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Interaction between Common and Siberian Chiffchaff in a contact zone

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The study of hybridization between closely related taxa of animals sheds light on many important issues of evolution biology and taxonomy. Common Chiffchaff (*Phylloscopus collybita abietinus*) and Siberian Chiffchaff (*Ph. c. tristis*) co-occur in an extended zone of sympatry in the area from the White Sea to the South Urals. In allopatric populations, these two races are well differentiated in the external appearance and song. Under sympatric conditions, individuals with intermediate appearance and vocalizations are found practically everywhere but the occurrence of hybridization has not been documented up to date. The article describes the results of our study of interrelations between Common and Siberian Chiffchaffs in mixed populations found in the Arkhangelsk Region, the Komi Republic, and the Southern Ural Mountains. Allopatric populations were studied in central Russia (Moscow and Kostroma regions) and central Siberia (Yenisey River and Sayan Mountains). In mixed populations, 30.2% of the individuals with species specific phenotype showed a phenotype/haplotype mismatch while there were no such mismatches in allopatric populations. 58.7% of the individuals with typical *abietinus* phenotypes carried *tristis* haplotypes while only 4.0% possessed the opposite phenotype/haplotype combination. Most of the individuals with intermediate phenotype had *tristis* haplotypes (97.6%). Only 9.8% of the individuals with known haplotypes performed mismatched songs. In mixed populations, 9 of 11 males clearly responded to the playback of the song of another taxa while in allopatry no such reactions were observed for the 14 males tested. Our results strongly suggest hybridization between *abietinus* and *tristis* in the mixed populations.

1. Introduction

Birds are widely recognized as an attractive target of research on microevolutionary processes, and such studies have commonly been conducted in contact zones (Hewitt 1988, Kryukov & Blinov 1994, Kvist & Rytkönen 2006). It is well known that reproductive isolation between closely related species of songbirds is largely ensured by differences in their territorial songs, which include both the genetic basis and some components acquired through learning (Catchpole & Slater 1995). In

In memory of Vladimir V. Leonovitch

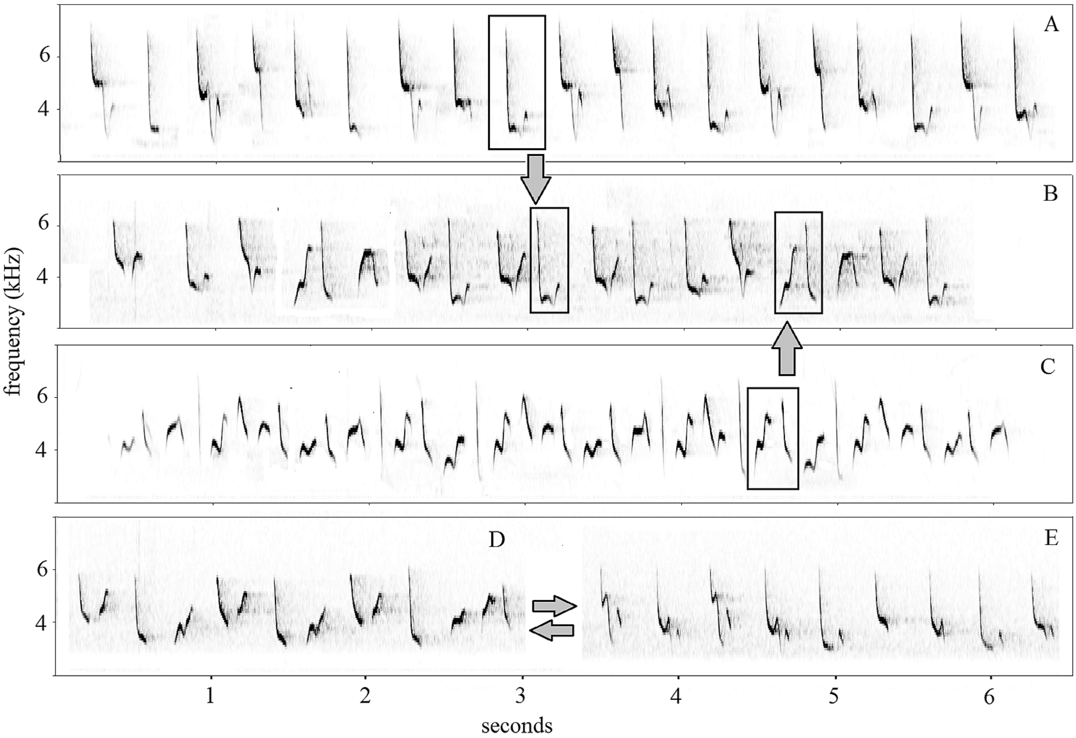

Fig. 1. Sonagrams of typical territorial songs of Chiffchaffs. A – Common Chiffchaff (*abietinus*), Kursk region, May, 2015; B – mixed singer, Southern Urals, Inzer, May, 2015; C – Siberian Chiffchaff (*tristis*), Krasnoyarsk region, Stolby Nature Reserve, June, 2009; D, E – bilingual singer with both *abietinus* and *tristis* phrases, alternating in the same song, Southern Urals, Starosubkhangulovo, May, 2015.

contact zones of closely related taxa the song can result in reproductive isolation and natural selection can lead to increasing divergence in the song of co-occurring species (Panov 1989, Salomon 1989, Eriksson 1991) through reinforcement (Dobzhansky 1940). Songs of closely related avian species may also converge through mutual learning ("mixed singing") (Helb *et al*. 1985, Sorjonen 1986, Vokurková *et al*. 2013) or gene flow (Helbig *et al*. 2001). The ability of songbirds to learn from other species makes it difficult to use songs as a hybridization marker and necessitates a parallel study of morphological and genomic characters.

The huge breeding range of *Phylloscopus collybita sensu lato* extends across nearly the entire Palaearctic and includes a varying number of taxa. Some of the Chiffchaff taxa meet and interact in contact zones. The relations between the taxa in such contact zones vary from almost complete reproductive isolation to intensive hybridization (Salomon & Hemin 1992, Helbig *et al*. 2001, Marova 1998, Shipilina & Marova 2013). The taxonomy of the "*Phylloscopus collybita* complex" was discussed by Clement & Helbig (1998).

The subject of our research were polymorphic populations of Chiffchaffs in the Southern Ural Mountains, the Komi Republic and the Arkhangelsk Region, where this species is represented by two well differentiated forms – the Common Chiffchaff (*Phylloscopus collybita abietinus*) and the Siberian Chiffchaff (*Ph.* (*c.*) *tristis*) and intermediate morphological and acoustic variations between them.

The two taxa have well differentiated songs and they differ also in plumage and biometric characters (Ticehurst 1938, Martens & Meincke 1989). The taxonomic relations between *abietinus* and *tristis* are not well understood. Molecular divergence between *abietinus* and *tristis* (1.7–2.0%) is not sufficient to confirm their status as separate species (Helbig *et al.* 1996).

The traditional interest of ornithologists in the relationships between *abietinus* and *tristis* is largely generated by the marked differences in their territorial songs, which are easily discernible to the human ear. These differences were first reported by Seebohm during his expedition to the Pechora River in 1875 (Seebohm 1882, 1890). In sonograms, the most noticeable difference is that *tristis* song inevitably contains elements with ascending frequency modulation, while such elements are absent in the songs of *abietinus*. In *abietinu*s song, each element starts on a high pitch and drops markedly (Martens & Meincke 1989, Marova & Leonovitch 1993). In the contact zone, many Chiffchaffs are "mixed singers" and sing a song containing elements of the typical songs of both taxa (Marova 2006, Marova & Alekseev 2008, Lindholm 2008, Komarova & Shipilina 2010) (Fig. 1).

More than a hundred years have passed since Sushkin (1897) first reported sympatry and intergradations between *tristis* and *abietinus* in the Southern Urals region. Later, the zone of sympatry was determined to extend over 1,500 km from here (Ufa) across the Komi Republic (Syktyvkar) to the Kanin Peninsula (Arkhangelsk) (Marova & Leonovitch 1993) (Fig. 2). However, evidence for hybridization between the two taxa was not pre-

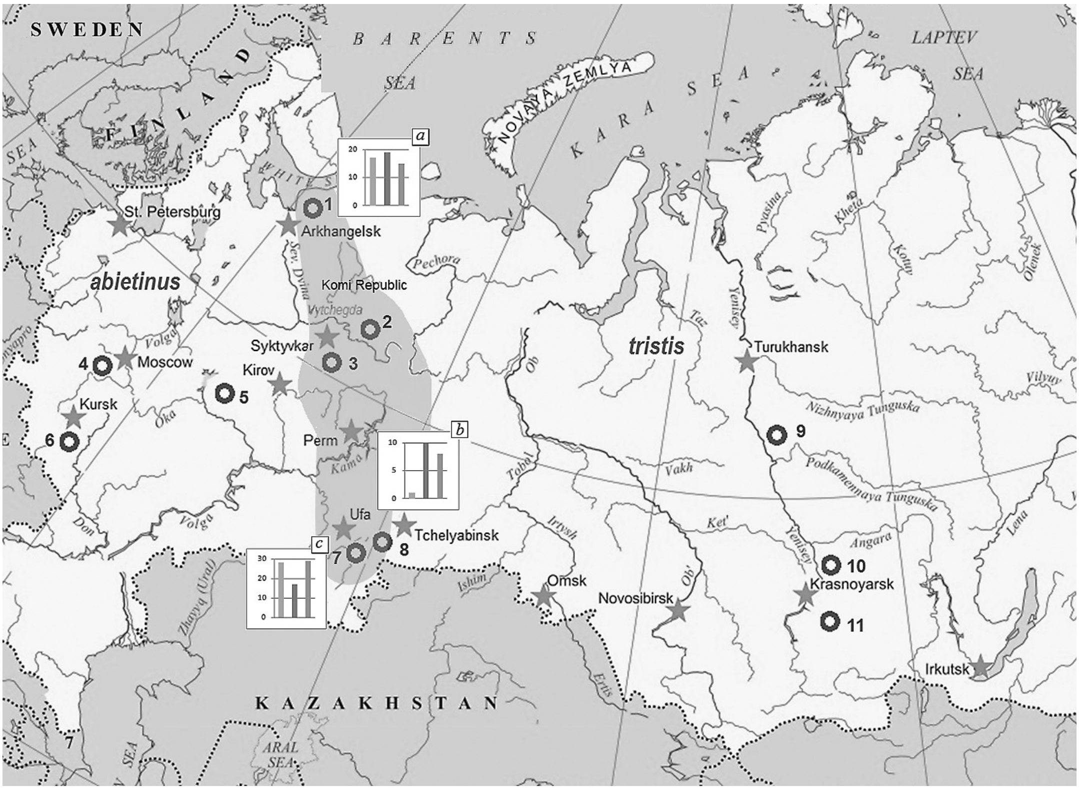

Fig. 2. The zone of contact and hybridization between Common (*abietinus)* and Siberian (*tristis)* Chiffchaffs. Black circles – locations where Chiffchaffs were captured and tape recorded: 1 – Arkhangelsk region, Pinezhsky Nature Reserve, 2 – Komi Republic, Vytchegda river, 3 – Komi Republic, Mordino, 4 – Moscow region, Zvenigorod Biological Station, 5 – Kostroma region, Manturovo, 6 – Kursk region, Central Chernozemny Nature Reserve, 7 – Inzer, South Uralsky Nature Reserve, 8 – Miass, Ilmensky Nature Reserve, 9 – mid-Yenisey, Mirnoe Biological Station, 10 – Yenisei ridge, Predivinsk, 11 – Eastern Sayan, Kuturchin and Stolby Nature Reserve. Grey figure indicates the zone of contact and hybridization. The diagrams in the white boxes indicate the distribution of phenotypes in different parts of the zone: *a* – Pinezhsky Nature Reserve, *b* – Ilmensky Nature Reserve, *c* –South Uralsky Nature Reserve. First column – *abietin*us phenotype, second column – intermediate phenotype, third column – *tristis* phenotype.

sented until many years later and is still questioned by some ornithologists (van den Berg 2009). Indeed, mixed singing is not a proof for hybridization and may be explained by mutual learning in a zone of sympatry (Helb *et al.* 1985).

In the present paper, we summarize our genetical, morphological, and bioacoustical data obtained in four regions in the contact zone of the Common and Siberian Chiffchaffs. We show that there is gene flow between the parental populations based on phenotype and haplotype, and dialect and haplotype. We also present the results of our field playback experiments in the zones of allopatry and sympatry. We show the important role of genetics in the formation of Chiffchaff songs. Lastly, we discuss the spatial distribution of *abietinus* and *tristis*, the geographical limits of the contact zone between the two taxa and possible directions of the seasonal migration of hybrids.

2. Material and methods

2.1. Study area and data set

Chiffchaffs were studied on their breeding grounds in May/June 2007–2012 and in 2016. Our data set includes 144 males from three populations found in the contact zone between *abietinus* and *tristis*: 74 males from the Southern Urals region (South Uralsky Nature Reserve and adjacent territory stretching for about 65 km along Ufa-Beloretsk road), 19 males from Transurals region (Miass, Ilmensky Nature Reserve) and 51 males from the Belomor-Kuloi Plateau (Arkhangelsk region, Pinezhsky Nature Reserve). Data on 53 males from the allopatric populations were collected in the Moscow, Vladimir and Kostroma regions (*abietinus, n* = 16) and in Central Siberia (Krasnoyarsk region, mid-Yenisey, Mirnoe Biological Station and Stolby Nature Reserve) (*tristis, n* = 37) (Fig. 2).

2.2. Tape recording and capturing method

Singing males were tape recorded and then captured in an Ecotone mist-net (6 × 2.5 m, mesh 16 mm) after being lured by the playback of their respective song type. Once photographed, measured and blood sampled (from *vena brachialis*) the birds were ringed and immediately released. We used digital sound recorders (Marantz PMD 660, PMD 620) with external condenser microphones (AKG C1000S, Sennheiser ME 66 with K6 module, and Philips SBC ME 570 with Sony PBR 330 parabolic reflector) for tape recording. Each recording lasted at least 3 minutes.

2.3. Vocalization analysis and playback tests

The sonograms were created in Syrinx 2.5s (J.M. Burt: http://syrinxpc.com) with settings FFT = 512 and Blackman window. As mentioned above, the most important difference between *tristis* and *abietinus* songs is the presence of elements that begin with ascending frequency modulation (ascending elements) in *tristis* song (Fig. 1). While such elements are obligatory and very distinctive in the songs of *tristis,* they are absent in *abietinus* songs. This difference can be calculated as a vocal index (VCI), which is defined as the proportion of ascending elements in the total number of elements in the song (Marova *et al.* 2009). VCI is zero for *abietinus* (no ascending elements), but it has always nonzero values for *tristis* and mixed singers.

We conducted 25 playback experiments mainly following the protocol of Martens & Meincke (1989). When a singing male was located, a loudspeaker was placed near the bird. We recorded the bird's reaction from a position 8 to 10 m away from the speaker. The song of one taxa was played for 3 minutes and the bird's behavior was recorded.

We played a song of one taxa to 11 males of the other taxa in the Southern Urals in a mixed *abietinus*-*tristis* population. We also made 7 translations of the *abietinus* song to the *tristis* males in an allopatric population in the mid-Yenisei, Mirnoe; and 7 translations of the *tristis* song to the *abietinus* males in an allopatric population in the Kursk Region (Central Russia).

2.4. Genetic analyses

We used 144 males from the sympatry zones and 53 males from the allopatry zones to analyze mitochondrial (mtDNA) cytochrome b *(cyt b*) gene. Total DNA was isolated from blood samples using the standard phenol-chloroform extraction technique. PCR product purification and sequencing were performed as described by Helbig *et al.* (1995). In the study by Helbig *et al.* (1996), sequences of 1041 nucleotides of *cyt b* were received for *abietinus* and *tristis* and they were shown to differ from each other at 15 nucleotides. We chose a site of the *cyt b* gene consisting of 389 nucleotides and analyzed this region because it has been previously published for the Chiffchaff (GenBank accession entries: Z73479.1, Z73482.1). Within this region five SNPs were found to be different between European and Siberian Chiffchaffs. For restriction analysis we chose two of the diagnostic SNPs (sites 474 and 495) making it possible to distinguish the haplotypes of *abietinus* and *tristi*s. The endonuclease Hinf I (GANTC) was used for restriction analysis. For each sample we used 0.3 µl of Hinf1 mixed with Buffer R (1 µl), bi-distilled water (4 µl) and extracted DNA (5 µl). The restriction reagent mix was held at 37 °C for 12 hours and fragment types were scored by regular polyacrylamide gel electrophoresis. We calculated mtDNA introgression level as the proportion of birds with mismatch between phenotype and haplotype.

2.5. Morphological analysis

All the males studied were caught in the middle of the nesting season and their plumage was characterized by a medium extent of wear. Primary identification of the captured males was carried out based on the plumage coloration. Common (*abietinus*) and Siberian (*tristis*) Chiffchaffs are similar in plumage but display varying degrees of olive, buff, grey and yellow (Ticehurst 1938, Svensson 1992, Dean & Svensson, 2005). We divided all captured males into three phenotypic groups: *abietinus, tristis* and an intermediate, based mainly on yellow coloration intensity on the ventral side of the body (throat, breast and belly). Chiffchaffs with maximum development of yellow on the underparts of the body were classified as *abietinus*. Typical *abietinus* possessed a yellow supercilium and eye ring, olive/greenish mantle, back and rump and yellow stripes on the ventral side of the body. The coloration of the underwings varied in intensity, but was usually bright yellow. Contrary to *abietinus*, typical *tristis* did not have any yellow on underparts, supercilium and eye ring. A subtle yellow hue could be found only on the underwings. We assigned to the intermediate group all birds with predominantly *tristis* appearance but possessing few or even a single yellow feather on the breast, belly and/or supercilium and eye ring (for more detailed description see Marova *et al*. 2013).

Wing length and tail length were measured on all captured males. The measurements were taken by the same persons (I.M. and D.Sh.). We also analysed the wing formula, i.e., the length of the second outermost primary compared with the 6th, 7th, and 8th ones. The comparison between primary lengths was done according to Svensson (1992). The wings of *abietinus* are known to be more pointed: the tip of the 2nd primary wing is usually localized between the 7th and 6th. In contrast, the *tristis* wing is more rounded: the tip of the 2nd primary wing is more often between the 7th and 8th (Ticehurst 1938).

3. Results

3.1. Genetic variation

In allopatry, the haplotypes of the individual birds captured always coincides with their external appearance (”*abietinus–abietinus*” or “*tristis–tristis*”) and no haplotypes/phenotypes mismatches (”*abietinus–tristis*”) have been observed. In the mixed populations from the contact zone, the interrelation between morphological and genetic traits was found to be much more complex. Firstly, we found both *abietinus* and *tristis* haplotypes in the Southern Urals (South Uralsky reserve) and Arkhangelsk (Pinezhsky reserve) regions. But in the Ilmensky reserve (200 km to the north-east from the South Uralsky reserve) only *tristis* haplotypes were registered (Fig. 2, Fig. 6). Secondly, a new, previously unidentified, haplotype was discovered in all three of the mixed populations. This haplotype closely resembled the typical *tristis* haplotype, differing by only one base pair. This new haplotype was not detected in allopatric *tristis* populations. In the sympatric zone, however, the new haplotype could be found nearly everywhere, although in different proportions, with a particularly significant proportion in the South Uralsky

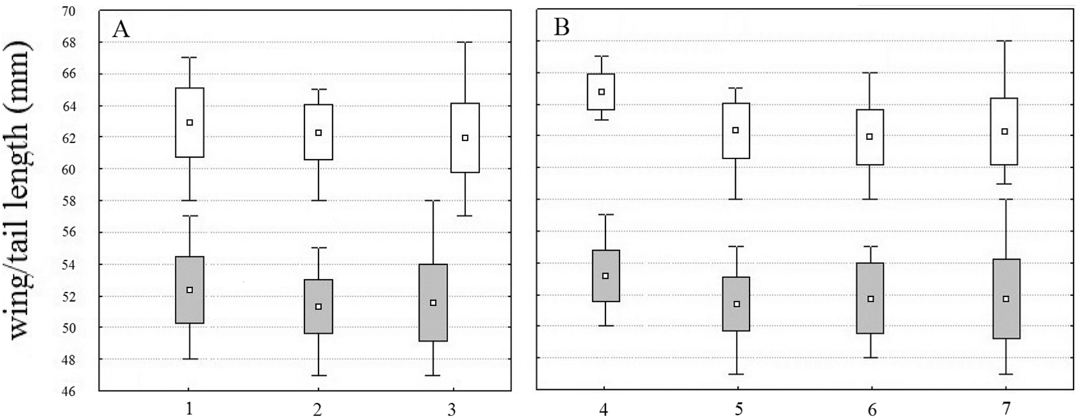

Fig. 3. The relationships between wing and tail length (mm) and phenotype (type of coloration)/haplotype. A – variation in wing and tail length of males assigned to different phenotype (type of coloration): 1 – *abietinus* phenotype, 2 – intermediate phenotype, 3 – *tristis* phenotype. B – variation in wing and tail lengths of males classified into four haplotype/phenotype groups: 4 – *abietinus* haplotype/*abietinus* phenotype, 5 – *tristis* haplotype/intermediate phenotype, 6 – *tristis* haplotype/abietinus phenotype, 7 – *tristis* haplotype/*tristis* phenotype. White boxes represent wing length data, dark boxes represent tail length data. Means, SD, minimal and maximal values are shown.

reserve (in about 21% of individuals). Below we analyze both these haplotypes together, comparing them with the European (*abietinus*) haplotype.

3.2. Morphological variation

A considerable diversity in phenotypes (plumage coloration) was detected in populations from Pinezhsky, Ilmensky and South Uralsky nature reserves (Fig. 2). In each of these populations, all three phenotypes were found in different proportions with a marked proportion of males possessing intermediate phenotypes. In contrast, no intermediate phenotypes were registered in any allopatric *abietinus* and *tristis* populations, where all of the examined individuals undoubtedly belonged to the typical phenotypes.

Fig. 3A (1, 2, 3) shows the variation in wing and tail lengths of males assigned to different phenotypes upon capture. The influence of phenotype was significant for both measurements (one-way ANOVA; $F = 4.1$, $P = 0.019$ for wing length; $F = 3.9$, $P = 0.02$ for tail length). The wings of typical *abietinus* were significantly longer than those of typical *tristis* (post-hoc Bonferroni test, $P = 0.011$, $df = 133$). Intermediate phenotypes did not differ significantly from the typical forms. Tail length did not show significant differences between the phenotypes, although differences between the typical *abietinus* and the intermediate phenotypes was close to being significant (Bonferroni corrected P = 0.056, df = 131).

Fig. 3B (4–7) illustrates the variation in wing and tail lengths of males classified into four haplotype/phenotype groups: 4) *abietinus* haplotype/*abietinus* phenotype; 5) *tristis* haplotype/intermediate phenotype; 6) *tristis* haplotype/*abietinus* phenotype; 7) *tristis* haplotype/*tristis* phenotype. As in the previous case, the influence of the group attribution factor (haplotype–phenotype) on the morphological measurements was significant (one-way ANOVA; $F = 11.1$, $P < 0.001$ for wing length; $F = 3.2$, $P = 0.02$ for tail length). As for paired comparisons (post-hoc Bonferroni-test) only one group – males with both *abietinus* coloration and *abietinus* haplotype – showed significant differences from all others at $P < 0.001$ (wing length, $df = 110$) and $P = 0.048$ (tail length, $df = 109$) (Fig. 3B, 1). Thus all males with *tristis* haplotypes were smaller, regardless of their coloration. In other words, many Chiffchaffs with *tristis* haplotypes were classified as *abietinus* (or intermediates) based on their coloration but did not differ in size from typical Siberian Chiffchaffs.

In mixed populations, the proportion of males having a longer 2nd primary was significantly higher among the individuals with *abietinus* phe-

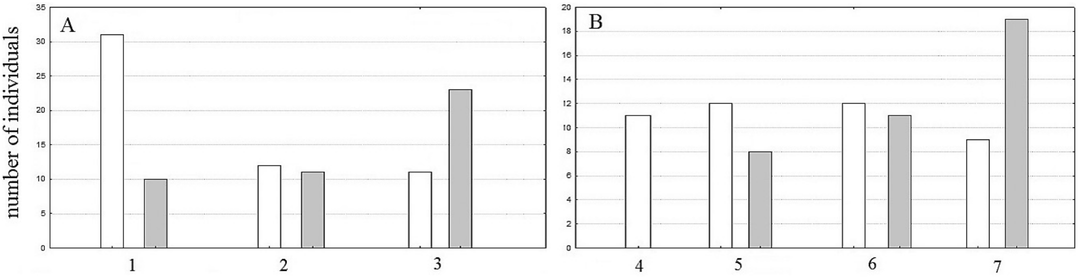

Fig. 4. The relationships between wing formula and phenotypes (type of coloration). A – variation in wing formula of males with the intermediate phenotype: 1 – *abietinus* phenotype, 2 – intermediate phenotype, 3 – *tristis* phenotype. B – variation in wing formula of males classified into four haplotype/phenotype groups: 4 – *abietinus* haplotype/*abietinus* phenotype, 5 – *tristis* haplotype/*abietinus* phenotype, 6 – *tristis* haplotype/intermediate phenotype, 7 – *tristis* haplotype/*tristis* phenotype. White boxes represent *abietinus* wing formula, whilst dark boxes represent *tristis* wing formula.

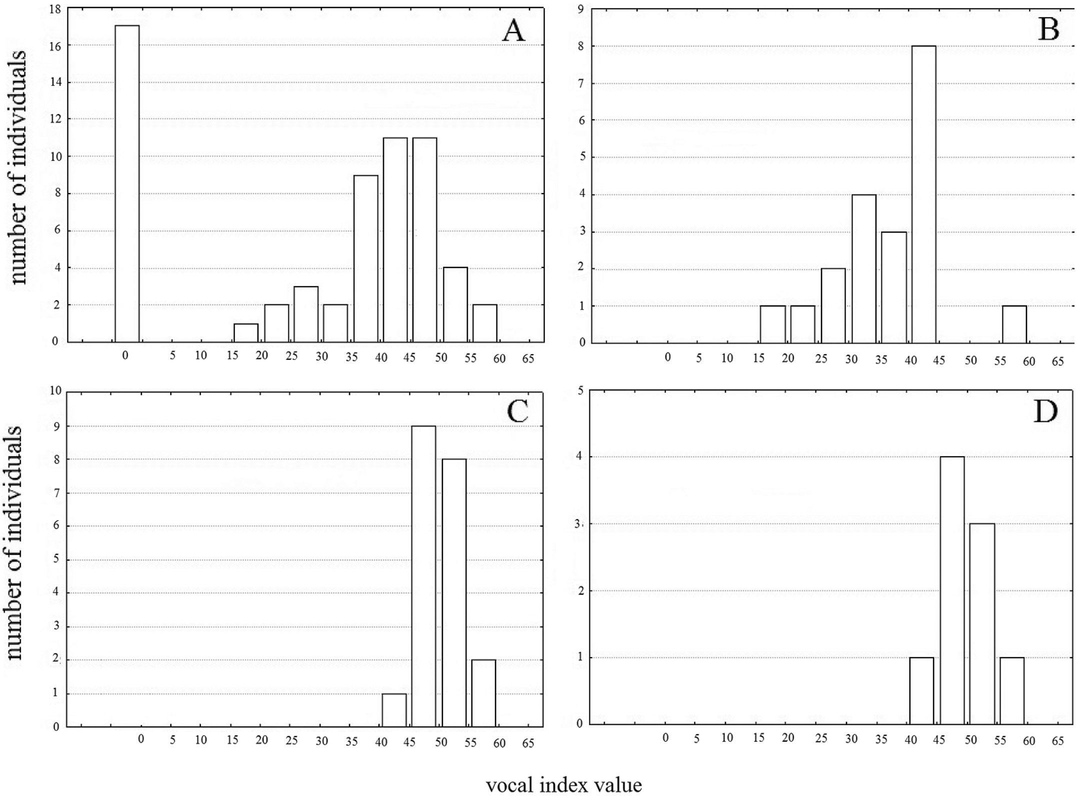

Fig. 5. Frequency distribution of vocal indexes (the fraction of ascending elements in the total number of elements in the song) in Chiffchaff from the allopatric and sympatric populations. A – South Uralsky Nature Reserve, B – Pinezhsky Nature Reserve, C – Central Siberia (mid-Yenisey and the Eastern Sayan), D – Ilmensky Nature Reserve. Horizontal axis – proportion of ascending notes within the song (%), vertical axis – number of males.

notype than among those with *tristis* phenotype (Fisher's exact test, two-tailed, $df = 1$, $P = 0.0002$). Chiffchaffs with intermediate coloration did not differ significantly from typical *abietinus* and *tristis* phenotypes (Fisher's exact test: intermediate phenotypes vs *abietinus* $P = 0.094$; intermedi-

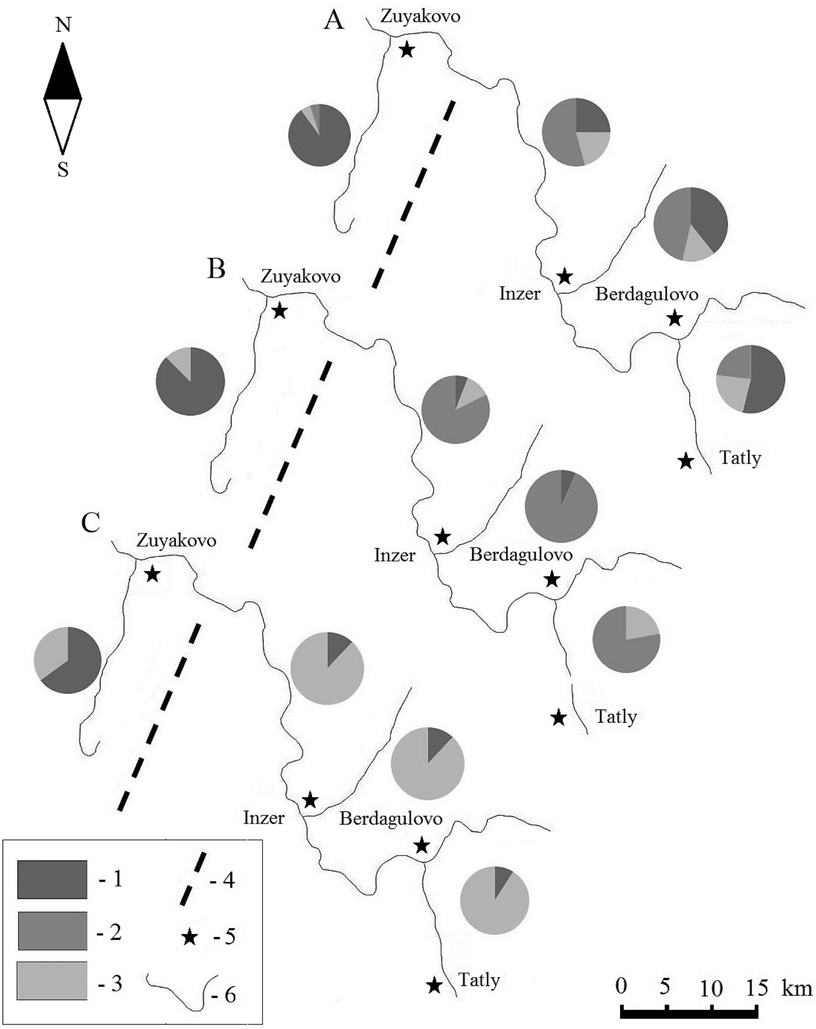

Fig. 6. Spatial distribution of Chiffchaffs with different dialects, phenotypes, and haplotypes across a hybrid zone in the South Uralsky Nature Reserve. A – phenotypes, B – vocal dialects, C – haplotypes. 1 – *abietinus* vocal dialect, phenotype or haplotype, 2 – intermediate phenotype or vocal dialect, 3 – *tristis* vocal dialect, phenotype or haplotype.

ate phenotypes vs *tristis* $P = 0.172$). It is noteworthy that the specimens with the intermediate phenotype could be divided into two approximately equal groups based on the wing formula (Fig. 4A).

When the specimens were grouped by haplotype and phenotype into four groups, as described above, the distribution of frequencies looked different (Fig. 4B). Firstly, all males from the "*abietinus* haplotype – *abietinus* phenotype" group showed a wing formula typical to European Chiffchaffs. This group differs significantly from all other groups (Fisher's exact test, two-tailed, $df = 1$, $P < 0.015$). Secondly, the Siberian-type wing formula could be found nearly twice as frequently as the European one in the "*tristis* haplotype – *tristis* phenotype" group. As for the two other variants (*5* and *6*), various types of wing formulas could be found almost equally often there. The differences between group 5, 6 and 7 were statistically insignificant (Fisher's exact test, two-tailed, $df = 1$, $P > 0.071$).

Thus in the contact zone, many Chiffchaffs with typical *abietinus* phenotype but *tristis* haplotypes, possessed metric characteristics (wing and tail length) and wing formula which are most often associated with *tristis.*

3.3. Vocalization

The most important difference between *tristis* and *abietinus* songs is the presence of elements begin-

Table 1. The results of playbacks conducted in the areas of allopatric (Kursk, Central Siberia) and sympatric (Southern Ural) distribution of Chiffchaffs.

Region	Dialect		Reaction	
	Recipient	Stimulus	yes	no
Kursk	*abietinus*	*abietinus*	7	0
Kursk	*abietinus*	*tristis*	0	7
Southern Ural	*abietinus*	*abietinus*	6	0
Southern Ural	*abietinus*	*tristis*	4	2
Southern Ural	*tristis*	*tristis*	5	0
Southern Ural	*tristis*	*abietinus*	5	0
Central Siberia	*tristis*	*tristis*	7	0
Central Siberia	*tristis*	*abietinus*	0	7

ning from ascending frequency modulation (ascending elements) in *tristis* song (Fig. 1). We calculated a vocal index (VCI) for the sympatric and allopatric populations (Fig. 5). The variability between songs in zones of contact was high. In the Southern Urals males with zero and nonzero VCI were recorded (Fig. 5 A, D). The vast majority of males with the typical European song (Fig. 5A) were identified in the westernmost part of the sympatric zone in the South Uralsky reserve and could be found throughout this territory, although their numbers rapidly decreased as one moved eastwards (Fig. 6B). Maximum VCI values (0.55–0.60) were predominantly registered in the easternmost mixed population in the Southern Urals region to the east of the Ural ridge (Miass, the Ilmensky reserve) (Fig. 5D) and in the allopatric *tristis* populations in Central Siberia (the middle reaches of the Yenisei River and the Eastern Sayan) (Fig. 5C). It is noteworthy that the frequency distributions of VCI in the population from the Ilmensky reserve (Fig. 5D) and in the population from Central Siberia (Fig. 5C) looks very similar in spite of their significant distance from one another.

Compared to the allopatric populations of *tristis*, the sympatric populations of the Pinezhsky and South Uralsky reserves showed a much broader range of non-zero VCI values. There were three clearly different groups within these populations: 1) the group of VCI = 0, corresponding to the European dialect; 2) the group of intermediate VCI values (0 < VCI ≤ 0.35); and 3) the group of VCI values corresponding to the Siberian dialect (0.35 < VCI ≤ 0.60). In the South Uralsky reserve, the boundary between the latter two groups was clearly defined while in the Pinezhsky reserve, this boundary was much less certain.

Another distinction between these two mixed populations was that in the South Uralsky reserve the Siberian song with highest VCI values (0.45–0.60) was presented by many males while in the Pinezhsky reserve such songs were extremely rare. It should be noted that males with the minimal non-zero VCI values were fairly rare in both mixed populations. Not a single male with a VCI < 0.15 was found and only 10 out of 65 males had VCI < 0.3.

3.4. Playback tests

All 11 males from the South Uralsky reserve vigorously responded to the conspecific song. Four out of 6 males with *abietinu*s song responded also to *tristis* song. All 5 males with *tristis* song responded to *abietinus* song; however, all 7 *tristis* males from the Siberian allopatric population did not show any response to the European song and all 7 *abietinus* males from the Kursk allopatric population did not respond to the Siberian song (Table 1).

3.5. Spatial structure of the contact zone in the Southern Urals

In the South Uralsky reserve, we observed clear changes in the distribution of different phenotypes, dialects and haplotypes from the west to the

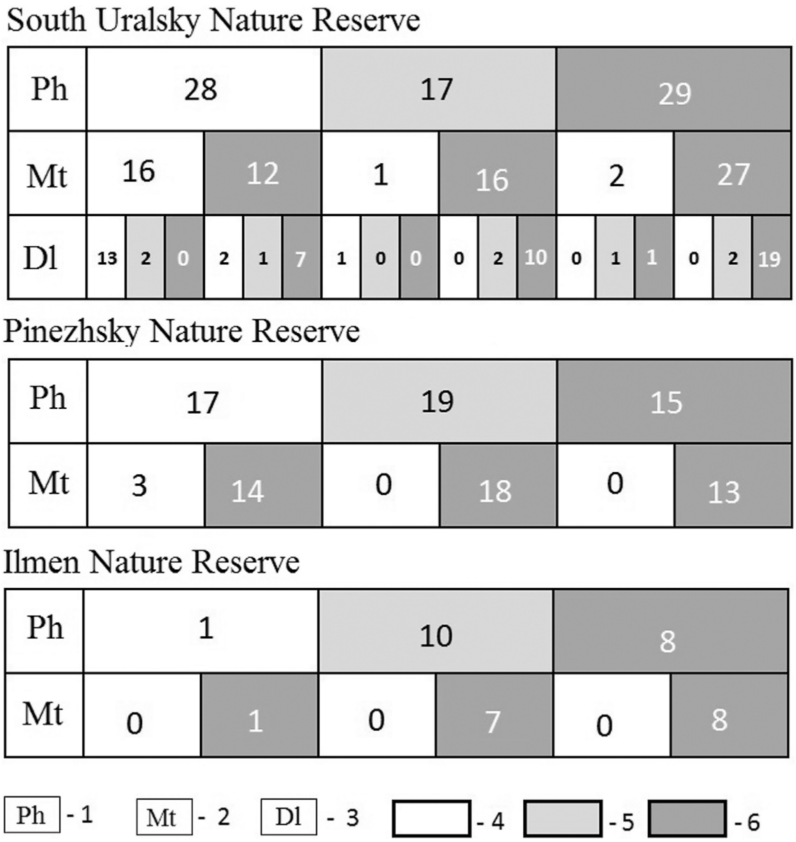

Fig. 7. The relationships between different phenotypes, haplotypes and vocal dialects in Chiffchaff males from the South Uralsky, Pinezhsky and Ilmensky Reserves. 1 – phenotype, 2 – haplotype, 3 – vocal dialect, 4 – *abietinus* phenotype, haplotype or vocal dialect, 5 – intermediate phenotype or vocal dialect, 6 – *tristis* phenotype, haplotype or vocal dialect.

east (Fig. 6). In this reserve, the boundary between the vocal dialects was most pronounced and coincided with the low mountain ridge Zilmerdak. The mean of VCIs increased at a distance of about 50 km from Zuyakovo in the Northwest to Tatly in the Southeast. European dialect clearly dominated in the populations found west of Zilmerdak ridge, but it was quickly replaced with Siberian dialect after crossing the ridge (Fig. 6B). Further east, the Siberian dialect dominated across all studied territory, including on the Ilmensky reserve, where all the males had typical Siberian dialect. The allocation of haplotypes closely resembled that of the dialects (Fig. 6C). To the west from Zilmerdak ridge, *abietinus* was the predominant haplotype, changing to *tristis* to the east of the ridge. Two Siberian haplotypes dominated in the southeast subpopulations.

The spatial separation of the phenotypes was less pronounced but it was in agreement with the border determined by dialect distribution. In the vicinity of Zuyakovo to the west of Zilmerdak ridge 90% of Chiffchaffs had an *abietinus* phenotype while on the Inzer river this value dropped to 25%. However further east the percentage of *abietinus* phenotypes rose once again making up almost half the population (Fig. 6A). Nevertheless in the Ilmensky nature reserve Chiffchaffs with an *abietinus* phenotype were extremely rare (5.3%) even though the individuals with intermediate phenotypes were still very common (52.6%).

It should be noted that in the easternmost parts of the Southern Urals contact zone all individuals with the European and intermediate phenotypes possessed the *tristis* haplotype. The European haplotypes were dominant in the vicinity of Zuyakovo (65%). However on the Inzer their proportions dropped to 12% and further east within the South Urals reserve their proportions remained at approximately the same low level. No single *abietinus* haplotype was found to the north-east of the Ural ridge in the Ilmensky reserve while more than half of the local specimens had an intermediate phenotype (52.6%).

4. Discussion

4.1. Evidences of hybridization

In our study, every recorded and captured Chiffchaff male has been classified according to a number of characters including plumage coloration (phenotype), wing and tail length, wing formula, dialect and haplotype. The key question is how do these characters match in different individuals? The results of the matching are summarized in Fig 7. For example out of 28 males with *abietinus* phenotype captured in the South Uralsky population 16 males had *abietinus* haplotype and 12 males – *tristis* haplotype. Out of the 16 males with *abietinus* phenotype and haplotype 13 males presented typical *abietinus* dialect and 2 males – a mixed dialect. There were no males with *tristis* dialect in this sample and one male has not been tape recorded.

The data clearly demonstrates the substantial number of mismatches between phenotype and haplotype in individual birds. In addition the mismatches were strongly asymmetrical. Indeed out of the 46 individuals with typical *abietinus* phenotypes 27 (58.7%) were found to have *tristis* haplotypes. In contrast out of 50 males that were classified as *tristis* upon capture, only 2 had *abietinus* haplotypes (4.0%). Among the 42 males of the intermediate phenotype, only one (2.4%) had *abietinus* haplotype. Thus, 29 out of 96 (30.2%) Chiffchaffs with typical *abietinus* or *tristis* phenotypes showed a mismatch between the phenotype and the haplotype. Most of the individuals with intermediate phenotype had *tristis* haplotypes (41 out of 42 males; 97.6%).

In our opinion the results indicate hybridization between *abietinus* and *tristis* in the mixed populations.

Our data indicates that in the contact zone many Chiffchaffs with typical *abietinus* plumage coloration but *tristis* haplotypes possess metric characteristics (wing and tail length) and wing formula which are most often associated with *tristis*. Thus we can conclude that the European phenotype (*abietinus* plumage coloration) shows an impressive stability in spite of the foreign (Siberian) mtDNA introgression.

In the hybrid Chiffchaff population in the Pyrenees (*Ph. collybita*, *Ph. ibericus*) a clear difference in the introgression between mitochondrial and nuclear DNA was found (Bensch *et al.* 2002). The authors attribute this result to the male-based gene flow and Haldane's rule (Bensch *et al*. 2002). A high mtDNA introgression level in the *abietinus-tristis* population (30.2%) in the South Uralsky reserve suggests that female hybrids probably have high fertility/viability. The usage of nuclear DNA markers confirmed an even greater introgression level (Shipilina *et al.* 2017).

4.2. Genetics and song

Our results indicate that in the sympatric zone (but not in the allopatric ones) males of European and Siberian Chiffchaffs responded to each other's songs and treat them as a relevant stimulus for response. In zones of contact between closely related avian species their mixed singing may result from hybridization or heterospecific song learning (Thielke & Linsenmair 1963, Helb *et al*. 1985, Vokurkov *et al*. 2013).

We have demonstrated a strong correspondence between haplotypes and dialects (Fig. 7). In the population of the South Uralsky reserve only two out of 43 males (4.6%) with *tristis* haplotypes performed *abietinus* songs, 5 males had mixed songs, while the rest sang songs matching the haplotype. Out of 18 males with *abietinus* haplotypes, only four (22.2%) showed mixed or *tristis* song. Thus, only six (9.8%) males out of 61 individuals with known haplotypes and dialects performed mismatched songs. The proportion of individuals with a song/haplotype mismatch was almost three times smaller than the proportion with a phenotype/haplotype mismatch (30.2%). The link between the dialect and haplotype seems to be even stronger in specimen with matching haplotype and phenotype. Out of 15 males with *abietinus* phenotype and haplotype and 21 males with the *tristis* phenotype and haplotype, not a single one performed the mismatching song and only 2 males were mixed singers.

Our data suggest that song dialects in Chiffchaffs are determined primarily on the genetic level of mitochondrial DNA. These data are also confirmed by our study of the nuclear DNA (Shipilina *et al.* 2017).

Also noteworthy in the hybrid zone between two other Chiffchaff taxa (*Ph. collybita*, *Ph. ibericus*) in the western Pyrenees only 5.3% (5 out of 94) of males demonstrated a mismatch between their dialect and haplotype (Helbig *et al*. 2001) and mixed singers were intermediate in microsatellite allele frequencies between *collybita* and *ibericus* (Bensch *et al*. 2002).

4.3. Spatial distribution of *abietinus-tristis* and geographical limits of the contact zone

In the Southern Urals the Zilmerdak ridge poses a clear border to the distribution of the two Chiffchaff taxa (Fig. 6). This is a relatively low ridge (about 900 m above sea level) completely covered with forest suitable for the nesting of Chiffchaff. But it is important that this ridge serves as a border between two forest types. At the western slope of the ridge deciduous (broad-leaved) forests are more common, while at the eastern slope mixed pine-leaf and spruce-fir forests predominate. Although both Chiffchaff taxa breed in a wide range of habitats there are obvious differences in their habitat choices. Siberian Chiffchaffs prefer dense coniferous "taiga" forests and usually do not inhabit pure deciduous forests, which are favourable for the European Chiffchaff. It seems that the ecological boundary limits a further westward expansion of *tristis* in this part of the contact zone.

We did not find any clear patterns in the spatial distribution of phenotypes, dialects or haplotypes in the Pinezhsky reserve in the north-eastern part of the contact zone (Arkhangelsk region). In this part of the contact zone exclusively secondary spruce forests with significant contribution of birch are uniformly distributed without any borders or ecological barriers. One important feature of this territory – namely its marked geographical isolation – must be taken into account. From the west and south-west the plateau is bounded by the White Sea. In the south it is delimited by a broad, densely populated by humans and almost treeless valley in the lower reaches of the Northern Dvina river. Such a position probably makes it difficult for *abietinus* to enter the territory from the west and south-west, whereas no natural or anthropogenic barriers occur for *tristis*, penetrating from the east. Perhaps as a result of the partial isolation the proportion of *abietinus* haplotypes is surprisingly low in the Pinezhsky reserve although the birds with *abietinus* phenotype occur here in a substantial number (Fig. 2).

In the central part of the sympatry zone located in the Komi Republic, *tristis* usually outnumbered *abietinus* although the abundance of Chiffchaffs in this area is subjected to yearly fluctuations (Selivanova *et al*. 2014). The fluctuations were especially clear for *abietinus* and in some years this taxa was completely absent in the area during the breeding season. According to our data the northern and north-eastern parts of the Komi Republic were inhabited exclusively by *tristis*, whereas in the south and south-eastern regions the population was mixed. The hybridization zone was located within the southern and south-eastern regions, in basins of the Vytchegda and Upper Pechora rivers (Fig.2) (for more details see Selivanova *et al.* 2014). Further west, Chiffchaff with mixed singing and intermediate phenotypes were found in the Kirov and Perm regions (Ushkov 1927, Vorontsov 1949, Sotnikov 2006).

Thus the hybridization zone between Common and Siberian Chiffchaffs occupies a large area. The zone extends from Arkhangelsk in the north-west through the southern and south-eastern regions of the Komi Republic, the Kirov and the Perm regions to the south-east in Ufa and Chelyabinsk (Fig.2). The eastern boundary of the hybridization zone has not yet been clarified. It was supposed to coincide with the Ob River (Koblik *et al.* 2006); however, there is currently no enough evidence to accept or reject this hypothesis.

Some researchers use the name *tristis* only for specimens from Eastern Siberia (East of the Yenisei river) while the specimens from the Urals to the Yenisei are assigned to "*fulvescens*" (Stepanyan, 1990, Glutz von Blotzheim & Bauer 1991, Cramp 1992). The latter was first described by Severtzov (1873) based on approximately 100 specimens collected during the autumn migration in "Turkestan" – the vast area from the Ural river to Baikal lake. Therefore, it is possible to assume that at least some individuals in this sample could be of hybrid origin from the contact zone between *abietinus* and *tristis.* Another reason that give rise to doubts about the validity of *fulvescens* is that populations composed solely of this phenotype was not found so far. All populations containing *fulvescens* phenotypes also include typical *tristis* phenotypes in a substantial number (Ticehurst 1938, Koblik *et al.* 2006, Marova *et al.* 2013). Our data suggest that the individuals described under the name "*fulvescens*" most probably are the result of hybridization between *abietinus* and *tristis*. In the practice of avian taxonomy, there are several cases when a hybrid population received special names. For example the subspecies *ludlowi* in the complex of Greenish Warbler is recognized to be the result of hybridization between two contacting subspecies, *viridanus* and *trochiloides* (Ticehurst 1938, Irwin *et al.* 2001). Italian sparrow *Passer italiae* is an another well known example (Hermansen *et al.* 2011). Nevertheless, the degree of morphological variability in the populations of "*fulvescens*" appears to be unacceptably broad to be qualified as a distinct subspecies.

4.4. Northern Chiffchaffs in Europe

It is well known that *abietinus* migrates to Africa while *tristis* winters in Pakistan, India and Nepal (Ticehurst 1938). What direction Chiffchaffs choose to migrate from the hybrid zone in Ural, Komi and Arkhangelsk remains unclear. Almost nothing is known about migration routes and wintering areas of hybrids and backcrosses.

From the beginning of the 19th century many Siberian Chiffchaffs were observed in Europe during the winter (Hartert *et al.* 1912). At least from the middle of the 19th century so called "Northern Chiffchaffs" with transitive characteristics were common in Britain. It was suggested that these mixed *tristis* and *abietinus* Chiffchaffs are drawn from a zone of contact between eastern and western birds in some part of north-east Europe or western Asia (Williamson 1954). Presently "*tristis*-like" Chiffchaffs, and some intermediate birds, are often observed in autumn and winter in many European countries (Boano & Bocca 1981, Noeske & Aumüller 2005, Noeske & Dean 2006, Dean *et al.* 2010). The geographical origin of these birds is unknown.

de Knijff *et al.* (2012) recently published a paper relevant to this subject. Twenty three Chiffchaffs which were caught in autumn at five Dutch ringing stations and identified by ringers as typical *abietinus* later were genetically analyzed and found to have the *tristis* haplotype (de Knijff *et al.* 2012). These birds were re-classified by the authors as Siberian Chiffchaffs based on mtDNA. Our research suggests that within the Chiffchaff breeding range specimens with *abietinus* phenotype but *tristis* haplotype are found exclusively in the contact zone and not in any allopatric populations. We assume that all specimen with mismatching phenotype and mtDNA – including those caught in the Netherlands – are of hybrid origin. It could mean that at least some hybrid Chiffchaffs from the *abietinus-tristis* contact zone migrate in autumn in the western direction (Marova & Shipilina 2015).

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Tiltaltin ja idäntiltaltin hybridisaatiosta

Lajien hybridisaation tutkiminen on keskeinen osa evoluutiobiologiaa ja taksonomiaa. Tiltaltti (*Phylloscopus collybita abietinus*) ja idäntiltaltti (*Ph. c. tristis*) elävät rinnakkain Vienanmerestä Uralvuorten eteläpuolelle ulottuvalla alueella. Allopatrisilla alueilla, missä vain toinen alalajeista elää, näiden alalajien laulu ja ulkoinen olemus (fenotyyppi) eroavat selkeästi. Sympatrisilla alueilla, missä molemmat alalajit esiintyvät, havaitaan yksilöitä joiden ääntely ja ulkoiset tuntomerkit ovat alalajien välimuotoa, mutta alalajien hybridisaatiosta tiedetään vain vähän.

Tässä artikkelissa tutkimme tiltaltin ja idäntiltaltin yhteiseloa sekapopulaatiossa Arkhangelskin

alueella, Komin tasavallassa ja Uralvuorten eteläosissa. Allopatrisia populaatioita tutkimme Moskovan ja Kostroman alueilla sekä Siperiassa (Jenisei-joki ja Sayan vuoristot). Sympatrisissa sekapopulaatioissa yksilöiden fenotyyppi ja haplotyyppi eivät sopineet yhteen 30.2 %:ssa yksilöistä, kun taas allopatrisissa populaatioissa fenotyyppi/haplotyyppi-yhteensopimattomuutta ei löytynyt. 58.7 % tyypillisistä *abietinus*-fenotyypeistä kantoi *tristis*-haplotyyppiä ja vain 4.0 % kantoi päinvastaista haplotyyppi/fenotyyppi-yhdistelmää.

Suurin osa välimuoto-fenotyypin yksilöistä (97.6 %) kantoi *tristis*-haplotyyppiä. Vain 9.8 % yksilöistä, joiden haplotyyppi tunnettiin, lauloi toisen haplotyypin laulua. Sympatrisessa populaatiossa 82 % koiraista vastasi toisen alalajin lauluun, mutta allopatrisessa populaatiossa tätä ei havaittu. Tuloksemme viittaavat *abietinus*- ja *tristis*-alalajien hybridisaatioon sekapopulaatioissa.

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