



# Phenotypic and genetic characterization of the East Siberian Willow Warbler (*Phylloscopus trochilus yakutensis* Ticehurst, 1935) in relation to the European subspecies

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## Abstract

Long-distance migrants with transcontinental breeding ranges are of particular interest for the study of local adaptation and geographic differentiation in birds. We compared phenotypes and genotypes between Far East Siberian Willow Warblers *Phylloscopus trochilus yakutensis* Ticehurst, 1935 with the European subspecies *P. t. trochilus* Linnaeus, 1758 and *P. t. acredula* Linnaeus, 1758. We found significant differences in mean body size and plumage colour, but intra-population variation overlapped extensively between the European and Siberian populations. We used stable isotope composition in winter-grown flight feathers as a proxy for wintering sites and found differences between all three subspecies, indicating different wintering grounds. Out of four nuclear loci analyzed (three of which are known to be substantially divergent between the European subspecies), none allowed to separate East Siberian *yakutensis* from North Scandinavian *acredula*. Hence, neither phenotypic traits nor the currently available genetic resources provide diagnostic criteria for confidently assigning individual Willow Warblers to a particular subspecies. Despite extensive overlap in phenotypes and genotypes, we propose that the subspecies names can still be used as biogeographical references to the three Willow Warbler populations that differ in migration strategies. We propose to use *yakutensis* for Willow Warblers breeding east of the Ural Mountains that presumably initiate autumn migration towards the southwest or west, in contrast to the genetically most similar *acredula* that start autumn migration towards the southeast or south. Future field studies are needed to elucidate whether the longitudinal variation in phenotype is a cline, or whether a clear contact zone between these subspecies can be identified.

**Keywords** Willow Warbler · *Phylloscopus trochilus yakutensis* · Subspecies · Genetic structure · Phenotypic variation · Clock gene · Stable isotopes

## Zusammenfassung

**Vergleichende phänotypische und genetische Charakterisierung der ostsibirischen (*Phylloscopus trochilus yakutensis* Ticehurst, 1935) und europäischen Fitis-Unterarten**

Langstreckenzieher mit trans-kontinentalem Verbreitungsgebiet eignen sich hervorragend zur Untersuchung lokaler Anpassung und geographische Differenzierung. Hier vergleichen wir Phänotyp und Genotyp der ostsibirischen Fitis-Unterart (*Phylloscopus trochilus yakutensis* Ticehurst, 1935) mit den europäischen Unterarten *P. t. trochilus* Linnaeus, 1758 und *P. t. acredula* Linnaeus, 1758. Unsere Ergebnisse zeigen signifikante Unterschiede der mittleren Körpergröße, sowie Gefiederfärbung, wobei die Bandbreite beider Messgrößen zwischen den Unterarten stark überlappen. Die zur Bestimmung der jeweiligen Überwinterungsgebiete genutzte stabile Isotopenkomposition der im Winter gemauserten

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Extended author information available on the last page of the article

Schwungfedern weist klare Unterschiede zwischen allen drei Unterarten auf. Zur populationsgenetischen Beschreibung wurden vier genomische Loci genutzt (wovon drei klare Unterschiede zwischen beiden europäischen Unterarten zeigen). Die resultierende populationsgenetische Struktur gruppiert die ostsibirische Unterart *yakutensis* klar zusammen mit der nordskandinavischen Unterart *acredula*. Unsere Ergebnisse erlauben weder auf Ebene des Phänotyps, noch auf Ebene des Genotyps eine klare diagnostische Abgrenzung der drei Unterarten. Trotz extensiver Überlappung der hier genutzten genotypischen und phänotypischen Marker halten wir dennoch weiter die Verwendung der bestehenden Nomenklatur als biogeographische Referenz im Kontext der verschiedenen von den Unterarten genutzten Zugrouten für sinnvoll. Um *yakutensis* von der genetisch eng verwandten Unterart *acredula* abzugrenzen empfehlen wir *yakutensis* für Fitispopulationen mit Brutgebiet östlich des Urals die im Herbstzug vermutlich in südwestlicher oder westlicher Richtung aufbrechen zu verwenden. Weiterführende Untersuchungen sind nötig um zu klären, in wie weit die beobachteten Unterschiede zwischen *yakutensis* und *acredula* bezüglich Längengrad einen graduellen Verlauf oder eine abrupte Änderung zwischen den Unterarten aufweisen.

## Introduction

Species with continent-wide breeding ranges typically show less diversity at higher than lower latitudes. For instance, the Chipping Sparrow (*Spizella passerina*) has five geographically structured mitochondrial lineages in total, but only two occurring at high latitudes (Carson and Spicer 2003; Martin and Tewksbury 2008). Despite occupying wide breeding ranges, several migratory species winter in relatively narrow and species- or even population-specific areas. For example, the Northern Wheatear (*Oenanthe oenanthe*) winters exclusively in sub-Saharan Africa, even though it breeds across the entire Holarctic (Bairlein et al. 2012). This pattern of restricted wintering ranges and much wider breeding distribution is best explained by rapid postglacial expansions from common refugia (Milá et al. 2006), combined with existing constraints on establishing new wintering areas. What makes these cases exceptionally interesting is that fast range expansions must have proceeded in parallel with locally adapted evolutionary changes in migration direction and timing. For example, the Common Rosefinch (*Carpodacus erythrinus*) colonized Scandinavia from central Russia only about 100 years ago (Pavlova et al. 2005), but keeps wintering in the same confined location in southern Asia. Hence, to end up in the same wintering quarters, Scandinavian Rosefinches must have changed their autumn migration direction from south to east southeast (Stach et al. 2016).

Similarly, the breeding range of the Willow Warbler spans from the coast of the Atlantic to the coast of the Pacific Ocean, with wintering grounds in tropical Africa for all subspecies (Shirihai and Svensson 2018). The Willow Warbler is currently classified in three subspecies; the nominal *trochilus* breeding in central and western Europe, *acredula* in northern Scandinavia/eastern Europe and *yakutensis* in eastern Siberia (Ticehurst 1938 mentioned in Williamson 1976). The general appearance of Willow Warblers is typical for a “leaf warbler” (Phylloscopidae), but shows substantial variation across its breeding range: a typical *trochilus*

has a bright yellow breast, yellow under-wing and under-tail coverts, and yellow chin and supercilium (Shirihai and Svensson 2018). In comparison, the subspecies *acredula* is on average longer-winged and has less yellow in the plumage, with a pale yellow supercilium and faintly streaked breast. The Siberian subspecies *yakutensis* is described as yet longer-winged with an even paler plumage, with hardly any trace of yellow (Williamson 1976; Shirihai and Svensson 2018). Shirihai and Svensson (2018) sketched the contact zone between *trochilus* and *acredula* relatively close to the hybrid zones characterized by Bensch et al. (2009). One notable difference, however, is that the populations breeding in southern Norway were assigned to *acredula* by Shirihai and Svensson (2018), whereas Bensch et al. (2009) assigned these to *trochilus*.

The breeding biology and phenotypic variation in European *acredula* and *trochilus* has been studied in great detail (Brock 1910; Tiainen 1983; Lawn 1984; Jakobsson 1988). Variation in plumage and size within the European subspecies is extensive, precluding subspecies identification of most, but the extreme individuals (Williamson 1976; Shirihai and Svensson 2018). Variation in genotypes and phenotypic traits in *trochilus* and *acredula* shows sharp clines across a narrow and well-defined contact zone in central Scandinavia, and a much wider diffuse cline east of the Baltic Sea (Bensch et al. 2009). This geographic distribution pattern suggests that the species expanded from a single glacial refugium and gradually evolved into the present phenotypes known as *trochilus* and *acredula* while colonizing Scandinavia from two directions (Bensch et al. 2009). However, robust population history inferences will require comprehensive data from the entire breeding range of the species, including populations from its easternmost breeding range. Our knowledge to date about the phenotypic characteristics of populations from the eastern range, which undertake the longest migratory journeys (Sokolovskis et al. 2018), is based almost exclusively on specimens from museum collections.

Here we characterize a *yakutensis* population from its extreme northeast breeding range based on morphology, genetic markers and migratory behaviour. We evaluate our novel results in the context of traits from the well-characterized European subspecies to discuss the phylogeography, population structure and taxonomy of this model species of migration research.

## Methods

### Sampling

The fieldwork was carried out in north-eastern Siberia in the Chaun River delta (68.80°N, 170.61°E) during June and July 2016. Territorial Willow Warbler males were lured into mist nets with song playback. The following morphological measurements were taken: total length of the bill to head; tarsus max length (to closest 0.1 mm), length of wing chord [“flattened wing” method as in Svensson (1992)], and whole tail length to closest 0.5 mm. Body mass was recorded with a Pesola LightLine balance, to the nearest 0.1 g. KS (sole data collector from Chaun) calibrated field measurements with SB to ensure that the data set from Chaun was comparable to data previously collected from Scandinavia. We combined all four morphological measurements into a principal component analysis (PCA) with R (version 3.5.1) package *factoextra* (Kassambara 2015). All phenotypic traits were also analysed individually using one-way analysis of variance (ANOVA), with subspecies as the explanatory factor, to test whether differences were significant. We then used Tukey’s honestly significant difference (HSD) test to see which of the subspecies were different (Tukey 1949).

Following a previously developed standard protocol (Bensch et al. 2009), plumage colour was scored by comparing all captured birds to three stuffed reference specimens representing the diversity in colouration of phenotypes across Scandinavia (Suppl. Figure 1). Plumage colour was scored from one to nine, where one translates to not even a hint of yellow and nine to bright yellow. All morphological and colour measurements taken for newly collected *yakutensis* were analysed with reference to the data set of *trochilus* and *acredula* presented in Bensch et al. (2009). In this study all birds classified as *trochilus* were sampled on breeding territories in Denmark and Sweden south of 60°N latitude, whereas birds classified as *acredula* were sampled on breeding territories in Finland, Norway and Sweden north of 65°N latitude (Suppl. Table 1).

We collected the innermost primary feather from every captured individual and preserved these in separate paper envelopes for stable isotope analysis. For DNA analysis we collected 20 µl of blood from each individual using a glass capillary tube, by puncturing the brachial wing vein

with a syringe needle. The blood was stored in SET buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M of EDTA, pH 8.0) at room temperature until brought to the lab for permanent storage at –20 °C. Willow Warbler is not included in Red Data Book of Russian Federation therefore no special permits were needed for our study. Metal rings were provided by Institute of Biological Problems of the North according to agreement with Bird Ringing Centre of Russia.

We did not analyse vocalization because our extensive field observations suggest that the Willow Warbler song is virtually undifferentiated throughout its vast breeding range and would unlikely contribute a clear characteristic to add pattern for classifying subspecies.

Information about sample sizes and geographic origins of all samples used for the statistical comparisons are listed in Supplementary Table 1.

### DNA extraction and genotyping

DNA was extracted from blood samples following an ammonium acetate protocol (as described in Richardson et al. 2001). Willow Warblers were genotyped at the following four loci; chromosome 1 (an intron of the *NBEA* gene by polymerase chain reaction (PCR) and Sanger sequencing as in Liedvogel et al. 2014), chromosome 3 (a biallelic marker “ww1” using PCR–RFLP as in Bensch et al. 2002), chromosome 4 (protein coding region of the *Clock* gene of one exonic poly-glutamine motif that varies in length, by PCR-fragment length analysis as in Johnsen et al. (2007) and chromosome 5 [intron of *C11orf41* gene by PCR and Sanger sequencing as in Liedvogel et al. (2014)]. On chromosome 4 we used a candidate gene approach to compare the number of glutamine (Q, encoded as CAA or CAG) repeats, a classical proxy for *Clock* gene diversity, following a protocol described in Johnsen et al. (2007). We sent PCR products of 36 *yakutensis*, 140 *acredula* and 47 *trochilus* samples to “Uppsala Genome Centre” for fragment length analysis of this variable *Clock* region. The number of repeats scored from the fragment length analyses were confirmed by Sanger sequencing of five randomly chosen Siberian Willow Warblers over the entire poly-Q repeat, allowing to precisely match the number of glutamine repeats to fragment length. To ensure that the analysed poly-Q region was not duplicated in the Willow Warbler genome, we blasted a consensus sequence of five sequences obtained from ABI sequencer (279 bp) with BLASTN 2.5.0 (Zhang et al. 2000) to a Willow Warbler genome assembly published in Lundberg et al. (2017). This sequence aligned within the annotated interval of the *Clock* gene. The genomic loci on chromosomes 1, 3 and 5 were chosen because they have been previously shown to differ between *trochilus* and *acredula* (Lundberg et al. 2013; Liedvogel et al. 2014). The locus on chromosome 4

(segment of the *Clock* gene) was included because of its putative role in migration timing (Saino et al. 2013).

All Sanger sequencing was carried out using a BigDye sequencing kit (Applied Biosystems, Austin, TX, USA), following standard protocols, on an ABI PRISM 3100 sequencer (Applied Biosystems, FL, USA) at the Department of Biology at Lund University. Primers and PCR conditions are listed in Supplementary Table 2. Sequences have been deposited at NCBI as PopSet: 1485777242 (chromosome 1) and PopSet: 1485777195 (chromosome 5).

## Molecular analysis

We used Geneious 6.1.8 (Kearse et al. 2012) and Aliview (Larsson 2014) to curate and trim sequences. The generated *yakutensis* sequences were aligned to existing reference sequences of *trochilus* and *acredula* (Suppl. Table 1). We used DnaSP Ver. 5.10.01 (Librado and Rozas 2009) to calculate Tajima's  $D$  and  $F_{st}$  and MEGA7 (Kumar et al. 2016) to calculate nucleotide diversity ( $\pi$ ). We inferred haplotypes using the PHASE algorithm implemented in DnaSP with default settings. NETWORK 5.0.0.1 (Bandelt et al. 1999) was then used to calculate median-joining haplotype network. We used Geneious 6.1.8 microsatellite analysis external plugin 1.4.0 to infer *Clock* allele lengths. We calculated  $F_{st}$  for poly-Q genotypes with Fstat 2.9.3.2 (Goudet 1995).

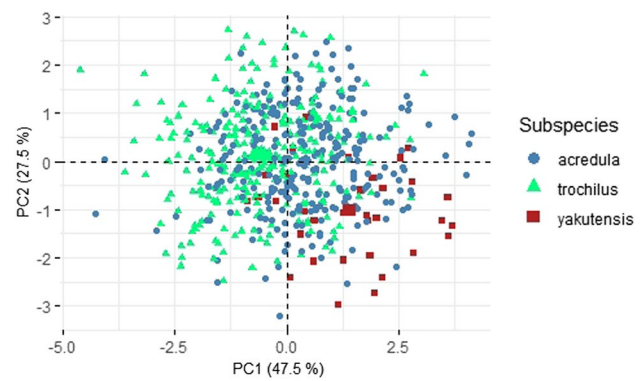
## Stable isotope analysis

Stable isotope ratios for  $\delta^{13}C$  and nitrogen  $\delta^{15}N$  were analysed for the innermost flight feather at the Stable Isotope Service Lab, Department of Biology, Lund University, Sweden ([www.biology.lu.se/services/instrumental-chemistry](http://www.biology.lu.se/services/instrumental-chemistry)). Stable isotope analyses were carried out for 20 male Willow Warbler males from the Chaun delta and ten males from southern Sweden sampled in the same year (2016). We analysed material (6 to 8 mg) from the tip of the innermost primary feather as described in Bensch et al. (2006a). We compared the samples collected in 2016 from *yakutensis* ( $n=20$ ) and *trochilus* ( $n=10$ ) with data in Bensch et al. (2006a) from breeding range samples of *acredula* ( $n=100$ ) and *trochilus* ( $n=100$ ) as well as winter samples of actively moulting birds collected from multiple countries in western ( $n=46$ ), eastern ( $n=26$ ) and southern ( $n=27$ ) Africa.

## Results

### Morphometrics and plumage

Male Willow Warblers sampled in Chaun were larger than conspecifics from Scandinavia (Figs. 1 and 2, Suppl. Tables 3 and 4). This was most prominent for tail length



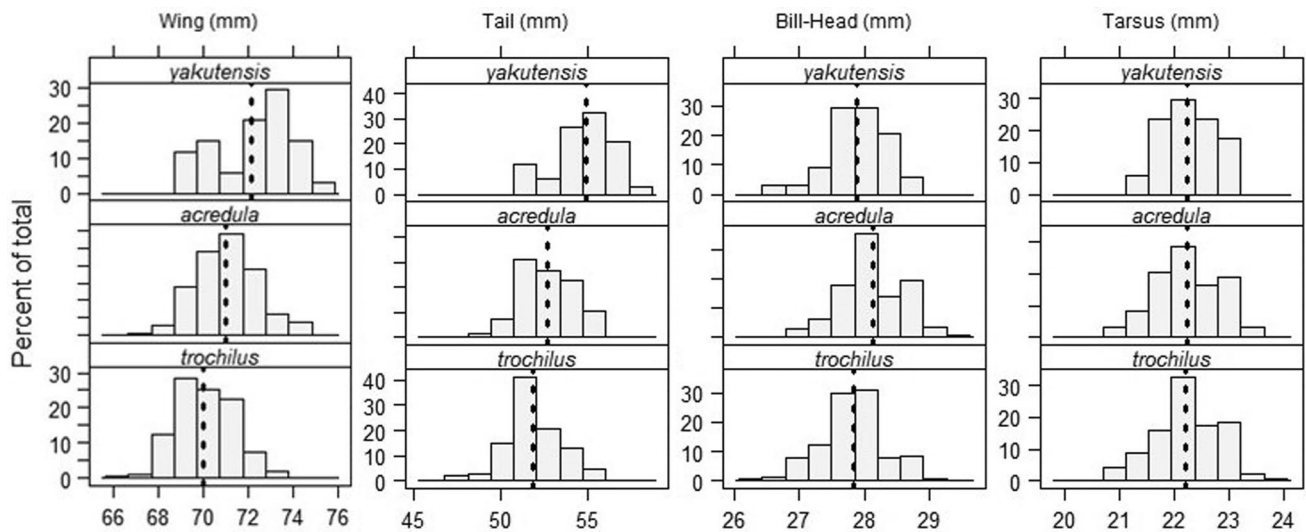
**Fig. 1** Principal component analysis of Willow Warbler morphology (males only). *trochilus*  $n=300$ , *acredula*  $n=291$ , *yakutensis*  $n=34$ . (See eigenvalues in Suppl. Table 3 and variable loadings in Suppl. Figure 2)

(one-way ANOVA,  $F_{2,622}=65.056$ ,  $P<0.001$ ; Tukey's HSD test,  $\alpha=0.05$ ) and wing length (one-way ANOVA,  $F_{2,622}=67.524$ ,  $P<0.001$ ; Tukey's HSD test,  $\alpha=0.05$ ). Mean wing length was 69.9 mm (SD 1.3) for *trochilus*, 71.0 mm (SD 1.4) for *acredula* and 72.2 mm (SD 1.6) for *yakutensis*. In the entire data set, tail length strongly correlated with wing length (Pearson's  $r=0.66$ ,  $P<0.05$ ). Tarsus length did not differ between the subspecies (one-way ANOVA,  $F_{2,622}=0.1169$ ,  $P=0.889$ ). Bill-head length differed significantly between the subspecies (one-way ANOVA,  $F_{2,622}=27.267$ ,  $P<0.001$ ). Post hoc Tukey's HSD test suggested that bill-head lengths of *acredula* was significantly longer compared to *trochilus* and *yakutensis*. However, we advise caution when interpreting the results as the observed difference is within possible measurement error. The breast colour scores differed significantly between all three subspecies (one-way ANOVA  $F_{2,622}=162.43$ ,  $P<0.001$ ). Although all *yakutensis* trapped in Chaun were grey/white on the breast with the yellow restricted exclusively to the under-wing coverts and auxiliaries, similarly grey birds do also occur at low frequency within the range of *acredula* (Fig. 3).

### Stable isotopes

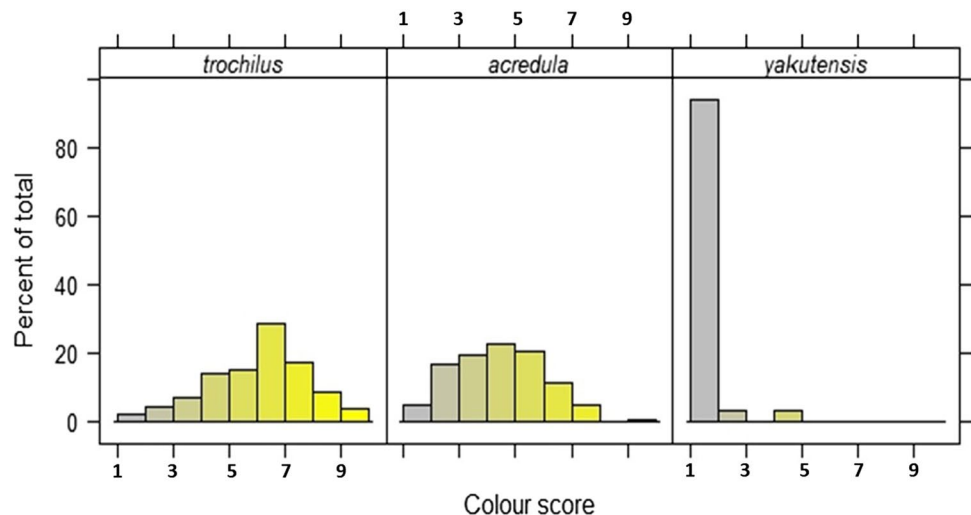
Feathers of *yakutensis* that had been moulted during the previous winter showed the highest  $\delta^{15}N$  values (mean  $12.30 \pm 1.79$ , one-way ANOVA  $F_{2,217}=101.18$ ,  $P<0.001$ , HSD-Tukey test with  $\alpha=0.05$ ) and the lowest  $\delta^{13}C$  values (mean  $-21.64 \pm 1.85$ , one-way ANOVA  $F_{2,217}=5.9$ ,  $P=0.003$ , HSD-Tukey test with  $\alpha=0.05$ ) compared to Scandinavian subspecies (Fig. 4). Overall, the  $\delta^{13}C$  and  $\delta^{15}N$  signatures of *yakutensis* were more similar to the signatures of Willow Warbler feathers moulted in southern





**Fig. 2** Comparison of tail length, wing length, bill and head length and tarsus length across all three Willow Warbler subspecies (males only). Dashed lines show mean values. *trochilus*  $n=300$ , *acredula*  $n=291$ , *yakutensis*  $n=34$

**Fig. 3** Breast colour of all three Willow Warbler subspecies. 1 refers to absolute lack of yellow colour, whereas 9 refers to a yellow breast. Only males are included. *trochilus*  $n=300$ , *acredula*  $n=291$ , *yakutensis*  $n=34$



Africa than to feathers moulted in western or eastern Africa (Fig. 4).

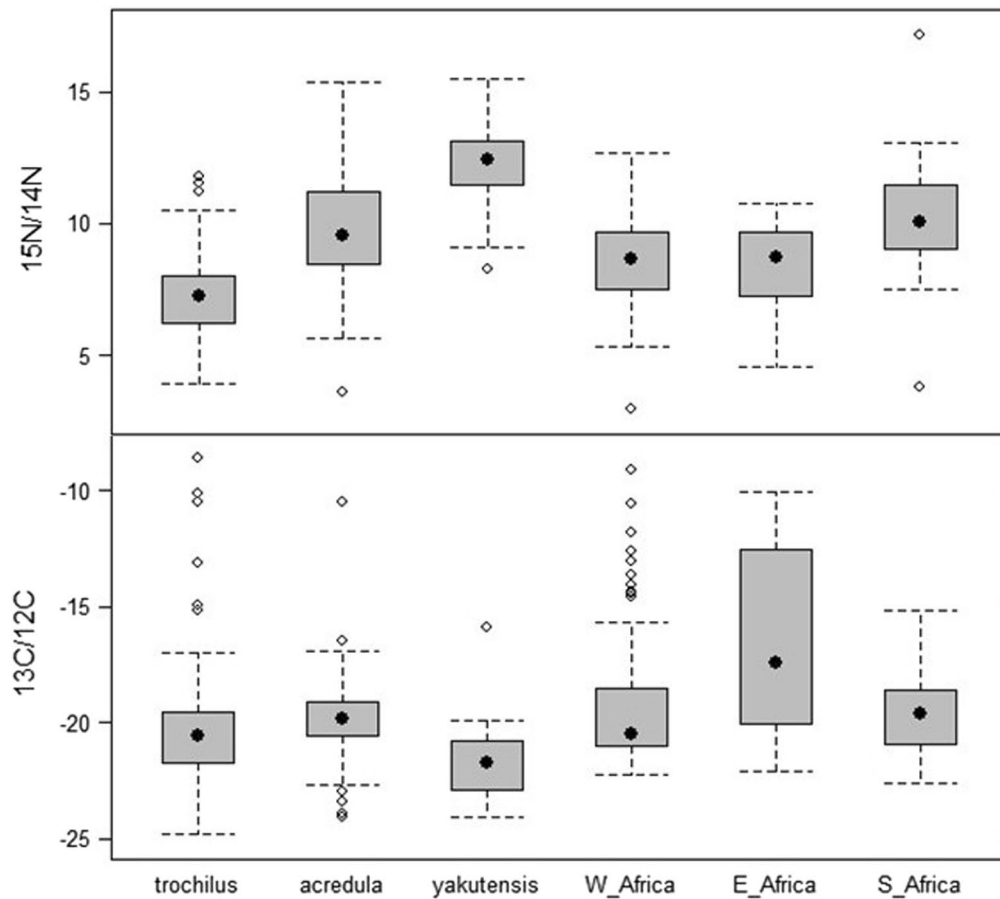
## Genotypes

We extracted DNA for genotyping samples from 36 *yakutensis*. While DNA extraction was successful for all individuals, clear genotyping results were obtained for all 36 birds for markers on chromosomes 3 (PCR–RFLP method) and 4 (fragment lengths analysis), but only for 26 individuals for the locus of chromosome 1, and 14 from the locus on chromosome 5. Most of these failures to successfully genotype those loci can likely be explained by the high density of indels in the Willow Warbler genome that creates many

frame shifts and makes Sanger sequencing notoriously difficult in this species (Lundberg et al. 2017).

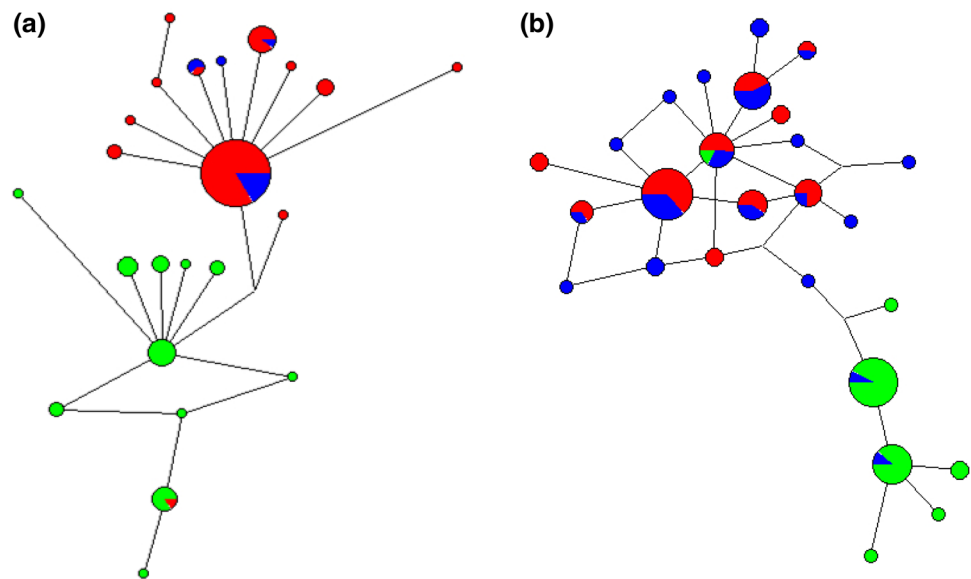
Across all the Willow Warbler subspecies, the sequenced region on chromosome 1 (214 bp) carried 19 SNPs (of which nine were singletons), 23 haplotypes and a nucleotide diversity ( $\pi$ ) of 0.007. The sequenced fragment on chromosome 5 (338 bp) had 21 SNPs (of which five were singletons), 26 haplotypes and a  $\pi$  of 0.01. All three subspecies showed negative values of Tajima's  $D$  for both loci although only significantly negative ( $-2.19$ ) for *yakutensis* on chromosome 1 (Suppl. Table 5).

For both Sanger sequenced loci, there was a clear separation between haplotypes primarily occurring among *trochilus* and haplotypes shared between *acredula* and *yakutensis* (Fig. 5). The pairwise  $F_{st}$  values were high ( $>0.6$ ) with



**Fig. 4**  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  ratios from Willow Warblers caught at breeding and wintering grounds. The isotope signatures for *trochilus* ( $n=100$ ), *acredula* ( $n=100$ ) and Africa (West  $n=47$ , East  $n=26$ , South  $n=27$ ) from Bensch et al. (2006a), *yakutensis* ( $n=20$ )

**Fig. 5** Haplotype networks for loci on chromosome 1 (a) and chromosome 5 (b). Each circle represents a unique haplotype. Colours show frequency of each subspecies carrying each haplotype. green: *trochilus*, blue: *acredula*, red: *yakutensis*. The size of the circle is proportional to the frequency of the haplotype and branch lengths are proportional to divergence between haplotypes. *trochilus*  $n=28(\text{chr1})/24(\text{chr5})$ , *acredula*  $n=12(\text{chr1})/28(\text{chr5})$ , *yakutensis*  $n=58(\text{chr1})/32(\text{chr5})$



**Table 1** Pairwise  $F_{st}$  values for all three subspecies for all four loci investigated

Locus	<i>Acr</i> vs <i>tro</i>	<i>Acr</i> vs <i>yak</i>	<i>Tro</i> vs <i>yak</i>	Total
Chrom 1	0.625	0.008	0.587	0.547
Chrom 3	0.468	0.268	0.906	0.511
Chrom 4	0.002	0.008	0.010	0.001
Chrom 5	0.621	0.009	0.729	0.565

Detailed account of sample size and origin in Suppl. Table 6

*Acr* *acredula*, *tro* *trochilus*, *yak* *yakutensis*

significant difference between *trochilus* and each of the other subspecies, but there were no differences ( $F_{st} < 0.01$ ) between *yakutensis* and *acredula* (Table 1).

For the bi-allelic locus on chromosome 3, 35 of the 36 *yakutensis* were homozygous for the “northern allele”, with only one individual carrying a heterozygous genotype. The latter individual was also heterozygous for the marker on chromosome 5. On chromosome 1, it was homozygous for the commonest *acredula/yakutensis* (northern) haplotype. The overall  $F_{st}$  for the locus on chromosome 3 was 0.51, with the lowest value between *acredula* and *trochilus*: 0.27 (Table 1).

For the *Clock* gene (chromosome 4) we found five alleles corresponding to variable numbers of glutamine repeats (9 to 12). All five alleles were present in all three Willow Warbler subspecies at similar frequencies (Fig. 6). The predominant genotype in all populations was homozygous for the most common allele with glutamine repeat length 11 (“11/11”) and the  $F_{st}$  value across all populations was 0.001. The level of heterozygosity (0.30 in *trochilus*, 0.33 in *acredula* and 0.19 in *yakutensis*) did not differ from what is expected under Hardy–Weinberg Equilibrium ( $P > 0.05$  Suppl. Table 6).

## Discussion

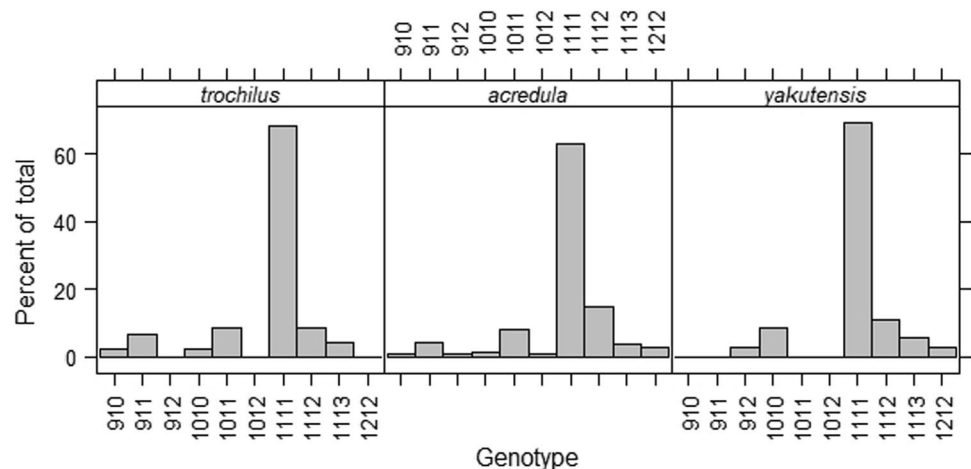
In this study we found *P. t. yakutensis* to be genetically inseparable from *P. t. acredula*. Meanwhile, the subspecies differ on average in size and plumage colouration.

As predicted from earlier work (Ticehurst 1936 in Williamson 1976; Shirihihi and Svensson 2018), Willow Warblers from the north-eastern edge of the breeding range are larger compared to the western and north-western edge of the range, with the wing being the single best trait discriminating between all three subspecies. Since *yakutensis* are migrating longer distances than *acredula* and *trochilus* (Sokolovskis et al. 2018) this pattern matches Seebohm’s rule, predicting that populations with longer migration routes have longer wings (Seebohm 1901). Unlike traits that directly facilitate flying (i.e., wing and tail length), the traits that are more exclusively associated with foraging (i.e., tarsus and bill to head length) did not differ between the Willow Warbler subspecies. Similarly, in Eurasian Blackcaps (*Sylvia atricapilla*), wing length differed between populations that migrate different distances, while tarsus did not (Rolshausen et al. 2009).

In Chaun, the plumage of Willow Warblers showed little variation, with grey upperparts, snow-white underparts, and yellow restricted to auxiliaries. In Scandinavia, the typical grey/white *acredula* phenotype can be found at low frequency within the breeding range of on average more yellow *trochilus* and vice versa. However, we neither caught nor visually observed any distinctly yellow *trochilus* type coloured individuals in north-eastern Siberia suggesting that grey/white *yakutensis* phenotype is not just a morph. We speculate that the frequency of yellow birds shows a gradual cline across the continent and decreases from west to east but we need additional samples to confirm it.

In contrast to other phenotypic traits, the migratory behaviour separates the European Willow Warbler

**Fig. 6** Frequency of *Clock* genotypes (chromosome 4) in all Willow Warbler subspecies.  $n=47$  for *trochilus*, 140 for *acredula* and 36 for *yakutensis*



subspecies relatively clearly, with little variation within populations but with clearly defined contact zones, referred to as migratory divides (Bensch et al. 2009). Birds breeding in the range of *trochilus* migrate to western Africa in autumn, whereas birds from the range of *acredula* migrate to eastern and southern Africa, supported by ring recoveries (Hedenström and Pettersson 1987), stable isotopes (Bensch et al. 2009) and tracking data for *trochilus* (Lerche-Jørgensen et al. 2017). In the Scandinavian contact zone, clines in morphology and migratory behaviours are largely overlapping however east of the Baltic, these are displaced from each other by 1500 km. Hence, in Southern Finland and the Baltic countries, Willow Warblers look mainly like *trochilus* at similar latitudes, but migrate like *acredula*. The birds breeding east of the Ural Mountains, presently classified as *P. t. yakutensis* have been supposed to migrate to southern Africa (Alerstam et al. 2003). Compared to isotope signatures of Willow Warbler feathers in active moult across Africa, the isotope composition of birds from Chaun suggested wintering locations in southern Africa. However, our recent geolocator study of three birds from Chaun revealed wintering areas in southeast Africa (Sokolovskis et al. 2018). It is important to note that the isoscape of Africa is very heterogeneous and assigning any single sample to correct geographic origin is difficult if not impossible (Hobson et al. 2012). The isotope study of Bensch et al. (2006a) did not include samples from this exact part of Africa, and because geolocator data are lacking for *acredula*, we do not yet know to what extent *acredula* and *yakutensis* might share wintering areas. The differences we found suggest that Willow Warblers from Chaun winter in a region with different  $\delta^{13}\text{C}$  and  $\delta^{15}\text{N}$  values than the Scandinavian *acredula*.

Using whole-genome resequencing, Lundberg et al. (2017) reported the presence of two putative inversions on chromosomes 1 and 5, harbouring most of the fixed differences between *acredula* and *trochilus* (in this study we sequenced parts of both these differentiating regions). Lundberg et al. (2017) also used a roughly 4000-SNP array to genotype approximately 1000 birds across their European breeding range, and also included four birds from far Eastern Siberia (Anadyr 64.71°N, 177.32°E) and four from Western Siberia (Yekaterinburg 56.97°N, 60.28°E) in the analysis. The combined genotypes of these Siberian birds from the range of *yakutensis* turned out to be inseparable from *acredula*. Our present study supports Lundberg et al. (2017) results by confirming that *acredula* and *yakutensis* are genetically extremely similar and differences, if any, are very small. The sequencing approach presented in this study allowed us to look for more variation in *yakutensis* that is not captured by the Lundberg et al. (2017) array, which was designed from SNPs that had originally been found in a contrast between *acredula* and *trochilus*. Yet, the same haplotypes seem to exist in *acredula* and *yakutensis*. Repeat

length variation (e.g., microsatellites) was not included in the SNP array used by Lundberg et al. (2017). One of the four genetic markers investigated in our study (*Clock*) shows length polymorphism. This locus was included in our analyses because previous studies on songbirds have found the number of glutamine repeats in the *Clock* gene to be correlated with migration distance and breeding latitude (Caprioli et al. 2012; Saino et al. 2015; Bazzi et al. 2016). Because the Willow Warbler populations differ substantially in these traits, we expected them to show differences in repeat length at the *Clock* gene. However, the size ranges and frequencies of different *Clock* alleles were almost identical across all three subspecies ( $F_{st}=0.001$ ). These results reject the hypothesis of an association between *Clock* repeat length and migration timing, distance or breeding latitude, at least in this species.

It has been estimated that there are 5.5 described subspecies for every continental bird species even though there are only 1.9 discrete mitochondrial lineages for the same species (Zink 2004). The three Willow Warbler subspecies all share the same mitochondrial haplotypes (Bensch et al. 2006b), which points out that mitochondria is not always informative for detecting very recent differentiation. In addition to the three subspecies discussed in this paper, minor variation in wing length has previously been used to describe *P. t. fitis* from Germany and *P. t. eversmanni* from southwestern Siberia (Salomonsen 1928). Haffer and Tiainen (in Glutz von Blotzheim 1991) call *eversmanni* a morph and question the separation of Willow Warblers in subspecies, but nevertheless acknowledges the existence of geographic structure in phenotype. More recent sources, such as Clements checklist of birds of the world (Clements et al. 2018) and Shirihaï and Svensson (2018), agree on the validity of the three Willow Warbler subspecies that we discuss in the current paper. It is worth mentioning that the curators at one of the largest existing bird skin collections, the Zoological Museum of Moscow State University, still distinguish *P. t. fitis* and *P. t. eversmanni* alongside *trochilus*, *acredula* and *yakutensis* based on thorough analysis of morphological differences (Yaroslav Redkin pers. comm.). Future research combining these yet unpublished results with genomic analyses is something to strive for.

If a strong phylogenetic signal is found when using a limited set of genetic markers, such as in the Arctic Warbler (*Phylloscopus borealis*) (Alström et al. 2018), it clearly supports a taxonomic split. Alternatively, failing to find evidence of a population structure when examining a limited set of genetic markers, does not mean that no genetic difference exists. Even very small and potentially overlooked local genomic differences can lead to strikingly different phenotypes [e.g., *Corvus corone corone* and *C. c. cornix* (Poelstra et al. 2014)]. Even though our study used candidate markers derived from full genome comparisons, genome assemblies



are never perfect and small differences can easily be missed. Commonly used neutral markers (e.g., majority of microsatellites) will mostly show differences among populations that occur because of drift. It is possible that any given subtle differentiation in phenotype is a result of recent point mutation and increases in frequency due to natural selection. In such case, even with a modern set of genomic tools, differences might be very difficult to find. Nevertheless, the true evolutionary history of each population is encrypted in genes, but still very difficult to extract. Examples of mismatching phenotypic and genetic structures in birds are accumulating (Harris et al. 2017; Schweizer et al. 2018); therefore, it is important to use detailed information of phenotypes together with genotypes in taxonomy. A serious revision of all currently acknowledged avian subspecies, integrating characteristics from various disciplines including genomics, tracking technology, and comparative morphology, remains an important task for modern ornithology because most of them have been described based on a handful of morphological characters only.

Some songbird species with large breeding ranges, such as the Siberian Rubythroat (*Calliope calliope*), are described as monotypic, while the Black Redstart (*Phoenicurus ochruros*), for example, has five distinct subspecies within a substantially smaller breeding range (Shirihai and Svensson 2018). Such differences between species in intraspecific variation ask for explanations and having described subspecies is a good starting point (Seebohm in Allen 1884). We agree with Fitzpatrick (2010) that the subspecies concept, although imperfect, is a useful aid when sorting samples and discussing geographic variation. In addition, by having subspecies as an entity means that we can save the well-defined biological entities as species and use subspecies for resolving and debating more ambiguously differentiated patterns. For example, thanks to trinomial taxonomy in the late nineteenth century in North America, 315 taxa were downgraded from full species to subspecies status (Mayr 1982).

A large continuous breeding range and minor clinal differentiation across Eurasia is a common pattern for songbirds [e.g., Bluethroat *Luscinia svecica*, and goldcrest *Regulus regulus* (Shirihai and Svensson 2018)]. In such species contrasts, the individuals from the extreme east and west might be apparent (as also illustrated by our study), but drawing a line between two morphs is arbitrary when variation changes over a gradual cline. In this regard, northern Eurasian songbirds might provide an excellent system for investigating early stages of local adaptation that may lead to parapatric speciation in the wild, which has not yet been clearly documented in vertebrates due to methodological difficulties, but might be a common speciation mechanism (Endler 1978), although this is still debated (Price 2008). The Willow Warbler in particular offers an excellent study system in this regard because, of

its well-defined secondary contact zone in central Scandinavia, a less distinct primary contact zone in the south-eastern Baltic region (Bensch et al. 2009) and very likely a 6000-km-long gradual cline from northern Scandinavia to the coast of the Pacific ocean (Lundberg et al. 2017).

We encourage other researchers working with birds, even if taxonomy is not their main field, to publish descriptive articles about the life history data of their study species. Wesołowski (2012) has pointed out that recent research trends have severely decreased quality of life history data in published papers. Each species is a unique entity, and generalizations of ecological or evolutionary concepts derived from theoretical models without knowledge of the specific life history nuances can lead to fundamentally wrong yet attractively titled high-profile publications.

We suggest that the taxonomic classification of Willow Warblers into three subspecies can still be used as a biogeographical reference to populations that show average but overlapping differences in phenotypes, but distinctly different migration patterns. Based on our results, we propose to use *yakutensis* for Willow Warblers breeding east of the Ural Mountains that initiate the autumn migration towards the southwest or west, in contrast to the otherwise most similar *acredula* that initiate the autumn migration towards the southeast or south from their breeding grounds in northern and eastern Europe. To more firmly locate the contact zone between *yakutensis* and *acredula*, which might occur around the Yenisei River (Seebohm 1901; Shirihai and Svensson 2018; Yaroslav Redkin pers. comm.), data from a longitudinal transect across Siberia will be required.

In conclusion, neither size nor plumage nor the molecular markers tested here allowed us to decipher a coherent population structure of the currently recognized Willow Warbler subspecies. However, taken together, stable isotope signatures, ring recoveries and tracking studies suggest that wintering sites and migration routes may serve as subspecies delimiting criteria in this species.

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