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Non-breeding season records of the Alpine Leaf Warbler *Phylloscopus occisinensis*

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Summary.—We present non-breeding season records of the recently named Alpine Leaf Warbler *Phylloscopus occisinensis* from Bangladesh (four individuals) and northern Thailand (one). Identification was based on mitochondrial DNA assay of feathers or blood from birds handled during ringing. Tickell's Leaf Warbler *P. affinis* (*sensu lato*) was abundant in scrub and scattered trees at the margins of wetlands in north-east Bangladesh, whilst the record from Thailand represents a significant eastwards extension of the previously recorded wintering range. Further sampling in South and South-East Asia will be necessary to resolve the winter ranges of the taxa *affinis* and *occisinensis*.

Within the 'Tickell's Leaf Warbler *Phylloscopus affinis* species complex', breeding around the margins of the Tibetan Plateau (which also encompasses the Sulphur-bellied Leaf Warbler *P. griseolus*), eastern populations of *P. affinis* were shown to constitute a distinct mitochondrial lineage that has been named Alpine Leaf Warbler *P. occisinensis* (Martens *et al*. 2008). Following this treatment, *P. affinis* breeds in the Himalayas, eastwards to Xizang, China, whereas *P. occisinensis* is distributed further east, beyond the Himalayan chain, in Yunnan, Sichuan, Qinghai and Kansu (China). Despite their deep mitochondrial divergence (15.8–16.0% in cyt*b*, Martens *et al*. 2008; *c*.7.8% for the complete mitochondrial genome, Zhang *et al*. 2019) the two taxa are scarcely distinguishable using morphology and nuclear DNA, whilst any difference in song appears to be clinal, with 'bioacoustic intermediacy' over a narrow contact zone in eastern Xizang (Zhang *et al.* 2019). The deep mitochondrial divergence has been attributed to 'ghost introgression' as an easterly distributed ancestor expanded west and interbred with another, unknown, and presumably extinct *Phylloscopus* species (Zhang *et al*. 2019). Treatment as two species is not universally followed, however (BirdLife International 2020).

Irrespective of whether *P. affinis* and *P. occisinensis* are treated as one or two species, it is of both faunal and possible future conservation interest that the winter (non-breeding) ranges of the two lineages are identified. The winter range of *P. affinis* (*sensu lato*) extends from northern and peninsular India (in the south-west Ghats) to Bangladesh and Myanmar (Rasmussen & Anderton 2005, Dickinson & Christidis 2014). Here we present records of overwintering birds sampled in north-east Bangladesh and northern Thailand.

Methods

Field collection.—Prior to their release, we collected feathers of three *P. affinis* (*sensu lato*) from a total of 28 individuals, mist-netted and ringed in low trees and scrub around two wetland sites in north-east Bangladesh during 2012–14 (Round *et al*. 2014), and from one additional individual of another 11 ringed at a third site during 2015 (Fig. 1).

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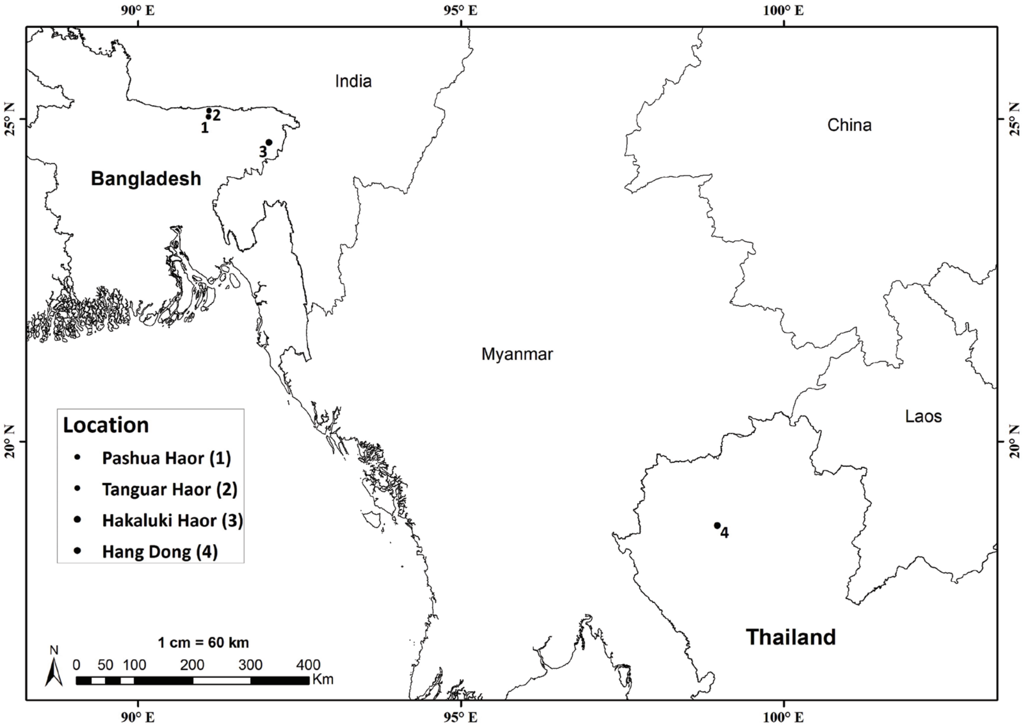

Figure 1. Sampling localities of *Phylloscopus affinis* (*sensu lato*) in Bangladesh and Thailand.

Figure 2. Alpine Leaf Warbler *Phylloscopus occisinensis*, before release, Hang Dong District, Chiang Mai Province, Thailand, 24 January 2020 (Sontaya Manawattana)

Additionally, a single *Phylloscopus* with yellowish underparts and supercilium, lacking wingbars, in Hang Dong District, Chiang Mai province, northern Thailand (Fig. 1), first found on 17 January 2020 by Thammarat Kaosombat, was tentatively identified from digital photographs as either Tickell's or Alpine Leaf Warbler by Wich'yanan Limparungpatthanakij

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TABLE 1
Biometrics (mm) and mass (g) of five wintering Alpine Leaf Warblers *Phylloscopus occisinensis* sampled. The first four individuals were trapped in Bangladesh, the fifth in Thailand.

Ring no.	Date	Location	Wing	Tail	Bill	Tarsus	Mass
AAA140	14 Feb 2012	Pashua Haor, Sylhet Division, 25º03′00″N, 91º06′00″E	53	40	12.9	19.6	5.9
AAA504	23 Feb 2014	Tanguar Haor, Sylhet Division, 25º04′59″N, 91º07′01″E	56	44	12.8	17.8	6.2
AAA509	23 Feb 2014	Tanguar Haor	54	43	12.5	17.5	6.0
AAA631	19 Feb 2015	Hakaluki Haor, Sylhet Division, 24º38′13″N, 92º01′34″E	56	44	12.8	20.4	6.7
A35121	24 Jan 2020	Hang Dong, Chiang Mai, 18º41′39″N, 98º58′34″E	55	43	12.6	18.9	5.9

a day or two after its discovery. This individual attracted particular interest as there were no previous records of *P. affinis* or *P. occisinensis* in Thailand or Indochina. On 24 January 2020, it was caught in a mist-net, ringed, measured, photographed in the hand (Fig. 2), and a blood sample taken on filter paper before release. The same bird remained at the site and was repeatedly observed until 9 February 2020. It was not heard to call during this period.

All individuals were caught in scrub in open marshy areas fringing wetlands on the plains, and mass and basic biometric data recorded (Table 1). The feathers were stored in labelled paper envelopes in a freezer at 0°C and the blood samples at -20°C.

Laboratory protocols.—Genomic DNA was extracted from feathers (the four Bangladesh individuals) and blood (the Thai bird) using NucleoSpin tissue kit (Macherey-Nagel), with 0.1% Dithiothreitol (DTT) added to increase the DNA yield (Olsson *et al*. 2005). Partial mitochondrial genes were amplified using primers: Bird F1 (5′-TTCTCCAACCACAAAGACATTGGCAC-3′) and Bird R1 (5′-ACGTGGGAGATAATTCCAAATCCTG-3′) for Cytochrome C oxidase subunit 1 (COI; Saitoh *et al*. 2015) and L14841 (5′-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3′) and H15547 (5′-AATAGGAAGTATCATTCGGGTTTGATG-3′) for cytochrome *b* (Cyt*b*; Helbig *et al*. 1995). Polymerase chain reaction (PCR) was performed using AccuStart II GelTrack PCR SuperMix (Quanta BioSciences) following the concentration of ingredients suggested by the manufacturer. The reactions for both genes were performed with the following steps: 94°C for three minutes followed by five cycles at 94°C for 30 seconds, 48°C for 30 seconds, 72°C for one minute, then 30 cycles at 94°C for 30 seconds, 51°C for 30 seconds, 72°C for one minute, and a final 72°C for five minutes (Saitoh *et al*. 2015) using an Eppendorf Mastercycler gradient thermocycler. PCR products were visualised with 1.5% agarose gel electrophoresis. The products successfully amplified were purified using NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) and sent for sequence analyses using the Applied Biosystems BigDye Terminator v3.1 Cycle Sequencing Kit protocol.

Genetic analyses.—Sequences of collected samples were deposited in GenBank. These sequences and those of other species downloaded from GenBank (Table 2) were aligned using ClustalW in MEGA7 v.7.0.21 (Kumar *et al*. 2016) and used to conduct Maximum Likelihood (ML) and Bayesian Inference (BI) phylogenetic analyses, with Yellow-streaked Warbler *P. armandii* serving as the outgroup. Phylogenetic trees for cyt*b* and COI were constructed separately because the sequences of both genes were not available for concatenative analyses. Kakusan4 (Tanabe 2007) was used to select the best-fit evolutionary model under Akaike information criterion (AIC; Akaike 1974) and Bayesian information criterion (BIC; Schwarz 1978) for ML and BI, respectively. The selected models for the ML

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TABLE 2
Ring numbers, localities and accession numbers for collected samples, and the samples in GenBank used in this study.

Ring no.	**Region**	**Accession no.**	
		COI	Cyt*b*
Collected samples			
AAA140	Bangladesh	MT921110	MT921114
AAA504	Bangladesh	MT921109	MT921113
AAA509	Bangladesh	MT921108	MT921112
AAA631	Bangladesh	MT921107	MT921111
A35121	Thailand	-	MT921115
Sequences downloaded from GenBank			
P. occisinensis	China	HQ608869	-
P. occisinensis	China	MK360490	-
P. occisinensis	China	-	HQ608829
P. occisinensis	China	-	MK360315
P. occisinensis	Tibet	-	EU815089
P. affinis	China	MK360452	-
P. affinis	China	MK360460	-
P. affinis	China	-	FJ155878
P. affinis	Nepal	-	EU851090
P. affinis	India	-	L77128
P. griseolus	Russia	GQ482433	-
P. griseolus	China	MK360478	-
P. griseolus	China	-	MK360295
P. griseolus	Russia	-	FJ155894
P. griseolus	Russia	-	EU851091
P. armandii	China	HQ608871	-
P. armandii	China	-	EU851092

trees were GTR Gamma (both COI and cyt*b*) and for the BI trees HYK85 Gamma (both COI and cyt*b*). The ML trees were constructed using RAxML v8.2.12 (Stamatakis 2014) on CIPRES Science Gateway V3.3 (Miller *et al*. 2010) with 1,000 bootstrapping iterations. A bootstrap value of 70% or higher was considered significant support. The BI trees were performed in MrBayes v3.2.6 (Huelsenbeck & Ronquist 2001) under a Metropolis-coupled, Markov chain Monte Carlo (MC-MCMC) approach, started from a random tree, run twice in parallel with a four-chain analysis for five million generations. The trees were sampled every 100 generations and 25% of these were discarded as 'burn-in'. We ran Tracer v1.7.1 (Rambaut *et al*. 2018) to evaluate stationarity, and Effective Sample Size (ESS) values >200 were obtained for all outputs. Consensus topology, branch length, and bipartition posterior probability were estimated from the remaining trees, with posterior probabilities of 95% or higher considered as significant support. The trees for both ML and BI approaches were visualised and edited in FigTree v1.4.3 (Rambaut 2009).

Results

The samples from all four Bangladesh-caught birds were successfully amplified for both COI and cyt*b* genes, whereas only cyt*b* was successfully amplified in the blood sample from the single northern Thai bird (Table 2). The alignments revealed 668 bp for

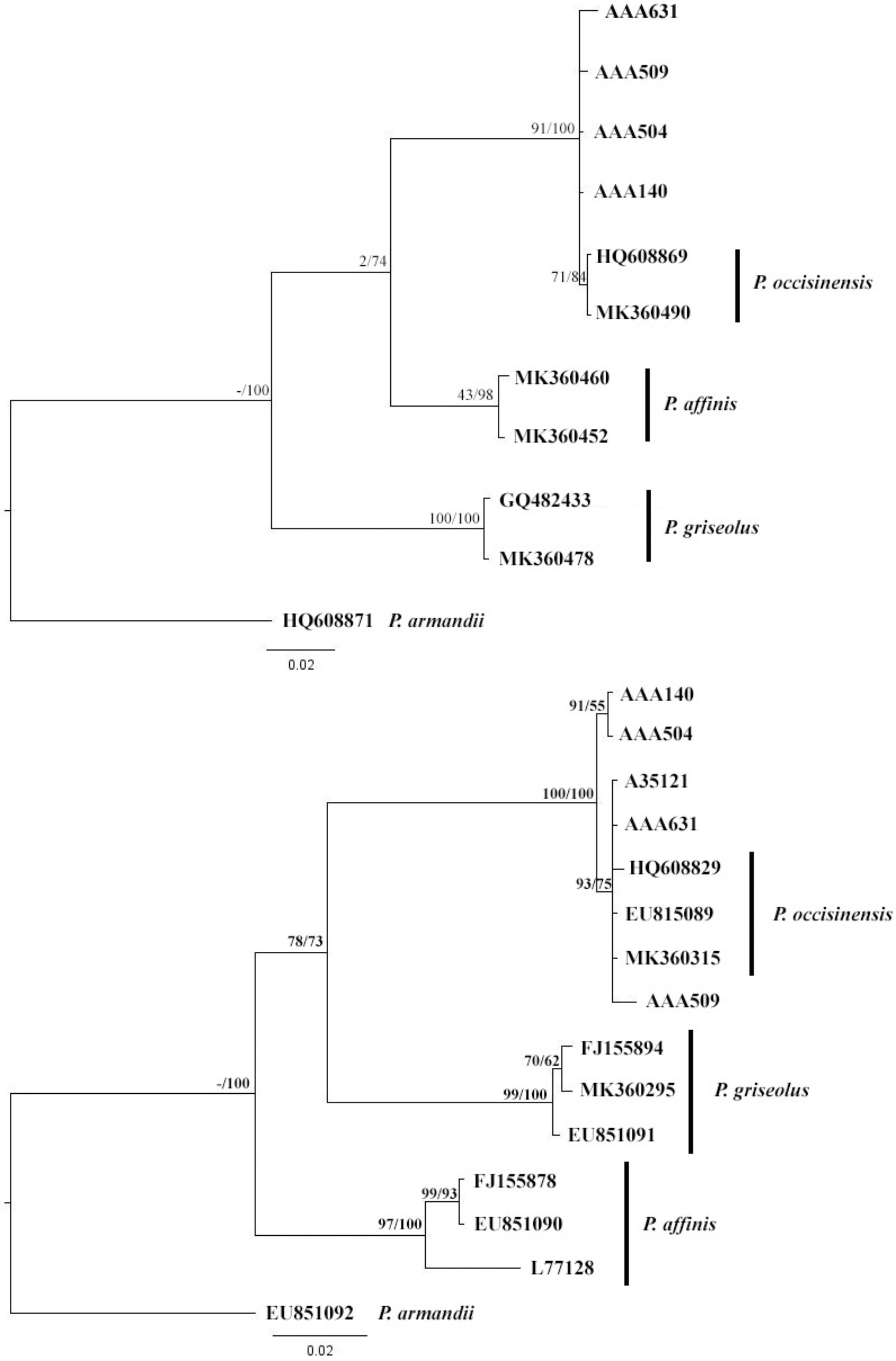

Figure 3. Bayesian Inference phylogenetic trees based on COI (A) and Cyt*b* (B) genes of collected samples and those downloaded from GenBank, with Yellow-streaked Warbler *Phylloscopus armandii* serving as an outgroup. Numbers on each node represent percent bootstrap values and posterior probabilities, respectively.

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COI (104 variable sites, 77 parsimony informative sites) and 673 bp for cyt*b* (115 variable sites, 84 parsimony informative sites). As the phylogenetic analyses based on ML and BI approaches provided similar results, only the BI trees are shown (Fig. 3). Phylogenetic trees for COI and cyt*b* were largely concordant in topology, and revealed the monophyly of all collected samples (AAA140, AAA504, AAA509, AAA631 and A35121) with *P. occisinensis*, with strong statistical support (COI: 91/100% and cyt*b*: 100/100% for bootstrap values and posterior probabilities, respectively). The *P. occisinensis* clade was distinct from both *affinis* and *griseolus*. However, whilst the *P. occisinensis* clade was resolved as sister to *P. affinis* in the COI tree, *occisinensis* appeared as sister to *P. griseolus* in the cyt*b* tree (Fig. 3). In the cyt*b* tree indicated by Martens *et al*. (2008) *affinis* was sister to *griseolus*, while in a concatenated tree of *t*RNA, *r*RNA and cyt*b* it was *occisinensis* and *griseolus* that appeared as sisters. Incongruities in mtDNA topologies among these taxa were discussed by Alström *et al.* (2018) and are not surprising as the mitochondrial divergence is almost identical among all three lineages (Zhang *et al*. 2019).

Discussion

Four wintering *P. affinis* (*sensu lato*) caught and ringed at wetlands in north-east Bangladesh, and another individual, a presumed vagrant, in northern Thailand, the easternmost South-East Asian wintering record known (Clement 2020), proved to lie in the recently distinguished Alpine Leaf Warbler *P. occisinensis* lineage.

Even considering the greater proximity of Bangladesh to the Himalayan breeding areas of the more westerly distributed *P. affinis* (*sensu stricto*), the discovery of *P. occisinensis* there was not especially surprising considering that north-east India and Bangladesh also harbour wintering populations of other species that breed in south-west China such as Firethroat *Calliope pectardens* (Rasmussen & Anderton 2005, Round *et al*. 2014). Given the abundance of *P. affinis* (*sensu lato*) in trees and scrub around the margins of northern Bangladesh wetlands in winter, and considering that so few individuals were sampled, the question arises whether *P. affinis* (*sensu stricto*) and *P. occisinensis* might winter alongside each other in the same areas as yet undetected. Clearly more sampling is needed to improve understanding of the winter distribution of these taxa across South Asia. Because *P. affinis* (*sensu lato*) winters as far south as the Western Ghats (Rasmussen & Anderton 2005) it might be productive to first sample individuals there to discover whether there is any major disparity in the wintering ranges of the two, the more southerly and westerly distributed birds perhaps being exclusively *P. affinis* (*sensu stricto*). It also remains to be investigated whether names applied to specimens currently synonymized with *P. affinis* (e.g., *Abrornis xanthogaster* Hodgson, 1844, *Phyllopneuste flaveolus* G. R. Gray, 1846) might in fact be applicable to the newly described taxon.

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