

**Phylogeny and species limits in the Palaearctic chiffchaff
Phylloscopus collybita complex:
mitochondrial genetic differentiation and bioacoustic evidence***

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Nucleotide sequences of the mitochondrial cytochrome b gene (1041 bp), analysis of vocalizations and behavioural evidence from zones of contact were used to reassess the species limits and phylogenetic relationships at the species and subspecies levels in the *Phylloscopus collybita* complex. A new classification is proposed which recognizes four biological species. *Phylloscopus brehmii* (Iberia) and *P. canariensis* (Canary Islands) are genetically and bioacoustically highly distinct. There is no mitochondrial gene flow between them or with *P. collybita*. The Mountain Chiffchaff *P. sindianus* (with subspecies *sindianus* and *lorenzii*) is equally distinct genetically from southwest Asian subspecies of *P. collybita* (*caucasicus*, *brevirostris*). The status of the Siberian form *tristis*, which shares potentially synapomorphic characters with the Mountain Chiffchaff (ascending song notes, grey-brown adult plumage) but genetically closely resembles *P. c. collybita* and *P. c. abietinus*, remains uncertain. In two zones of secondary contact between taxa with "greenish" (*P. collybita*) and brownish plumage, hybridization is either unrecorded (*caucasicus* v *lorenzii*, Caucasus Mountains) or its extent is insufficiently known (*abietinus* v *tristis*, west of Ural Mountains). A phylogeny reconstructed from nucleotide sequences agrees with one based on song and some morphological characters in identifying *P. brehmii* as the oldest branch within the chiffchaff group. Of the remaining taxa, four fall into a clade with greenish plumage (*P. collybita* ssp.), two into one with brownish plumage (*P. s. sindianus*, *P. s. lorenzii*), while the position of *P. canariensis* with respect to these two clades is uncertain. Molecular and phenotypic phylogenies contradict each other regarding the affinities of *P.* (ssp.?) *tristis*.

Within their nearly trans-Palaearctic distribution range (Fig. 1), a varying number of taxa have traditionally been placed within the polytypic species *Phylloscopus collybita*, most ranking as allopatric subspecies (Ticehurst 1938, Vaurie 1954). Most recent accounts accept *P. collybita* and *P. sindianus* as full species within the chiffchaff complex (Sibley & Monroe 1990, Cramp 1992). *Phylloscopus collybita* comprises the subspecies *collybita* (W and central Europe), *abietinus* (N and easternmost Europe), *brehmii* (Iberia and N. Africa), *canariensis*, *exsul* (Canary Islands) and *tristis* (Urals to E Siberia). Four other subspecies have been described and were synonymized by later authors as follows: *fulvescens* west of Ural Mountains included in *tristis* (Martens &

Meincke 1989, Cramp 1992); *menzbieri* from northeastern Iran and Turkmeniya included in *fulvescens* by Vaurie (1954) and in *tristis* by Cramp (1992); *brevirostris* (Watson 1962, Haffer 1991) with uncertain position (Cramp 1992); *caucasicus* newly described by Loskot (1991), not discussed by other authors so far. *Phylloscopus sindianus* is composed of the disjunct subspecies *sindianus* (Central Asia) and *lorenzii* (Caucasus, NE Turkey mountains; Williamson 1976, Cramp 1992). The latter was assigned status of a monotypic species by Stepanyan (1978, 1983) without further details.

Species limits and phylogenetic relationships within the chiffchaff group are poorly understood. Morphologically, all of these forms are very similar, although slight differences in colouration, wing shape and body proportions do exist (Ticehurst 1938, Gaston 1974, Cramp 1992, Martens & Eck 1995). Acoustic differences between several of the taxa

* Dedicated to Ernst Mayr on the occasion of his 90th birthday.

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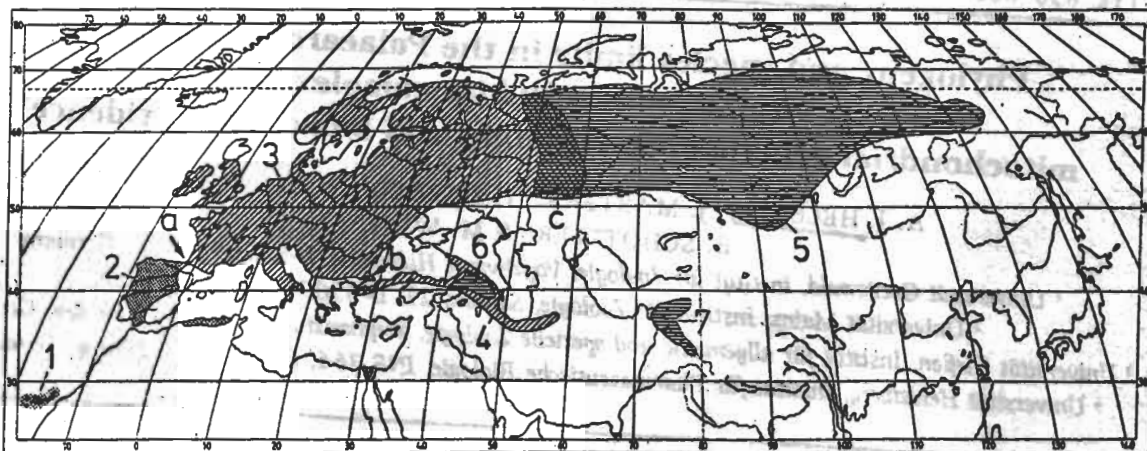


Figure 1. Breeding distribution of the chiffchaff complex (*P. [collybita]*): (1) *P. canariensis*; (2) *P. brehmii*; (3) *P. collybita*, northern subspecies *collybita* and *abietinus*; (4) *P. collybita*, southern subspecies *brevirostris*, *caucasicus* and *menzibleri* (W to E order, geographical limits not yet defined); (5) *P. (ssp.?) tristis*; (6) *P. s. sindianus lorenzii*; (7) *P. s. sindianus*. Zones of secondary contact and overlap. (a) Between *P. brehmii* and *P. c. collybita*; (b) Between *P. c. collybita* and *P. c. abietinus*; (c) Between *P. c. abietinus* and *P. (ssp.) tristis*.

mentioned, however, are much more pronounced as is typical within the species-rich but morphologically homogeneous genus *Phylloscopus* (Thielcke & Linsenmair 1963, Martens & Hänel 1981, Martens 1982, Salomon 1987, Martens & Meincke 1989). Full or semispecies status of the Iberian form *brehmii* is supported by its well-differentiated calls, territorial song (Thielcke & Linsenmair 1963, Salomon 1987) and mitochondrial DNA (Helbig *et al.* 1993). The song of the Canary Island Chiffchaff *P. c. canariensis* differs so markedly from mainland forms that it may also be specifically distinct. The distribution ranges of many of the forms discussed here are allopatric or disjunct, creating problems with the application of the biological species concept (Mayr & Ashlock 1991). So far, with the exception of *P. brehmii* (Helbig *et al.* 1993), no genetic studies are available that would help to delineate species limits within this complex.

In this paper, we compare two independent sets of characters, DNA sequences of the mitochondrial cytochrome b gene and vocalizations, of nine chiffchaff taxa; three more taxa are discussed for which no sequence data are available. Our goals were to assess the genetic distinctiveness of each taxon, compare the magnitude of genetic v bioacoustic differentiation, search for evidence of gene flow or barriers to gene flow between them and reconstruct their phylogenetic relationships.

Nucleotide sequences of the mitochondrial cytochrome b gene have been used extensively in studies of avian phylogeny (Edwards & Wilson 1990, Edwards *et al.* 1991, Quinn *et al.* 1991, Richman & Price 1992, Helm-Bychowski & Cra-craft 1993, Kornegay *et al.* 1993, Seibold *et al.* 1993) and population differentiation (Baker 1992, Birt-Friesen *et al.* 1992, Taberlet *et al.* 1992) and have revealed at least two previously unrecognized species (Smith *et al.* 1991, Helbig *et al.* 1995). Within the mitochondrial genome, the cytochrome b gene is a fairly conservative region, which reflects

evolutionary divergence over large time spans (in the range of several hundred thousand to millions of years). For theoretical and practical reasons, e.g. the availability of near-universal primers for amplification and sequencing (Kocher *et al.* 1989), it has become a reference gene for phylogenetic studies.

Recent evidence is reviewed from contact zones where some of the chiffchaff taxa meet and interact in complex ways. Based on the combined evidence now available, a new classification of the group is proposed.

MATERIAL AND METHODS

Mitochondrial DNA sequences

The avian cytochrome b gene comprises 1143 nucleotides, 1041 of which were sequenced in eight chiffchaff taxa (Table 1). The published cytochrome b sequence of *P. s. sindianus* (910 nucleotides; Richman & Price 1992) was also included. We used sequences of the Willow Warbler *Phylloscopus trochilus*, Wood Warbler *Phylloscopus sibilatrix*, Blackcap *Sylvia atricapilla*, Sedge Warbler *Acrocephalus schoenobaenus* and Fan-tailed Warbler *Cisticola juncidis* as out-group taxa. With the exception of *P. collybita caucasicus*, of which only two individuals were available, full or partial sequences (allowing unequivocal assignment to genotype) were obtained from 26 *P. collybita collybita* from southern Germany and France, four *P. c. abietinus* from Israel (migrants) and Finland, 21 *P. (c.) brehmii* from southwestern France and northern Spain and one from Tunisia, seven *P. (sindianus) lorenzii* from the Caucasus, ten *P. (c.) tristis* from the Mirnoye Biological Station, Yenesei River (Siberia), and 24 *P. (c.) canariensis* from the Canary Islands (seven each from La Palma and El Hierro, six from Tenerife, four from Gran Canaria; Table 1). All birds were identified by song or calls or both. Most individuals (except *tristis*) were singing

Table 1. *Phylloscopus* taxa, of which cytochrome *b* gene has been sequenced in this study. The origin, total number of individuals (*n*) and the number of birds of which 300, 600, 900 or 1041 nucleotides were sequenced are given

Taxon	<i>n</i>	Origin	Nucleotides sequenced			
			300	600	900	1041
<i>P. c. collybita</i>	26	S Germany	7	—	—	1
		SW France	3	10	1	4
<i>P. c. abietinus</i>	4	Israel (migrants)	2	—	—	1
		Finland*	—	—	1	—
<i>P. c. brevirostris</i>	3	Uludag, Turkey	2	—	—	1
<i>P. c. caucasicus</i>	2	Caucasus	1	—	—	1
<i>P. (ssp.) tristis</i>	10	Yenessei, Siberia	1	4	4	1
<i>P. brehmii</i>	22	SW France	2	4	4	2
		N Spain	1	7	—	1
		Tunisia	—	1	—	—
<i>P. canariensis</i>	24	Teneriffe	5	—	—	1
		El Hierro	5	—	—	2
		La Palma	6	—	—	1
		Gran Canaria	4	—	—	—
<i>P. s. lorenzii</i>	7	Caucasus	2	3	1	1
<i>P. trochilus</i>	5	Finland*, Germany	2	1	1	—
		Greece (migrant)	—	—	—	1
<i>P. sibilatrix</i>	1	Greece (migrant)	—	—	—	1

* Sequence taken from Richman and Price (1992).

males on the breeding grounds, which were attracted by tape recordings and caught in mist nets.

Total DNA was isolated from blood samples (c. 50 µl) preserved in NaF-EDTA buffer (Arctander 1988). Polymerase chain reactions (PCR), product purification and sequencing were performed as described elsewhere (Helbig *et al.* 1995). Sequencing primers were spaced such that large overlaps in readable sequence resulted, giving us an opportunity to verify sequences obtained from independent reactions.

Sequence analysis and reconstruction of phylogeny

Sequences were aligned visually, which was possible without introducing gaps or insertions. The phylogeny was analysed using the maximum parsimony (PAUP 3.1.1; Swofford 1993) and neighbour-joining methods (Saitou & Nei 1987, program package MEGA by Kumar *et al.* 1993). To estimate the amount of phylogenetic information content of the data matrix, the skewness of the tree length distribution was assessed for a random sample of 1000 'trees' using the G-test (Hillis & Huelsenbeck 1992, 'random trees' option in PAUP). Pairwise genetic distances were calculated according to Kimura's (1980) two-parameter model and as the rate of synonymous base substitutions (i.e. mutations not leading to an amino acid replacement) per site according to the weighted pathway method (Li 1993). Bootstrap tests (1000 replicates) were run using either parsimony (heuristic

search, random sequence addition) or neighbour-joining searches to assess the robustness of the clades identified.

Nucleotide substitutions can be either transitions (changes between two purines or between two pyrimidines) or transversions (changes between a purine and a pyrimidine). Among closely related species, transitional differences are about 20 times more common than transversions (see Results, compare also Edwards *et al.* 1991). Transversions therefore retain much more phylogenetic information, while transitions, especially at third codon positions where most substitutions occur, rapidly approach saturation levels (Haeuser *et al.* 1993). To ensure that the different probabilities of substitutions did not affect our phylogenetic conclusions, transversions were weighted 1:1, 5:1 or 20:1 over transitions in the parsimony analysis (see Results).

Bioacoustics

Tape recordings were taken in the field (mainly by J.M.) as follows: *abietinus*: Moscow, Russia, 1991; *tristis*: Novosibirsk area, Russia, 1986; *sindianus*: Leh and Shey, Ladakh, India, 1976; *lorenzii*: Zakatali Reserve, Azerbaijan, 1981; Elbrus area and Kurush, Dagestan, Russia, 1991; *caucasicus*: Kislovodsk and Elbrus area, Russia, 1991; Central Elbrus Mountains and near Tabris, Iran, 1978; *collybita*: various localities in Germany; *canariensis*: Canary Islands: Gran Canaria, Tenerife, La Gomera, La Palma, El Hierro, 1981 (J.M.), 1990 and 1991 (E.H., B.S.); *brehmii*: Gibraltar, Spain, 1961

Table 2. Pairwise genetic distances between chiffchaff taxa and related *Phylloscopus* species (outgroup taxa) based on 1041 nucleotides of the mitochondrial cytochrome b gene. Above diagonal: proportion of nucleotides differing between taxa (in %); below diagonal: absolute numbers of nucleotide differences

Taxon	1	2	3	4	5	6	7	8	9	10	11
1 <i>P. c. collybita</i>		1.0	1.7	1.5	1.5	4.6	3.7	3.6	4.6	9.2	11.5
2 <i>P. c. abietinus</i>	10		1.7	1.3	1.3	5.0	3.5	3.6	4.6	9.3	11.5
3 <i>P. (c.) tristis</i>	18	18		1.9	1.9	4.8	4.0	3.5	4.3	9.4	11.6
4 <i>P. c. caucasicus</i>	16	14	20		0.2	5.4	3.7	3.9	4.5	9.7	11.7
5 <i>P. c. brevirostris</i>	16	14	20	2		5.3	3.7	3.8	4.5	9.7	11.8
6 <i>P. brehmii</i>	48	52	49	56	55		5.0	4.3	4.5	10.1	11.2
7 <i>P. canariensis</i>	38	36	41	38	38	52		3.4	4.1	10.3	12.5
8 <i>P. s. lorenzii</i>	37	37	36	41	40	45	35		1.7	10.3	12.2
9 <i>P. s. sirdianus</i>	42	42	39	41	41	41	37	15		11.1	13.2
10 <i>P. trochilus</i>	95	96	96	100	100	105	107	107	100		13.5
11 <i>P. sibilatrix</i>	120	120	119	122	123	117	130	127	120	140	

(G. Thielcke); Tunisia, 1992 (F.H., B.S.); *menzbieri*: Kopet Dagh, Turkmeniya, 1985 (I. Marova, P. Tomkovich); *brevirostris*: various localities in northern Turkey, 1990 (P. S. Hansen). As recording equipment we used a Uher 4200 tape recorder, Nagra SN, Walkman Sony WM-D3 with Sennheiser Directional microphone ME 88/K3N, Sennheiser MD 211 N in parabolic reflector 80 cm diameter, parabolic reflector Telinga Pro III. Sonographic analysis was performed with a Kay DSP 5500 Sonagraph and 300-Hz filter. Original sonograms are presented in Figures 3, 4 and 6.

RESULTS

Mitochondrial DNA

Characterization of molecular variation

The cytochrome b nucleotide sequences of all taxa studied are shown in the Appendix. Within the chiffchaff complex (nine taxa), variation occurred at 112 nucleotide positions (10.8%) of 110 codons (out of 347). Of these, derived character states at 50 nucleotide positions were "phylogenetically informative", i.e. they were shared by at least two taxa. Among the 110 variable codons, only eight amino acid replacements were inferred from the nucleotide data. In other words, 92.9% of the molecular variation observed consisted of "silent" mutations, which are selectively neutral, because they do not result in an altered phenotype. For these very closely related taxa, such a low number of substitutions actually affecting the phenotype was expected. Only 4 out of the 112 base substitutions were transversions, all others were transitions. This confirms the extreme transition/transversion bias (28/1 in this data set) typical of the avian cytochrome b gene, which must be taken into account when using such sequences to construct phylogenetic trees (see below).

Genetic differentiation and species limits

Absolute and percentage values of cytochrome b sequence divergence (Table 2) among the (sub)species studied show that all taxa are clearly differentiated genetically. From these data, it is immediately obvious that *P. c. collybita*, *abietinus*, *brevirostris*, *caucasicus* and *tristis* on the one hand, and *P. s. sirdianus* and *lorenzii* on the other hand, are genetically most closely related. Within these groups, pairwise sequence divergence values range from 1.0% to 2.0% except for *brevirostris* v *caucasicus*, which differ by only 0.2%. Evidence for mitochondrial gene flow was found between *P. c. collybita* and *abietinus*: a recently fledged bird from southwestern Germany, caught on 7 June 1992 near Radolfzell (S Germany), i.e. far southwest of the range of *abietinus*, carried the *abietinus* genotype. In contrast, all other *collybita* ($n = 26$), *brehmii* ($n = 21$), *canariensis* ($n = 24$), *lorenzii* ($n = 7$) and *tristis* ($n = 10$) exclusively carried mitochondrial haplotypes restricted to their respective (sub)species. Regarding the first three taxa, sample sizes were large enough for mitochondrial gene flow to be detected if it occurred. However, no such evidence was found, which is especially remarkable in the case of *collybita* and *brehmii*, whose sequences differed by 4.6%. These two taxa form a narrow zone of overlap in southwestern France and northern Spain, where hybridization has been recorded, based on identification by calls and songs of adults feeding nestlings (Salomon 1987, 1990). Since mitochondrial DNA is inherited maternally, the lack of mixing between *collybita* and *brehmii* haplotypes means that female hybrids or their offspring must be sterile. Furthermore, among breeding birds on the Canary Islands, no haplotype other than the ones typical of *P. c. canariensis* were detected, even though *collybita* (singing males recorded on Fuerteventura, February 1991 [F.H., B.S.]), *P. (c.) brehmii* (specimens in Museum A. Koenig, Bonn; S. Eck in litt.) and perhaps *abietinus* (Glutz von Blotzheim & Bauer 1991) reach these islands on migration. *Phylloscopus (c.) canariensis* was

surprisingly well differentiated from all other forms and differed even more from its nearest breeding range neighbour, *P. (c.) brehmii* (5.0%), than from nominate *collybita* (3.7%; Table 2). All seven *lorenzii* specimens from the Caucasus, where this taxon occurs in parapatry with *P. c. caucasicus*, carried the same cytochrome b haplotype. The sequence divergence between *lorenzii* and *caucasicus* was 3.9%, i.e. about as large as between *collybita*, *brehmii* and *canariensis*. Individual haplotypes of *tristis* differed from *abietinus* by 1.7–2.0%.

Intrataxon genetic variation

It was not a primary goal of this study to investigate the amount and extent of intrataxon sequence variation. However, for four taxa (*collybita*, *tristis*, *brehmii*, *canariensis*) we have large enough sample sizes (Table 1) to provide minimum estimates of intrataxon variability. Among 25 individuals of *collybita* (excluding the one with *abietinus* genotype), there were eight variable positions defining seven haplotypes. Within the other taxa, variable nucleotide positions were 11 among 22 *P. brehmii* (eight haplotypes), eight among 24 *canariensis* (nine haplotypes) and nine among ten *tristis* (7 haplotypes). Of the 31 nucleotide positions that were found to vary within taxa, 30 involved transitions and only one (in *canariensis*) involved a transversion. To calculate genetic distances (Table 2) and to reconstruct phylogenetic trees, only the most frequently occurring haplotypes were used. This is justified because within each taxon haplotypes differed by only one or two substitutions (0.1–0.2%) from the respective dominant type. The only exceptions were one *P. (c.) tristis* and one *P. (c.) brehmii* from Tunisia, both of which differed by four substitutions from the respective commonest haplotype. A strongly deviant cytochrome b sequence detected in one *brehmii* from southwestern France (Helbig *et al.* 1993) could not be amplified further and is not considered here. In conclusion, sequence divergence within taxa ranged from zero to 0.2% (0.4% in *tristis* and *brehmii*), i.e. an order of magnitude lower than between taxa (except *brevirostris/caucasicus*; see Table 2).

In the Canary Island Chiffchaff, the occurrence of derived haplotypes (those differing from the commonest type) on various islands was of special interest because call notes are island specific (Henning *et al.* 1994) and suggest a high degree of interisland differentiation. While the main cytochrome b haplotype occurred on all four islands (Gran Canaria, El Hierro, Tenerife, La Palma), six of the eight derived types were restricted to single islands. Although sample sizes are too small to test for interisland differences in haplotype frequency, this result not only demonstrates a remarkable haplotype diversity but also some interisland differentiation at the mitochondrial genetic level.

Phylogenetic relationships

For reconstructing the phylogenetic tree, Willow Warbler and Wood Warbler were used as outgroup taxa relative to

the chiffchaff complex. Sedge Warbler, Blackcap and Fan-tailed Warbler were included as successively more distant outgroup taxa. The tree length distribution based on sequences of 11 *Phylloscopus* taxa (nine "chiffchaff" taxa plus Willow Warbler and Wood Warbler) was significantly left-skewed, no matter whether transversions were weighted 5:1 over transitions ($G_1 = -1.688$, $P < 0.01$) or remained unweighted ($G_1 = -0.769$, $P < 0.01$). This indicates the presence of strong phylogenetic signal in the data set, a prerequisite for any phylogenetic analysis to yield meaningful results (Hillis & Huelsenbeck 1992). Maximum parsimony (PAUP 3.1.1.) and neighbour-joining (MEGA 1.0) yielded largely congruent trees, which grouped the "chiffchaff" taxa as a monophyletic group with the Willow Warbler as their closest relative (Fig. 2).

Unweighted parsimony yielded two most parsimonious trees (length 601 steps, consistency index 0.702), differing only in the placement of one of the outgroup taxa (*Acrocephalus*). Transition–transversion (TS/TV) weighting yielded the tree topology shown in Figure 2, which was independent of the strength of the weighting (1/5, 1/10, 1/20). The neighbour-joining analysis yielded identical trees with Kimura two-parameter distances (including TV and TS; Kimura 1980) or with rates of synonymous substitutions per site (weighted pathway method; Li 1993). When distances were calculated using transversions only (to better resolve phylogenetically old branches), the *collybita/abietinus* group could not be resolved (there are no transversion differences between these taxa).

The bootstrap tests strongly supported the grouping of *P. c. collybita*, *abietinus*, *brevirostris*, *caucasicus* and *tristis* as a monophyletic group (99%). The most surprising result was that *P. (c.) canariensis* was not closely related to either *brehmii* or *collybita*. In parsimony reconstructions it clustered with the western and central Asian mountain forms *sindianus* and *lorenzii*, although this association was not significantly supported by the bootstrap test (44–70%). *Phylloscopus (c.) brehmii* emerged as the most ancient of the chiffchaffs (bootstrap 91% with TV:TS = 20:1) and formed the sister group of the other two clades, contrary to the intuitive expectation that it should be a close relative of either *collybita* or *canariensis*, its parapatric and allopatric breeding range neighbours (Fig. 2).

The neighbour-joining tree differed from the parsimony tree only in that *collybita* and *abietinus* appeared as sister taxa and *tristis* was placed outside the *collybita-abietinus-brevirostris* group. However, neither of these differences was supported by high bootstrap values, indicating that there was no real conflict between the trees. From the overall congruence between the two phylogenetic methods and the bootstrap test results, we conclude that most branches of the reconstructed phylogeny are well supported by the sequence data. Details like the exact position of *abietinus* (sister taxon of either *collybita* or *caucasicus/brevirostris*) and *canariensis* (sister taxon of either *sindianus/lorenzii* or the *collybita* group) remain to be more firmly established.

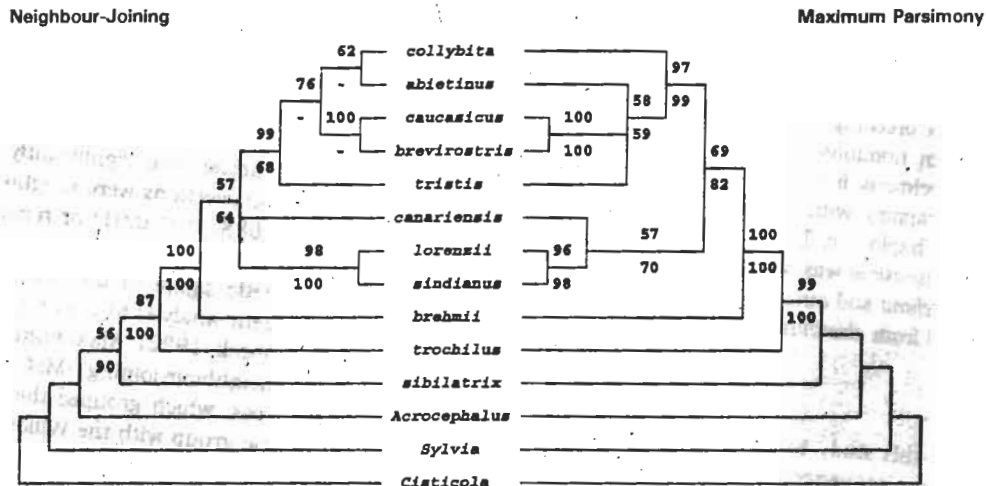


Figure 2. Phylogeny of nine taxa of the chiffchaff complex. Willow and Wood Warbler, plus Sedge Warbler, Blackcap and Fan-tailed Warbler as outgroup taxa. The trees are based on 1041 nucleotides of the cytochrome b gene (910 for *sindianus*; cf. Appendix). Branches with less than 50% bootstrap support are shown as unresolved polytomies. Numbers indicate the frequencies (in %) with which each clade was identified in 1000 bootstrap replicates. (Left) Neighbour-joining tree based on Kimura two-parameter distances including both transversions (TV) and transitions (TS; bootstrap values above branch) and using TV only (below branch); (Right) Maximum parsimony (heuristic search), unweighted (values above branch) and with TV weighted 5:1 over TS (below branch).

Bioacoustics

Territorial song (Fig. 3)

Within continental Eurasia, chiffchaff songs fall into four major groups:

Group 1: *collybita*, *abietinus*, *brevisrostris*, *caucasicus*, *menzbieri*. Each song note starts on a high pitch (8.0–9.2 kHz), drops markedly to 2.4–3.0 kHz within 80–90 ms, ascends again to 4–5 kHz and ends with a highly variable appendix (details in Becker *et al.* 1980, Martens & Meincke 1989). Often, the first descending part of the note is characterized by a small "knee", i.e. a short, 10–30-ms horizontal part, resulting in the typical "descent-and-knee" note (see Fig. 3) of the northern *collybita* and *abietinus* as well as the south-west Asian taxa *brevisrostris*, *caucasicus* and *menzbieri* (Thielcke & Linsenmair 1963, Becker *et al.* 1980, Martens & Hänel 1981, Martens 1982). Geographically, this note type covers most of Europe (except Iberia) plus the whole southern, partially disjunct chiffchaff range of Turkey, lower parts of the Caucasus, Elburs Mountains and east to Kopet Dag Mountains in Turkmeniya.

Group 2: *tristis*, *sindianus*, *lorenzii*. Within each taxon, songs are more varied and are composed of several note types, some resembling those of Group 1, others ascending from a low starting frequency (3–4 kHz) to about 5–6 kHz with a "knee" interspersed and ending in a variable finishing part (Fig. 3m). Most important are variations of the "descent-and-knee" note, the second part of which may rise again to the initial pitch (Fig. 3m, o) or may be transformed to similar notes with a low frequency range (Fig. 3n). Consequently, the songs of this group are much richer in note

types and create a more vivid acoustic impression. All taxa of this group, *lorenzii* (Caucasus), *sindianus* (Himalayas) and *tristis* (E Palearctic), are very similar in their grey-brown plumage, largely lacking green or olive tones (Ticehurst 1938, Vaurie 1954, 1959, Cramp 1992).

To the human ear, songs of *sindianus* and *lorenzii* on the one hand and *collybita*, *abietinus*, *brevisrostris*, *caucasicus* and *menzbieri* on the other are almost indistinguishable. Syntax of song strophes, especially intervals between notes and length of notes, which are important characters to the human ear, are almost identical. The Siberian *tristis*, on the other hand, has short pauses between the notes, a lower frequency range and relatively many ascending notes. This results in a quite distinct acoustic impression of mostly long, warbling verses, not chiffchaff-like at all.

Group 3: *canariensis*. *Canariensis* song consists predominantly of notes falling rapidly in pitch within a broad frequency range (4.5 kHz); the second note part may ascend nearly as abruptly, often reaching the initial frequency. Small "knees" may be included in the first, descending part of a note (cf. group 1); however, they are not horizontal but ascending in frequency (Fig. 3a, b). Notes starting with an ascending branch also occur but are uncommon and perhaps not homologous to ascending notes of Group 2 taxa. To the human ear, *canariensis* song has a very distinct acoustic quality: it sounds harsh, atonal and irregular as a result of the rapid frequency changes and the varying lengths of notes and the pauses between them. The song is quite different from that of all other taxa and contrasts most strongly with the warmer and more melodious quality of *tristis*.

Group 4: *brahmii*. The song of *P. (c.) brahmii* from Iberia

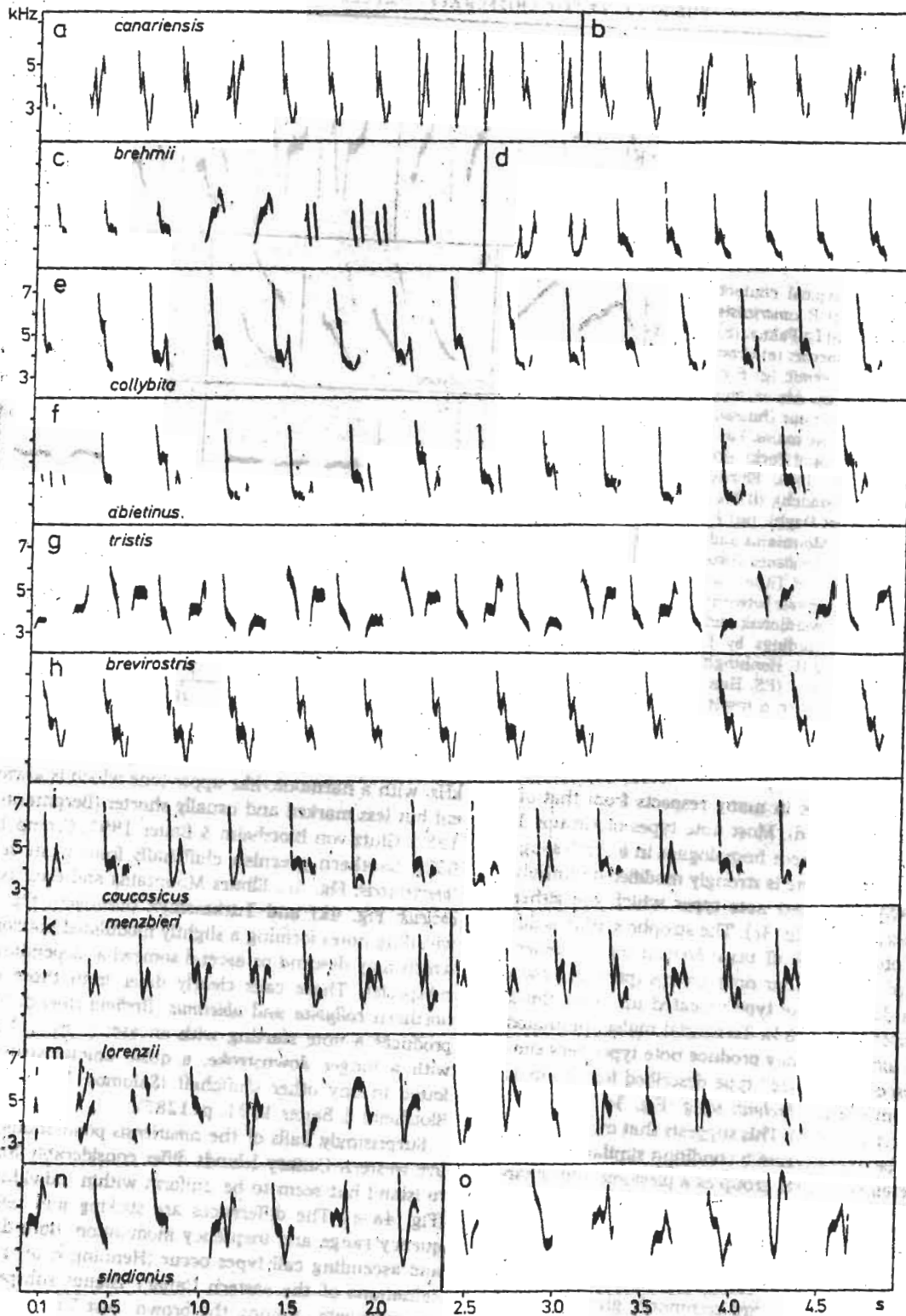


Figure 3. Sonograms of typical territorial songs of 9 chiffchaff taxa compiled from various sources and own field studies. (a, b) *P. canariensis* (Gran Canaria, March 1981); (c, d) *P. brehmii* (Gibraltar, April 1961; Tunisia, Tabarka, May 1992); (e) *P. c. collybita* (Germany, Bergen/Dümme, May 1991); (f) *P. c. abietinus* (Russia, Moscow, June 1991); (g) *P. (ssp.) tristis* (Russia, Novosibirsk, May 1986); (h) *P. c. brevirostris* (Turkey, Ulu Dagh, July 1990); (i) *P. c. caucasicus* (Russia, Caucasus, Kislovodsk, June 1991); (k, l) *P. c. menzbieri* (Turkmeniya, Kopet Dagh, May 1985); (m) *P. s. lorenzii* (Russia, Caucasus, Elbrus area, June 1991); (n, o) *P. s. sindianus* (India, Ladakh, Shey, June 1976). Recordings by J. Martens except c (G. Thielcke); d (B. Schotter, F. Henning); h (P.S. Hansen); k, l (I. Marova, P. Tomkovich).

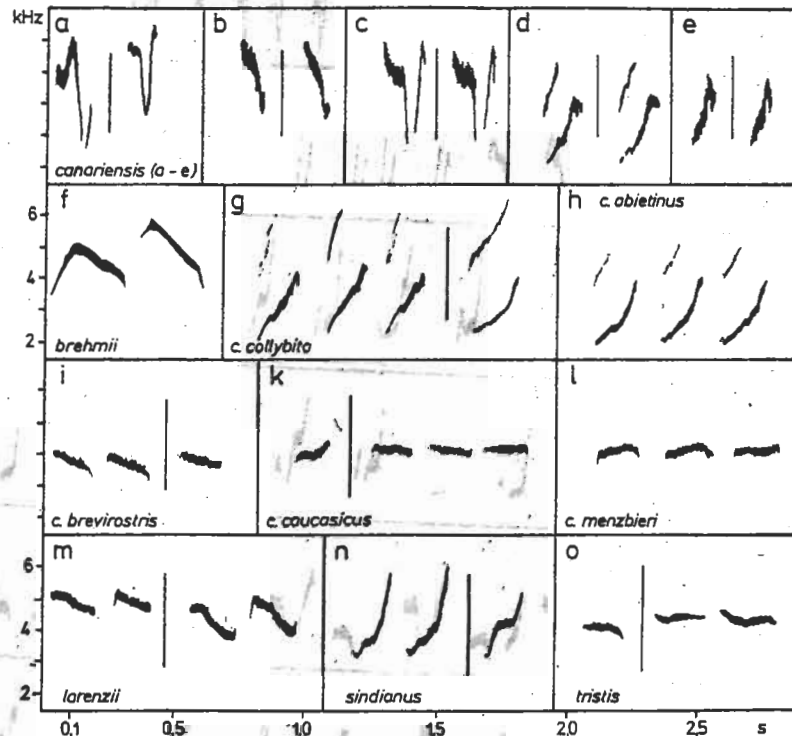


Figure 4. Sonograms of typical contact calls of chiffchaff taxa. (a–e) *P. canariensis canariensis*, Canary Islands. (a) La Palma; (b) El Hierro; (c) Gomera; (d) Tenerife; (e) Gran Canaria (two males); (f) *P. brehmii*; (g) *P. c. collybita* (two males; Germany, Mainz and Bergen/Dümme); (h) *P. c. abietinus* (Russia, Moscow); (i) *P. c. breviostris* (two males; Turkey, Ulu Dag and Ilgaz National Park); (k) *P. c. caucasicus* (two males; Iran, Elbrus Mous, Dasht-Nazir and S Alamdeh); (l) *P. c. menzbieri* (Turkmeniya, Kopet Dag); (m) *P. s. lorenzii* (two males; Elburs Mountains and Kurush/Daghestan); (n) *P. s. sindianus* (two males; India, Ladakh, Kargil and Tikse), (o) *P. (ssp.) tristis* (two males, intervals between notes shortened; Russia, W Swerdlowsk and 70 km E Krasnoyarsk). Recordings by J. Martens except a (left), b, c, e (F. Henning); f (M. Salomon); h (I. Marova); i (P.S. Hansen); j (I. Marova, P. Tomkovich); n (right) (P.S. Hansen). o (A. Blinov).

and northern Africa differs in many respects from that of the other groups (Fig. 3c, d). Most note types of Groups 1 and 2 songs have no apparent homologues in *brehmii* song. The "descent-and-knee" note is strongly modified or entirely replaced by newly developed note types which are either ascending or descending (Fig. 3c). The strophe syntax is different from all other chiffchaff taxa: *brehmii* song is generally composed of three or four note groups (phrases), each consisting of a distinct note type repeated up to six times (Thielcke & Linsenmair 1963). Territorial males stimulated by playback of local song may produce note types very similar to the "descent-and-knee" type described for Group 1, i.e. different from normal *brehmii* song (Fig. 3d; sonagram also in Thielcke *et al.* 1978). This suggests that modern *brehmii* song could be derived from a condition similar to Group 1 songs and retained in that group as a plesiomorphic character.

Calls (Fig. 4)

Here we refer to calls which are commonly given by adult birds when slightly irritated during song display or, generally, during events requiring attention. Within the whole chiffchaff group, calls are simple structures. Differences between males and females have not been studied in detail. The following call forms have been recorded. Northern greenish chiffchaffs from central, northern and eastern Europe (nominate *collybita*, *abietinus*; Fig. 4g, h) use an ascending note *hweet*, *hüüd*, ranging from about 2 kHz to 5

kHz, with a harmonic-like upper tone which is always present but less marked and usually shorter (Bergmann & Helb 1982, Glutz von Blotzheim & Bauer 1991, Cramp 1992, p. 632). Southern greenish chiffchaffs from western Turkey (*brevirostris*; Fig. 4i), Elburs Mountains and Caucasus (*caucasicus*; Fig. 4k) and Turkmeniya (*menzbieri*; Fig. 4l) use whistling notes forming a slightly modulated horizontal bar, which may descend or ascend somewhat depending on the subspecies. These calls clearly differ from those of adult northern *collybita* and *abietinus*. *Brehmii* (Iberia, N Africa) produces a note starting with an ascent, then descending with a longer downstroke, a quite unique structure not found in any other chiffchaff (Salomon 1987, Glutz von Blotzheim & Bauer 1991, p. 1287).

Surprisingly, calls of the *canariensis* populations from the five western Canary Islands differ considerably from island to island but seem to be uniform within individual islands (Fig. 4a–e). The differences are striking and refer to frequency range and frequency modulation. Both descending and ascending call types occur (Henning *et al.* 1994). Vocalizations of the eastern Canary Islands subspecies *exsul* are unknown. Among the brown, east Eurasian taxa, *lorenzii* (Fig. 4m) and *tristis* (Fig. 4o) have whistling calls with **only slight frequency modulations**, descending in *lorenzii* and **nearly horizontal** in *tristis*. These calls are similar to those of the southern greenish chiffchaffs (Fig. 4i–l). *Sindianus* (Fig. 4n), however, utters ascending notes with a **broad frequency range**, often with a short descending introductory part, sometimes even with a "knee" interspersed.

Biological significance of differences in vocalizations

The differences in songs described above (Groups 1–4) are biologically important characteristics of the populations concerned and potentially act as reproductive isolating mechanisms where vocally divergent populations meet. There is some evidence for acoustic character displacement between *collybita* and *brehmii* in the area of sympatry (Salomon & Hemim 1992). The western Palaearctic group of predominantly greenish forms (excluding *brehmii* and *canariensis*) have the most simple song structure within the chiffchaff complex. Every note displays all of the information crucial for species recognition, i.e. the constant shape and change in frequency of the first note part, which is always descending (for details see Becker *et al.* 1980). Among the southern subspecies at least, *caucasicus* has a slightly richer repertoire. Central European populations respond to most of its song note types (Martens & Hänel 1981). On the other hand, a high percentage of *brehmii* males—despite their marked acoustic differences in syntax and note shape—are attracted by *collybita* song of central European birds, but not *vice versa* (Thielcke *et al.* 1978, Salomon 1989). Both *canariensis* and the eastern Palaearctic group of predominantly grey and brownish chiffchaffs have considerably more complex song repertoires including ascending notes (few in *canariensis*), to which western greenish chiffchaffs do not respond (Martens & Hänel 1981, Martens & Meincke 1989). In *canariensis*, song notes contain rapid and complex frequency changes to which neither *collybita* responds in playback experiments nor *vice versa* (Thielcke *et al.* 1978, Henning *in litt.*).

We assume that characteristics of song syntax and song elements evolved independently of each other. If this is correct, chiffchaff taxa can be clustered according to song characters using a parsimony algorithm. This was done in Figure 5, using presence or absence of 16 different note types and four syntax characteristics (phrases, high element diversity, long song elements, short pauses). This analysis clearly illustrates two points: (1) the songs of *brehmii* and *canariensis* are highly distinctive compared with all other chiffchaff taxa and (2) the three brownish, eastern forms, *lorenzii*, *sindianus* and *tristis*, share song characteristics that set them apart from all others. The phenogram in Figure 5 should not be interpreted as a phylogenetic tree (there is no outgroup to judge polarity), but it does support the above separation of chiffchaff taxa into four distinct groups based on song characteristics.

Proposed new classification

Based on the combined evidence of mitochondrial DNA sequences, comparative bioacoustics, morphology and population interactions in contact zones, we propose the following new classification of the chiffchaff complex:

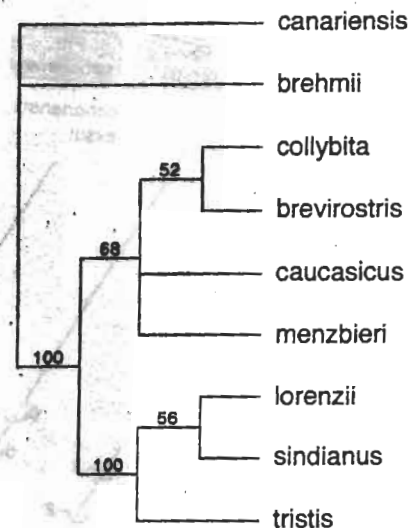


Figure 5. Parsimony phenogram of nine chiffchaff taxa based on presence or absence within their songs of 16 types of song elements (cf. Fig. 3) and four syntax characters (phrases, short pauses between notes, long song elements, high element diversity). The unrooted phenogram is a majority rule consensus of 25 equally parsimonious trees found by ten heuristic searches using PAUP 3.1.1. Numbers are frequencies (in %) with which each clade occurred in the 25 trees.

Genus *Phylloscopus* Boie 1826

Superspecies *Phylloscopus* [*collybita*]

Phylloscopus brehmii (Homeyer 1871)

Phylloscopus canariensis (Hartwig 1886)

Phylloscopus canariensis canariensis (Hartwig 1886)

Phylloscopus canariensis exsul Hartert 1907

Phylloscopus collybita (Vieillot 1817)

Phylloscopus collybita collybita (Vieillot 1817)

Phylloscopus collybita abietinus (Nielsson 1819)

Phylloscopus collybita breviostris (Strickland 1836)

Phylloscopus collybita caucasicus Loskot 1991

Phylloscopus collybita menzbieri Schestoperov 1937

Phylloscopus sindianus Brooks 1880

Phylloscopus sindianus sindianus Brooks 1880

Phylloscopus sindianus lorenzii (Lorenz 1887)

Phylloscopus (ssp.?) *tristis* Blyth 1843

The new classification reflects the large sequence divergence and the lack of mitochondrial gene flow between the first four species proposed, takes into account the evidence for mitochondrial gene flow between *P. c. collybita* and *P. c. abietinus* and the close genetic similarity between *P. c. breviostris* and *P. c. caucasicus* as well as between *P. s. sindianus* and *P. s. lorenzii*. The true status of *P.* (ssp.?) *tristis* remains uncertain because interactions with *abietinus* in the zone of contact and overlap are insufficiently known and phenotypic and molecular data lead to conflicting conclusions about its phylogenetic affinities (Figs. 2 and 6; see Discussion).

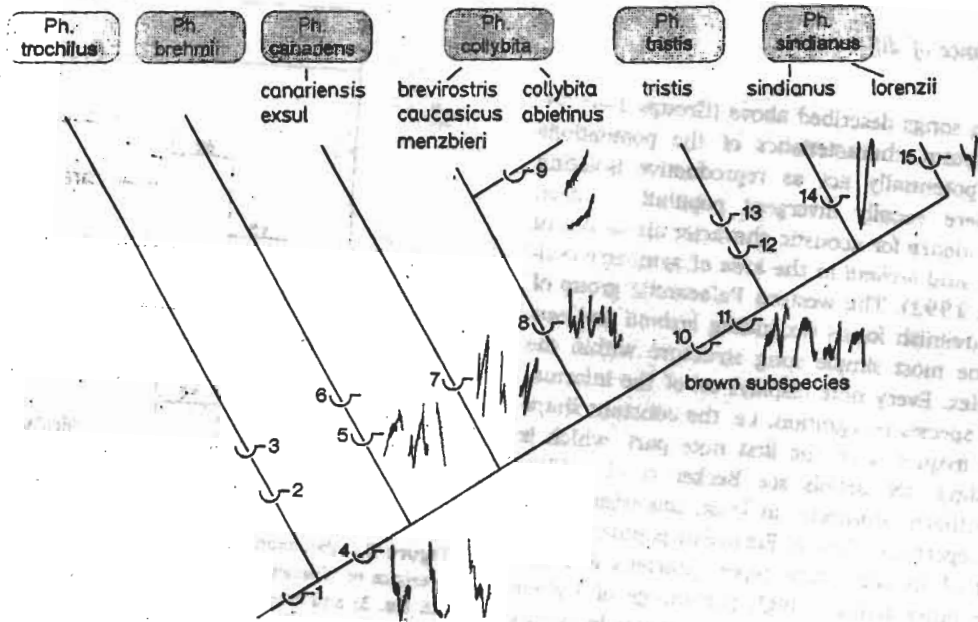


Figure 6. Phylogeny of the chiffchaff complex plus its sister species, the Willow Warbler, derived from weighted acoustic characters, mainly song note types, some calls and a few morphological characters. The acquisition of hypothetical phenotypic synapomorphies (plumage colour, song elements, morphological characteristics) is indicated on the branches of the cladogram. (1) Common ancestor, nondescript green-yellowish plumage; (2) Descending pitch of song verse; (3) Phrases of song verse; (4) "Descent-and-knee" note; (5) Loss of "descent-and-knee" note; (6) Phrases within invariable short verse; (7) Steep, nearly atonal song notes; (8) Variable "descent-and-knee" notes; (9) Ascending call note with harmonic; (10) Grey-brown plumage; (11) Ascending song notes; (12) Low frequency range; (13) Rhythm, independently developed phrases; (14) Broad frequency song notes; (15) Narrow frequency song notes with distinct finishing part.

The superspecies *Phylloscopus [collybita]* thus consists of two allopecies (*canariensis*, *sindianus*) and two (*brehmii*, *collybita*), possibly three (*tristis*), parapatric semispecies. The low frequency of hybridization and the lack of mitochondrial gene flow in the small zone of overlap between *P. brehmii* and *P. collybita* show that they behave as semispecies (as defined by Short 1969) and taxonomically have to rank as full species (see also Amadon & Short 1976, 1992).

DISCUSSION

Most taxa of the chiffchaff complex are distributed allopatrically or parapatrically (Fig. 1), which makes the assessment of species limits difficult. In this study, we combined evidence from mitochondrial genetics, bioacoustics and behaviour in zones of secondary contact (see below) in an effort to understand the levels of differentiation and evolutionary relationships among members of this complex. Within taxa, chiffchaff songs show little variation over large geographic areas, but marked differences exist between some, but not all, of the named (sub)species (Thielcke & Linsenmair 1963, Martens & Hänel 1981, Martens & Meincke 1989). Song characters in this group appear to be stable for long periods of time and probably evolved in isolated refuge areas during Pleistocene glaciations, from where they spread after climatic conditions became more

favourable. Thus, vocal differences, like mitochondrial DNA sequences, reflect evolutionary differentiation within the chiffchaff complex over long time spans much better than does morphology, which is relatively uniform.

Characteristics of mtDNA sequences

Mitochondrial DNA sequences offer a number of advantages when studying the occurrence (or lack) of gene flow between taxa and the phylogeny of closely related species. In warm-blooded vertebrates, mitochondrial substitution rates are approximately ten times higher than in nuclear genes (Miyata *et al.* 1982, Wilson *et al.* 1985), resulting in a greater temporal resolution of evolutionary events than is reflected in nuclear encoded protein genes. Inheritance of mitochondrial DNA is clonal (no recombination), which means that events of fertile hybridization and back-crossing, even if they occur rarely or have occurred in the relatively distant past, may potentially be reflected in the mitochondrial genetic make-up of a species. Several cases of mitochondrial gene transfer across species boundaries have been documented or suspected (Wilson *et al.* 1985, Tegelström 1987, Aubert & Solignac 1990, Seibold *et al.* 1993), although the majority of such events, at least if they are relatively rare, will probably remain undetected because of stochastic extinction of mitochondrial lineages.

Inheritance of mitochondrial DNA is generally maternal.