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Molecular and acoustic evidence support the species status of *Anthus rubescens rubescens* and *Anthus* [*rubescens*] *japonicus* (Passeriformes: Motacillidae)

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Abstract

The Buff-bellied Pipit *Anthus rubescens* comprises two allopatric subspecies groups: *A. r. rubescens* and *A. r. alticola* in North America and *A.* [*r.*] *japonicus* in north-east Asia. Despite their great morphological resemblance in breeding plumage, most individuals can be assigned to one or the other subspecies group in non-breeding plumage. Allopatric distributions, morphological differentiation and previously reported molecular divergence suggested the need for additional taxonomic study to assess the rank of these two populations. To resolve the taxonomy of the Buff-bellied Pipit species complex we analysed i) two mitochondrial DNA (mtDNA) loci and ii) nine bioacoustic parameters across 69 sound recordings (338 flight calls) recovered from public databases using principal component analysis and Euclidean distance measures. By comparing our mtDNA and call divergence measures with similar values measured between long-recognised species pairs of the genus, we show that the level of mitochondrial and acoustic divergence between the two Buff-bellied Pipit subspecies groups is typical of species-level divergence in the genus *Anthus*. Therefore, we recommend splitting the Buff-bellied Pipit species complex into two species: *Anthus rubescens* (American Pipit) and *Anthus japonicus* (Siberian Pipit). Our results also suggest that the Water Pipit *A. spinoletta* deserves taxonomic reassessment as its lineages are highly divergent in acoustics and mtDNA, while mtDNA relationships suggest paraphyly relative to the Rock Pipit *A. petrosus*. Our work highlights the crucial importance of integrative approaches in taxonomy and the usefulness of bioacoustics in studying cryptic diversity.

Key words: American Pipit, Buff-bellied Pipit, Beringia, Bird, Speciation, Palearctic, Nearctic

Introduction

Traditionally, taxonomy has been based on morphological criteria, as these were the only criteria that could be studied on museum specimens. However, avian species delimitation is now typically based on a body of evidence integrating morphological, acoustic, genetic and other data (e.g., Alström *et al.* 2007, 2008, 2015a, 2016, 2021a, b; van Els & Norambuena 2018; Sangster 2022). Vocalizations are especially important in avian systematics as they can reflect species divergence and differentiation (Alström & Ranft 2003) but also directly contribute to reproductive barriers (Price 2008), although differences in vocalizations are not systematically linked to evolutionary divergences (Potvin *et al.* 2013). In passerines, both song, which is largely learned (Päckert 2018) and calls, which are considered to be usually innate (Marler 2004) but can also be learned (e.g., Porter & Benkman 2019), have been found to reflect

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genetic divergence and to provide results congruent with other data sets in species delimitation (Päckert *et al.* 2004; Ng *et al.* 2016; Gwee *et al.* 2019; Alström *et al.* 2021a, b; Sangster 2022; Sin *et al.* 2022). In some cases, calls can even provide information on species relationships: in 47 species of the large American wood warbler radiation, acoustic distance in flight call was significantly correlated with phylogenetic distance (Farnsworth & Lovette 2008). A growing number of deeply divergent lineages or newly recognised species have also been "discovered" thanks to DNA sequence data (e.g., Battey & Klicka 2017; Collinson *et al.* 2017; Pertiera *et al.* 2020; Pavia *et al.* 2021), even though true cryptic species which do not exhibit any diagnostic morphological or acoustic differences are not yet recognized in birds. Although single-locus approaches, such as analyses of mitochondrial DNA (mtDNA) sequences, are rightly seen as unreliable for species delimitation when used alone (Rubinoff & Holland 2005; Dufresnes & Jablonski 2022), mtDNA sequence data, in combination with other sources of information, can still potentially provide meaningful taxonomic information on reproductive isolation, timing of population divergence and hence systematics, at least when coupled with other criteria such as acoustics or morphology in an integrative taxonomic framework (Padial *et al.* 2010). In this study, we combine available and newly generated mtDNA sequence data and new acoustic data to review the taxonomy of an avian species.

The Water Pipit *Anthus spinoletta* complex was long treated as a single polytypic species. The species complex was subsequently split into the three species Water Pipit *Anthus spinoletta* (Linnaeus), Rock Pipit *Anthus petrosus* (Montagu) and Buff-bellied Pipit *Anthus rubescens* (Tunstall), based on local sympatry without apparent hybridization between *A.* [*r.*] *japonicus* and *A. s. blakistoni* in Siberia, combined with differences in plumage, biometry and breeding ecology (reviewed in Alström & Mild 2003). The comprehensive revision of Alström & Mild (2003) recognised three polytypic species: *A. spinoletta* (with subspecies *A. s. spinoletta*, *A. s. blakistoni* Swinhoe and *A. s. coutellii* Audouin), *A. petrosus* (with subspecies *A. p. petrosus* and *A. p. littoralis* Brehm) and *A. rubescens* (with subspecies *A. r. rubescens*, *A. r. alticola* Todd and *A.* [*r.*] *japonicus* Temminck & Schlegel). A fourth subspecies of *A. rubescens*, *A. r. pacificus* Todd, is recognised by some checklists (e.g., Clements *et al.* 2007, 2022) but lumped with the nominate subspecies by Alström & Mild (2003), followed here and by e.g. Gill *et al.* (2023). Zink et al (1995) suggested that differences in mtDNA restriction fragment profiles between Siberian (*A.* [*r.*] *japonicus*) and North American (*A. r. rubescens*/*alticola*) subspecies groups were consistent with species-level divergence. Similarly, Garner *et al.* (2015) suggested that *coutellii* and *blakistoni* are sufficiently divergent in plumage, acoustics and mtDNA from *spinoletta* to represent different species, although they refrained from formally recommending this split.

Anthus rubescens occurs in two major biogeographical areas, the Eastern Palearctic and the Nearctic. North America is occupied by *A. r. rubescens*, which breeds in the Arctic Circle between Alaska and Labrador south to Washington State in the northwestern USA and disjunctly in the northeastern USA and winters from the southern USA to southern Mexico and (rarely) northern Central America; and by *A. r. alticola* which breeds in a restricted and fragmented high-altitude range mainly in the Rocky Mountains from British Columbia and Montana south through California and New Mexico (Alström & Mild 2003; Hendricks & Verbeek 2020). The wintering grounds of *alticola* are poorly understood because its separation from *rubescens* outside the breeding season is problematical (Alström & Mild 2003). These two, which together form the Nearctic subspecies group of *A. rubescens* (hereafter referred to as "*rubescens*"), are very similar phenotypically, although in summer plumage *alticola* is more deeply coloured and less streaked below (Alström & Mild 2003; Hendricks & Verbeek 2020). In the eastern Palearctic, *A.* [*r.*] *japonicus* (hereafter "*japonicus*") breeds in north-eastern Russia between the Yenisei River and the Bering Strait and winters mainly in Japan, Taiwan and southeastern China but also in small numbers west to Arabia and the Middle East (Figure 1; Alström & Mild 2003; Shirihai & Svensson 2018). Nearctic and Palearctic taxa are not safely separable in breeding plumage, but they differ consistently in their respective winter plumage, mostly due to the paler underparts and larger, darker and more clearcut streaking on the breast and flanks and whiter and more clearcut wing-bars of *japonicus* compared to *rubescens* (Alström & Mild 1996, 2003; Lee & Birch 2000; Shirihai & Svensson 2018; Hendricks & Verbeek 2020). The tarsi and toes are generally paler in *japonicus* than *rubescens* in both summer and winter plumage, although there is overlap (Lee & Birch 2000; Alström & Mild 2003; Shirihai & Svensson 2018; Hendricks & Verbeek 2020). The calls of both subspecies group are also known to differ, but no comprehensive analysis has been undertaken, and some potential geographical variation has been noted within at least *japonicus* (Alström & Mild 2003; Garner *et al.* 2015).

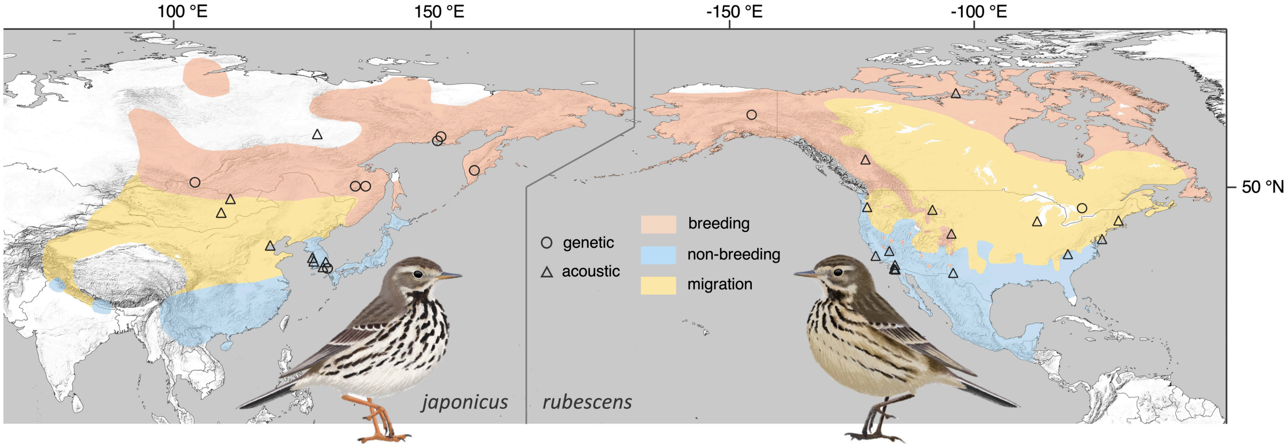

FIGURE 1. Breeding, migrating and wintering distributions of Palearctic *Anthus* [*rubescens*] *japonicus* and Nearctic *Anthus rubescens rubescens*/*alticola* subspecies groups (from BirdLife International 2022; illustration @Andrew Birch). Circles indicate origins of sequenced individuals and triangles indicate origins of analysed recordings of calls. Localities outside of the usual range of the species complex (e.g., Ireland, Oman and Israel) are not figured here.

Because of their mostly allopatric distributions, plumage and acoustic differences, and mtDNA restriction fragment divergence, *rubescens* and *japonicus* have previously been suggested to be better treated as two species (Zink *et al.* 1995; Garner *et al.* 2015), but this proposal has not yet been accepted by any of the major avian taxonomic checklists. In this study, we examine the species limits in the *Anthus rubescens* species complex using genetic divergence of mtDNA and divergence in flight call. We evaluate whether the level of genetic and acoustic divergence, combined with the known plumage differences, reflect species-level differentiation between the Asian and American subspecies groups or not, using the general principle that allopatric populations that diverge as much as valid closely related species should be afforded species rank (see details in Discussion).

Material & Methods

Phylogenetic analyses

Sequences from a 694 bp fragment of the cytochrome c oxidase subunit 1 (COI) gene from most of the Eurasian *Anthus* species and subspecies (*A. richardi* Vieillot, *A. berthelotii* Bolle, *A. trivialis* (Linnaeus), *A. hodgsoni* Richmond, *A. gustavi* Swinhoe, *A. cervinus* (Pallas), *A.* [*rubescens*] *japonicus, A. rubescens rubescens*, *A. pratensis* (Linnaeus), *A. spinoletta coutellii*, *A. spinoletta blakistoni*, *A. spinoletta spinoletta*, *A. petrosus*) were retrieved from GenBank and BOLD (barcodingoflife.org; Accession numbers in Sup. Mat. 1). A 998 bp fragment of the control region (CR) from most of the Eurasian *Anthus* species and subspecies (*A. hodgsoni*, *A. trivialis*, *A. roseatus* Blyth, *A. cervinus*, *A.* [*rubescens*] *japonicus*, *A. rubescens rubescens*, *A. pratensis*, *A. spinoletta coutellii*, *A. spinoletta blakistoni*, *A. spinoletta spinoletta*, *A. petrosus*) produced by the late Anders Ödeen in collaboration with P.A. was obtained as follows (Accession numbers in Sup. Mat. 1): Total genomic DNA from feather calamus, tissue or blood was extracted using the DNeasy Tissue Kit (Qiagen) following recommended procedures. PCR amplifications using the primers MCRI1 (5'-CAGGTACCATACAGCCCAATTT-3') and MCRI2 (5'-GAAGCGCGATCAATAGATAACC-3'; Alström & Ödeen 2002) were carried out in 25 µl volumes containing 1 X amplification buffer with 15 mM MgCl2, 0.625 units Taq polymerase, 0.2 mM of each dNTP, 0.4 µM of each primer and 0.1–2.5 ng DNA. PCR cycles involved 3 min 94°C denaturation, 35 cycles of 15 s 94°C denaturation, 30 s 63°C annealing and 1 min 72°C extension, and 72°C 10 min extension. 5 µl of the PCR products were run on a 2% agarose gel to check length and specificity of the amplifications and the remaining 20 µl were purified for cycle sequencing with Microcon PCR and YM-100 Centrifugal Filter Devices (Millipore). All amplified fragments were sequenced in both directions with the primers used for PCR amplification—primarily as internal primers—with BigDye Terminator Ready Reaction kit (Applied Biosystems). The products were run on ABI-prism 310 and 377 automated sequencers. The resulting electropherograms were analysed with Sequencing Analysis 3.3 software (Applied Biosystems). For both loci, sequences were aligned by eye. Maximum Likelihood (ML) phylogenetic trees were inferred for both markers separately using the HKY+G substitution model, which was selected as the best-fitting substitution model using defaults settings in Mega-X (Kumar *et al.* 2018). We did not attempt to build a tree by concatenating the two genes as different individuals were sequenced for each. Tree robustness was estimated by 1000 bootstrap replicates. We calculated mean inter-group (species/subspecies) genetic divergence using the Tamura 3-parameter model, the pairwise deletion option and rate variation among sites modelled with a gamma distribution in Mega-X (Kumar *et al.* 2018). Following Nabholz *et al.*'s (2016) "analysis 2" and "analysis 4" methods (depending on the fossil calibration set used) and based on the approximate 20 g mass of the Buff-bellied Pipit species complex (Cramp & Simmons 1983), we used mean inter-group divergence to infer estimates of the divergence time between *japonicus* and *rubescens* for both CR and COI markers.

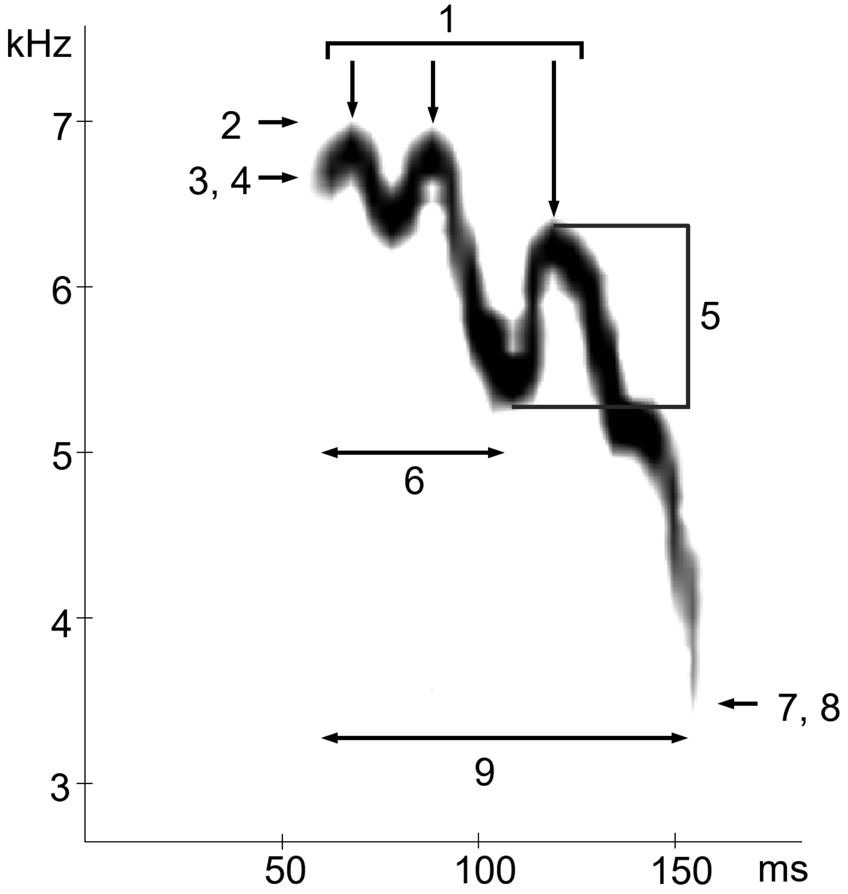

FIGURE 2. Call measurements: 1) number of frequency peaks; 2) maximum frequency; 3) rising or falling call; 4) start frequency; 5) amplitude of the largest modulation; 6) duration up to the largest modulation; 7) minimum frequency; 8) end frequency; 9) call duration.

Acoustic analyses

In order to measure the divergence between flight calls of both Buff-bellied Pipit subspecies groups we started by examining visually a selection of flight call sonograms of *rubescens* and *japonicus*, and we then selected nine acoustic variables that captured the differences apparent between these two taxa. These nine characters were measured on sonograms using Raven Lite 2.0.1 (Center for Conservation Bioacoustics 2016) as follows: rising or falling start of the call (1 / 0, compare Fig. 5C with 5B), call duration (ms), start frequency (kHz), end frequency (kHz), maximum frequency (kHz), minimum frequency (kHz), number of frequency peaks, duration up to the modulation with largest amplitude (ms), amplitude of the largest modulation, i.e. with largest amplitude (kHz; measure details on Figure 2). We measured these variables on flight calls from 30 recordings of *A. r. rubescens* and 39 recordings of *A.* [*r.*]

japonicus. Between one and eight calls were selected from each recording based on sonogram clarity. Because calls from several different individuals may occur on the same recording, we estimate that we have measured call parameters of a minimum of 30 *A. r. rubescens* and a minimum of 39 *A.* [*r.*] *japonicus.* We measured a total of 155 calls of *A. r. rubescens* and 183 calls of *A.* [*r.*] *japonicus.* To compare the call divergence between *japonicus* and *rubescens* with call divergence between other *Anthus* taxa we measured the same acoustic variables following the same protocol for *A. petrosus*, *A. pratensis*, *A. cervinus*, *A. spinoletta spinoletta*, *A. spinoletta coutellii* and *A. spinoletta blakistoni* (15 recordings corresponding to 15 individuals and n = 15 calls for each taxa). All recordings were retrieved from xeno-canto.org and The Sound Approach collection (recording list and measurements in Sup. Mat. 2).

We performed various Principal Component Analysis (PCA) on call variables using the R package *ade4* (Dray & Dufour 2007). First, a PCA was performed using calls from *A. r. rubescens*, *A.* [*r.*] *japonicus*, *A. petrosus*, *A. pratensis*, *A. s. spinoletta*, *A. s. coutellii* and *A. s. blakistoni* for general call divergence visualisation. To compare the differentiation between both *A. rubescens* subspecies and between *A. spinoletta* and *A. petrosus*, additional PCAs were performed for: 1) both types of calls given by *rubescens* and *japonicus* (see below); 2) common calls of *rubescens* and *japonicus*; 3) *A. petrosus* and *A. s. spinoletta*, *A. s. coutellii* and *A. s. blakistoni*; and 4) *A. petrosus* and *A. s. spinoletta*. We also computed the mean pairwise Euclidean distances between all taxa using *meandist* function on *vegan* R package (Oksanen *et al.* 2019) and all nine acoustic variables. For visualisation, we used the distance matrix to infer a Neighbor-Joining (NJ) tree using Mega-X (Kumar *et al.* 2018). Call measurements were standardized (mean = 0 and variance = 1) before PCA and distance computation.

Results

MtDNA phylogeny and genetic divergence

The ML phylogenetic trees reconstructed from both CR and COI sequences (Fig. 3 & 4) recovered all the *Anthus* taxa represented by multiple samples as well-supported monophyletic groups (but note that *A. r. alticola* was not represented in our data set). At the species level, all of the species except *A. spinoletta* were also recovered as monophyletic. *Anthus r. rubescens* and *A.* [*r.*] *japonicus* formed a well-supported monophyletic clade in both trees. In both trees, *A. pratensis* was recovered with moderate bootstrap support (90 or 91) as sister to the *A. spinoletta*/*A. petrosus* group, with the *A. rubescens* species complex sister to these species. In both trees, *A. petrosus* was sister to *A. s. spinoletta*, with *A. s. coutellii* and *A. s. blakistoni* outside of this clade, rendering *A. spinoletta* paraphyletic, although the support was low (COI) to moderate (CR).

We found genetic divergences between *rubescens* and *japonicus* of 2.2% in CR and 2.5% in COI (Tables 1 & 2). Importantly, the amount of divergence between these two taxa is comparable with those of undisputed pairs of species: 1.3% in CR and 0.9% in COI for the pair *spinoletta* and *petrosus*/*littoralis*; and 2.9% in CR and 4.4% in COI for the pair *A. trivialis* and *A. hodgsoni* (Tables 1 & 2). The mtDNA lineage divergence between *rubescens* and *japonicus* from a most recent common ancestor dates back to around 1.64 Mya [1.31–1.99] to 2.67 Mya [1.51–3.33] for COI and 1.47 Mya [1.17–1.79] to 2.40 Mya [1.36–3] for CR, based on Nabholz *et al.*'s (2016) "analysis 2" and "analysis 4" methods, respectively.

Acoustics

The calls of *japonicus* fall into two types, an M-shaped call and a second type of call similar to (with only minor average differences from) the calls of *rubescens* (Fig. 5 and 6B). M-shaped calls can be separated from the common type shared by *japonicus* and *rubescens* by reduced amplitude, with the maximum and minimum frequencies varying very little over the duration of the call (except for the falling end of the call where the energy rapidly fades out). Despite extensive research on the many available recordings, we failed to find any M-shaped calls in *A. r. rubescens*. For the rest of the analyses, calls were visually attributed to the M-shaped type or the common type and treated as separate groups in PCAs and other analyses. While M-shaped calls of *japonicus* are easy to separate from *rubescens*, the type of call common to the two taxa is similar, but, on average, *japonicus* common-type calls are longer and lower-pitched, with a median number of peaks of 3 against 2 for *rubescens* and greater amplitude of the largest modulation (Table 3).

TABLE 1. Mean inter-group (species/subspecies) genetic divergence calculated from the COI mtDNA marker.

	A. berthelotii	*A. cervinus*	*A. gustavi*	*A. s. blakistoni*	*A. s. spinoletta*	*A. s. coutellii*	*A. trivialis*	*A. hodgsoni*	*A. petrosus*	*A. pratensis*	*A. r. rubescens*	*A.* [*r.*] *japonicus*
A. richardi	0.0888	0.139	0.1163	0.1434	0.1408	0.1328	0.1164	0.1246	0.1389	0.1523	0.1383	0.1315
A. [*r.*] *japonicus*	0.1281	0.0759	0.099	0.0505	0.0486	0.0519	0.1004	0.0936	0.0483	0.0508	0.025	NA
A. r. rubescens	0.1222	0.0737	0.095	0.0558	0.0506	0.0519	0.0926	0.09	0.0458	0.05	NA	
A. pratensis	0.1495	0.0676	0.108	0.0355	0.036	0.0328	0.1142	0.1068	0.0349	NA		
A. petrosus	0.1466	0.0714	0.1006	0.0144	0.0094	0.0122	0.1124	0.1132	NA			
A. hodgsoni	0.1409	0.1166	0.1034	0.1136	0.1146	0.1051	0.0443	NA				
A. trivialis	0.1295	0.1137	0.0898	0.1182	0.112	0.105	NA					
A. s. coutellii	0.1457	0.0678	0.0986	0.0116	0.0106	NA						
A. s. spinoletta	0.1555	0.0694	0.1017	0.0134	NA							
A. s. blakistoni	0.1493	0.0742	0.1109	NA								
A. gustavi	0.1223	0.1089	NA									
A. cervinus	0.1634	NA										

TABLE 2. Mean inter-group (species/subspecies) genetic divergence calculated from the CR mtDNA marker.

	A. s. coutellii	*A. s. blakistoni*	*A. petrosus*	*A. s. spinoletta*	*A. pratensis*	*A.* [*r.*] *japonicus*	*A. r. rubescens*	*A. roseatus*	*A. cervinus*	*A. hodgsoni*
A. trivialis	0.0722	0.072	0.0704	0.0786	0.0739	0.0687	0.0703	0.0531	0.063	0.0288
A. hodgsoni	0.075	0.0743	0.0701	0.0764	0.0749	0.0664	0.069	0.0539	0.0624	NA
A. cervinus	0.0542	0.0422	0.0478	0.0485	0.0426	0.0415	0.0418	0.0397	NA	
A. roseatus	0.052	0.0463	0.0504	0.0517	0.0482	0.0505	0.0469	NA		
A. r. rubescens	0.0354	0.0278	0.0363	0.0359	0.0331	0.0224	NA			
A. [r.] japonicus	0.04	0.0333	0.0427	0.0414	0.0468	NA				
A. pratensis	0.0283	0.0262	0.0306	0.0319	NA					
A. s. spinoletta	0.0203	0.0169	0.0135	NA						
A. petrosus	0.0196	0.0147	NA							
A. s. blakistoni	0.0099	NA								

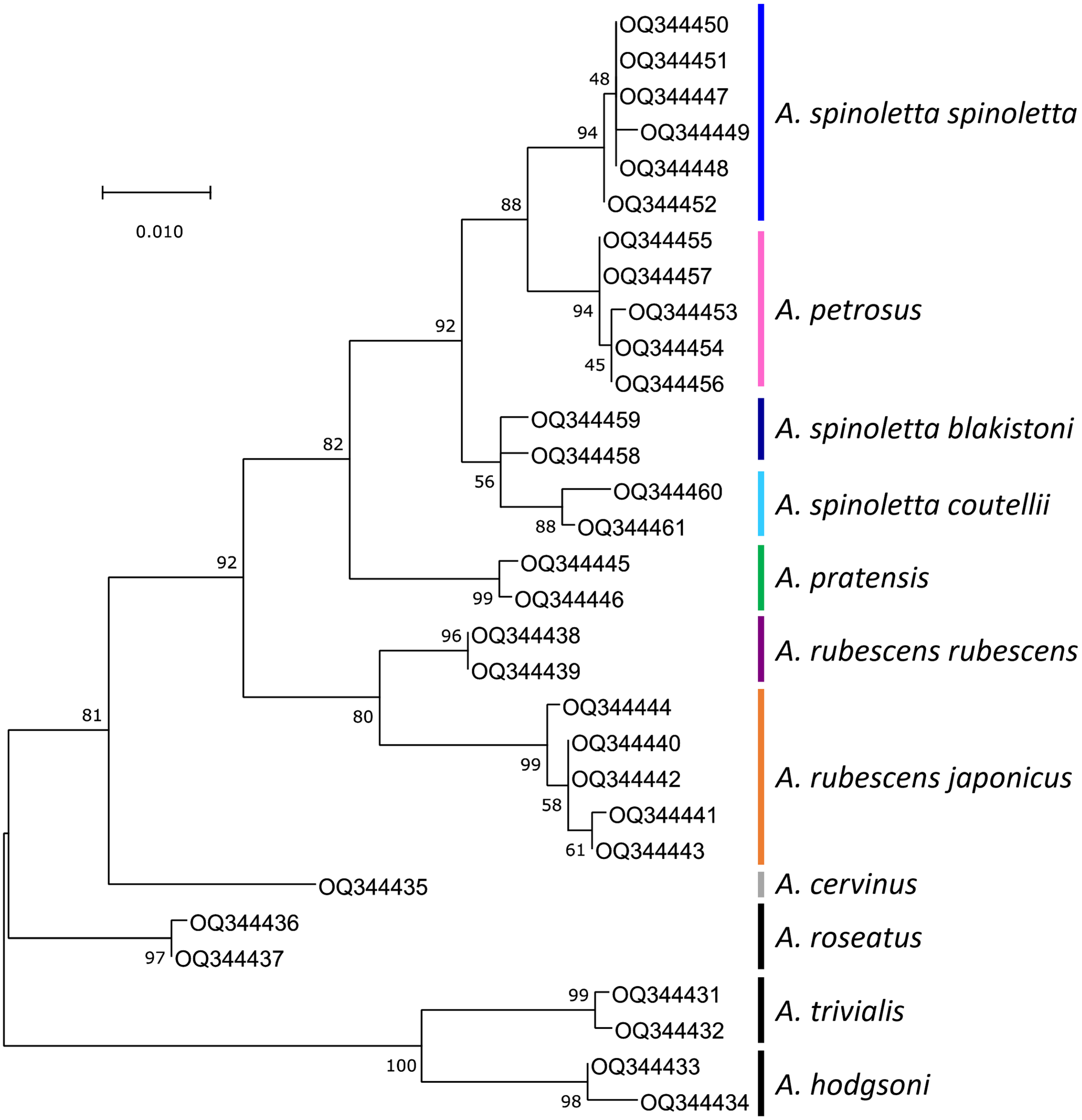

FIGURE 3. Maximum-likelihood tree inferred from 998 bp of CR using a HKY+G substitution model implemented in MEGA-X (Kumar *et al.* 2018). Bootstrap values are indicated on the nodes.

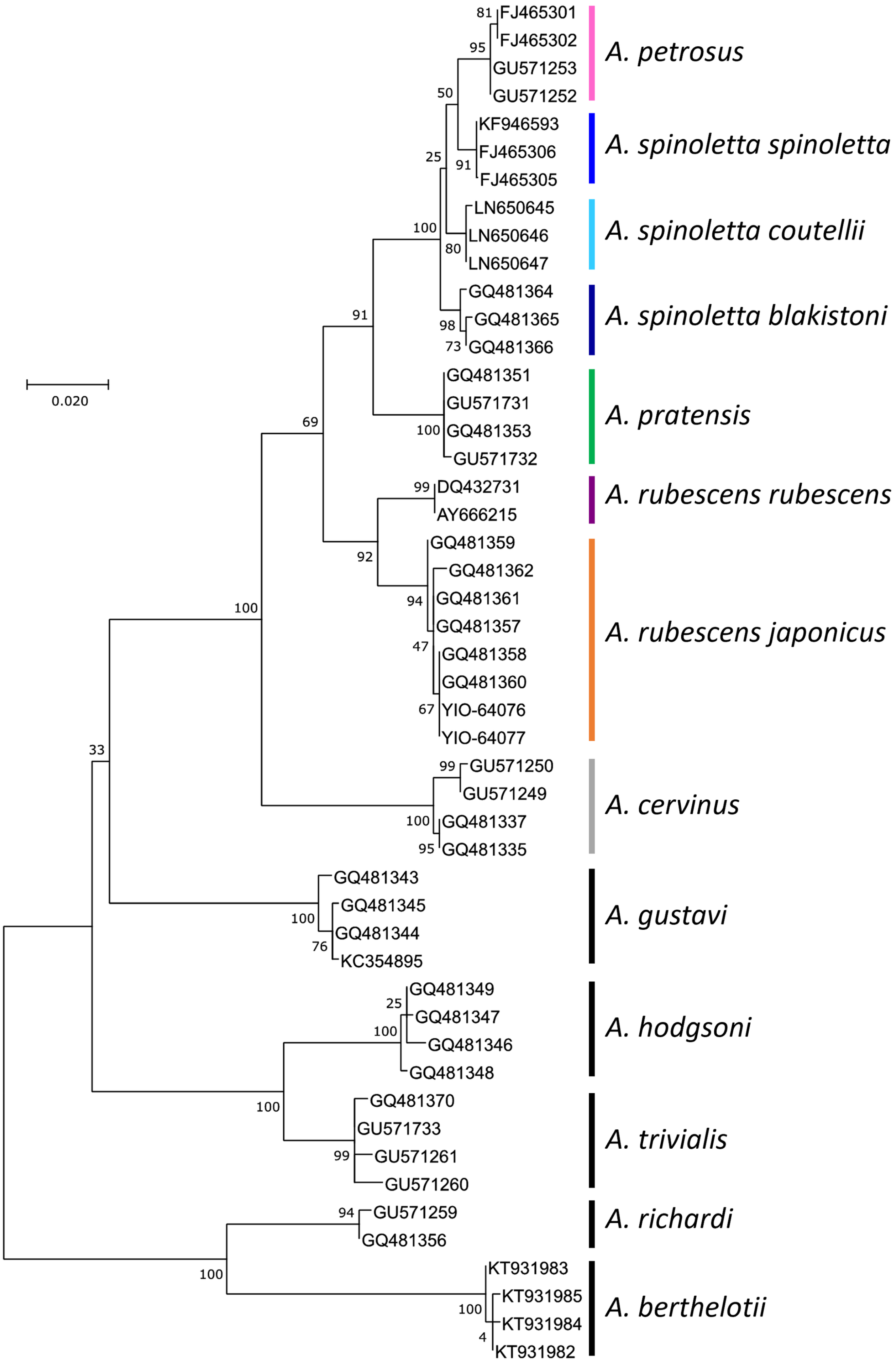

FIGURE 4. Maximum-likelihood tree inferred from 694 bp of COI using a HKY+G substitution model implemented in MEGA-X (Kumar *et al.* 2018). Bootstrap values are indicated on the nodes.

TABLE 3. Comparison of call measurements between *Anthus* [*rubescens*] *japonicus* (common and M-shaped type of calls) and *A. rubescens rubescens*. For each taxon and variable mean ± SD; min–max are reported.

	Rising or falling (0 or 1)	Number of frequency peaks	Call duration (ms)	Start frequency (kHz)	Maximum frequency (kHz)	Minimum frequency (kHz)	End frequency (kHz)	Amplitude of the largest modulation (kHz)	Duration up to the largest modulation (ms)
A. r. rubescens (n=155)	0 ± 0 ; 0–0	1.79 ± 0.72 ; 0–4	68.82 ± 8.81 ; 48.9–93.0	8.21 ± 0.59 ; 6.65–9.42	8.21 ± 0.59 ; 6.65–9.42	4.37 ± 0.49 ; 3.08–5.6	4.41 ± 0.56 ; 3.08–6.2	0.68 ± 0.24 ; 0–1.15	34.39 ± 9.89 ; 1.1–5.5
A. [*r.*] *japonicus* (n=152)	0.04 ± 0.19 ; 0–1	2.85 ± 0.65 ; 1–5	79.94 ± 9.55 ; 54.5–100.8	7.68 ± 0.59 ; 6.35–9.17	7.72 ± 0.56 ; 6.51–9.17	3.54 ± 0.70 ; 2.55–5.47	3.54 ± 0.70 ; 2.55–5.47	0.99 ± 0.36 ; 0.42–2.41	39.18 ± 12.76 ; 0.83–6.4
A. [*r.*] *japonicus* M-shaped (n=31)	0.16 ± 0.37 ; 0–1	2.77 ± 0.66 ; 2–4	72.82 ± 7.21 ; 58.7–85.8	5.95 ± 1.28 ; 3.25–7.51	6.62 ± 0.93 ; 4.37–7.66	4.03 ± 0.86 ; 2.79–6.67	3.96 ± 0.70 ; 2.79–5.11	1.35 ± 0.32 ; 0.74–2.2	28.48 ± 13.37 ; 0.94–5.39

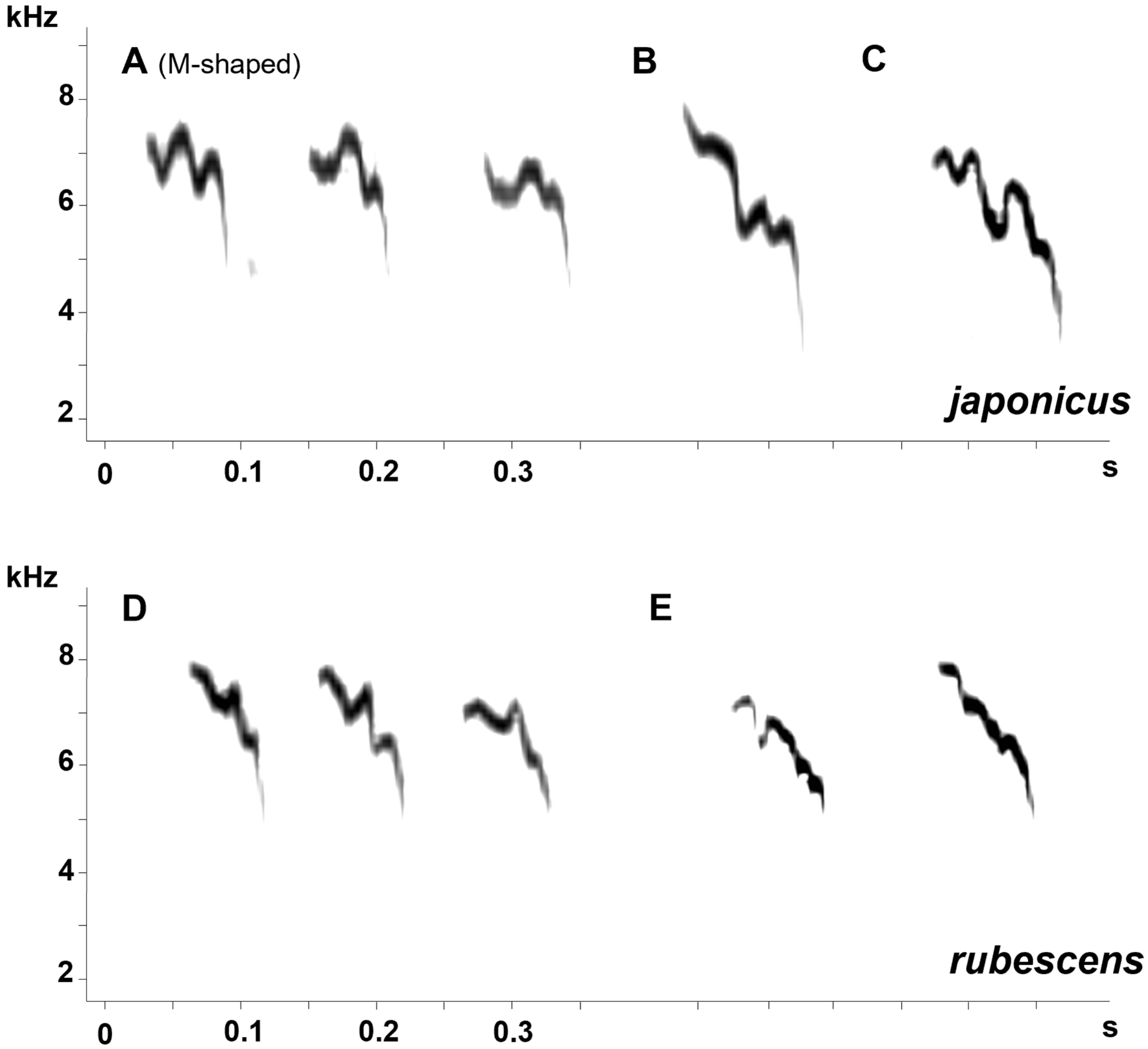

FIGURE 5. Sonograms of various calls from *Anthus* [*rubescens*] *japonicus*: A) M-shaped calls (xeno-canto.org: XC267502); B) and C) common calls (xeno-canto.org: XC437043 & The Sound Approach: 02.050. MR. 01938.02) and *A. r. rubescens*: D and E common calls (xeno-canto.org: XC598639, XC599314).

The PCA based on call parameters that includes *A. pratensis*, *A. petrosus*, *A. spinoletta* and the *A. rubescens* species complex reveals two main clusters: the first one comprising both subspecies groups of the Buff-bellied Pipit and a second one comprising *A. pratensis*, *A. petrosus* and *A. spinoletta* (Figure 6A). In a second PCA on *rubescens* and both call types of *japonicus*, the M-shaped calls of *japonicus* appear to be well-differentiated from the common calls shared by *rubescens* and *japonicus* (Figure 6B). When considering only common calls, only a limited overlap can be observed between *rubescens* and *japonicus* (Figure 6C). For comparison, we also provide the PCAs made with *A. petrosus* and *A. spinoletta*, and the PCA made with only *petrosus* and *spinoletta*. In both cases, there is also overlap between *A. petrosus* and either the three *A. spinoletta* subspecies (Figure 6D) or *A. s. spinoletta* only (Figure 6E). The PCA analyses therefore showed a similar amount of overlap in typical call parameters between the two *A. rubescens* subspecies groups as between *A. petrosus* and *A. spinoletta*.

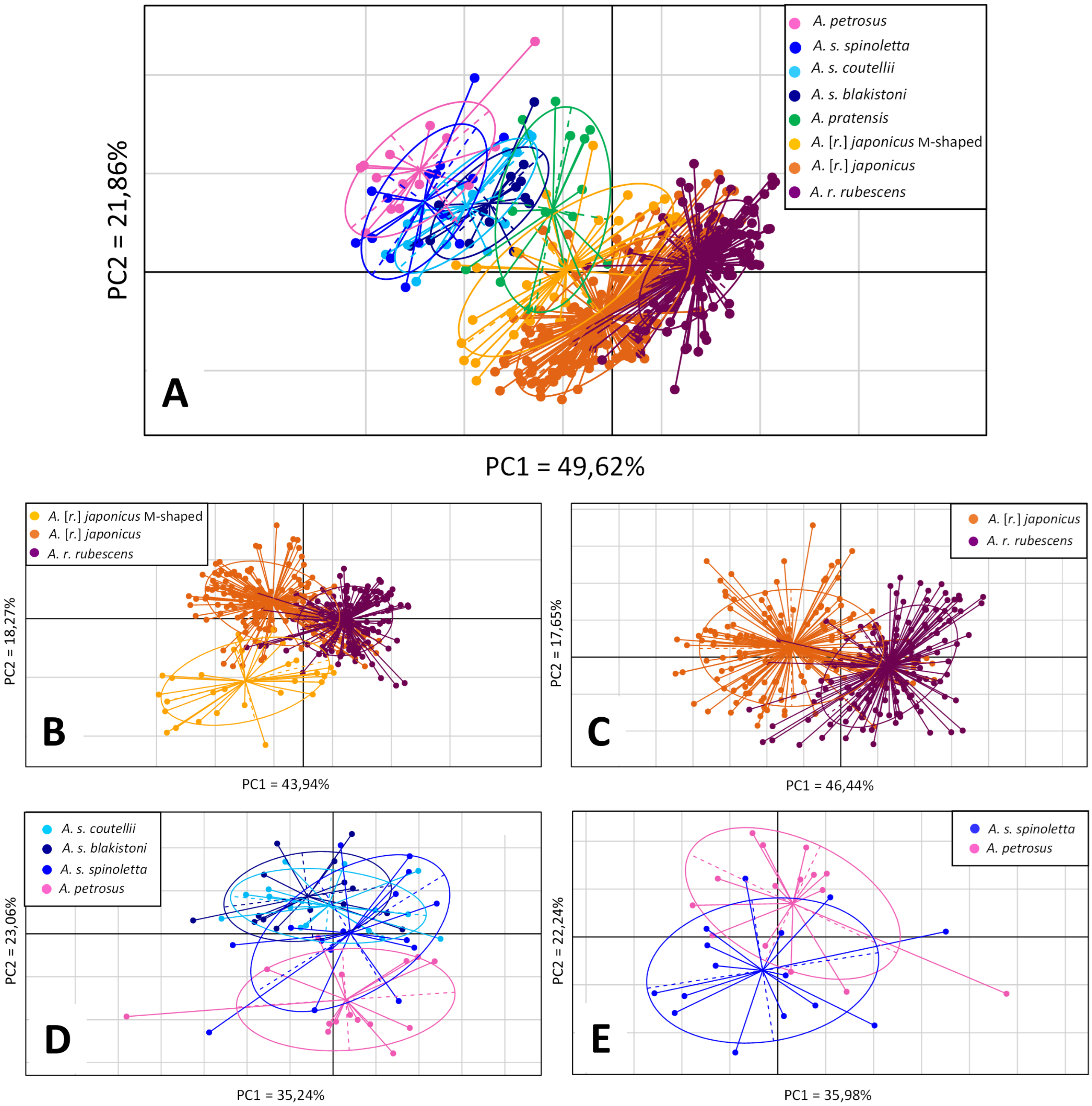

FIGURE 6. Principal component analysis of the nine call measurements for various species selections. A: all small *Anthus* species except *A. cervinus*; B: two types of calls of *A. r. rubescens* and *A.* [*r.*] *japonicus*. C: only common-type calls of *A. r. rubescens* and *A.* [*r.*] *japonicus*; D: *A. petrosus* and different *A. spinoletta* subspecies; E: *A. petrosus* and *A. s. spinoletta*.

The distance matrix based on call measurements and the NJ tree obtained from it showed divergence in call parameters of the same order of magnitude within and between currently accepted species (Table 4; Sup. Mat. 3). For example, acoustic distances between *A. pratensis* and *A. s. blakistoni* (2.93) or between *A. petrosus* and *A. spinoletta* (3.58) are comparable to the distances between *blakistoni* and *spinoletta* (3.39) or between common calls of *rubescens* and *japonicus* (3.30) (Table 4; Sup. Mat. 3). The divergence in calls between *japonicus* and *rubescens* is therefore comparable to the divergence measured between *A. petrosus* and *A. spinoletta* and just slightly less than the distance between *A. pratensis* and *A. spinoletta* or *A. petrosus*.

TABLE 4. Pairwise Euclidian distances between *Anthus* taxa based on call parameters.

Mean Euclidean distance	*A. s. blakistoni*	*A. cervinus*	*A. s. coutellii*	*A.* [*r.*] *japonicus*	*A.* [*r.*] *japonicus* M-shaped	*A. petrosus*	*A. pratensis*	*A. r. rubescens*
A. cervinus	7.459							
A. s. coutellii	2.661	6.75						
A. [*r.*] *japonicus*	4.624	8.041	4.649					
A. [*r.*] *japonicus* M-shaped	4.287	8.448	4.372	3.956				
A. petrosus	3.935	5.745	3.703	5.695	5.826			
A. pratensis	2.929	7.477	3.252	3.972	3.967	4.432		
A. r. rubescens	5.287	8.461	5.391	3.305	4.298	6.421	4.253	
A. s. spinoletta	3.388	6.471	3.139	5.25	5.042	3.582	4.048	6.217

Discussion

In this paper, we adhere to the principles of the Biological Species Concept in viewing species as evolutionary lineages that are able to coexist in close geographical proximity without entirely mixing their genomes, i.e., that have evolved intrinsic barriers to reproductive isolation preventing them from "melting down" if they are not separated by geography (Mayr 1942; Mallet 2008). Ranking of allopatric taxa in species-level systematics is notoriously difficult, especially under this species concept. In line with other authors (e.g., Helbig *et al.* 2002; Speybroeck *et al.* 2020), we use a consistency approach to allopatric taxa and treat as species allopatric taxa that are as divergent as closely related valid species that are known to be reproductively isolated. Ideally, divergence should be assessed using multiple lines of evidence. Here, we use flight call recordings and mtDNA data to assess divergence in acoustics and genetics between the essentially allopatric taxa *Anthus r. rubescens* (including *A. r. alticola*) and *A.* [*r.*] *japonicus*. We also discuss the biogeography of these two taxa to assess whether they are entirely isolated by geography.

Divergence between **rubescens** *and* **japonicus**

First, phylogenetic reconstructions based on mtDNA showed the absence of mtDNA haplotype sharing and a clear genetic differentiation between *japonicus* and *rubescens*. We found that the genetic divergence exhibited by both mtDNA markers, which admittedly are clonally inherited, are comparable to divergence values of well-recognised congeneric species. Divergence estimation time between both taxa is around 2 Mya (between 1.3 Mya and 3 Mya, depending on the mitochondrial marker and the fossil calibration method used) which is in the range of many other species-level divergence times in birds (Nabholz *et al.* 2016).

Second, call analyses revealed the same pattern of relative divergence compared to other closely related species pairs: divergence between the calls of Nearctic and Palearctic *A. rubescens* subspecies groups appears to be comparable to the divergence observed between closely related taxa that are unanimously treated as separate species (e.g., between *A. spinoletta* and *A. petrosus*). In addition, *japonicus* includes in its repertoire a M-shaped call which is not emitted by *rubescens* and sounds distinctly different. The lack of fully diagnostic characters between the common calls of *rubescens* and *japonicus* is not unique among this group: the same pattern was found between *A. petrosus* and *A. spinoletta spinoletta* (Fijen 2014) or (with less overlap) between *A. trivialis* and *A. hodgsoni* (Martin 2013).

Despite extensive research on online libraries and through direct enquiries with ornithologists or birders, we could not collect a sufficient number of song recordings for *japonicus* (only one good quality song was available). We therefore were unable to test whether differences exist between the songs of *japonicus* and *rubescens*. The songs of pipits of the genus *Anthus* are generally complex and consist of a series of quickly repeated, high-pitched notes which are arranged in a few phrases (Alström & Mild 2003). The phrases emitted during the song seems to strongly depend on the behaviour of the bird (in flight versus perched). Furthermore, it has been shown in *A. spinoletta*, *A. petrosus* and *A. pratensis* that songs are individually highly variable, and the songs of the two latter can be especially difficult to distinguish (Alström & Mild 2003). It would therefore not be surprising if the songs of *japonicus* and *rubescens* were very similar or even indistinguishable in most individuals. This hypothesis should however be verified when more samples become available.

Plumage differences

The plumage-based separation of Asian and Nearctic populations of the Buff-bellied Pipit has previously been discussed in the identification literature. They are very similar in breeding plumage, although *alticola* is generally separable from *rubescens* by its deeper and less-streaked underparts, but in non-breeding plumage, the combination of several plumage criteria allows the assignment of most individuals to subspecies group (the main criteria are summarised in Table 5; see also Alström & Mild 2003; Garner 2015; Shirihai & Svensson 2018; Hendricks & Verbeek 2020). As morphology is strongly conserved in the genus *Anthus* (Alström & Mild 2003), other pairs of closely related species in this genus are known to be difficult to separate based on plumage alone (e.g., *A. richardi*, *A. rufulus* Vieillot and *A. godlewskii* (Taczanowski); *A. spinoletta* and *A. petrosus* in non-breeding plumage). These identification difficulties, however, do not prevent their recognition as valid species. To reiterate, the levels of difference in plumage and bare parts coloration between the two *A. rubescens* subspecies groups in non-breeding plumage are similar to the levels of differences between *A. spinoletta* and *A. petrosus*.

TABLE 5. Summary of the known phenotypical differences between *A. r. rubescens* and *A.* [*r.*] *japonicus*.

Period	Plumage trait	*A. r. rubescens*	*A.* [*r.*] *japonicus*	Remarks
Year-round	Leg colour	Ruddy-brown to blackish	Pinkish to pale reddish-brown	
	White tip on P5	Most of the tip	Limited to external shaft	Large overlap
Breeding	Breast streaking	Finely and weakly streaked	Rounder and more abundantly steaked	Both taxa very similar in breeding plumage
Non-breeding	Breast streaking	Brown to dark brown, narrow, short and thin, evenly spaced, thinner along the flanks	Brown-black to black, large, clearcut and contrasting, reach far and merge on the upper breast, often forming a dark necklace	*japonicus* can show few and small streaks on the breast or thin streaks on the flanks and some *rubescens* dark or blackish streaks
	Underparts colour	Warm buffish	Pale buffy	Both become white/whitish with feather wear, some whitish *rubescen*s exist
	Upperparts streaking	Almost plain, weakly streaked	Rather conspicuous and dark streaking	
	Upperparts colour	Grey-brown	Dark olive-brown	
	Malar patch	Dark brown, ill-defined shape	Large and dark, triangular shape	
	Median and greater coverts tips	Pale brownish (rarely whitish with wear), form a diffuse wing bar	Pale brownish when fresh and whitish from mid-October, form a conspicuous and crisp wing bar	

Biogeography

All the species most closely related to the *A. rubescens* species complex occur only in Eurasia (cf. Alström & Mild 2003; Alström *et al.* 2015b; Pietersen *et al*. 2019), so the ancestor of the species most likely colonised North America from Eurasia. Colonization from Europe across the Atlantic Ocean seems unlikely, as this is rarely seen in birds [but see Bairlein *et al.* 2012 for the case of the Northern Wheatear *Oenanthe oenanthe* (Linnaeus)]. Therefore, the ancestor of the *A. rubescens* species complex probably colonized North America across the Bering Strait. Based on our data, it is not possible to determine whether it colonized North America from Siberia before or after splitting into two taxa, or whether it colonized Siberia from North America after having diverged from other Palearctic *Anthus* species in America. Whatever the scenarios of dispersal, the Bering Strait currently separates the *rubescens* and *japonicus* subspecies groups, even though we do not know whether they are fully allopatric there or not (Alström & Mild 2003). During the succession of glacial and interglacial periods that occurred throughout the Pleistocene, sea levels regularly rose and fell, resulting in the opening and closing of the Bering land bridge (Hopkins 1959). This land connectivity alteration was probably coupled with southward distributional contractions of both Asian and American bird populations which were followed by distributional expansions during interglacial periods (Hopkins 1959; McLaughlin *et al.* 2020). Repeated periods of land discontinuity and distribution splits between Asia and North America during the Pleistocene are therefore probably strongly implicated in vicariance mechanisms responsible for the current divergence between taxa inhabiting each side of the Bering Strait.

Two main levels of divergence have been described between closely related avian taxa or populations distributed in Asia and America, on either side of the Bering Strait (Zink *et al.* 1995; McLaughlin *et al.* 2020). First, shallow population or subspecies levels of divergence [*Hirundo rustica* Linnaeus, *Clangula hyemalis* (Linnaeus), *Luscinia svecica* (Linnaeus), *Phylloscopus borealis* (Blasius), *Motacilla tschutschensis* Gmelin; Pavlova *et al.* 2003; Reeves *et al.* 2008; Dor *et al.* 2010; Saitoh *et al.* 2010; Safran *et al.* 2016; Drovetski *et al.* 2018; Harris *et al.* 2018; McLaughlin *et al.* 2020] are compatible with recent colonisation of northern North America or with strong dispersal abilities maintaining gene flow. Second, strong, arguably species-level divergence [*Mareca penelope* (Linnaeus)/ *M. americana* (Gmelin)*, Anas crecca* (Linnaeus)/*A. carolinensis* Gmelin, *Numenius phaeopus* (Linnaeus)/*N. hudsonicus* Latham, *Pica pica* (Linnaeus)/*P. hudsonia* (Sabine), *Picoides tridactylus* (Linnaeus)/*P. dorsalis* Baird and *Tringa brevipes* (Vieillot)/*T. incana* (Gmelin); Zink *et al.* 2002; Peters *et al.* 2012; Winker *et al.* 2013; Song *et al.* 2018; Tan *et al.* 2019; McLaughlin *et al.* 2020] is potentially linked to older colonization of North America or to stronger impact of the Bering Strait on speciation in these groups because of ecological components. The Bering Strait therefore appears to have a variable propensity to cause speciation depending on species' ecology and timing of crossing. The existence of a strong migratory divide, with Asian populations generally migrating south-west/north-east versus American populations generally migrating south-east/north-west, could well enhance genetic divergence and reproductive isolation, as hybrids might take unfavourable routes and hence be selected against (Irwin *et al.* 2005; Delmore & Irwin 2014; Turbek *et al.* 2022). *Anthus* [*r.*] *japonicus* migrates from eastern Siberia to its wintering grounds in Japan, Taiwan and southeastern China and therefore uses a southward migratory orientation (and possibly southwest towards the Middle East; Alström & Mild 2003; Porter & Aspinall 2013). In contrast, *rubescens* migrates from Alaska and Arctic Canada to the southern United States and Mexico (rarely Central America) through a south-southeast migratory orientation (Alström & Mild 2003; Figure 1). The situation of the Asian and Northern American subspecies groups of Buff-bellied Pipit hence corresponds to that described in species distributed on both sides of the Bering Strait which exhibit strongly different migratory orientations and wintering grounds.

More importantly, the long-distance migratory behaviour of both subspecies groups of *A. rubescens*, the close proximity of their nearest breeding locations (the species even breeding on islands in the Bering Strait, where the exact location of the contact zone between *japonicus* and *rubescens* is unknown, Fig. 1, Alström & Mild 2003), and the recent migration of multiple avian species across the Bering Strait suggest that it is highly unlikely that this barrier alone has been sufficient to isolate *japonicus* and *rubescens* for a long time. Even though the lack of nuclear DNA data or information on the subspecies occupying the islands of the Bering Sea does not allow us to reach definitive conclusions on the level of reproductive isolation between these taxa, the absence of mtDNA allele sharing in our dataset suggests a strong restriction (or absence) of female gene flow between *rubescens* and *japonicus* subspecies groups that almost certainly required a substantial amount of reproductive isolation and probably could not have been achieved purely by geographic isolation.

Taxonomic recommendations

The American and Siberian subspecies groups of the *A. rubescens* species complex are currently mostly allopatric, although parapatry or even narrow sympatry in the Bering Strait area cannot be excluded (Alström & Mild 2003). We have shown here that their level of mtDNA divergence is larger than or similar to other valid species in the same genus and is typical of avian species-level divergence (Zink & Barrowclough 2008; Nabholz *et al.* 2016). Similarly, divergence in plumage and calls are of similar magnitude to those found between other closely related species pairs in the genus *Anthus*. We thus formally recommend treating these two groups as separate species under the Biological Species Concept. We also note that the same conclusion would apply under the General Lineage Concept of de Queiroz (2007) or within the framework of integrative taxonomy (Padial *et al.* 2010). This two-way split would lead to the recognition of the monotypic Siberian (Buff-bellied) Pipit *Anthus japonicus* and the polytypic American (Buff-bellied) Pipit *Anthus rubescens* comprising both *A. rubescens rubescens* and *A. rubescens alticola*. Both species show large distributions and, even if accurate estimation of their population size is impossible due to isolated breeding grounds, neither currently seem to match any of the IUCN threat criteria and should therefore keep Least Concern IUCN status, though they would likely be vulnerable to impacts of climate change in future.

We acknowledge that it would be preferable to have genetic samples from both sides of the Bering Strait and to assess gene flow across the contact zone with multilocus or genomic data. However, we believe that taxonomic decisions should be based on the data at hand, without necessarily having to wait for the perfect data set, in order to provide a more accurate catalogue of species diversity on earth, providing the approaches and decisions are sound. The species status of *japonicus* and *rubescens* subspecies groups is the most likely hypothesis given the data we have. Should we maintain the status quo (which is based on the least likely hypothesis) while waiting to obtain further data that will take a long time to obtain, or should we promote a change in taxonomy which reflects the most likely hypothesis? We prefer the second option, as we feel strongly that taxonomies should reflect species diversity as closely as possible in order to provide relevant information for biodiversity studies and conservation. As sampling through the contact zone will probably not happen in the near future, we recommend a taxonomic change now.

We refrain from making formal taxonomic recommendations here for the various subspecies of *Anthus spinoletta* because our samples of calls and mtDNA are small, but we note that they are also allopatric taxa with a similar amount of divergence in calls and mtDNA as *A. spinoletta* and *A. petrosus*. A dedicated study on this complex, including more call recordings, songs, breeding and non-breeding plumage, a larger sampling of mtDNA and (ideally) nuclear DNA would probably reach a similar conclusion and support a three-way split (*spinoletta* vs *coutellii* vs *blakistoni*), as already recommended for *coutellii* by Garner *et al.* (2015).

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Supplementary Materials. The following supporting information can be downloaded at the DOI landing page of this paper: BB_Pipit_Sup_Mat_1_Accession_Number; BB_Pipit_Sup_Mat_2_Call_Measures; BB_Pipit_Sup_Mat_3_Call_Distance_Tree.