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Use of DNA Barcode Sequences for Distinguishing the Three Species in the Arctic Warbler (*Phylloscopus borealis*) Species Complex

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The Arctic warbler (*Phylloscopus borealis*) species complex is commonly present in the Palearctic region. By 2014, the three bird subspecies were split into three species, Arctic warbler (*P. borealis*), Japanese leaf warbler (*P. xanthodryas*), and Kamchatka leaf warbler (*P. examinandus*), based on different breeding areas and distinct vocalization. However, their similar coloration and body size make it difficult to distinguish these species in nonbreeding season. Taiwan is located in the potential migration routes of the Arctic warbler species complex; however, no confirmed record of *P. xanthodryas* and *P. examinandus* exists. In this study, we first compared the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) sequences of samples from breeding sites during the breeding season and confirmed that the three species could be distinguished based on CO1 gene sequences. We then examined the species of the Arctic warbler species complex samples collected from eastern Taiwan. For the first time, we confirmed that all three species visited Taiwan during migration season. In the Taiwanese samples, no clear distinction could be made between species based on plumage coloration and size, indicating that both these traits are not reliable for species identification. Reassessment of the CO1 gene sequences of the three species deposited in the Barcode of Life Data System revealed that the taxonomic status needs to be updated for 31.8% of the samples.

Key words: Arctic warbler species complex, DNA barcode, *Phylloscopus borealis*, *Phylloscopus xanthodryas*, *Phylloscopus examinandus*

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BACKGROUND

Understanding the species list of a region is the first step in biodiversity conservation. A complete species list can be used as a basis for assessing species survival, migration, and threats; this in turn contributes to biodiversity conservation and natural resource management (Mace 2004; Kirby et al. 2008; Rutt et al. 2019; Cazalis et al. 2020; Exantus and Cézilly 2023). *Phylloscopus borealis* is a bird species belonging to the family Phylloscopidae. It is widely distributed in the Palearctic region. Historically, three subspecies were identified: *P. b. borealis* (Blasius, 1858), *P. b. xanthodryas* (Swinhoe, 1863), and *P. b. examinandus* (Stresemann, 1913) (Vaurie 1954). Because these subspecies cannot be easily identified based on morphology, they are collectively referred to as the Arctic warbler species complex or superspecies (Martens 2010). In addition to differences in body size (Saitoh et al. 2008), genetic differentiation has been reported between Arctic warbler populations or subspecies. Reeves et al. (2008) found that the birds breeding on Sakhalin Island and the Kamchatka Peninsula differed from the birds on Palearctic/Beringian in terms of mitochondrial ND2 sequence. Saitoh et al. (2010) and Withrow et al. (2016) reported significant differences in mitochondrial cytochrome *b* gene sequences between the three subspecies. Based on allopatry in breeding areas, differences in vocalization, and divergence of mitochondrial gene sequences, Alström et al. (2011) argued that reproductive isolation exists in the Arctic warbler species complex, and that this complex should be split into three distinct species. In the Clements Checklist of Birds of the World in 2014, the Arctic warbler species complex was formally split into three species: Arctic warbler (*P. borealis*), Japanese leaf warbler (*P. xanthodryas*), and Kamchatka leaf warbler (*P. examinandus*) (Clements et al. 2014).

The three species of the Arctic warbler species complex have distinct breeding ranges: The Arctic warbler breeds in northern Europe, northern Siberia, Russian Far East, Mongolia, northeastern China, and Alaska; the Japanese leaf warbler breeds between Honshu and Kyushu, Japan; and the Kamchatka leaf warbler breeds in the Kamchatka Peninsula, Sakhalin, Kuril Islands, and Hokkaido (del Hoyo et al. 2020a b; Lowther and Sharbaugh 2020). After breeding season, these species migrate southward for wintering via different routes. The Arctic warbler migrates along eastern China and eastern and southeastern Asia and winters in Borneo and Indonesia (Lowther and Sharbaugh 2020). A recent study revealed that the Alaskan population further migrates through the Philippines and winters in the Palau region (Adams et al. 2022). The Japanese leaf warbler is

speculated to winter from Taiwan to Java, and the Kamchatka leaf warbler possibly migrates through Japan and South Korea and winters in the Philippines (del Hoyo et al. 2020a b). The Arctic warbler species complex can be found in in Taiwan in nonbreeding season. According to Severinghaus et al. (2012), the Arctic warbler and Japanese leaf warbler winter in Taiwan. However, as per a recent checklist, the Arctic warbler is regarded as a common winter visitor in Taiwan. So far, no confirmed records of the Japanese leaf warbler and Kamchatka leaf warbler exist in Taiwan (Ding et al. 2023).

During breeding season, the three species can be distinguished by their distinct breeding areas and vocalization (Alström et al. 2011). After breeding season, they can only be distinguished based on morphology at migratory stopovers or wintering sites. Minor differences exist in plumage coloration between the species: the Arctic warbler has the grayest upperpart and whitest underpart, the Japanese leaf warbler has even yellow coloration throughout, and the Kamchatka leaf warbler has a yellowish strip from the central throat to underparts (Hsiao and Li 2017). Lowther and Sharbaugh (2020) and del Hoyo et al. (2020a b) have noted that the Arctic warbler is plain olive on top (tinged grayish and brownish) and paler and whitish below, with a grayish wash or streaks on the breast side and flanks; the Japanese leaf warbler has a bright olive green crown and upperparts, variably yellowish throat and underparts, and white or whitish belly; and the Kamchatka leaf warbler has an olive green, less bright crown and upperparts, with a duller, less bright, yellow throat and underside. In addition to plumage coloration, Saitoh et al. (2008) compared samples from breeding areas and noted differences in body size in the species complex. In general, the Arctic warbler is the smallest, whereas the Japanese leaf warbler is the largest. The body size of the Kamchatka leaf warbler differs between populations: the body size of the Kamchatka population is similar to that of the Japanese leaf warbler, and the body size of the Hokkaido and Sakhalin populations is similar to that of the Arctic warbler. In summary, the three species show minor morphological differences: the Arctic warbler is smaller and has grayish plumage, the Japanese leaf warbler is larger and has yellowish plumage, and the body size and plumage coloration of the Kamchatka leaf warbler is intermediate between those of the Arctic warbler and Japanese leaf warbler. As morphological differences are minor and overlap among species, no reliable method exists to distinguish the three species by appearance.

Molecular markers provide an alternative method for species identification. Mitochondrial DNA (mtDNA) sequences represent such markers. Because of their maternal inheritance (Dawid and Blackler 1972), small genome size (Moritz et al. 1987), and high mutation rate (Brown et al. 1979), mtDNA sequences can reveal genetic differentiation between species (Castro et al. 1998); therefore, they are widely used in phylogenetic studies. DNA barcoding is a global tool for specimen identification. Sequencing a fragment of the mtDNA gene and comparing the sequence

with known sequences from a database can enable the accurate identification of species (Hebert et al. 2003). In birds, the mitochondrial cytochrome *c* oxidase subunit 1 (CO1) gene is used for DNA barcoding. The size of the CO1 gene used for DNA barcoding is approximately 648 bp. It is widely used for avian species identification and taxonomy research because of its small size, high success rate of sequencing, and low sequencing cost in addition to the availability of universal primer pairs (Hebert et al. 2004; Dove et al. 2008; Lijtmaer et al. 2011; Tavares et al. 2011; Colihueque et al. 2021). The Barcode of Life Data Systems (BOLD, https://www.boldsystems.org/) is an online database hosting the DNA barcode data. Currently, more than 6000 bird species have sequences been submitted to the database. However, as taxonomic status changes through more researches, some species classifications in the database may need to be updated (van den Burg and Vieites 2023).

During wintering, birds of the Arctic warbler species complex rarely sing; therefore, species identification by vocalization is not feasible for wintering birds. The plumage coloration of wintering birds is generally less bright. The occurrence of feather wear during migration further makes plumage coloration less species distinctive. In this study, we analyzed Arctic warbler species complex samples collected from a bird banding program. We used mitochondrial CO1 gene sequences derived from blood samples for species identification. For the first time, we confirmed that all three species visited Taiwan. Morphological measurements and photographs of the samples revealed that neither body size nor plumage coloration is a reliable trait for distinguishing the species of the Arctic warbler species complex. In addition, we reanalyzed the CO1 gene sequences of the curated Arctic warbler species complex and found that many taxonomic statuses of the curated samples need to be updated.

MATERIALS AND METHODS

Bird banding and sample collection

Samples were collected from a bird banding program in Taroko National Taiwan, eastern Taiwan, that has been conducted since 2009. The environments are mainly grassland along forest edge. We set up mist nets to catch birds. After capture, each bird was first fitted with a numbered metal ring in the tarsus. We then measured the weight, maximum wing length, tail length, bill length, and tarsus length of the birds. We collected approximately 20 μl blood by venipuncture from the brachial vein, and the blood samples were stored in absolute alcohol. We took pictures of each bird against a gray background, which was later used as a color reference to correct for white

balance of the pictures. All the procedures could be completed in eight min. Subsequently, we immediately released the birds at the same site. Information on the samples, including sampling dates, locations, photographs, and sex, is provided in Table S1 and the Barcode of Life Data System (ID: NDHUP001-23 to NDHUP036-23). Bird banding and sample collection were approved by Taroko National Park, Hualien Forest District Office, Forestry Bureau (now Hualien Branch, Forestry and Nature Conservation Agency, Department of Agriculture) and the Hualien County Government.

Downloading CO1 gene sequences of the Arctic warbler species complex from the database

We searched the CO1 gene sequences of the Arctic warbler species complex in the BOLD database by using *Phylloscopus borealis*, *Phylloscopus xanthodryans*, and *Phylloscopus examinandus* as keywords. We downloaded a total of 73 sequences from the database. After removing six sequences of poor quality and one sequence that was possibly a repeat sequence from the same individual, we obtained 66 sequences. Among these, 3 sequences were from the Japanese leaf warbler, 13 sequences were from the Kamchatka leaf warbler, and 50 sequences were from the Arctic warbler (Table S2). We identified collection sites and sampling dates from the database or from the original references. Before the Arctic warbler species complex is categorized into three species, all the birds are classified as the same species (*P. borealis*); therefore, some early submitted sequences of *P. borealis* may be derived from different species. In this study, to avoid potential mistakes in species identification, we selected 21 sequences that were collected during the breeding season (June to August, Billerman et al. 2022) and noted the collection sites. We then identified the species of these samples according to the breeding/collection sites. We identified a total of 12, 4, and 5 sequences in Arctic warbler, Japanese leaf warbler, and Kamchatka leaf warbler samples, respectively.

DNA extraction, amplification, and sequencing

We used the FavorPrep^{TM} Blood/Cultured Cell Genomic DNA Extraction Mini Kit (Favorgen, Taiwan) to extract genomic DNA from the blood samples of the Arctic warblers according to the manufacturer's instructions. We then performed polymerase chain reaction (PCR) to amplify the DNA fragments of sex chromosomes and the mitochondrial CO1 gene by using the extracted DNA as a template. For molecular sexing, we amplified a fragment of the chromodomain helicase DNA-binding protein (CHD) gene from sex chromosomes by using the primer pair of 2550F (5'-GTTACTGATTCGTCTACGAGA-3') and 2718R (5'-ATTGAAATGATCCAGTGCTTG-3')

(Fridolfsson and Ellegren 1999). The reaction volume was 10 µl; it consisted of 0.5 µl DNA, 0.6 µl dNTP (2.5 mM of each nucleotide), 0.4 µl 2550F primer (10 mM), 0.4 µl 2718R primer (10 mM), 1.0 µl 10 × PCR buffer, 7.02 µl ddH_2O and 0.08 Taq DNA polymerase (5 U/µl, Protech, Taiwan). The PCR program was as follows: 3 min at 95°C; 35 cycles of 30 s at 95°C, 40 s at 46°C, and 50 s at 72°C; and a final extension step at 72°C for 5 min. We dyed the PCR products using EZ-Vision® Two, DNA Dye-as-Loading Buffer 6× (Protech, Taiwan) and then performed electrophoresis on 1.5% agarose gel (100 V, 30 min) using a Bio-100™ DNA Ladder (Protech) as a size reference. We examined the gels under ultraviolet light. Moreover, we identified the sex of the birds based on the presence or absence of the female-specific DNA fragment of the CHD-W gene.

We used the primer pair of AWCF1 (5'-CGCYTWAACAYTCYGCCATCTTACC-3') and AWCR3 (5'-ATGCTCGGGTGTCTACGTCTAT-3') developed by Patel et al. (2010) to amplify a fragment of the mitochondrial CO1 gene sequence. The reaction volume was 20 µl; it consisted of 1.0 µl template DNA, 1.3 µl dNTP (Ex Taq™ dNTP, 2.5 mM, Takara), 0.6 µl AWCF1 primer (10 mM), 0.6 µl AWCR3 primer (10 mM), 2.0 µl 10X PCR buffer (Ex Taq™ buffer, 10X, Takara, Japan), 1.3 µl $MgCl_2$ (25 mM), 12.9 µl ddH_2O, and 0.3 µl Taq DNA polymerase (Takara Ex Taq™ polymerase, 5 U/µl, Takara, Japan). The PCR program was as follows: 3 min at 94°C; 35 cycles of 30 s at 94°C, 40 s at 63°C, and 60 s at 72°C; and a final extension step at 72°C for 5 min. The PCR products were sequenced at the Cancer Progression Research Center, National Yang Ming Chiao Tung University. The sequences were aligned and edited using Sequencher 5.4.5 software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

CO1 gene sequence analyses

All sequences, including those downloaded from BOLD and those obtained from samples collected in this study, were aligned using Sequencher 5.4.5 software. DnaSP version 6.1203 (Rozas et al. 2017) was used to identify haplotypes and to calculate haplotype diversity (H_d). Network 10 (Fluxus Engineering) was used to construct a haplotype network by using a medium-joining network algorithm (Bandelt et al. 1999).

Species identification by plumage coloration

The plumage coloration of the banded Arctic warbler species complex was examined using photographs taken during the banding process. The photographs were first corrected for white balance, using PhotoDemon software (version 9.0). For some pictures without gray background, we used the same software to automatically correct white balance. Colors of upper regions (head, back,

and wing) and underparts (throat and breast) were used to identify the species. For each bird, the upper regions were denoted as having the coloration of olive green, grayish olive green, and light olive green by using the photographs provided by Round et al. (2016) as reference. The underparts were denoted as having the coloration of white, yellow, and light yellow. Figure 1 illustrated some plumage coloration. Species identification was performed based on coloration (del Hoyo et al. 2020a b; Lowther and Sharbaugh 2020; Hsiao and Li 2017). All coloration assignment and species identification procedures were performed by SJC. The results were compared with those of genetic analyses.

Fig. 1. The plumage coloration of the Arctic Warbler species complex, showing olive green upperparts (A), grayish green upperparts (B), light olive-green upperparts (C), paler and whitish underparts (D), variably yellowish throat and underparts (E), and bright yellow throat and underparts (F).

Morphological measurements

According to the results of molecular sexing and CO1 species identification, we explored sexual dimorphism in the measurements of Arctic warblers. Two samples had missing values for some traits; we therefore excluded them from the analysis. We performed principal component analysis (PCA) to assess the differences in the size of the samples.

RESULTS

A total of 36 birds from the Arctic warbler species complex were banded. Molecular sexing

revealed 17 male birds and 19 female birds.

Species identification by CO1 gene sequences

In this study, 607 bp fragments of CO1 gene sequences were obtained from 102 samples, including 66 sequences downloaded from BOLD and 36 sequences obtained from Taiwanese birds. The sequence set contained 49 variable sites, of which 44 were located at the third nucleotide of a codon. In total, 27 haplotypes were identified.

Based on the 21 CO1 gene sequences of the Arctic warbler species complex from BOLD, which were collected from the breeding area during the breeding season, three haplogroups were identified. The haplogroups matched well with the sampling sites (Fig. 1). This indicates that CO1 gene sequences are an ideal marker for distinguishing Arctic warblers, Japanese leaf warblers, and Kamchatka leaf warblers.

Using the haplotype network, we identified the species of the 36 samples collected in this study. Specifically, thirty-two of them were Arctic warblers (14 males and 18 females), two were Japanese leaf warblers (both males), and two were Kamchatka leaf warblers (one male and one female) (Fig. 2, Table S1).

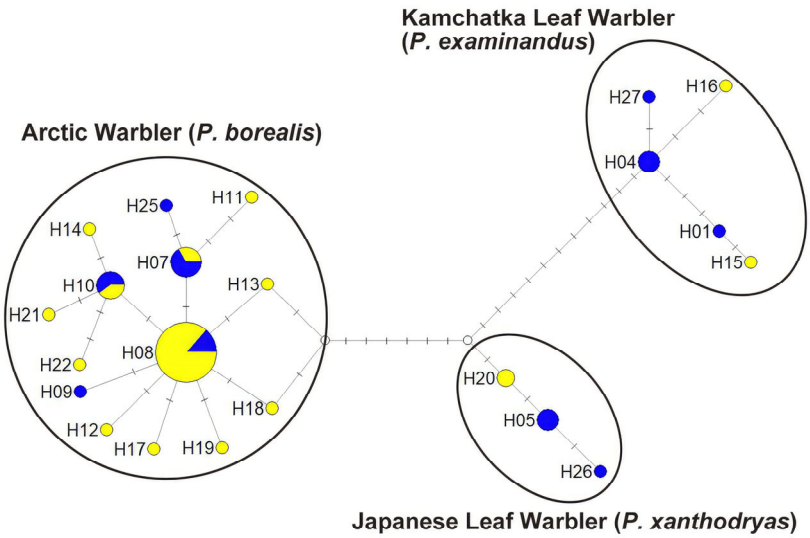

Fig. 2. Haplotype networks of the Arctic warbler species complex (*Phylloscopus borealis* spp.) constructed using fragments of mitochondrial CO1 gene sequences (607 bp). Blue indicates samples from BOLD, which were collected from breeding areas during the breeding season. Yellow represents the Taiwanese samples obtained from this study. The size of the circle is proportional to the sample size.

Reassessment of the CO1 gene sequences of the Arctic Warbler species complex deposited in BOLD revealed that the species name of many samples needs to be updated. Three Japanese leaf warbler samples in BOLD should be reclassified as Arctic warblers. Of the 50 Arctic warbler samples in BOLD, five should be reclassified as Japanese leaf warblers, and 13 should be reclassified as Kamchatka leaf warblers. The 13 Kamchatka leaf warblers in BOLD are all correctly classified. In total, the taxonomic status of 31.8% of the samples (21/66) needs to be updated (Table S2).

On combining sequences from the BOLD database and the Taiwanese samples, sixteen of 27 haplotypes were identified from Arctic warblers (n = 67, H_d = 0.767). Moreover, four haplotypes were identified from Japanese leaf warblers (n = 7, H_d = 0.81), and seven were identified from Kamchatka leaf warblers (n = 28, H_d = 0.743) (Table S1, Table S2).

Species identification by plumage coloration and size

Using the results of genetic species identification, we examined the plumage coloration and morphological measurement data of the three species. Both traits showed extensive overlap among species; therefore, they cannot be used for species identification. Of the 30 samples for which photo records were available, the species of only 50% (15/30) were correctly identified by plumage coloration (Table 1, Table S2). Identification errors occurred for all three species.

Table 1. Comparison of species identification by mitochondrial CO1 gene sequences and plumage coloration. Numbers in bold indicate the number of birds that were identified as the same species by using both methods

		Species identified by plumage coloration		
Species identified by CO1 gene sequences	N	Arctic warbler	Japanese leaf warbler	Kamchatka leaf warbler
Arctic warbler	26	**13**	2	11
Japanese leaf warbler	2	1	**1**	0
Kamchatka leaf warbler	2	1	0	**1**

We noted sexual dimorphism in the morphological measurements of Arctic warblers. Male birds were significantly larger than female birds in all measurements, except for the bill length (Table 2). Because only two Japanese leaf warblers (both male birds) and two Kamchatka leaf warblers (one male and one female) were identified, we could not compare sexual dimorphism for both species. In PCA, both PC1 and PC2 explained 71.8% of the variation (44.9% and 26.9% for PC1 and PC2, respectively). PCA of all samples revealed that the measurements of the three species

greatly overlapped. However, Kamchatka leaf warblers tended to be larger in both PC1 and PC2, whereas Japanese leaf warblers tended to be smaller in PC2 (Fig. 3).

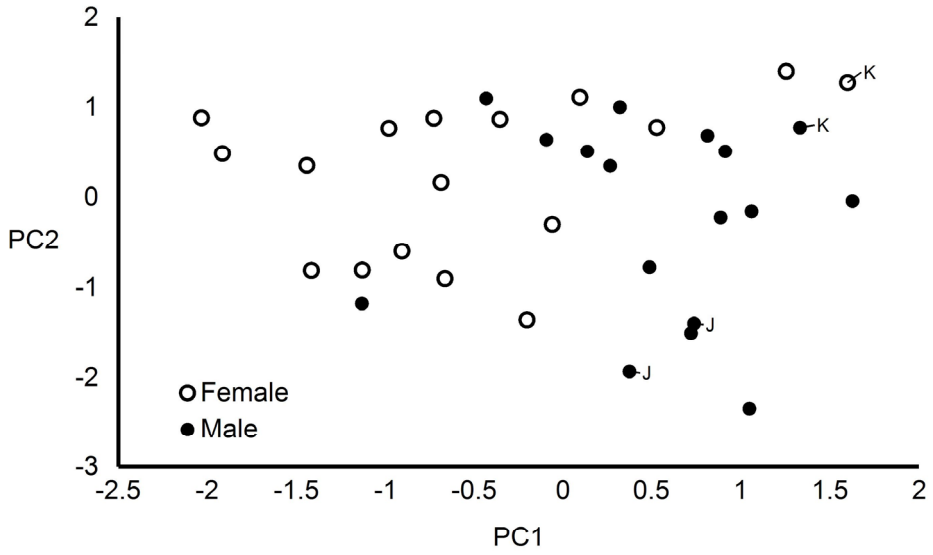

Fig. 3. Principal component analysis of measurement data of the Arctic warbler species complex. Samples marked with "J" are Japanese leaf warblers, those marked with "K" are Kamchatka leaf warblers, and the rest are Arctic warblers.

Table 2. Morphological measurements of the Arctic warbler species complex samples collected from Taiwan

Measurements	Species					
	Arctic warbler			Kamchatka leaf warbler		Japanese leaf warbler
	Male (n = 14)	Female (n = 16)	t test	Male (n = 1)	Female (n = 1)	Male (n = 2)
Weight (g)	9.67 ± 1.09	8.93 ± 1.10	$t = 1.85$, $p = 0.04$	10.4	11.8	9.15 ± 0.78
Maximum wing length (mm)	66.11 ± 2.65	62.56 ± 2.57	$t = 3.70$, $p < 0.001$	69.0	66.0	67.50 ± 0.71
Tail length (mm)	47.46 ± 3.23	44.63 ± 3.21	$t = 2.41$, $p = 0.012$	44.5	48.0	51.25 ± 0.35
Bill length (mm)	9.43 ± 0.46	9.29 ± 0.41	$t = 0.91$, $p = 0.19$	9.67	10.19	8.65 ± 0.29
Tarsus length (mm)	19.32 ± 0.53	18.78 ± 0.63	$t = 2.57$, $p = 0.008$	20.35	19.71	19.61 ± 0.89

DISCUSSION

Taiwan is located in the middle of the East Asian-Australasian Avian Flyway. The annual latitudinal migration of nearly 700 species of birds occurs along this flyway (Kirby et al. 2008). According to the latest checklist, no confirmed record of the Japanese leaf warbler and Kamchatka leaf warbler exists in Taiwan at present (Ding et al. 2023). In this study, we successfully identified the three species of the Arctic warbler species complex based on mitochondrial CO1 gene sequences. Our results present strong evidence of their presence in Taiwan.

All our Japanese leaf warbler and Kamchatka leaf warbler samples were banded during the migration season: three were collected in October and one was collected in May. Given their low number and appearance in the migration season, both species appear to be rare transients at our study site (eastern Taiwan). More samples from other regions of Taiwan would help reveal their population status in Taiwan.

During nonbreeding season, the Arctic warbler species complex cannot be distinguished by species-specific songs. Although several studies have reported differences in plumage coloration (del Hoyo et al. 2020a 2020b; Lowther and Sharbaugh 2020), we failed to distinguish the three species based on the plumage coloration of our samples. A similar result was reported by Round et al. (2016); they found that the plumage coloration of some wintering Arctic warblers was very similar to that of the Kamchatka leaf warbler. For birds in migration, feather abrasion, changes in food composition, and different molting stages may make species identification by morphology more difficult.

According to the results of PCA, we did not find considerable disparity in body size among the three species. The Kamchatka leaf warbler tended to be larger than the Arctic warbler and Japanese leaf warbler. This does not fully conform to the findings of previous studies (Saitoh et al 2008; Round et al. 2015 2016), which have reported that the body size of the Kamchatka leaf warbler is intermediate of the size of the Arctic warbler and Japanese leaf warbler. For migratory birds, their body weight may fluctuate greatly, owing to fat deposition and depletion along the migratory routes. Therefore, there may be high variation in weight measurement either within or among species. In addition, we noted significant sexual dimorphism in the body size of Arctic warblers. However, we did not have sufficient samples to test whether such dimorphism exists in the other two species. If all three species exhibit sexual dimorphism in their body size, species identification by body size will become more unreliable.

For closely related species, incomplete lineage sorting and introgression through secondary

contact may lead to the three species not necessarily presenting reciprocal monophyly. Given the fact that the three species are not sympatric during breeding season, and they have distinct specie-specific vocalizations, genetic introgression through hybridization among seems less likely. In addition, in a study on the phylogeny of Phylloscopidae warblers, Alström et al. (2018) did not find evidence of incomplete lineage sorting in mitochondrial cytochrome b gene. However, they found evidences of incomplete lineage sorting in nuclear genes in some lineages. In our samples, the three species cannot be distinguished by morphology, therefore we cannot rule out the possibility of incomplete lineage sorting among them.

In addition to examining the Taiwanese samples, we reassessed the sequences deposited in BOLD, and the results revealed a high proposition (31.8%) of the samples need to revise their species names. Nearly all the Japanese leaf warblers and Kamchatka leaf warblers misidentified as Arctic warblers in BOLD were submitted before the splitting of the Arctic warbler species complex into the Arctic warbler, Japanese leaf warbler, and Kamchatka leaf warbler (Clements et al. 2014). Regarding the revision of taxonomy, their species names need to be updated. Three Arctic warblers have been misidentified as Japanese leaf warblers in BOLD. These samples were collected from South Korea (Park et al. 2011), where the Arctic warbler species complex is regarded as transient (it does not breed or winter there) (del Hoyo et al. 2020a b; Lowther and Sharbaugh 2020). These samples may be identified by morphology, which was proven to be ambiguous for species identification in this study.

A biological database serves as an invaluable resource for biodiversity research. However, as the amount of data increases, outdated taxonomic statuses are a potential problem. For example, in a recent study, van den Burg et al. (2020) found that approximately 13% of amphibian cytochrome *b* records in GenBank are taxonomically outdated. Päckert (2022) found that some Charadriidae CO1 gene sequences in GenBank may not correspond to their taxon names, which may lead to inaccurate classification results. We propose that while extracting data from BOLD, taxonomic updates should be considered.

CONCLUSIONS

In this study, we confirmed that the CO1 gene sequence can effectively identify the three species of the Arctic warbler species complex. Applying this method to samples collected from eastern Taiwan, for the first time, we confirmed the presence of the Japanese leaf warbler and Kamchatka leaf warbler in Taiwan, adding two new species to the avifauna of Taiwan. In addition, our results highlighted that the morphological (both measurement data and plumage coloration)

differences among species, as described in the literature and guidebooks, are not reliable for distinguishing the species during the migration/wintering season. Moreover, as the taxonomic status may change over time, caution should be taken when extracting sequence data from a database (such as BOLD).

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Authors' contributions: Both authors contributed equally to field work, data analyses, and manuscript writing. SJC performed morphological measurements and DNA sequencing.

Competing interests: The authors declare that they have no competing interests.

Availability of data and materials: Sequence data are submitted to BOLD. The metadata of the samples are attached in the Supplementary Materials.

Consent for publication: All the authors consent to the publication of this manuscript.

Ethics approval consent to participate: Bird banding and sample collection were approved by Taroko National Park, Hualien Forest District Office, Forestry Bureau (now Hualien Branch, Forestry and Nature Conservation Agency, Department of Agriculture), and the Hualien County Government.

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Supplementary Materials

Table S1. Information of the Arctic warbler species complex samples collected in Taiwan that were used in this study. (download)

Table S2. Information of the Arctic warbler species complex samples downloaded from the Barcode of Life Data System. (download)