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*Published in:*  
Journal of Avian Biology

*DOI:*  
[10.1034/j.1600-048X.2000.1310414.x](https://doi.org/10.1034/j.1600-048X.2000.1310414.x)

2000

[Link to publication](#)

*Citation for published version (APA):*  
Hansson, M., Bensch, S., & Brännström, O. (2000). Range expansion and the possibility of an emerging contact zone between two subspecies of Chiffchaff *Phylloscopus collybita* ssp. *Journal of Avian Biology*, 31(4), 548-558. <https://doi.org/10.1034/j.1600-048X.2000.1310414.x>

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## Range expansion and the possibility of an emerging contact zone between two subspecies of Chiffchaff *Phylloscopus collybita* ssp.

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Hansson, M. C., Bensch, S. and Brännström, O. 2000. Range expansion and the possibility of an emerging contact zone between two subspecies of Chiffchaff *Phylloscopus collybita* ssp. – J. Avian Biol. 31: 548–558.

The Chiffchaff *Phylloscopus collybita* is represented in Sweden by two different subspecies; the northern well-established *abietinus* and the southern recently established *collybita* which has expanded its range northward during the past two decades. At present, an area approximately 500 km wide separates the two subspecies. In order to document differences between the northern and southern populations we compared morphology, vocalisation, habitat choice, and neutral genetic variation in mitochondrial (mt) DNA and at four microsatellite loci of 30 male Chiffchaffs from each subspecies. Our results show significant differences in several morphological traits and in song. Playback experiments revealed a significant difference in aggressive behaviour depending on which population-specific song that was played to the birds. Mitochondrial DNA was geographically structured with ~90% of the birds carrying a mt haplotype matching their sample population. No allelic differences at the microsatellite loci were found between populations. Our data demonstrate a substantial differentiation between the northern and southern populations despite gene flow, clearly separating them into the subspecies *abietinus* and *collybita*.

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Ever since the end of the last glaciation in northern Europe, approximately 10000 years ago, the Scandinavian peninsula has been recolonised by thousands of species, animals as well as plants. One important path of colonisation was from the south, over a former land bridge connecting it with what is now Denmark (Ekman 1922). The other main colonisation path was from the northeast, through Finland and north of the Gulf of Botnia, with a subsequent southward spread through Scandinavia. Traces of these two colonisation routes might be detected even today as secondary contact zones between different subspecies across central Sweden (Ekman 1922) or as mitochondrial haplotype lineages (Jaarola and Tegelström 1995, Taberlet et al. 1998). For example, the Willow Warbler *Phylloscopus trochilus* exhibits a contact zone centred around 62°N where morphology and migratory direction differ between populations (Hedenström and Pettersson 1987, Bensch et al. 1999). In an unknown number of cases the two forms that once recolonised Scandinavia from the

south and north, respectively, may have merged completely, leaving no distinct contact zone behind.

Until about 30 years ago the Chiffchaff *Phylloscopus collybita* was only represented in Sweden by the subspecies *abietinus* which is distributed from northern Lapland south to 60°N. This suggests a northern route of post-glacial colonisation. From about 1970 the nominate subspecies *collybita* has expanded its range from Denmark and is presently a common breeding bird in southern Sweden (Staab and Fransson 1991, Hagemeyer and Blair 1997). An area approximately 500 km wide is not yet inhabited by Chiffchaffs; however, considering how quickly *collybita* has invaded the southern parts of Sweden, it may not be long until it will spread north to the southern range limit of *abietinus*.

The two subspecies differ slightly in plumage and body measurements; *collybita* is on average smaller and greener than the larger and greyer *abietinus* (Cramp 1992), however single individuals are often impossible to separate even in the hand (Svensson 1992). The

genetic differentiation between *collybita* and *abietinus* has earlier been estimated to be 1.0% at the mitochondrial cytochrome b gene (Helbig et al. 1996). Of 26 birds sampled in Germany (within the range of *collybita*) and typed for mitochondrial DNA haplotype, one (3.8%) carried an *abietinus* mt haplotype. Hence, because *abietinus* haplotypes already exist well within the range of *collybita*, gene flow and mixing have to some extent already occurred, possibly across the contact zone in NE Poland (Cramp 1992).

Several species of bird and mammal exhibit hybrid zones running latitudinally across Scandinavia, probably reflecting post-glacial colonisation routes from both the south and the north (Taberlet et al. 1998). The gap in distribution presently shown by Swedish Chiffchaffs suggests that this scenario is going to be repeated in yet another species. This would allow us, for the first time, to study how morphology, genetics and behaviour change over time and space as two differentiated populations gradually merge into each other. It is therefore important to document the present conditions because, as the distance between the populations shrinks, increasing gene flow might start affecting the characters of the two subspecies.

The aim of this study was to establish the current level of differentiation between the South Swedish population of *collybita* and the North Swedish population of *abietinus*. We captured male Chiffchaffs from both populations, and compared habitat choice, morphology and neutral genetic variation in mitochondrial (mt) DNA and at four microsatellite loci. We recorded the song from males and used sonagrams to look for diagnostic differences between males from the two regions. A playback experiment with song from both regions tested for differences in aggressive behaviours depending on which song the males were exposed to.

## Materials and methods

### The species

The Chiffchaff is an insectivorous passerine, mainly breeding in forests and woodlands although showing extensive geographical variation in habitat requirements (Hagemeijer and Blair 1997, Clement and Helbig 1998). Subspecies *abietinus* of northern Sweden breeds preferably in old spruce (*Picea*) forests while the southern continental subspecies *collybita* mainly breeds in deciduous forests, often with rich herb and bush layers such as found close to marshland areas. Ulfstrand and Högstedt (1976) estimated the Swedish Chiffchaff population at some 200 000 pairs.

The subspecies *abietinus* has been common in northern Sweden for at least a couple of hundred years (Nilsson 1858) and possibly much longer. However, it has never been clear why southern Scandinavia has

remained unpopulated for such a long time (Voous 1960). To quote the Swedish ornithologist Erik Rosenberg (translated from Swedish) "One may ask why the Chiffchaff does not occur in southern Sweden, because it is such a common species in Britain and the other parts of Western Europe. The explanation is that we are dealing with two subspecies, one from the east and one from the west. It is the former that has invaded Sweden from the North and has settled in its habitat, the boreal coniferous forest. Southern Sweden seems more suitable for the western subspecies. In the future, this subspecies may colonise this vacant space" (Rosenberg 1972). It did not take more than a decade until Rosenberg's prediction was confirmed. During the late 1970s *collybita* was spreading north from Denmark into southern Sweden. Today the subspecies is a relatively common breeder in all of southern Sweden, reaching approximately 57°N (Hagemeijer and Blair 1997).

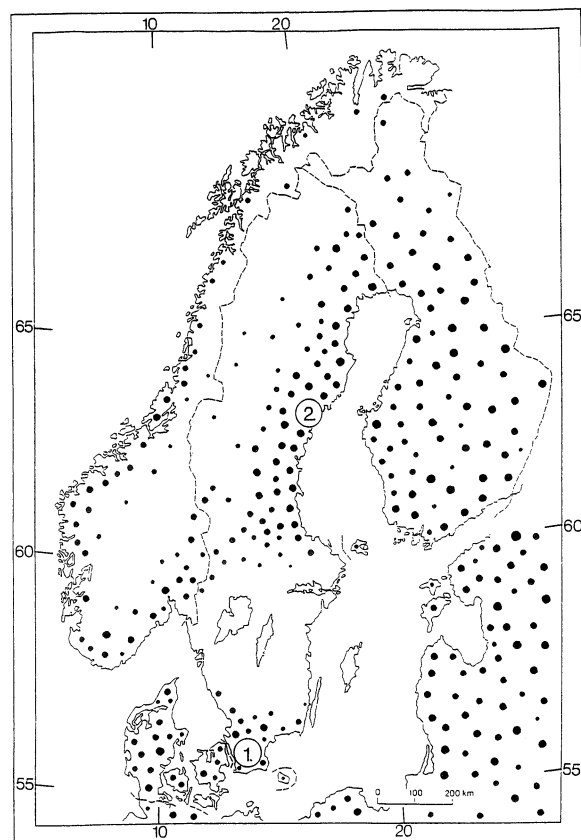


Fig. 1. The present distribution of the Chiffchaff *Phylloscopus collybita* in northern Europe, according to Hagemeijer and Blair (1997). (1) marks the study site of the southern population (*Ph. c. collybita*) in Sweden and (2) that of the northern population (*Ph. c. abietinus*).

## The study areas

In Skåne, southern Sweden (Fig. 1) 30 males were captured (details below) between 8 and 29 May 1998. Most of them were caught in the woodlands bordering Lake Krankesjön and in Häckeberga and Torup. In Ångermanland, northern Sweden (Fig. 1) 30 males were captured and studied between 4 and 15 June in the same year. The birds were caught in a coastal woodland area located 20–50 km south of Örnsköldsvik.

In order not to assume that all birds of the southern population of Sweden were *collybita* and all of the northern ones were *abietinus*, we refer to the two different samples as *southern* and *northern*, respectively.

## Morphology and plumage

### Measurements

We collected the following measurements according to Svensson (1992) if not otherwise stated: length of the left wing (to the nearest 0.5 mm) according to method 3, length of primaries (numbered one to ten from distal to proximal wing) relative to the longest one, tail length to the nearest mm (by inserting a small ruler between the outermost and second outermost tail feather), right and left tarsus (to the nearest 0.1 mm) according to Alatalo et al. (1984), bill length (to the distal edge of the nostril), bill width, bill height and the total length of bill and head, all to the nearest 0.1 mm. Pointedness of the wing (WP) was calculated according to the formula in Salomon et al. (1997):

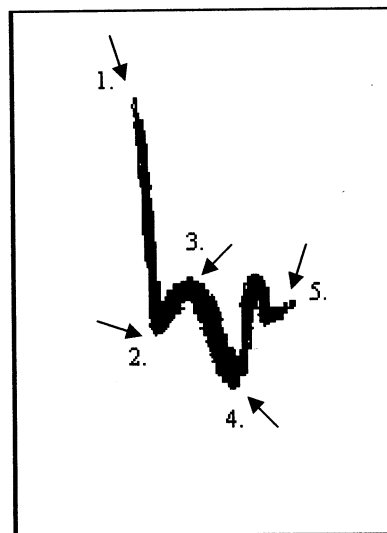
$$WP = [(P3 - P10)/WL] \times 100$$

where P3 is the difference in length between the 3rd primary and total wing and P10 is the difference between the 10th primary and total wing. A high value corresponds to a more pointed, and a lower value to a more rounded wing. Body mass was measured to the nearest 0.1 g on a Pesola spring balance (50 g). All birds were measured by both M.C.H. and O.B., and if the measurements differed, we used the mean in the following analyses. To test for significant differences between the two populations, Student's t-tests were performed on all of the variables.

## Bioacoustics and song recordings

The song of the Chiffchaff is characterised by a typical "descent-and-knee" note (Fig. 2). Every song note is initiated on a high pitch but drops rapidly. Often, the song is preceded by a low churring sound before the long and rhythmic alternation between the distinct chiffs and chaffs begins.

## Frequency (kHz)



## Time (s)

Fig. 2. A sonographic illustration of a syllable from a Chiffchaff song. Arrows and numbers indicate where the five measurements for the song analyses were taken. The characteristic knee begins at position 2.

Songs from 10 males in Skåne and 10 males in Ångermanland were recorded with a Telinga Pro 3 parabolic microphone, 58 cm in diameter, connected to a Sony Walkman WM-D6C tape recorder. Every recording lasted between 2 and 5 min. The song recordings were later copied to another tape and multiplied in order to obtain 10 min of continuous song from each recorded bird for later use in the playback experiment.

To establish whether males from southern and northern Sweden responded equally strongly to song from either area, 20 southern and 20 northern birds were subjected to playback testing. Each song was used only once in each of the populations (see Kroodsmma 1989). Ten of the birds were exposed to song from a southern bird and ten were exposed to song from a northern bird in an alternating pattern. During the 10-min broadcasts, the level of aggressiveness was recorded as the number of times the male flew over the tape recorder, how closely he approached the tape recorder and whether he started singing. Before each test, we made sure that the male was in fact in his territory, by hearing him sing at a maximum distance of 25 m from us.

From each recorded individual, three groups of three syllables were randomly selected for further sonographic analyses. For every syllable, the frequency (kHz) was taken at five different time positions to characterize each male's song (Fig. 2). This included estimating the highest and lowest frequency, length of the syllable (the short "chiff" syllable and the longer "chaff" syllable) and the time interval to the next syllable.

## Genetic analyses

### Blood sampling

A blood sample of 20  $\mu$ l was taken from the brachial vein and transferred to a test tube containing 500  $\mu$ l SET-buffer (0.15 M NaCl, 0.05 M TRIS, 0.001 M EDTA). Samples were kept cool, but not frozen, for 1–30 days, and then stored at  $-80^{\circ}\text{C}$  until later analyses. Total DNA was isolated using phenol-chloroform extraction (Sambrook et al. 1989). The DNA concentration was measured on an UV spectrometer as OD at 260 nm. Samples were diluted in ddH<sub>2</sub>O to a concentration of 25 ng/ $\mu$ l.

### MtDNA

A portion of the 3' end of the cytochrome b gene of the mitochondria was amplified with the primers CytX (5'-ACCCTGAGGGCAAATATCATTTC-3') and CytY (5'-ATGATGATGAATGGGTGTTTCG-3') designed from known sequences of the two subspecies (Helbig et al. 1996) to give a PCR product of a 650 nt. After cutting with the endonuclease TaqI, these fragments would enable a separation between *abietinus* and *collybita* mtDNA using agarose gel electrophoresis, as the former is expected to be cut into three fragments (500, 128 and 22 nt) and the latter into four fragments (400, 129, 100 and 21 nt).

Polymerase chain reactions (PCR) were performed in a total volume of 25  $\mu$ l, including 50 ng of template DNA, 0.4  $\mu$ M of each primer, 0.125 mM of dNTPs, 1.5 mM MgCl<sub>2</sub> and 0.5 units of Taq DNA polymerase (Perkin Elmer). The amplifications started by 2 min of initial denaturation at  $94^{\circ}\text{C}$  and were followed by 30 cycles at  $94^{\circ}\text{C}$  for 30 s,  $55^{\circ}\text{C}$  for 30 s and  $72^{\circ}\text{C}$  for 2 min. A control blank containing no DNA was included in every run to test for contamination. Eight  $\mu$ l of the PCR product was digested with 10 U TaqI in a total volume of 20  $\mu$ l for two hours at  $65^{\circ}\text{C}$ . The cut PCR products were separated on a 2% agarose gel, and stained in ethidium bromide and scanned in a FluorImagerSI, Vistra™. The degree of population geographical substructure in mtDNA was tested using the program AMOVA (Analysis of Molecular Variance; Excoffier et al. 1992).

### Microsatellites

The 60 individuals were typed for allelic length variation at four heterologous microsatellite loci. POCC1 and POCC6 were developed for *Phylloscopus occipitalis* (Bensch et al. 1997), PHTR1 for *Phylloscopus trochilus* (Fridolfsson et al. 1997) and LS2 for *Lanius ludovicianus* (Mundy and Woodruff 1996). PCR reactions of 10  $\mu$ l included 25 ng of total genomic DNA, 0.125 mM of each nucleotide, 1.5 mM MgCl<sub>2</sub>, 0.4  $\mu$ M of forward fluoroscein-labelled primer, 0.4  $\mu$ M of reverse primer

and 0.5 units of Taq DNA polymerase. The PCR amplifications started by initially heating the samples to  $94^{\circ}\text{C}$  for 2 min followed by 28 cycles at  $94^{\circ}\text{C}$  for 30 s,  $51^{\circ}\text{C}$  (POCC1) or  $55^{\circ}\text{C}$  (PHTR1, POCC6 and LS2) for 30 s and  $72^{\circ}\text{C}$  for 1 min. The reaction was terminated by a 10-min step at  $72^{\circ}\text{C}$ . The PCR products were separated on 6% polyacrylamide gels. The allele sizes were determined by comparison with three previously identified Chiffchaff individuals, used as reference. The gels were scanned in a FluorImagerSI, Vistra™ for detection of the separated allele bands. An estimate of genetic differentiation from the microsatellite data was obtained with the software R<sub>ST</sub> Calc according to Goodman (1997).

## Results

### Habitat

At our southern study site, birds inhabited rich, deciduous forests, preferably older stands of *Betula*, *Fagus*, *Corylus*, *Ulmus*, *Quercus* and *Alnus*. No birds were found in pure beech forest, coniferous forest or in very young deciduous forest. Most of the forests were situated along marshlands or near lakes.

At our northern study site we found the birds in old coniferous forests (*Picea*, *Pinus*) at low altitudes near the coastline, often in or near marshlands. We found few or no individuals in inland areas or at higher elevations than 200 m.

### Morphological measurements

Males sampled at the northern study site were on average larger than males in southern Sweden (Table 1, Fig. 3) and the differences were most pronounced in wing length, tail length and body mass. Northern males also had significantly more pointed wings than southern males. No significant differences were found in tarsus length, bill length, bill height, bill width or bill and skull.

### Bioacoustics

The sonagrams of two different singing individuals from each of the two populations are shown in Fig. 4. Males in the southern population initiated and ended the syllables at significantly higher frequencies than males in the northern population (Table 2). There was no significant difference in the length of the time interval between syllables; however, southern birds tended to show a larger variation in this interval than did northern birds.

The playback experiment (Fig. 5) demonstrated that both southern and northern males flew more times over the tape recorder when exposed to song from their own population than when exposed to song from the other population (northern males:  $t = 2.59$ ,  $P < 0.01$ ; southern males:  $t = 4.87$ ,  $P < 0.0001$ ). There was no significant difference in how close the birds came to the tape recorder during the two different song exposures indicating that they reacted to both types of song but responded more aggressively when subjected to song from their own population. Most often, the exposed birds started to sing themselves during the playback experiment. This song was different from that of unprovoked males, being much faster and more intense.

### Genetic analyses

The analysis of the cytochrome b gene demonstrated that 26 of the 30 southern males carried a *collybita* mitochondrial haplotype while four carried an *abietinus* haplotype. Among the northern males, 27 out of a total of 30 birds carried an *abietinus* mt haplotype and three individuals a *collybita* haplotype. Hence, seven individuals carried a mt haplotype not matching the population in which they were sampled. The association between morphology and mt haplotype was less strong than that between morphology and sample population (Fig. 6). Six of the birds with a mismatch between mt haplotype and sample population showed a wing length/tail length combination that completely matched the sample population. However, the morphology of one bird was characteristic for its mitochondrial haplotype rather than its sample population. This bird, captured in the north, carried a *collybita* mt haplotype and had a short wing (60 mm) and tail (51 mm) well outside the range of those traits among males in the north.

The analysis of genetic variance of mt haplotypes between the populations resulted in a  $\text{PHI}_{\text{ST}}$  value of 0.732 ( $P < 0.001$ ) suggesting that 73% of total variance in mtDNA is due to variance among populations and 27% is due to variance within populations. For the four

analysed microsatellite loci, the allele frequencies for the southern and northern males were very similar and almost all alleles were present in both populations (Table 3). Among the southern males we found two alleles at the locus LS2, seven alleles at PHTR1, five at POCC1 and 9 at POCC6. The corresponding figures for the northern males were two alleles at LS2, five at PHTR1, seven at POCC1 and 10 at POCC6. The calculation of population structure indicated that the two groups form a near panmictic population at these loci ( $R_{\text{ST}} = -0.0064$ , NS).

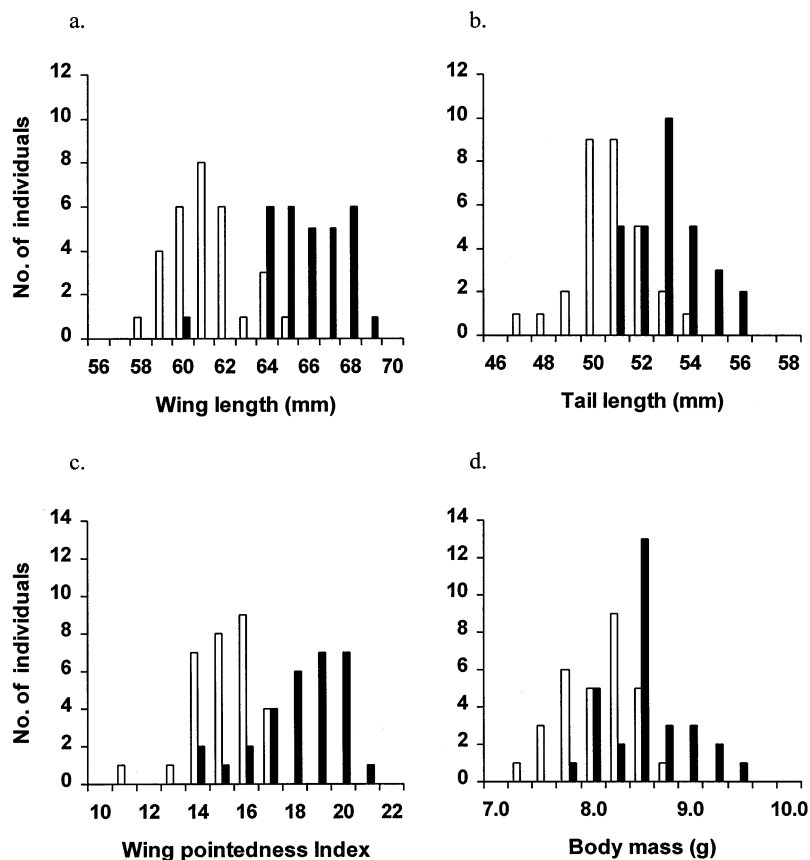
### Discussion

The recent colonisation of southern Sweden by Chiffchaffs of the southern subspecies *Ph. c. collybita* offers the opportunity to follow the formation of a contact zone, given that the northward spread of *collybita* will continue and reach the southern limit of *Ph. c. abietinus*. These two subspecies are substantially separated in several morphological and behavioural traits likely to be under selection. Hence, a contact zone will probably result in a hybrid zone. Such a zone is likely to expand in width initially until dispersal into the zone is balanced by selection against hybrids (Barton and Hewitt 1989, Barton and Gale 1993). However, even if many contact zones appear to be fixed in space, suggesting a balance between dispersal and selection against hybrids, this assumption has rarely been tested. In North America, the hybrid zone of the Blue-winged Warbler and Gold-winged Warbler (*Vermivora chrysoptera* and *V. pinus*), and that of the Hermit and Townsend Warbler complex (*Dendroica occidentalis* and *D. townsendi*), have been shown to be moving as a consequence of a competitive asymmetry between the parental species (Gill 1980, Rohwer and Wood 1998). Hence, even if *collybita* and *abietinus* will establish a contact zone of stable width in central Sweden, the northward-spreading *collybita* might push the distribution of *abietinus* further to the north.

Table 1. Mean values ( $\pm$ SD), t-values and P-values of the analysed morphological variables for each of the two populations of Chiffchaffs. Differences are tested with t-tests.

	Southern pop. (n = 30)	Northern pop. (n = 30)	t-value	P-value
Wing length (mm)	61.05 ( $\pm$ 1.64)	65.69 ( $\pm$ 1.85)	10.27	<0.0001
Wing pointedness	15.20 ( $\pm$ 1.32)	18.22 ( $\pm$ 2.81)	7.77	<0.0001
Tail length	50.49 ( $\pm$ 1.40)	52.90 ( $\pm$ 1.43)	6.58	<0.0001
Tarsus (r) length (mm)	21.93 ( $\pm$ 0.46)	21.86 ( $\pm$ 0.41)	0.57	0.29
Tarsus (l) length (mm)	21.84 ( $\pm$ 0.56)	21.88 ( $\pm$ 0.44)	0.26	0.40
Bill height (mm)	2.03 ( $\pm$ 0.07)	2.04 ( $\pm$ 0.07)	0.72	0.24
Bill length (mm)	5.92 ( $\pm$ 0.29)	5.87 ( $\pm$ 0.23)	0.84	0.20
Bill width (mm)	2.32 ( $\pm$ 0.18)	2.35 ( $\pm$ 0.17)	0.67	0.25
Bill and skull (mm)	26.56 ( $\pm$ 0.44)	26.75 ( $\pm$ 0.44)	1.65	0.05
Weight (g)	8.05 ( $\pm$ 0.38)	8.53 ( $\pm$ 0.40)	4.72	<0.0001

Fig. 3. Distributions of wing and tail length, wing pointedness and body mass in the two populations of Chiffchaff. White columns indicate the southern population (n = 30), black columns indicate the northern population (n = 30).



### Habitat and phenotypic differentiation

Presently, there are obvious differences in habitat choice between Chiffchaffs breeding in southern and northern Sweden but it is not clear whether this only reflects general habitat differences between the two regions. There is, however, vast coniferous forests which appear suitable for *abietinus* south of its present range limit at 60°N, suggesting that habitat preferences alone cannot explain why *abietinus* has not reached southern Sweden. Whether the two subspecies will form a hybrid zone depends on to what extent *collybita* individuals will remain in deciduous forest even at 60°N or whether they will also colonise coniferous forest. Differential habitat choice by the two subspecies would substantially limit hybridisation even if they establish a geographical contact zone. Our results suggest, however, that pure *collybita* males may establish territories in the coniferous forests when they come into the range of *abietinus* as exemplified by the male with both *collybita* morphology and mt haplotype encountered at the northern study site.

At the border between SW France and NE Spain, in the western Pyrenees, the subspecies *Ph. c. collybita* forms a contact zone with another subspecies, *Ph. c. brehmii* (Salomon et al. 1997). Despite no clear differ-

ence in habitat choice between these two subspecies, this contact zone is less than 50 km across (Salomon et al. 1997). No information seems to be available about dispersal distances in the Chiffchaff; however, in general migratory passerines show mean natal dispersal distances >20 km (Paradis et al. 1998). Hence, a balance between selection and dispersal likely maintains narrow contact zones such as the one between Pyrenean Chiffchaffs (Salomon et al. 1997).

In many species of birds, wing length increases with latitude probably reflecting longer migratory distances of northern populations (Leisler and Winkler 1986). Long-distance migrants have more pointed wings than short-distance migrants (Marchetti et al. 1995, Mönkkönen 1995), probably because high wing pointedness results in a high aspect ratio which reduces the cost of transport by allowing faster and more energy-efficient flight (Rayner 1988, Norberg 1989). We found wings to be significantly longer and more pointed in northern than southern male Chiffchaffs, suggesting that the former's wintering grounds are farther away from the breeding grounds. However, very little information exists on the wintering areas of *collybita* and *abietinus* and the few ringing recoveries suggest that both subspecies winter in the Mediterranean area (Zink 1987). Even though the two subspecies may have the

same wintering area, *abietinus* breeds further to the north, and will on average have a migratory route several thousand km longer than *collybita* (Gaston 1974).

Assuming that a more pointed wing is required for the longer migration, could this constrain the northward spread of the relatively blunt-winged *collybita*? Kirkpatrick and Barton (1997) recently described theoretically how gene flow from the centre of a range to populations at the range limit can constrain formation of local adaptations and hence limit further range expansion. Under certain conditions there will be a balance between gene flow and selection for local adaptations that will limit the species' range. That wing length of *collybita* is relatively constant across its distribution range (Fig. 7) despite northern populations of *collybita* (like those in southern Sweden) having substantially longer migration distances than southern populations (south France) supports a high level of homogenising gene flow.

On average *abietinus* is less yellow and more greyish than nominate *collybita* (Cramp 1992). The reason for this could be their different habitat use; *abietinus* breeds mainly in coniferous forest whereas *collybita* breeds mainly in deciduous forest, the latter having more rich-green light conditions. Supporting the habitat hypothesis for colour differences is the fact that a similar differentiation in colour is shown by the Willow Warbler of the two subspecies *Ph. t. trochilus* and *Ph. t. acredula* with a contact zone across Scandinavia just north of the present southern range limit of *abietinus* (Cramp 1992, Bensch et al. 1999). A divergence in plumage, as well as in song, may however be due to sexual selection (Price 1998) where females prefer males with the characteristics of their own subspecies. A preference in *abietinus* females for a more greyish look may prevent the more yellowish males of *collybita* from mating and hence limit the migration of *collybita* genes into the *abietinus* population of northern Sweden.

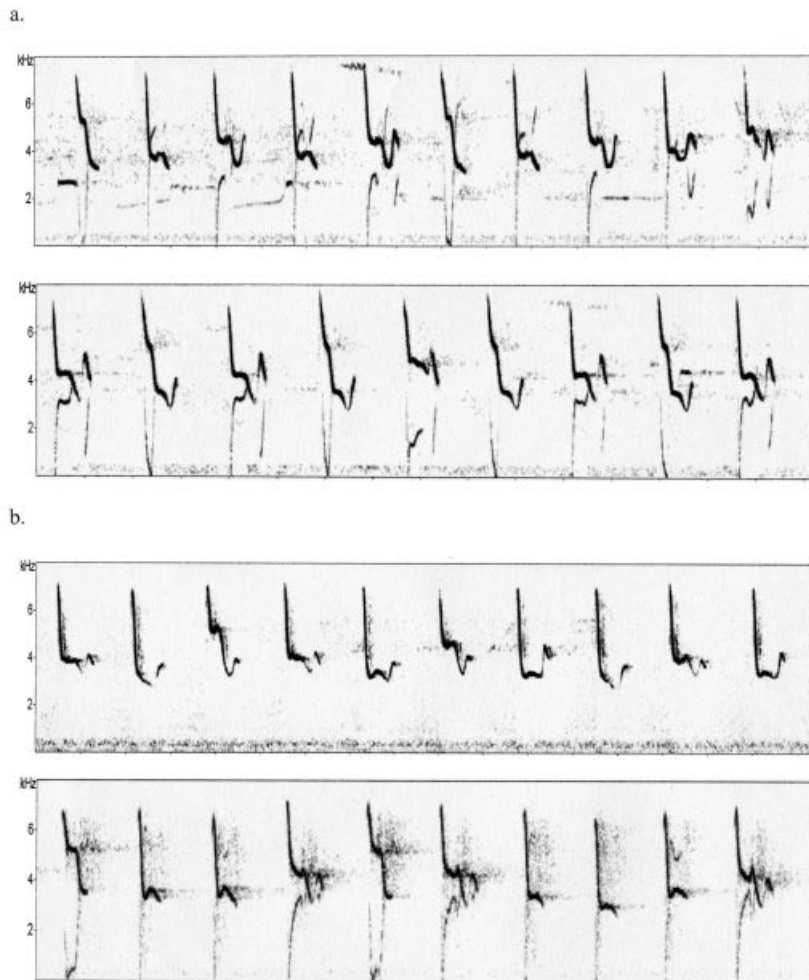


Fig. 4. Sonograms of songs from two different individuals in (a) the southern population and (b) the northern population.



Table 2. Mean values ( $\pm$  S.D.), t-values and P-values of the analysed variables from the song sonograms of ten southern birds and ten northern birds. Positions for frequency measurements are illustrated in Fig. 2.

Variable	Southern pop. (n = 10)	Northern pop. (n = 10)	t-value	P-value
Time between syllables (s)	0.326 (0.017)	0.329 (0.013)	0.43	0.34
Frequency (kHz), pos. 1	6.96 (0.19)	6.55 (0.18)	4.97	<0.0001
Frequency (kHz), pos. 2	4.57 (0.24)	4.06 (0.33)	3.95	<0.0005
Frequency (kHz), pos. 3	4.68 (0.30)	4.14 (0.36)	3.67	<0.001
Lowest frequency (kHz), pos. 4	3.51 (0.23)	3.34 (0.26)	1.49	0.08
End frequency (kHz), pos. 5	3.92 (0.38)	3.65 (0.19)	1.98	<0.05
Length of the "chiff" note (s)	0.088 (0.017)	0.086 (0.013)	0.59	0.28
Length of the "chaff" note (s)	0.146 (0.015)	0.143 (0.014)	0.90	0.19

Some features of the song, such as its range in frequency, are known to be adapted to the physical characteristics of the environment, suggesting a role for habitat in song divergence (Morton 1975). The fact that we found major habitat differences along with the acoustic differences may be an indication of such a relationship. An alternative hypothesis is that the differences in song result from the differences in size between northern and southern males (see Appleby and Redpath 1997). Our playback experiment showed that the males reacted significantly more strongly to the song of males from their own population than to song from the other population (Fig. 5). Such a differential response to song from their own compared to song from the other population could decrease gene flow if females also respond in the same way as males; however, an experiment with females would be extremely hard to carry out in the field.

### Genetic differentiation

The results of the analysis of the mitochondrial cytochrome b gene revealed that most of the birds carried the population-specific sequences. In the northern population 90% had the *abietinus* haplotype and in the southern population 87% had the *collybita* haplotype. This mix is only marginally larger than that reported from central Europe where 96% had the *collybita* haplotype (Helbig et al. 1996). The two haplotypes differ by approximately 1% sequence divergence (Helbig et al. 1996). This corresponds to a separate evolutionary history of several hundred thousand years, suggesting divergence in separate refugia during the Pleistocene glaciations (Awise and Walker 1998). However, we found no evidence of differentiation between the southern and northern populations at the four microsatellite loci. The contrasting results for the mtDNA and microsatellites might be explained by one or several mechanisms, which we presently cannot separate. First, if more males than females disperse between populations this would result in a higher nuclear than mt gene flow. In birds, however, males generally seem to be the philopatric sex (Greenwood 1980), although there are several exceptions (e.g. Bensch and Hasselquist 1991)

and no information exists on sex-dependent dispersal distances in Chiffchaffs. Second, according to Haldane's rule (Wu and Davis 1993) a higher nuclear than mt gene flow may also suggest reduced fitness of hybrid females, because in birds the female is the heterogametic sex. This has been suggested as a mechanism for limited mtDNA gene flow between Pied Flycatcher *Ficedula hypoleuca* and Collared Flycatcher *F. albicollis* (Tegelström and Gelter 1990).

Size traits such as wing and tail length and body mass tend to have relatively high levels of heritability ( $h^2 = 0.5$ ) in populations of wild passerines (Gustafsson 1986). The fact that the birds with a "mismatched" mitochondrial haplotype morphologically resembled the local population suggests that these birds were not first-generation immigrants or hybrids, but rather back-

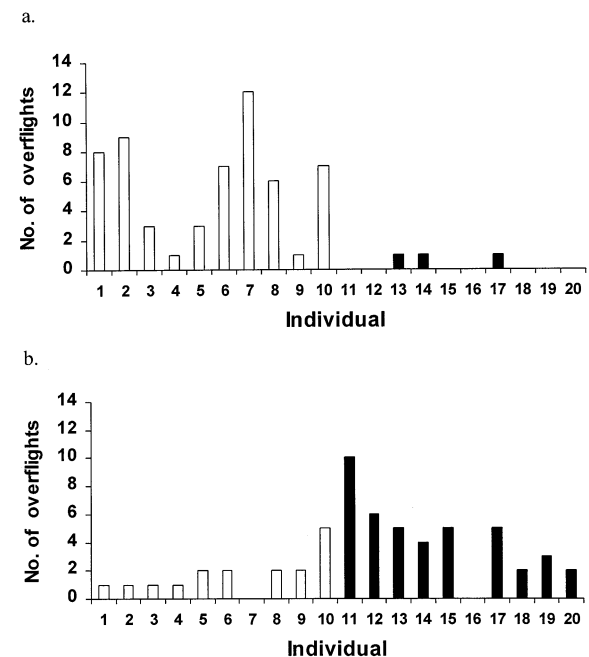


Fig. 5. Number of flights over the tape recorder during the playback experiments. (a) The response of the southern individuals and (b) the response of the northern individuals. White columns represent the reaction of birds exposed to songs from the southern population and black columns represent the reaction of birds exposed to songs from the northern population.

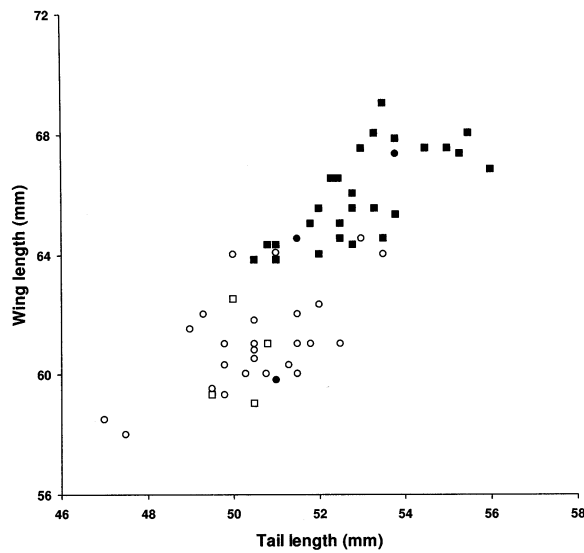


Fig. 6. The relationship between tail length and wing length in the male Chiffchaffs. Mitochondrial cytochrome b haplotype is also shown. Individuals captured in the southern population are indicated by open symbols, individuals captured in the northern population by filled symbols. A circle indicates that the bird carried *collybita* haplotype, a square indicates *abietinus* haplotype.

crosses. However, one bird caught in northern Sweden appeared to be a first-generation immigrant because it undoubtedly was a *collybita*, both according to morphology and mitochondrial haplotype.

If *abietinus* and *collybita* will form a contact zone in Sweden it may either lead to the two populations becoming totally panmictic, or to a stable interaction between these two subspecies. Stable hybrid zones are known in several species of birds including both resident (Carrion and Hooded Crows *Corvus corone* ssp., Saino and Villa 1992; the *Larus glaucescens-occidentalis* complex, Bell 1996) and migratory species (Pied and Collared Flycatchers, Alatalo et al. 1982; Red- and Yellow-shafted Flickers *Colaptes auratus*, Moore and

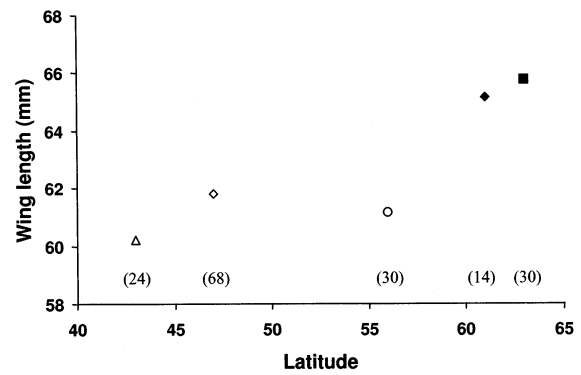


Fig. 7. Mean values for wing lengths of Chiffchaffs at four different study sites in Europe. Open triangle represents Bayonne, France (Salomon et al. 1997); open diamond = SW Germany (Hanski and Tiainen 1991); open circle = Skåne, S. Sweden (this study); filled diamond = southern Finland (Hanski and Tiainen 1991); filled square = Ångermanland, N. Sweden (this study). Sample sizes from the different sites are given in parentheses.

Buchanan 1985; Chiffchaffs, Salomon et al. 1997; Willow Warblers *Phylloscopus trochilus* ssp., Bensch et al. 1999). The level of gene flow across such hybrid zones determines the extent to which each local population of a species is an independent evolutionary unit (Slatkin 1994). Considering the strong differentiation of several traits between the two populations of Chiffchaff in the present study, the equilibrium state will most likely not lead to one panmictic population of Chiffchaffs in Sweden. Rather, we speculate that a zone of contact will form somewhere along 60°N, continuing to uphold the geographic separation with *collybita* in the south and *abietinus* in the north. On a longer term, however, the location of the contact zone may not be stable even if its width reaches an equilibrium because of dispersal and selection against hybrids. This could result if there is competitive asymmetry between the two subspecies (e.g. Rohwer and Wood 1998), e.g. if one is more aggressive than the other.

Table 3. Allele frequencies at four microsatellite loci presented per population. Allele size is indicated in base pairs (bold). N = 60 for all.

Locus	Region	Alleles (named by size in bp) and their proportions									
LS2	South	<b>188</b>	<b>189</b>								
	North	0.78	0.22								
PHTR1	South	<b>84</b>	<b>86</b>	<b>88</b>	<b>90</b>	<b>92</b>	<b>94</b>	<b>96</b>	<b>98</b>		
	North	0.02	0.00	0.05	0.02	0.60	0.22	0.08	0.01		
POCC1	South	0.03	0.00	0.05	0.00	0.47	0.37	0.08	0.00		
	North	<b>201</b>	<b>203</b>	<b>205</b>	<b>207</b>	<b>209</b>	<b>211</b>	<b>213</b>	<b>215</b>	<b>217</b>	
POCC6	South	0.00	0.03	0.68	0.22	0.02	0.05	0.00	0.00	0.00	
	North	0.02	0.00	0.67	0.18	0.03	0.07	0.02	0.00	0.01	
		<b>174</b>	<b>176</b>	<b>178</b>	<b>180</b>	<b>182</b>	<b>184</b>	<b>186</b>	<b>188</b>	<b>190</b>	<b>198</b>
	South	0.17	0.07	0.03	0.08	0.28	0.17	0.13	0.02	0.05	0.00
	North	0.08	0.17	0.02	0.28	0.18	0.10	0.10	0.02	0.03	0.02

*Acknowledgements* – We are very grateful to Sören Svensson for help with the choice of study site in northern Sweden and to Falsterbo Bird Observatory for pointing out morphological differences between the Chiffchaff subspecies. A. Helbig and S. Rohwer provided helpful comments on a previous version of this paper. The study was financially supported by the Swedish Natural Science Research Council (NFR).

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(Received 21 June 1999, revised 6 December 1999, accepted 28 December 1999.)