

Distinguishing between Sakhalin Leaf Warbler *Phylloscopus borealoides* and Pale-legged Leaf Warbler *P. tenellipes* on call

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The Sakhalin Leaf Warbler *Phylloscopus borealoides* has only recently been confirmed as a frequent passage migrant and probable winter visitor in South-East Asia, alongside the long-recognised Pale-legged Leaf Warbler *P. tenellipes*. The great morphological similarity of these two species renders them very hard to distinguish either in the field or in the hand. We sampled both species caught on passage at coastal sites in Thailand, extracting and amplifying the mitochondrial cytochrome c oxidase I (COI) gene from tail-feathers. Call-notes of birds were recorded on release. The results from bioacoustics and genetic analyses in a sample of 33 birds were 100% concordant. We were able to confirm that the call-notes of Pale-legged Leaf Warblers (n = 14 individuals) were consistently higher than those of Sakhalin Leaf Warblers (n = 19 individuals), having mean peak frequencies of $5,729.29 \pm 77.23$ Hz and $4,786.58 \pm 191.08$ Hz for the two species respectively, with no overlap. This confirms previous assertions that the call-notes, though similar, differ significantly in frequency, so that most individuals that are heard to call may be assigned to one species or the other by experienced observers. This will greatly facilitate future assessment of the relative status of the two species from field observations.

INTRODUCTION

Distinguishing between the two sibling species of leaf warblers, Pale-legged Leaf Warbler *Phylloscopus tenellipes* and Sakhalin Leaf Warbler *P. borealoides*, is particularly difficult, even in a genus whose members pose no shortage of identification challenges. Although first described as a distinct species by Portenko (1950), the Sakhalin Leaf Warbler until recently was regarded by most authors as a subspecies of the Pale-legged Leaf Warbler. The two are almost identical in plumage and bare-part colours and, while both sexes of Sakhalin Leaf Warbler are on average longer-winged than those of Pale-legged Leaf Warbler, the overlap in biometrics and wing formula is large, so that most (unsexed) individuals lie in an overlap zone between male Pale-legged and female Sakhalin and cannot easily be attributed to one species or the other, even when examined in the hand by ringers (Round *et al.* 2016). The distributions of the two on their breeding grounds are allopatric, with Pale-legged Leaf Warbler breeding in south-east Russia, north-east China and North Korea (Clement 2017), and the Sakhalin Leaf Warbler on Sakhalin Island, Russia, and the Kuril Islands, Russia, and Japan (Clement & de Juana 2017). The songs of the two are markedly different, as is habitat use on the breeding grounds. Sakhalin Leaf Warblers feed mainly in the crowns of coniferous trees, while Pale-legged Leaf Warblers mostly frequent broad-leaved understorey and middle storey vegetation (Martens 1988, Weprincoew *et al.* 1989). Both authors indicated that the short, metallic *tsink* call-notes of the two species, although similar, were separable, with that of Sakhalin Leaf Warbler being lower-pitched than that of Pale-legged Leaf Warbler. Both species are long-distance migrants and, although Pale-legged Leaf Warbler has long been considered to winter in Thailand and neighbouring South-East Asian countries (Deignan 1963, King *et al.* 1975, Lekagul & Round 1991, Dickinson & Christidis 2014), only recently have records of both Sakhalin Leaf Warbler and Pale-legged Leaf Warbler in Thailand been substantiated by DNA assay. DNA extracted from tail-feathers revealed 12 Sakhalin Leaf Warblers during spring and autumn passage seasons, and nine passage and wintering Pale-legged Leaf Warblers, among birds caught and ringed during 2008 to 2012 (Round *et al.* 2016). At least 15 Sakhalin Leaf Warblers were further identified unequivocally on measurements among a sample of 75 individuals of both species caught and ringed on spring migration in Thailand during April 2013 (Robson 2013, Round & Limparungpatthanakij 2013). However, the first regional records of Sakhalin Leaf Warbler were of passage migrants in Hong Kong, China,

where 32 trapped birds were considered on measurements to be that species, alongside 42 Pale-legged Leaf Warblers and 73 others that were indeterminate (Carey *et al.* 2001). The identity of two Sakhalin Leaf Warblers was confirmed by DNA assay along with five Pale-legged Leaf Warblers (P. J. Leader *in litt.* to PDR, July 2017).

Yap *et al.* (2014) made observations in the field of two presumed Sakhalin Leaf Warblers wintering in Singapore (from mid-December 2013 to mid-March 2014), whose identity was confirmed when sub-song was heard from one or both individuals. An analysis of the call-notes from one of these was compared with those of Pale-legged Leaf Warblers on the breeding grounds and from presumed Pale-legged Leaf Warblers on passage, and the calls were found to be markedly lower-pitched, thus agreeing with the descriptions of Sakhalin Leaf Warbler's call previously published in Martens (1988) and Weprincoew *et al.* (1989).

OBJECTIVES AND METHODS

Our objective was to obtain a significantly large sample of *P. borealoides* and *P. tenellipes* caught on migration in order to examine whether we could reliably correlate identification based on DNA with call-notes recorded on release. One or two tail-feathers were taken from migrant individuals caught for ringing, chiefly on Ko Man Nai (Man Nai Island), Klaeng district, Rayong province, east Thailand (12.613°N 101.688°E), on northwards spring passage during March–April 2013–2016. A few individuals caught on autumn passage in southern Thailand—three from Khao Dinsor, Chumphon province (10.633°N 99.286°E), on 30 September 2014, 3 October 2014 and 4 October 2015, and one from Khao Ramrom, Nakhon Si Thammarat province, southern Thailand (8.234°N 99.806°E), on 28 October 2015—were also included in the analysis. Biometrics (at least wing, tail, bill and tarsus, and sometimes also full wing formula) were recorded from birds in the hand. Call-note on release was recorded with a Sony PCM D 50 Linear Recorder and a Sennheiser ME 66 microphone. Call parameters were visualised in spectrograms using Adobe Audition 3.0 software (Adobe Systems Inc., San Jose, CA, USA). Call duration was measured directly from the spectrogram and peak frequency measured following a Fast Fourier Transformation of the spectrogram into frequency and amplitude domain. At most, three calls were heard from each individual as the bird flew off: only the first call, when the bird was nearest the microphone, was measured. Many birds did not utter any call on release.

Molecular assay

Genomic DNA was extracted from feathers using QIAamp Mini Kit or QIAamp DNAeasy Kit (QIAGEN), with 30 μ l of 0.1% Dithiothreitol (DTT) added to increase the DNA yield (Olsson *et al.* 2005). The samples were incubated in a water bath at 55°C for 24 hours. Amplification of 695 base pairs of mitochondrial cytochrome c oxidase I gene (COI) was accomplished by using a pair of universal primers, Bird F₁ (5'-TTCTCCAACCACAAAGACATTGGCAC-3') and Bird R₁ (5'-ACGTGGGAGATAATTCCAAATCCTG-3') (Saitoh *et al.* 2015). Polymerase chain reaction (PCR) was performed in a 25 μ l reaction volume, and each reaction contained 12.5 μ l of Toptaq Master Mix, 5 pmol of BF₁ primer, 5 pmol of BR₁ primer, 5.5 μ l of H₂O and 3 μ l of DNA templates. PCR was done in a thermal cycler (Mastercycler[®] nexus gradient). PCR amplification followed the protocol from Saitoh *et al.* (2015): 94°C for 3 minutes followed by 5 cycles at 94°C for 30 seconds, 48°C for 30 seconds, 72°C for 1 minute, then 30 cycles at 94°C for 30 seconds, 51°C for 30 seconds, 72°C for 1 minute and a final 72°C for 5 minutes. PCR products were checked by electrophoresis before sequencing, using an ABI 3730xl automatic sequencer following the ABI PRISM BigDye Terminator Cycle Sequencing protocol. Both strands were sequenced using the same primers as for PCR. Each pair of strands was aligned and checked manually in MEGA 7 (Kumar *et al.* 2016).

A phylogenetic tree with bootstrap values of 100 replications was constructed from the COI sequences using the best fit HKY+G model and maximum likelihood method. Sequences of *P. tenellipes* (GQ482452, GQ482453) and *P. borealoides* (AB843688, GQ482415) from GenBank were used as references, with *P. magnirostris* (HQ608867) and *P. borealis* (KC354945, JN801360) as out-groups.

RESULTS

Feather samples from a total of 48 individuals were obtained, of which 45 yielded DNA sufficient for assay. The total included 27 Sakhalin Leaf Warblers and 18 Pale-legged Leaf Warblers (Figure 1). All sequences were deposited in GenBank (Accession nos. KY627686–KY627730). Of these, recorded call-notes from 33 individuals—19 Sakhalin Leaf Warblers and 14 Pale-legged Leaf Warblers—were available for bioacoustic analysis.

There was 100% concordance between results obtained from DNA assay and from bioacoustic data. The two taxa clustered distinctly from each other when Fast Fourier Transformed Peak Frequency was plotted against duration (Figure 2), with no overlap in call-note frequency. The mean frequency of the call-note for Sakhalin Leaf Warbler was 4786.58 ± 191.08 Hz, compared with 5729.29 ± 77.23 Hz for Pale-legged Leaf Warbler (Table 1). The difference was highly significant (T-test; $P = 0.000$). The highest frequency for Sakhalin Leaf Warbler was 5254 Hz, this being 387 Hz lower than the lowest frequency recorded for Pale-legged Leaf Warbler (5641 Hz). The two were therefore readily separable on call-note frequency.

Table 1. Peak frequency and duration of calls of Sakhalin Leaf Warbler *Phylloscopus borealoides* and Pale-legged Leaf Warbler *P. tenellipes* (mean \pm standard deviation).

		peak frequency (Hz)	duration (sec)	n
<i>P. borealoides</i>	mean	4,786.58 \pm 191.08	0.084 \pm 0.017	19
	range	(4,521–5,254)	(0.063–0.125)	
<i>P. tenellipes</i>	mean	5,729.29 \pm 77.23	0.066 \pm 0.012	14
	range	(5,641–5,857)	(0.046–0.87)	



Figure 1. Maximum likelihood tree based on 695 base-pairs sequences of the mitochondrial COI gene compared with sequences from GenBank of *P. tenellipes* (GQ482452, GQ482453) and *P. borealoides* (AB843688, GQ482415). The tree was rooted with *P. magnirostris* (HQ608867) and *P. borealis* (KC354945, JN801360) as out-groups. Bootstrap values (100 replicates) were indicated at nodes. Low freq and High Freq indicate individuals from which the call-note was recorded.

There was also a small but significant difference in call durations (T-test; $P = 0.002$), although this was scarcely detectable by the human ear.

DISCUSSION

By means of recording vocalisations from 33 individuals (19 Sakhalin Leaf Warblers and 14 Pale-legged Leaf Warblers) whose identities were confirmed by DNA assay, we can confirm a marked difference in the frequencies of the call-notes of the two species. Indeed, an observer with prior familiarity with the calls of both taxa would have little difficulty in separating most individuals by ear. Recently archived and labelled recordings of the call-notes of both species on www.xeno-canto.org illustrate this difference

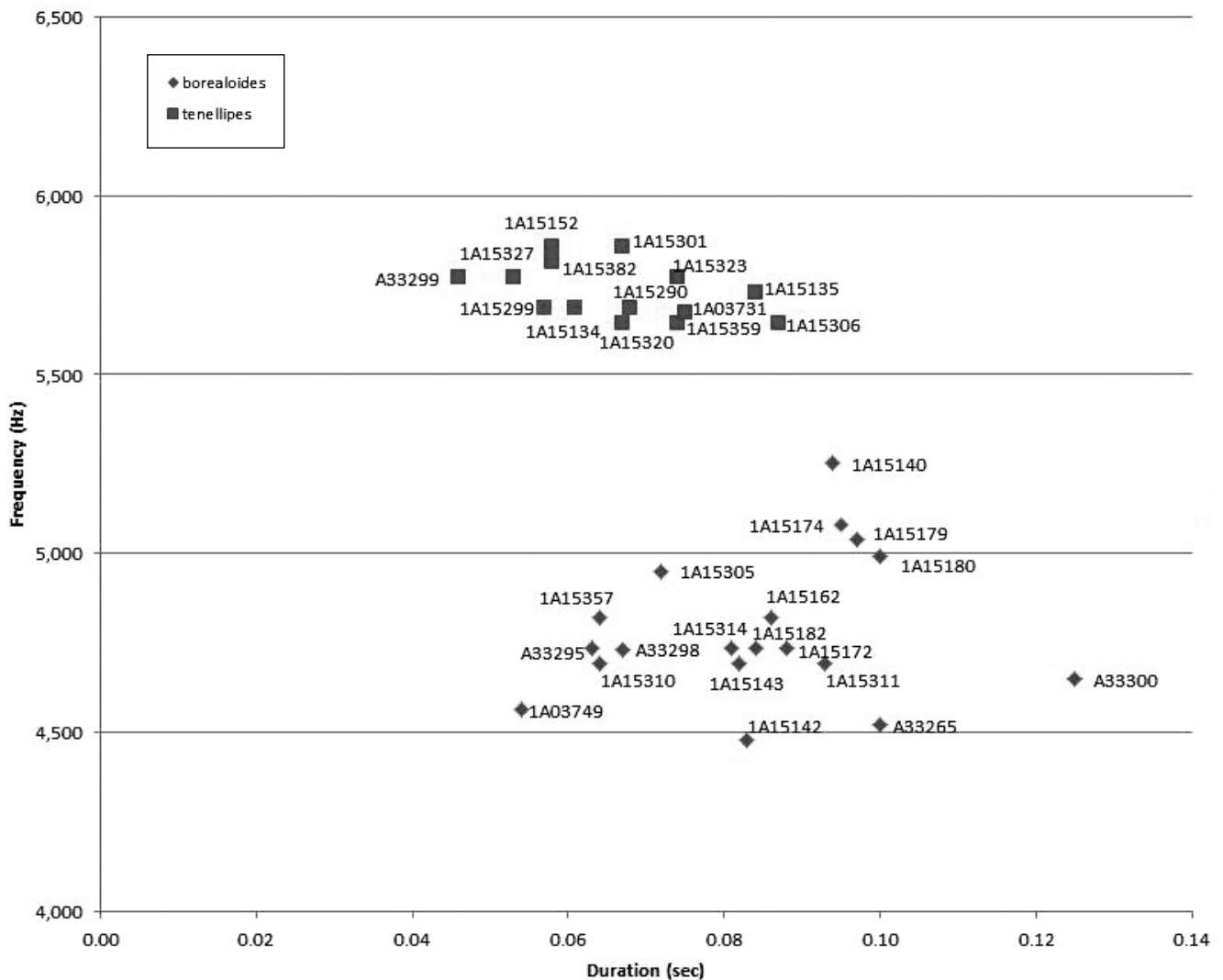


Figure 2. Plot of Fast Fourier Transformed peak frequency of calls of Sakhalin Leaf Warbler and Pale-legged Leaf Warbler against call duration. Each data point is identified by the ring number of the relevant individual.

well. Previous descriptions of call-note differences (Martens 1988, Weprincew *et al.* 1989, Yap *et al.* 2015) were based on small or undefined samples.

Vocalisations—call-notes or song—may be the only reliable way of distinguishing between these two taxa in the field away from their breeding areas. Even when individuals are examined in the hand, the majority may be not be readily diagnosable by either wing-length or wing formula. Improved knowledge of call-note differences will be important in unravelling the migratory ecology and annual cycle of these two sibling species. A small number of mid-winter records of Sakhalin Leaf Warbler from Bangkok (D. Gandy *in litt.* to PDR) and several others from Peninsular Thailand, archived on xeno-canto, point to a wintering area that extends into, and possibly mainly centred on, the Sunda subregion. A companion paper (Round *et al.* in prep.) will focus on the timing of passage and relative abundance of the two species in Thailand.

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