence data would fall within the variation observed in *dumetorum*, so that *Acrocephalus orinus* could be safely removed from the list of extant or extinct bird species. To our surprise, this hypothesis proved to be wrong.

MORPHOLOGY AND MEASUREMENTS

The specimen is poorly prepared, so that the underparts are 'cramped' and badly displayed. The tail is twisted, with the feathers broken off on one side, but with a full set examinable on the other. It appears to be completing moult with sheaths still present at the base of some of the underpart feathers, but we were unable to confirm the presence of sheaths on the outer primaries of either wing. The secondaries and tail feathers all appear to be fully grown.

The upperparts are rich olive-brown, with a slight rufous tinge, especially on the upper tail-coverts. The underparts are strongly washed with olive-buff, although a degree of staining may be involved. The sides are more olive brown, and the throat is paler creamy olive. A pale superciliary stripe is evident in the specimen but does not seem to be strongly pronounced. Coloration is close to that of freshly moulted *A. dumetorum* specimens from Indian wintering grounds, but these are somewhat more olive, less rufous-tinged, and slightly paler. The upper mandible is dark, but the cutting edges and entire lower mandible are pale. The tarsi, toes and claws appear pale brown.

Measurements and wing structure details of the specimen are given in Table 1, and compared with those of *dumetorum* and some other potentially related taxa. *A. orinus* clearly has a longer tail and larger feet and claws than *dumetorum*. The bill is longer, stronger and slightly broader than in *dumetorum* and tapers less toward the tip (Fig. 1a,b). As pointed out by Vaurie (1955) the rictal bristles are shorter and weaker.

Table 1. Measurements and structure of the *Acrocephalus orinus* type specimen compared with those of *A. dumetorum*, *A. concinens haringtoni*, *A. australis sumbae* and *A. stentoreus brunnescens*. Reported are mean (range) for 10 of each (males and females). All measurements (in mm) taken by D.P.

	orinus	dumetorum	haringtoni	sumbae	brunnescens
wing	61	62.2 (60–64)	57.4 (55–59)	68.2 (65–70)	88.8 (87–91)
tail	57	51.0 (48–52)	56.3 (53–59)	62.1 (60–64)	80.6 (77–83)
tarsus	23.5	22.3 (22–23)	21.9 (21.5–22.5)	24.3 (23.5–25)	29.8 (29–31)
bill (to skull)	19.5	17.5 (16.5–18)	14.9 (14–15)	20.0 (19.5–21)	25.4 (24.5–27)
bill (to rear of nostril)	12.2	11.0 (10–11.5)	9.7 (9–10)	13.4 (12.5–14)	16.6 (16–17)
bill width (across rear of nostril)	4.6	4.2 (4.0–4.6)			
hindclaw	7.2	5.2 (5–5.5)	6.4 (6–7)	8.6 (8–9)	9.3 (9–10)
hallux	8.5	7.1 (7–7.5)	7.5 (6.5–8)	9.9 (9–10.5)	11.4 (11–11.5)
tip of 2nd p. relative to tips of other pp.	9th–10th	5th–7th	8th–9th (7th–10th)	6th–8th	5th–6th
extension of 1st p. beyond primary coverts	+2	–0.4 (–3 to +3)	+4.9 (+3 to +7)	–2.9 (–1 to –5)	–4.4 (–3 to –7)
primaries emarginated	3rd–5th	3rd–4th (3rd–5th)	3rd–5th (3rd–6th)	3rd–5th	3rd–4th (3rd–5th)
outer rectrix tip to tail tip	9	5.7 (4–7)	12.2 (9–16)	9.3 (8–11)	13.1 (10–16)

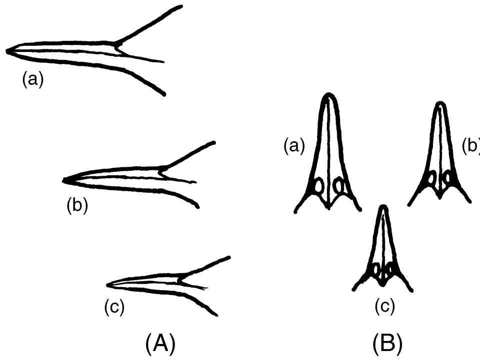

Figure 1. Bill of (a) *Acrocephalus orinus*, (b) *A. dumetorum* and (c) *A. concinens* as seen from the side (A) and above (B).

Figure 2. Wing formula of *Acrocephalus orinus*.

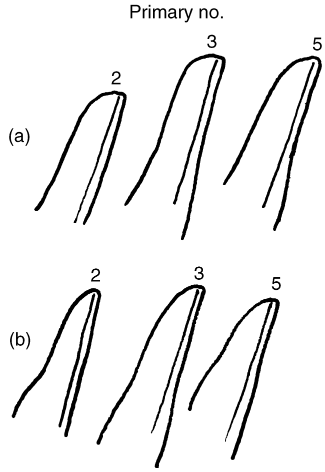

Figure 3. Shape of primary tips in (a) *Acrocephalus orinus* and (b) *A. dumetorum.*

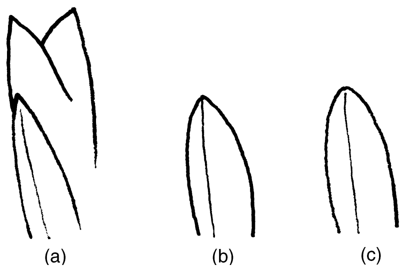

Figure 4. Shape of tail feather tips in (a) *Acrocephalus orinus*, (b) *A. dumetorum* and (c) *A. concinens.*

Full wing formula details (see also Fig. 2) are as follows (primaries numbered from outermost inwards, measurements in mm): P5 longest; P6 0.5, P7 3.5, P8 6.5, P9 7.5, P10 9 shorter than P5; P4 2, P3 4, P2 9 shorter than P5; tip of P2 near tip of P10 on closed wing; P1 very small, narrow and pointed, 2 mm longer than longest primary covert; P2–P5 emarginated on outer web; P2 with notch on inner web 16 mm from tip; secondary tips 12–13 short of tip of P5. This is of course quite different from the wing formula of *dumetorum* in which the longest primary is P3 and the tip of P2 falls at P5–P7. The short second primary seems generally rather smaller and weaker than the other leading primaries. The shortness of the third and fourth primaries relative to the fifth is unusual. It suggests that these feathers may not be fully developed, and could be up to 2–3 mm short of their full length. However, active growth of the outer primaries as reported by Williamson (1968) could not be confirmed, and indeed seems unlikely in a bird with fully grown inner secondaries. The primary tips are broad and rather 'square' compared with the narrower primary tips of *dumetorum* (Fig. 3). The tail is more graduated than in *dumetorum* and the feathers strikingly pointed (Fig. 4).

Whilst superficially similar to *dumetorum*, the Sutlej Valley bird thus differs in a number of characters: plumage colour, length and shape of the bill, wing and tail structure, shape of the wing and tail feathers, and foot and claw size.

Species in the *agricola* group (*A. concinens*, *A. agricola* and the Manchurian Reed Warbler *A. tangorum*) are all smaller than *orinus*, with shorter wing and tail measurements and much smaller bills (e.g. Alström *et al.* 1991). *A. concinens* (see Table 1), the least migratory of the three, has a rounded wing like *orinus* with the second primary about equal to the ninth, a well-graduated tail and similarly large feet and claws. It is a paler bird, however, more rufous-brown (less olive) above and warmer buff below, and has rounded tail feathers and a larger first primary. *A. tangorum* has rather pointed tail feathers, but is generally much more tawny or rufous than *orinus* with a more pointed wing (second primary usually longer than the seventh).

The various forms in the *stentoreus* complex (now treated under either Clamorous Reed Warbler *A. stentoreus* or the Australian Reed Warbler *A. australis*) are all larger than *orinus*, the smallest of these, the resident *A. australis sumbae* of northern Australasia having a wing length of 65–70 mm. They have relatively long tails like *orinus* and the second primary falls between P5 and P8, but the first primary (even in resident forms) is minute, falling well short of the primary covert tips. Bills are long and strong, but typically more attenuated near the tip than in *orinus*. Tarsi, toes and claws are relatively more robust than in *orinus* and usually dark greyish. Some of the Indian Ocean and Australian forms resemble *orinus* in coloration, but the form breeding nearest to the Himalayas, the large *A. stentoreus brunnescens* (see Table 1), is rather pale and greyish olive above.

Comparison of the morphology and measurements of the Sutlej Valley bird with those of known forms in the genus *Acrocephalus* does therefore indicate that it represents a distinct species. But we cannot exclude the possibility of the bird being a hybrid on this basis alone (see Beier *et al.* 1997).

MITOCHONDRIAL CYTOCHROME B GENE

DNA extraction, PCR and molecular methods

In a first trial, we attempted to isolate DNA from *A. orinus* from the base of contour feathers using a chelex extraction protocol according to Ellegren (1992). However, we were not able to amplify DNA from this extract. Hence, for the further studies of *A. orinus* and the other three museum specimens analysed, we isolated DNA from skin fragments (*c.* 0.5 × 0.5 × 3 mm) from the ventral side of the foot (registration nos. of the specimens in Table 4). Each skin fragment was placed in 100 μL lysis buffer (0.1 M Tris, 0.005 EDTA, 0.2% SDS, 0.2 M NaCl, pH 8.5) with 3 μL proteinase K (10 mg/mL) for 3 h of digestion at 55 °C (Laird *et al.* 1991) followed by standard ethanol precipitation. The precipitates were suspended in 50 μL ddH_2O and the DNA concentration checked on a spectrophotometer as the optical density at 260 nm. The sample from *A. orinus* showed a DNA concentration of 40 ng/μL.

The PCR was performed in volumes of 25 μL and included 1 μL of template DNA, 0.125 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.6 μM of each primer and 0.5 units of Taq DNA polymerase. The PCR amplifications were initiated by heating the samples to 94 °C for 3 min followed by 35 cycles consisting of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. The reaction was terminated by a 10-min step at 72 °C. We used 2.5 μL of the final reaction product to run on a 2% agarose gel in 0.5× TBE buffer to check the success of the reaction. All PCR reactions involving the museum specimens only included a single sample from a Great Reed Warbler *A. arundinaceus* as a positive control and two blank reactions as negative controls, to check for any cross contamination of DNA from other warbler species. Fragments selected for sequencing were cloned using TA-cloning kit (Invitrogen) according to the manufacturer's instructions. We amplified inserted DNA from 10 colonies per plate using standard M13 primers and evaluated the length of the amplified products on 2% agarose gels. Fragments of expected lengths from three to six of the amplified colonies were precipitated with ammonium acetate/ethanol and sequenced with dye terminator cyclic sequencing on an ABI PRISM™ 310 (Perkin Elmer). Phylogenetic analyses were done with the program MEGA using a Kimura 2-parameter genetic distance (Kumar *et al.* 1993).

Results of cytochrome *b* gene sequencing

Initial trials, using the universal primer pair for the 5′ end of the cytochrome *b* gene, L14841 and H15149 (Kocher *et al.* 1989), yielded blank reactions. Similarly, negative results were obtained when using primers for the control region which works in most warbler species (Bensch & Härlid 2000). However both these fragments are 300–400 nt long, and because of the age of the *A. orinus* specimen, the DNA might have been so degraded that only very short fragments were retrievable (e.g. Krings *et al.* 1997).

By using the four primer pairs shown in Table 2 we obtained four novel fragments of lengths 63, 71, 76 and 110 nt (excluding the lengths of the primers). The separate sequences obtained from the same original PCRs were in most cases identical. However, two of the sequences obtained differed from the

Table 2. Sequences of primers used (listed in the 5′ to 3′ direction) in amplifying the 5′-end of the cytochrome *b* gene in *Acrocephalus orinus*.

Name	Position[1]	Primer-pair[3]	Sequence
L14841[2]	14,990	1F	CCATCCAACATCTCAGCATGATGAAA
cytz	15,055	1R	GAGGTGTCTGCTGTGTAGTG
orinus1	15,052	2F	ACAGGGCTCCTATTAGCCA
cyt3	15,124	2R	GAGGTTGCGGATTAGTCA
orinus2	15,116	3F	CACGTATGCCGAGACGTACA
cyt4	15,193	3R	GATCCGTAGTAGAATCCTCG
orinus3	15,187	4F	CATCTGCATTTACTTTCACA
H15149[2]	15,298	4R	GCCCCTCAGAATGATATTTGTCCTCA

[1]The numbers refer to the position of the 3′ base of the primer in the chicken mitochondrial genome (Desjardins & Morais 1990). [2]Kocher *et al*. (1989). [3]F forward and R reverse relative the light strand sequence.

other five sequences at one base pair, suggesting PCR errors (fragments one and three in *A. orinus*). In these two cases we used the majority rule to determine a consensus sequence for the fragment, and in both cases this resulted in conserved positions relative to the sequence of other small and unstreaked *Acrocephalus* warblers. Each of the four fragments was tested against the GenBank International Nucleotide Sequence Database using the BLAST search routine. All fragments gave the best fit to cytochrome *b* sequences of other *Acrocephalus* warblers, but none matched completely any of the known sequences. Compared to corresponding stretches of cytochrome *b* of *A. dumetorum*, the *A. orinus* fragments differed by 6, 3, 5 and 10 substitutions. The four fragments obtained overlapped with 2–7 nt and produced an aligned sequence of 306 nt (EMBL accession number AF317712).

We next employed a neighbour joining analysis including *A. orinus* and 19 species/subspecies of *Acrocephalus* warblers, using three species of related genera as outgroups. The *A. orinus* sequence showed the closest association with *A. dumetorum* (Fig. 5). The bootstrap values for most branches were relatively low. Note that the tree obtained is very similar to the one obtained using the full cytochrome *b* gene (Leisler *et al*. 1997, Helbig & Seibold 1999), clearly separating the species into three clades, *small unstreaked*, *large unstreaked* and *small streaked*. All three subspecies of *A. stentoreus* for which cytochrome *b* data are available cluster tightly together within the well-supported (96%) clade of large unstreaked *Acrocephalus*. This analysis therefore rejects the hypothesis that *A. orinus* is a member of the *stentoreus* group (e.g. Williamson 1968).

The 306 nt fragments from *A. orinus* and *A. dumetorum* showed a nucleotide divergence of 7.8% (Table 3). Of the 24 pair-wise differences, 22 were transitions and two transversions (positions 19 and 294). The distribution of these differences relative to the three codon positions was as follows: five at the first, none at the second and 19 at the third (Table 3). Comparing their amino acid sequences (102 codons), *A. orinus* and *A. dumetorum* were identical except at codon 7 where *A. orinus* had isoleucine and *A. dumetorum* leucine, caused by the transversion at nucleotide position 19 (Table 3). All the other small, unstreaked *Acrocephalus* had the same amino acid (isoleucine) as *A. orinus* at codon 7, but they differed at one to three other codons.

The sequence of cytochrome *b* from *A. orinus* was obtained from the toe pad of a specimen over 100 years old, whereas all the other *Acrocephalus* sequences analysed in Fig. 5 and Table 3 were obtained from blood samples less than 10 years old. To check for possible bias resulting from either the age of the specimen and/or the source of DNA template, we analysed toe pad samples from similarly old specimens of two *A. dumetorum* and one *A. agricola* (Table 4) using the primer pair 3 (Table 2). Both the two *A. dumetorum* and the *A. agricola* specimen resulted in sequences characteristic of their respective species. Hence, the novel sequence obtained from *A. orinus* cannot be explained by the age or source of the material.

At present, there are 10 cytochrome *b* sequences available from *A. dumetorum*, including the two partial sequences obtained from the old specimens described above. These samples cover a large part of the species' range (Table 4). We could identify at

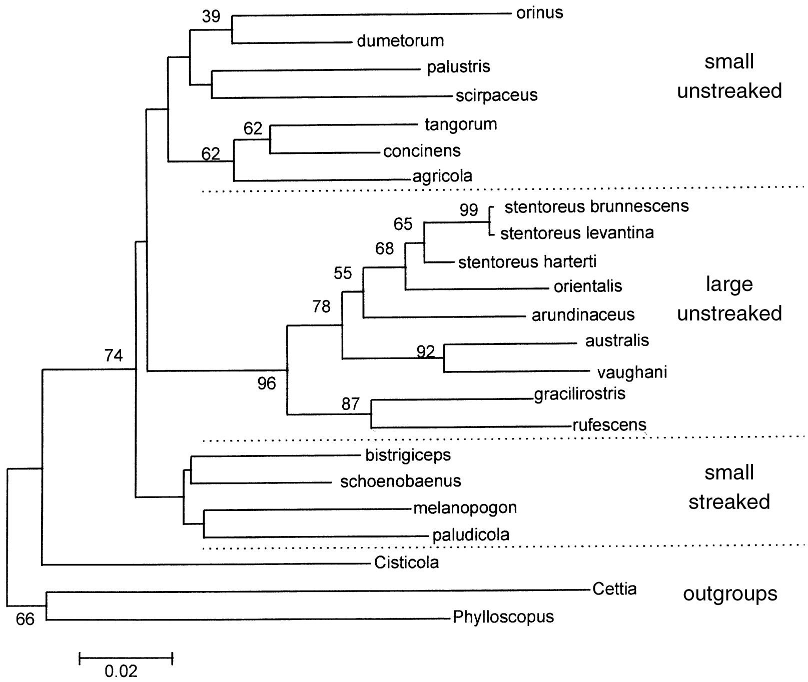

Figure 5. Phylogeny of *Acrocephalus* warblers from partial cytochrome *b* sequences (306 nt). The tree was estimated using the neighbour joining method with a Kimura-2 parameter genetic distance. Bootstrap values are indicated for branches with a support > 50% and for the common branch of *A. dumetorum* and *A. orinus*.

least five different haplotypes, which differed from each other at a maximum of two nucleotide substitutions. Hence, we found no indication of the *A. orinus* sequence being a member of any of the mt haplotypes existing in *A. dumetorum*.

Mitochondrial DNA distances and species status

Contrary to expectation we found the type specimen of *A. orinus* to carry a unique mitochondrial haplotype that showed a sequenced divergence of 7.8% relative to *A. dumetorum*. Mitochondrial DNA difference is often a poor guide to species status. For example, there are well-accepted species showing an mt DNA cytochrome *b* divergence of less than 0.5%, like the falcons *Falco rusticolus* and *F. cherrug* (Helbig *et al.* 1995) and the skuas *Catharacta skua* and *Stercorarius pomarinus* (Cohen *et al.* 1997). On the other hand, cytochrome *b* differences within species in *Acrocephalus* warblers can sometimes be as high as 4.5% (Leisler *et al.* 1997). But given that the documented differences in mt DNA within species rarely exceed 3% (Quinn *et al.* 1991) while those between avian sister species are on average 5% (Johns & Avise 1998), the divergence of 7.8% between the *A. orinus* type and *A. dumetorum* clearly supports the recognition of the former as a species of its own. This conclusion rests on the assumption that the *A. orinus* sequence obtained indeed represents the cytochrome *b* gene of this particular specimen. We feel confident that this is the case since we can reject the possibility that it is (1) a PCR artefact, (2) an 'numt', i.e. a nuclear copy of a mitochondrial gene or (3) the result of a cross specimen contamination, as outlined below.

First, all pairwise nucleotide differences between *A. orinus* and *A. dumetorum* are either at the first or third codon position. This indicates strongly that the sequence obtained has evolved in a functional gene rather than in a PCR tube, as the latter would have resulted in a random distribution of differences relative to codon position. The age of the specimen (132 years) is obviously not a problem because when we use similarly old *A. dumetorum* and *A. agricola* specimens, we obtain their species-specific sequences.

Secondly, in PCR-based studies of mt DNA genes, there is always a risk of amplifying a nuclear copy of an mt DNA gene, a so-called numt (Sorenson & Quinn 1998). This is particularly risky when the DNA template is obtained from blood because avian

Table 3. Partial cytochrome *b* sequences of seven *Acrocephalus* taxa (306 nucleotides). The data correspond to position 14 992–15 297 of the chicken mt genome (Desjardins & Morais 1990). The first nucleotide of each row is numbered (in bold) relative to the first nucleotide of the presented sequence.

	1																
orinus	TTC	GGC	TCA	CTT	CTA	GGC	ATC	TGC	CTA	GTT	ACC	CAA	ATT	GTC	ACA	GGG	CTC
dumetorum	...	...	...	..C	...	..T	C..	...	...	...	...	...	..C	...	...	...	...
agricola	...	...	...	..C	...	...	...	...	...	..C	...	...	..C	..T	...	..A	...
tangorum	...	...	...	..?	...	...	...	...	...	..C	...	...	..C	..T	...	..A	...
concinens	...	...	...	..C	...	...	...	...	T..	..C	...	...	..C	...	...	..A	...
palustris	...	...	...	..C	...	...	..T	...	...	ACC	...	...	...	...	...	..C	...
scirpaceus	...	...	...	..A	...	..T	..T	...	..G	A.C	...	...	..C	...	...	..A	..T
	52																
orinus	CTA	TTA	GCC	ATA	CAC	TAC	ACA	GCA	GAC	ACC	TCC	CTA	GCA	TTT	GCT	TCC	GTC
dumetorum	T..	C..	...	...	...	...	...	...	...	...	...	...	...	..C	..C	..T	...
agricola	...	C..	...	...	...	...	...	...	...	..T	...	...	..G	..C	..C	...	..A
tangorum	...	C..	...	...	...	..T	...	...	...	...	...	...	...	..C	...	..T	..A
concinens	...	C..	..T	...	...	...	...	...	...	...	...	...	...	..C	..C	...	..A
palustris	...	C..	...	...	...	...	...	...	...	...	...	...	...	..C	..C	...	...
scirpaceus	..G	C..	...	..G	...	...	...	...	...	...	...	...	...	...	..C	...	...
	103																
orinus	GCC	CAC	GTA	TGC	CGA	GAC	GTA	CAA	TTC	GGA	TGA	TTG	ATC	CGC	AAC	CTC	CAC
dumetorum	...	...	...	...	...	...	...	...	...	...	...	C.A	...	...	...	...	...
agricola	...	...	...	..T	...	...	..T	...	...	...	...	C.A	...	...	...	...	...
tangorum	...	...	...	...	...	...	...	...	...	...	...	C.A	...	...	...	...	...
concinens	...	...	...	..T	...	...	...	...	..T	...	...	C.A	...	...	...	...	...
palustris	...	...	...	...	..G	...	...	...	..T	...	...	C.A	...	...	...	...	..T
scirpaceus	...	...	...	...	...	...	...	...	..T	...	...	C..	...	...	...	...	...
	154																
orinus	GCA	AAT	GGA	GCC	TCT	TTC	TTC	TTC	ATC	TGC	ATT	TAC	TTT	CAC	ATC	GGC	CGA
dumetorum	...	..C	...	..T	..C	...	...	...	...	...	...	...	...	...	...	...	...
agricola	...	..C	...	...	..C	...	...	...	...	...	..C	...	..C	...	...	...	...
tangorum	...	..C	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	..C
concinens	...	..C	...	...	..C	...	...	...	...	...	..C	..T	...	...	...	...	...
palustris	...	..C	...	..T	..C	...	...	...	...	...	...	...	..C	..T	...	...	...
scirpaceus	...	..C	...	...	...	...	...	...	..T	...	...	...	..C	...	...	...	...
	205																
orinus	GGG	TTT	TAC	TAT	GGA	TCG	TAC	TTA	AAC	AAA	GAA	ACC	TGA	AAC	ATC	GGC	GTT
dumetorum	..A	..C	...	...	...	...	...	C..	...	...	...	...	...	...	...	...	...
agricola	...	..C	...	...	..G	..A	...	C..	...	...	...	...	...	...	G..	...	..C
tangorum	..A	...	..T	...	...	..A	...	C..	...	...	...	...	...	..T	G..	...	...
concinens	..A	...	...	..C	...	..A	...	C..	...	...	...	...	...	..T	G..	...	..C
palustris	...	..C	...	..C	...	...	...	C.G	...	...	..G	...	...	...	...	...	..C
scirpaceus	...	...	...	..C	...	..A	...	..G	...	...	...	...	...	...	...	...	..C
	256																
orinus	GTC	CTT	TTA	CTA	ACT	CTC	ATA	GCA	ACC	GCC	TTT	GTA	GGC	TAT	GTC	CTG	CCC
dumetorum	...	..C	...	...	..C	...	...	...	...	..T	..C	...	..A	..C	...	..A	..?
agricola	...	..C	C..	..G	..C	...	..T	...	...	..T	G.C	...	..A	..C	...	...	...
tangorum	..T	..C	C..	...	...	...	...	...	...	...	..C	...	..A	..C	...	...	...
concinens	...	..C	C..	...	G..	...	...	...	...	...	..C	...	..A	..C	...	...	...
palustris	...	..A	...	...	..C	...	..G	...	...	..T	..C	...	..A	..C	...	..A	...
scirpaceus	A..	..C	C..	...	..C	...	...	...	...	.T.	..C	...	..A	..C	...	..A	...

erythrocytes lack mitochondria. For the *A. orinus* sequence we amplified DNA extracted from skin, which presents a relatively low risk of obtaining an numt. In any case, the base composition of the sequence also supports a mitochondrial origin: (1) it shows the mitochondrial characteristic transition/transversion bias, (2) no stop codons and (3) an amino acid sequence almost identical to *A. dumetorum*.

Table 4. Variable sites at the first 306 nt of the mitochondrial cytochrome *b* gene in specimens of *Acrocephalus orinus*, *A. dumetorum* and *A. agricola*.

Taxa	Locality	Tissue	Variable sites[9]
			111111 1111122222 2222222222 222233
			1113344558 9999012335 6689901122 3456667788 889900
			2890928251 0369243689 5862570926 1751270625 684736
orinus[1]	Himachal Pradesh, India, November 1867	toe pad	TCATTCGCTC ATTCCCATGT CTTTCGTAGT CATTTATACC TTCTGC
dumetorum[2]	Kazakhstan	blood	CTC....TC. .CCT...CAC TC...AC..C ...C..C.TT .C????
dumetorum[2]	Finland	blood	CTC.C..TC. .CCT...CAC TC...AC..C ...C..C..T .CACA?
dumetorum[3]	Sweden, June 1992	blood	CTC....TC. .CCT...CAC TC...AC..C T..C..C.T? ??????
dumetorum[3]	Punjab, India, April 1997	blood	CTC...?TC. .CCT...CAC TC...AC..C T..C..C??? ??????
dumetorum[4]	SE Finland, breeding, two individuals, 1992	blood	CTC....TC. .CCT...CAC TC...AC.AC T..C..C.T. ..A.AT
dumetorum[5]	Crimea, migration, two individuals, 1992	blood	CTC....TC. .CCT...CAC TC...AC.AC T..C..C.T. ..A.AT
dumetorum[6]	Punjab, India, August 1913	toe pad	?????????? ???????CAC TC...????? ?????????? ??????
dumetorum[7]	SE Ural, Russia, August 1872	toe pad	?????????? ???????CAC TC..T????? ?????????? ??????
agricola[2]	Ukraine	blood	C..CCTA.CT GCC.ATTCAC .CCC..CGAC .GCCCGCT.T GCAC..
agricola[8]	Mysore, India, February 1883	toe pad	?????????? ???????CAC .CCC.????? ?????????? ??????

[1]Specimen 1886.7.8. 1742 at the Natural History Museum. [2]Sequence from Leisler *et al.* (1997). [3]Sequence obtained for the present study. [4]Sequence from Helbig and Seibold (1999). [5]Sequence from A. Helbig (pers. comm.). [6]Specimen 1949. Whi.1.11427 at the Natural History Museum. [7]Specimen 1898.12.12.762 at the Natural History Museum. [8]Specimen 1883.8.1.20 at the Natural History Museum. [9]Position numbering as in Table 3.

Hence, there is no indication that the sequence obtained from *A. orinus* represents an numt.

Thirdly, the specimen has probably been in contact with many other bird skins over the past 132 years, and possibly also blood from other specimens at the time the skin was prepared. In theory, this could have resulted in contamination of DNA from other specimens. However, this cannot explain the novel *A. orinus* sequence because all *Acrocephalus* species which are similar to the *A. orinus* specimen have been sequenced, and none fits the *A. orinus* sequence obtained. The closest match is with *A. dumetorum*.

Accepting that the *A. orinus* sequence represents a unique mitochondrial lineage, we finally ask whether it can be a divergent haplotype within the variation of *A. dumetorum* (cf. Nordborg 1998). In a within-species study, the major mitochondrial branches should be detected after sequencing a handful of individuals (Saunders *et al.* 1984). We have information from 10 *A. dumetorum*, collected from most of its Palaearctic breeding range as well as from wintering areas close to where *A. orinus* was collected. The mitochondrial DNA of these 10 specimens shows a tight cluster, well separated from the *A. orinus* sequence. Hence, we consider it highly unlikely that the *A. orinus* haplotype can be a member of the *A. dumetorum* group.

CONCLUSION

In the most recent complete reference work on the birds of the Indian subcontinent (Grimmett *et al.* 1998), *A. orinus* is referred to as being synonymous with *A. stentoreus*. This is in clear contrast to the results from our re-examination of the Sutlej Valley bird and determination of part of its mitochondrial DNA sequence, both of which indicate that *Acrocephalus orinus* deserves its species status. Its morphology, with a relatively rounded wing, suggests it represents a resident or short-distance migratory species. Its mitochondrial DNA sequence suggests that its closest relatives are among the other small, unstreaked *Acrocephalus* warblers, and not within the *A. stentoreus* group. In order to confirm whether it is a sister species of *A. dumetorum* as the present study suggests would require the analysis of a longer mt DNA fragment than presently available. The species *Acrocephalus orinus*, represented by a single individual collected in the Himalayas, has existed in the taxonomic shadow lands for more than a century. It may still exist in the wild and there may be other overlooked specimens in Museum collections.

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