

# Lost and found: the enigmatic large-billed reed warbler *Acrocephalus orinus* rediscovered after 139 years

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We present compelling evidence of the continued existence of the large-billed reed warbler *Acrocephalus orinus*, hitherto known only from the unique type specimen collected in NW India 139 years ago. Morphological and genetic analyses of an unusual *Acrocephalus* warbler mist-netted south-west of Bangkok, Thailand, on 27 March 2006, confirmed its identity as *A. orinus*, and revealed that it was heterozygous at four out of eight microsatellite markers, indicating the continued existence of a viable population whose breeding and wintering areas are still unknown.

The provenance of the handful of bird species each described from a single type specimen has sometimes been questioned because they might be aberrant examples, or hybrids between already known species (Collar and Fishpool 2006).

The status of the large-billed reed warbler *Acrocephalus orinus* perplexed taxonomists for more than a century (Williamson 1968), until an examination of the mitochondrial DNA of the type specimen, collected in the Sutlej Valley, Himachal Pradesh, NW India, on 13 November 1867, confirmed its identity as a valid species (Bensch and Pearson 2002). Without any other records, its behaviour, ecology, breeding and wintering range remained unknown.

A small, unstreaked *Acrocephalus* warbler captured ca. 3,100 km from the type locality on 27 March 2006 at Laem Phak Bia, Phetchaburi Province, SW Thailand (13° 03'N; 100° 05'E) showed a rounded wing and a particularly strong and long beak and other morphological features (Fig. 1) which invited comparison with the recently re-examined type specimen of *A. orinus* (Bensch and Pearson 2002). In this paper, we examine the morphology, phylogenetic position and multilocus heterozygosity of this newly caught specimen to suggest

hypotheses for the migratory pattern, distributional range and current population size of *A. orinus*.

## Materials and methods

### Study area

The Laem Phak Bia Environmental Research and Development Project, a royally initiated pilot wastewater treatment project, lies on 1.6 km<sup>2</sup> of land in Ban Laem District, Phetchaburi Province, Thailand, about 120 km SW of Bangkok. It is situated in an otherwise brackish water area, in which the dominant land-use is salt pans, and is separated from the coast by a ca. 500 m-wide belt of regenerating mangroves dominated by *Avicennia* spp. Roughly 90% of the site is occupied by five large water treatment ponds, lacking fringing vegetation, while approximately 4% of the area is occupied by grass filter beds. Due to the year-round input of water rich in organic pollutants, it is a highly productive artificial freshwater ecosystem, attracting many resident and migrant birds that feed on fishes and invertebrates.



Fig. 1. The large-billed reed warbler *Acrocephalus orinus* captured at Laem Phak Bia, SW Thailand, on 27 March 2006. Photo by P. D. Round.

## Methods

Ring studies of migrant and resident birds have been carried out at Laem Phak Bia by one of us (PDR) since September 2000, in collaboration with the Wildlife Research Division, Department of National Parks, Wildlife and Plants Conservation. Birds were caught in mist-nets during 1–2 day visits year-round (at least 1–2 times per month during spring and autumn migration periods; less frequently at other times of year). Nets were placed in grass filter beds, on a low embankment separating a 5 m × 100 m wide bed of *Cyperus corymbosus* from an equal-sized bed of *Vetiveria zizanioides*. To the north the area was bordered by a further four filter beds containing other grasses, and *Typha angustifolia*, and to the south by mangroves. All birds caught were ringed, examined for stage of moult and wear, and their biometrics, weights and the status of fat and muscle recorded. Digital photography was used to document the features of the scarcer species, and plucked or shed feathers of some were retained for DNA analysis.

To extract DNA from the putative *A. orinus* we used the basal 5 mm of a tail feather following the protocol in Laird et al. (1991). For each polymerase chain

reaction (PCR) we used approximately 5 ng of DNA and reagent concentrations as described in Bensch and Pearson (2002). Partial cytochrome *b* sequences were sequenced from amplifications with the primer L14841 and H15149 (Kocher et al. 1989), using an annealing temperature of 52°C, and the primers L14995 and H16065 (Helbig et al. 1995), using an annealing temperature of 45°C. Molecular sexing was done by amplifying introns of the CHD1Z/W gene with the primers 0057 F (5'-CGTCAATTTCCATTTCAGG-TAAG-3') and 002 R (5'-TTATTGATCCATCAA-GTCTC-3'), modified by D. Arlt (unpubl. data) from the primers 2550F and 2718R first described by Fridolfsson and Ellegren (1999). We used an annealing temperature of 49°C and we have confirmed that this protocol produces accurate sexing of other *Acrocephalus* warblers (B. Hansson and S. Bensch, unpubl. data). We tested the level of heterozygosity of the above-mentioned Laem Phak Bia bird at eight microsatellite loci (Ase8, 9, 11, 12, 19, 34, 56 and 58) first isolated in the Seychelles warbler *A. sechellensis* (Richardson et al. 2000), and subsequently tested for variation in that species and in three other *Acrocephalus* warblers (*A. arundinaceus*, *A. griseldis* and *A. palustris*; Hansson and Richardson 2005). For comparison, we also genotyped six Blyth's reed warblers *A. dumetorum* (collected in Kazakhstan in 2001) at the same eight microsatellite loci as this is the suggested closest relative to *A. orinus*. Microsatellites were amplified in 10 µl reaction volumes using 6-FAM-labelled forward primers and an annealing temperature of 60°C (with the exception of Ase8, which was run in a touchdown cycle following Richardson et al. 2000). The amplified microsatellite alleles were separated in acrylamide gels and detected in a Typhoon imaging system 9200 (Amersham Biosciences).

The phylogenetic analysis was based on the longer cytochrome *b* sequence (1080 bp) and was run using the program MrBayes (Ronquist and Huelsenbeck 2003), implementing a GTR+I+G model of molecular evolution as first selected by the program MrModeltest (Nylander 2004). The Bayesian phylogeny was obtained by using four heated and one cold mcmc chain, which was sampled every 200 generations over 20 million generations generating 100,000 trees. After visualizing the parameters we discarded the first 25% of the trees as the burn-in period. The remaining 75,000 trees were used to construct a majority consensus tree. The phylogenies were visualised using MEGA 3.0. (Kumar et al. 2004).

For the calculation of the effective population size ( $N_e$ ) we assumed that the population size had remained constant from the first record of the species until the present and that the generation time was two years. The average population heterozygosity ( $H_{pop}$ ) is predicted to decline by  $1/2N_e$  per generation (Kimura 1983). After

X generations, the remaining  $H_{\text{pop}}$  should therefore be reduced by  $(1 - 1/2N_e)^X$ . Hence, a species with an effective population size of 50 individuals will lose ca 20% of its original heterozygosity after 20 generations (Amos and Harwood 1998).

## Results

An unfamiliar *Acrocephalus* warbler netted on 27 March 2006, at approximately 10:00 h, was initially thought to be a blunt-winged warbler *A. concinens* until it was realised that both the bill length and wing-length were well outside the range for that species. Measurements and a wing formula were recorded and the bird was ringed, weighed and photographed with a digital camera. Immediately before release the two central rectrices were removed and placed in a small ziploc polythene bag for DNA assay.

The features of the newly trapped bird corresponded closely with the morphology of the *A. orinus* type specimen as described in Bensch and Pearson (2002). Unlikely as this seemed – *A. orinus* was hitherto known only known from a single specimen collected no closer to Thailand than NW India and, moreover, 139 years ago – identification of the bird as *A. orinus* was confirmed after a DNA assay was

performed on a single rectrix and compared with that of the type specimen.

## Morphology

The upperparts were uniform warm olive-brown, lacking rufescent tones, with slightly warmer, lighter brown edges to the greater coverts, tertials and inner secondaries. There was a short creamy whitish supercilium, extending as far as the hind margin of the eye; a creamy-white crescent below the eye and a short blackish eyeline behind and in front of the eye, which did not extend on to the lores. The underparts were whitish with a buffy wash across the breast, on the flanks and undertail-coverts. The rectrices showed no white edging or tipping. The rectal bristles were prominent. The orbital ring was slaty grey; the iris dull brown with a slight grey tinge; the upper mandible blackish, strongly ridged on the midline. The lower mandible was fleshy pink to the tip. The legs were brownish-grey with dull yellowish-horn soles to the feet (Fig. 1).

Measurements are given in Table 1. The wing was strongly rounded with the primary projection only slightly more than 1/3 the length of the exposed tertials, and the tips of six primaries visible beyond the tertials. The wing formula closely resembled that of *A. concinens*

Table 1. Measurements and structure of the large-billed reed warbler *A. orinus* trapped at Laem Phak Bia compared with the type specimen and with some congeners. Measurements are given in mm. Wings were measured maximum chord. Bill width was measured proximally (at the posterior edge of the nostrils). Primaries are numbered ascendantly; comparison of p2 is made relative to the wing point and to the tips of the inner primaries. The outermost primary is compared in relation to the longest primary coverts (pc). †Estimated from photographs.

| Character                                 | <i>A. orinus</i><br>(Laem Phak Bia) | <i>A. orinus</i> type (Bensch<br>and Pearson 2002, or<br>D.J. Pearson own data*) | <i>A. c. concinens</i><br>(P. D. Round own data) | <i>A. dumetorum</i><br>(Williamson 1968, or<br>D.J. Pearson own data*) |
|-------------------------------------------|-------------------------------------|----------------------------------------------------------------------------------|--------------------------------------------------|------------------------------------------------------------------------|
| Wing                                      | 64                                  | 61                                                                               | 54.9 (53–59; n=49)                               | 61.2 (58–66; n=117)                                                    |
| Tail                                      | 60                                  | 57                                                                               | 57.1 (52–60.5; n=31)                             | 51.9 (45–58; n=116)                                                    |
| Outer rectrix to tail-tip                 | 6.7                                 | 9.0                                                                              | 11.0–11.8 (n=2)                                  | (4–8.5)                                                                |
| Wing point                                | p4 (p5 subequal)                    | p5                                                                               | p4 or p4=p5; p5 (once)                           | p3=p4                                                                  |
| P2                                        | 6.7 mm<br><wingpoint; =p8–p9        | 9 mm<br><wing point; =p9–p10                                                     | mainly p8–p9;<br>(range =p7–p8 to<br>p10–ss)     | 4–5 <wing<br>point; =p5–p7                                             |
| P1                                        | = pc                                | 2 >pc                                                                            | 5.6 >pc (3.0–7.0<br>>pc; n=8)                    | 3 <pc–3 >pc                                                            |
| Bill (to skull)                           | 20.6                                | 19.5                                                                             | 16.6<br>(15.9–17.8; n=14)                        | 16.7<br>(15.5–18.0; n=43)                                              |
| Bill width                                | 4.8                                 | 4.6                                                                              | 3.9 (3.3–4.6; n=8)                               | 4.2 (4.0–4.6; n=10)*                                                   |
| Bill depth<br>(anterior edge of nostril)  | 3.7†                                | 3.6*                                                                             |                                                  | 2.8–3.2 (n=4)*                                                         |
| Bill depth<br>(posterior edge of nostril) | 4.1†                                | 4.0*                                                                             |                                                  | 3.2–3.4 (n=4)*                                                         |
| Tail:wing ratio                           | 0.94                                | 0.93                                                                             | 1.04 (0.95–1.12;<br>n=31)                        | 0.82 (0.78–0.85;<br>n=10)*                                             |
| Bill:wing ratio                           | 0.32                                | 0.32                                                                             | 0.30 (0.28–0.32;<br>n=14)                        | 0.28 (0.27–0.29;<br>n=10)*                                             |
| Hind claw                                 | 7.5†                                | 7.2                                                                              |                                                  | 5.2 (5.0–5.5; (n=10)*)                                                 |

(Table 1), though differed in the minute outermost primary – equal in length to the longest primary coverts. The outer web of p3, and (distally) the outer webs of p4 and p5 were emarginated. The weight was 9.5 g when the bird was weighed at 10:44 h. Fat was scored as 0 and muscle as 2 (Bairlein 1995). The bird was slightly worn, and all flight feathers appeared to be of a uniform age class, indicating that it had probably undergone a complete moult earlier during the winter. The dull grey-brown iris was suggestive of a bird in its second calendar year, although the age cannot be stated with certainty.

### DNA assay

Analyses of the mitochondrial cytochrome b gene from the plucked tail feather confirmed an almost perfect match to the 1867 type specimen (0.3% sequence divergence for the 305 bp), thus confirming the identity of the Laem Phak Bia bird as *A. orinus*. While the DNA of the type specimen was substantially degraded, complicating DNA amplification (Bensch and Pearson 2002), the fresh DNA of the Laem Phak Bia bird made it possible to amplify longer fragments. Amplification of a 1080 bp-long cytochrome b sequence, and phylogenetic analyses using Bayesian statistics (Ronquist and Huelsenbeck 2003) supported the sister relationship of *A. orinus* with *A. dumetorum* (Fig. 2) from which it showed a cytochrome b divergence of 9.9%.

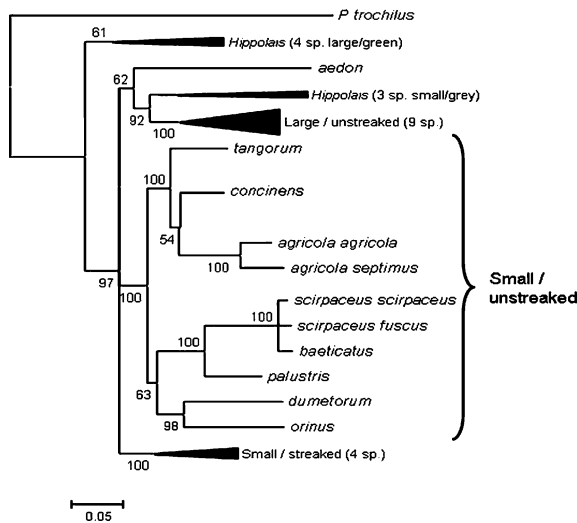


Fig. 2. Phylogenetic position of large-billed reed warbler *Acrocephalus orinus* and other related warblers. A Bayesian phylogenetic tree of the cytochrome b gene including all taxa of *Acrocephalus* and *Hippolais* published in GenBank. Branches supported by less than 50% posterior probabilities were collapsed.

The sequence of the Laem Phak Bia bird differed from the type specimen (BMNH Registration no. 1886.7.8. 1742) at one synonymous transition and has been deposited in the GenBank data base (Accession No. DQ681065). Molecular sexing techniques revealed that the bird was a male, which may be important when comparing its biometrics with potential future records.

To estimate the possible size, and thus likely viability, of the population, we examined the Laem Phak Bia bird at eight microsatellite loci. It was confirmed heterozygous at four of the eight loci, a level of diversity comparable to that of other *Acrocephalus* warblers for which we have data, including the sister species, *A. dumetorum* (Fig. 3). Since we do not know whether the observed heterozygosity of the Laem Phak Bia bird (0.5) is representative for the current population, we assumed that  $H_{ind}$  was binomially distributed around  $H_{pop}$ . This simplifying assumption would be correct if all investigated loci had the same expected heterozygosity and the population was in Hardy-Weinberg equilibrium. Using the binomial distribution, we can then calculate that finding an individual that is heterozygote at four out of eight loci ( $H_{ind} = 0.50$ ) would be substantially unlikely ( $P = 0.1$ ) when  $H_{pop} < 0.24$ . Second, if we assume that when *A. orinus* was discovered, in 1867,  $H_{pop}$  was 0.60 (the present level for these loci in its closest relative, *A. dumetorum*; Fig. 3), a loss of heterozygosity from 0.60 to 0.24 in 70 generations would correspond to a mean effective population size of approximately 40 individuals [ $H_{pop}^{x=70} = H_{pop}^{x=0} \times (1 - 1/2N_e)^x$ ]. A more conservative estimate of 26 individuals would result if we instead assume  $H_{pop} < 0.20$  ( $P = 0.05$  to find an individual

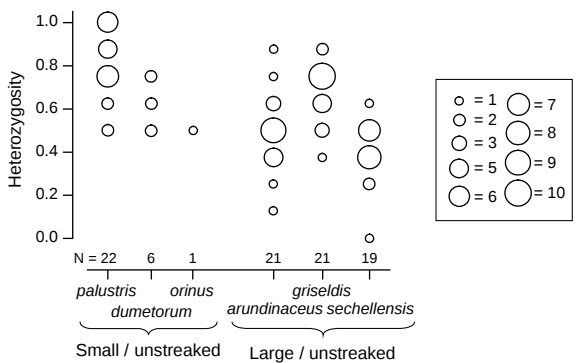


Fig. 3. The level of heterozygosity in the Laem Phak Bia *A. orinus* compared to levels of diversity present in *A. dumetorum* (this study) and four other *Acrocephalus* warblers (Hansson and Richardson 2005) typed for variation at the same eight microsatellite loci isolated in the Seychelles warbler *A. sechellensis*. The number of individuals tested for each species are given above the X-axis. The sizes of the circles correspond to the number observed (1–10) for each level of heterozygosity.

with heterozygosity of 0.50) and an original heterozygosity of 0.80 (as in *A. palustris*, Fig. 3).

## Discussion

On plumage *A. orinus* most closely resembles *A. concinens*, though differs in its shorter supercilium. It differs from *A. dumetorum* by its richer, more deeply olive (rather than greyish olive-brown or cold olive) upperparts, and proportionately shorter, more rounded wing. It differs from both by its markedly longer, stronger bill and from *A. concinens* by its minute outermost primary.

The measurements of both *A. orinus* individuals (the Laem Phak Bia bird and the type specimen) corresponded very closely (Tables 1 and 2). Additionally the hind claw of the *A. orinus* type specimen (7.2 mm) and the Laem Phak Bia bird (7.5 mm, estimated from photographs) were larger than in *A. dumetorum* (Table 1). Both the *A. orinus* type specimen and the Laem Phak Bia bird had broader, more square primary tips, and slightly narrower, more pointed, tail feathers than *A. dumetorum*.

A detailed comparison of the wing formula of the Laem Phak Bia bird with the *orinus* type specimen (Table 2) indicates that, as suggested both by Williamson (1968) and Bensch and Pearson (2002), the outermost primaries of the latter were not full-grown (though the short outermost primary of the type specimen, which accorded with that in the Laem Phak Bia bird, was probably fully grown). This combination of a short first primary with a strongly rounded wing may be diagnostic for *A. orinus* among small, unstreaked *Acrocephalus* warblers, as it is otherwise found only in a few of the larger, primarily resident species such as *A. stentoreus*.

The unique cytochrome *b* haplotype of *A. orinus*, 9.9% divergent from its closest relative, *A. dumetorum*, excludes the possibility that the distinct morphology seen in the type and the Laem Phak Bia bird is the result of hybridization between known species of *Acrocephalus*. Williamson (1968) considered that *A. orinus* “might represent a rare and isolated form of the widely and patchily distributed (clamorous reed warbler) *A. stentoreus*”, which is usually a resident or short distance migrant. Since *A. orinus* was not subsequently found at the type locality, this might imply that the

species had already been extirpated, discouraging active searches elsewhere. However, since both the type specimen and the present individual were recorded during the northern temperate winter period, and since their closest relative (*A. dumetorum*) is a long distance migrant, it is possible that *A. orinus* likewise breeds in the Palaearctic and winters in southern Asia, almost certainly to the west of Thailand, possibly in Myanmar or the Indian subcontinent. Conceivably, it could winter widely alongside any of the three subspecies of *A. concinens*, or *A. dumetorum*, and yet have remained overlooked owing to its morphological similarity to those taxa.

*Acrocephalus orinus* may be only an extreme vagrant in Thailand. It was the only *Acrocephalus* other than *A. bistrigiceps* (175 ringed) and *A. orientalis* (204 ringed) that was caught at Laem Phak Bia during 2000–2006, and surveys (including ringing surveys) of lowland aquatic habitats, including beds of *Phragmites* reeds and other waterside vegetation, have been previously conducted elsewhere in the country without revealing *A. orinus*. Further surveys of aquatic habitats (and perhaps drier grassland or scrub habitats) in S and SE Asia should be mounted in efforts to locate further individuals, since the core range and habitat of *A. orinus* remains unknown. It is almost certainly rare, but huge areas in Asia are poorly studied and it is nevertheless possible that a substantial population exists. Our estimate for the lower limit of the effective population size (26–40 birds) only holds if the population size has remained constant over the past 139 years. It is not possible to detect recent population declines or increases from estimates of heterozygosity obtained from a single individual.

This rediscovery of the large-billed reed warbler, an event distant both in time and space from the only previous record, draws attention to the continued existence of the species, but yields very little new information on its distribution, beyond indicating that it could potentially be quite widespread in winter in S and SE Asia. Hopefully this will encourage active searches for its unknown breeding and wintering areas. The rediscovery additionally highlights the need to search for other presumed extinct species in less studied regions, and the need to conserve ecologically suitable habitats for such species in perpetuity.

Table 2. Comparison of the detailed wing formula of two large-billed reed warblers *Acrocephalus orinus*. Measurements (mm) give shortfall of primaries (numbered ascendantly) and secondaries in relation to the wing point. (‡ Estimated from photographs).

|               | p2  | p3 | p4 | p5  | p6  | p7   | p8  | p9  | p10  | SS     |
|---------------|-----|----|----|-----|-----|------|-----|-----|------|--------|
| Type specimen | 9   | 4  | 2  | 0   | 0.5 | 3.5  | 6.5 | 7.5 | 9    | 12–13  |
| Laem Phak Bia | 6.7 | 1‡ | 0  | 0.5 | 3‡  | 4.5‡ | 7‡  | 8‡  | 9.5‡ | 10–11‡ |

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