Male-biased gene flow across an avian hybrid zone: evidence from mitochondrial and microsatellite DNA

Helbig, AJ; Salomon, M; Bensch, Staffan; Seibold, I

Published in:
Journal of evolutionary biology

DOI:
10.1046/j.1420-9101.2001.00273.x

2001

Male-biased gene flow across an avian hybrid zone: evidence from mitochondrial and microsatellite DNA

A. J. HELBIG,* M. SALOMON,† S. BENSCH‡ & I. SEIBOLD*

*University of Greifswald, Vogelwarte Hiddensee, Kloster, Germany
†Université de Rennes I, UFR SVE Campus de Beaulieu, Rennes, France
‡Molecular Population Biology Lab, Department of Animal Ecology, University of Lund, Lund, Sweden

Keywords:

Haldane's rule;
hybrid zone;
leaf-warblers;
microsatellites;
mtDNA;
reproductive isolation.

Abstract

Mating pattern and gene flow were studied in the contact zone between two morphologically very similar Chiffchaff taxa (*Phylloscopus collybita, P. brehmii*) in SW France and northern Spain. Mating was assortative in *brehmii*, but not in *collybita*. Mixed matings were strongly asymmetric (excess of *collybita* male × *brehmii* female pairs), but did produce viable offspring in some cases. Sequence divergence of the mitochondrial cytochrome *b* gene was 4.6%. Haplotypes segregated significantly with phenotype (only five 'mismatches' among 94 individuals), demonstrating that mitochondrial gene flow was very restricted. The estimated proportion of F_1 hybrids in the reproductive population was significantly lower than expected under a closed population model, indicating strong selection against hybrids. Genetic typing of 101 individuals at four microsatellite loci also showed significant population differentiation, but nuclear gene flow was estimated to be 75 times higher than mitochondrial gene flow. This strong discrepancy is probably due to unisexual hybrid sterility (Haldane's rule). Thus, there is a strong, but incomplete, reproductive barrier between these taxa.

Introduction

In the Palearctic bird fauna there are many cases of contact zones, where two phenotypically similar but distinct taxa come into contact and hybridize to a limited extent (Haffer, 1989, 1992; Hewitt, 1996; Martens, 1996). These hybrid zones are generally interpreted as zones of secondary contact of closely related taxa which diverged in geographical isolation, but have not reached the state of complete reproductive incompatibility. Even if the frequency of hybridization has been assessed, the extent of gene flow across such zones is generally not known, because hybrid viability and hybrid fitness are difficult to measure in nature. The fact that phenotypes on both sides of a hybrid zone remain distinct is generally taken to indicate that there is a barrier to gene flow. Mechanisms constituting such barriers may be pre-zygotic (positive assortative mating among parental phenotypes) or post-zygotic (reduced fitness in one or both sexes of the hybrids).

In interspecific crosses, the situation is quite frequent that only one sex among the hybrids is less viable, sterile or has strongly impaired fertility. Haldane (1922) pointed out that in such cases it is the heterogametic sex which is sterile, a fact that has come to be known as 'Haldane's rule' (review: Wu *et al.*, 1996). In organisms where the heterogametic sex is the female, as in birds and lepidoptera, unisexual hybrid sterility should constitute a much stronger barrier to mitochondrial than to nuclear gene flow (Sperlich, 1993). In bird taxa which differentiated (in allopatry) with respect to their mtDNA, such differentiation should be maintained in zones of secondary contact even in the face of ongoing (nuclear) gene flow. This situation has indeed been well documented in two geographically separate contact zones between the Collared *Ficedula albicollis* and the Pied

Correspondence: Andreas J. Helbig, University of Greifswald, Vogelwarte Hiddensee, D-18565 Kloster, Germany.
Tel.: (49)-38300–212; fax: (49)-38300–50441;
e-mail: Helbig@mail.uni-greifswald.de

Flycatcher *F. hypoleuca* (Tegelström & Gelter, 1990; Gelter *et al.*, 1992; Sætre *et al.*, 1997a,b; 1999). Mating between these partially sympatric species is assortative, but they hybridize frequently on the island of Gotland (Sweden) and, more rarely, elsewhere (Haffer, 1993). Male hybrids were shown to be generally fertile, although some fitness reduction relative to males of the parental phenotypes does exist (Saetre *et al.*, 1999). Female hybrids, in contrast, appeared to be sterile, as none were ever found to reproduce in the wild and no mitochondrial gene flow was detected between the two taxa.

In the present paper we examine the extent of interbreeding and gene flow across another avian hybrid zone, that between two Chiffchaff taxa of the species-rich leaf–warbler genus *Phylloscopus* (Sylviidae). Based on previous studies (Helbig *et al.*, 1996; Salomon *et al.*, 1997) we regard the common Chiffchaff *P. [c.] collybita* and the Iberian Chiffchaff *P. [c.] brehmii* as members of the superspecies *Phylloscopus [collybita]*, which includes further allospecies on the Canary Islands (*canariensis*), in the Caucasus and Himalayas (*sindianus*) and in Siberia (*tristis*). *P. collybita* and *P. brehmii* form a narrow zone of contact and local sympatry in extreme SW France and northern Spain. They differ mostly in vocalizations, both territorial songs and calls, which allow unambiguous field identification of pure phenotypes of either sex. Morphologically the two forms are very similar, although males in the contact zone can be distinguished in the hand by a combination of three measurements (Salomon *et al.*, 1997). In areas where both occur, *collybita* and *brehmii* males are interspecifically territorial. Mixed pairs have been noted in the past, but the composition of pairs in the contact zone has only been studied based on small sample sizes (Salomon, 1987). Mitochondrial DNA sequences (cytochrome *b* gene) between the two forms differed by 4.6%, and a limited sample from the contact zone (21 *brehmii*, 19 *collybita*) did not reveal any mixing of haplotypes between the taxa (Helbig *et al.*, 1993, 1996).

We here examine the extent of gene flow between *collybita* and *brehmii* based on three sources of information: (1) the composition of breeding pairs in a large part of the contact zone based on field observations over several years; (2) the extent of congruence between phenotype (determined acoustically) and mitochondrial haplotype in a large sample of individuals; and (3) the degree of nuclear genetic differentiation using microsatellite markers. Use of two independent genetic marker systems is required, because levels of mitochondrial and nuclear gene flow are expected to differ. The results contribute to an understanding of the relative roles of pre-zygotic and post-zygotic isolation as mechanisms maintaining shape, width and geographical placement of hybrid zones.

Materials and methods

Field investigations

The *collybita – brehmii* contact zone was mapped by M. Salomon (with help of Joel Bried) in the years 1981–87, 1991–92 and 1995–97 in SW France and northern Spain. Within the zone of sympatry, where both taxa occur (Fig. 1), the composition of mated pairs was studied during the breeding season. Chiffchaffs usually form socially monogamous pairs and both sexes are strongly territorial at the height of the nesting season. Territories were located by listening for the typical song. Males were then lured into mist nets by song playback from a recorder placed underneath the net. Each individual was ringed and a blood sample was taken from the ulnar vein (years 1991–92,1995 only). Females did not respond as well to song playback, but were usually easy to identify as they flew around the singing male giving their taxon-specific contact calls (see sonogram in Salomon, 1987). Females calling in close proximity to a territorial male were assumed to be paired to that male. In almost all cases, only a single female was noticed on any given territory. Some pairs were observed when feeding nestlings, when both partners frequently uttered their diagnostic contact calls.

Some males utter a song containing elements of the typical songs of both taxa. These are called 'mixed-singers'. The phenomenon is well known among closely related passerine bird species (Helb *et al.*, 1985; Sorjonen, 1986). In general, song development in oscine passerines involves a prominent learning component (Catchpole & Slater, 1995). Mixed singers may be hybrids, or they may have acquired their song by partially copying a heterospecific tutor. Chiffchaffs in the field were classified into one of three acoustic phenotypes: *collybita, brehmii* or mixed singer. Classification of males was usually based on territorial song. Both sexes could also be identified by their contact calls. Male and female 'mixed singers' give distinct calls different from either pure phenotype (sonograms see Salomon, 1987; 1989). Although female chiffchaffs do not sing, we use the term 'mixed singers' also for females in order to have a uniform label for birds of both sexes producing mixed vocalizations. In this way we determined the composition of a total of 260 mated pairs spread over approximately 2000 km^2 in the area of sympatry.

Analysis of mating pattern

The contact zone between *collybita* and *brehmii* in SW France and northern Spain was divided into sectors of 5×5 km each (Fig. 1; cf. Salomon, 1987). In order to test whether the mating pattern differed from expectations under the assumption of a random mating process, we

defined seven categories of sectors depending on the relative frequencies of the phenotypes:
– allopatric areas = only one taxon present (*collybita*-allopatric; *brehmii*-allopatric)
– 50/50 sympatric = both taxa occur together at proportions not significantly different from parity (binomial test; see Schwartz, 1963).
– *collybita*-prevailing = sympatry with >50% (but less than 95%) *collybita* (binomial test: $P < 0.05$)
– *brehmii*-prevailing = sympatry with >50% (but less than 95%) *brehmii* (binomial test: $P < 0.05$)
– accidental sympatry = rarer taxon comprising <5% of all individuals (*'collybita* accidental'; *'brehmii* accidental').

From the 1980s to the 1990s we observed a widening and a south-westward shift of the contact zone at the expense of *brehmii*. Therefore the allocation of 25 km^2 sectors to the above categories was adjusted for the 1980s and the 1990s according to the relative abundance of the two taxa.

From the frequencies of the three phenotypes (*collybita*, *brehmii*, mixed singer) in each of these categories of sectors we calculated the frequencies of pairings expected under the random mating hypothesis and compared them with the observed frequencies. In the zone of sympatry (= all sectors where both taxa occurred), the phenotypic composition of a total of 260 mated pairs was investigated.

Mitochondrial genetics

Total cellular DNA was isolated from blood samples (50 μL) preserved in EDTA buffer. The cytochrome *b* gene was PCR amplified as a contiguous fragment 1100 bp long (as described by Helbig *et al*., 1995) and parts of this gene (300–1041 bp) were sequenced in 19 *collybita* and 21 *brehmii* from the contact zone in SW France. Two haplotypes, differing by 4.6% sequence divergence, were found (accession no. Z73476, Z73487). From the sequences a restriction site was determined which enabled us to distinguish between *brehmii* and *collybita* haplotypes using the restriction endonuclease Alu I. This enzyme cuts a 300-bp amplification product (primers A and B in Helbig *et al*., 1995) from *brehmii* mtDNA into two fragments of about equal length, but does not cut fragments amplified from *collybita* mtDNA. With this RFLP method we screened a further 64 individuals from the contact zone (26 *collybita*, 28 *brehmii*, nine mixed singers). Thus the mitochondrial haplotypes of a total of 103 individuals from the contact zone (94 pure phenotypes, nine mixed singers) were determined. For comparison, we also screened by RFLP 19 *collybita* (France, Germany) and 14 *brehmii* (southern Spain, Tunisia) sampled far away from the contact zone. Analysis of molecular variance was conducted with the program ARLEQUIN (Schneider *et al*., 1998).

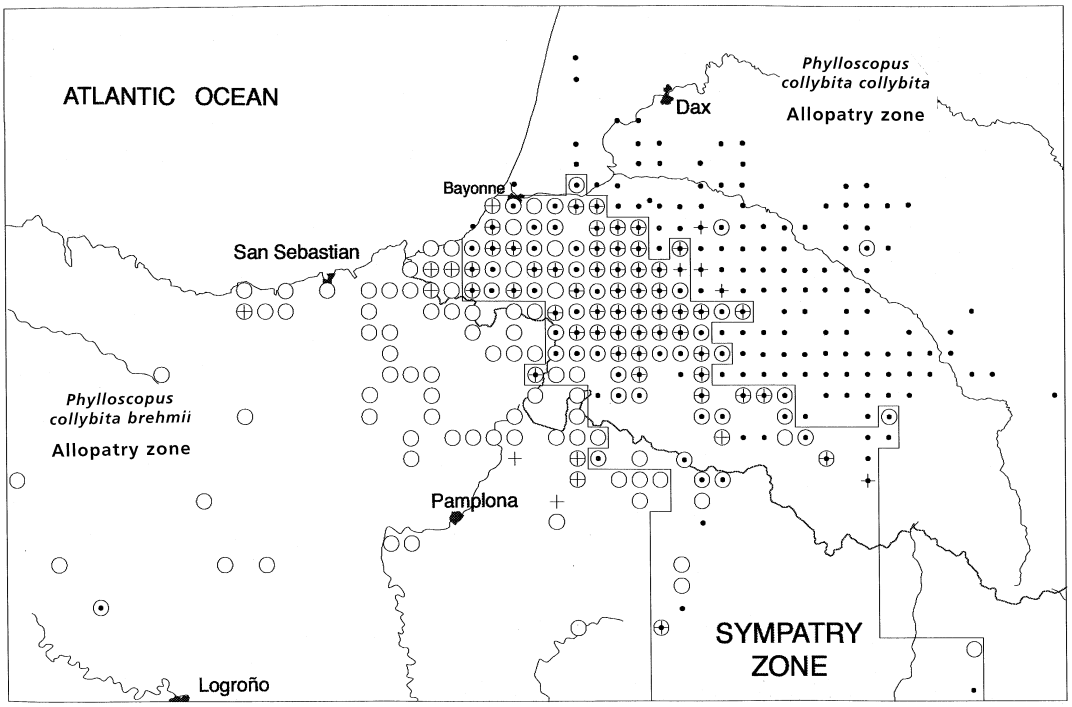

Fig. 1 Map of contact zone (5 × 5 km sectors) between two Chiffchaff taxa in SW France and northern Spain. The occurrence of singing males during the core breeding season (mid-May-June) in the years 1981–87, 1991–97 is indicated as follows; full dots = *collybita*; open circles = *brehmii*, crosses = mixed singers.

Pairing (*m* × *f*)	C > 95	C > 50	50 : 50	B > 50	B > 95	Total	χ^2
col × col	11	48	15	3	0	77	0.314
	12.02	*50.31*	*16.82*	*2.41*	*0.004*	*81.56*	
bre × bre	0	6	20	60	4	90	12.49***
	0.058	*3.352*	*10.97*	*44.36*	*3.399*	*62.14*	
bre × col	1	15	18	8	0	42	0.566
	0.833	*12.99*	*13.59*	*10.34*	*0.115*	*37.87*	
col × bre	2	4	2	2	0	10	21.25***
	0.833	*12.99*	*13.59*	*10.34*	*0.115*	*37.87*	
col × mix	0	3	1	1	0	5	3.19
	0.119	*6.201*	*2.660*	*1.223*	*0.006*	*10.21*	
mix × col	0	13	4	2	0	19	8.45**
	0.119	*6.201*	*2.660*	*1.223*	*0.006*	*10.21*	
bre × mix	0	1	0	2	0	3	4.156*
	0.008	*1.601*	*2.148*	*5.245*	*0.173*	*9.175*	
mix × bre	0	5	4	1	0	10	0.191
	0.008	*1.601*	*2.148*	*5.245*	*0.173*	*9.175*	
mix × mix	0	1	1	2	0	4	3.97*
	0.001	*0.764*	*0.421*	*0.620*	*0.009*	*1.815*	
Total	14	96	65	81	4	260	

Table 1 Observed and *expected* frequencies of pair combinations in the zone of sympatry. Sectors in the zone were assigned to five categories according to the relative abundance of *collybita* (C) and *brehmii* (B) (see text).

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

Microsatellites

A total of 105 individuals from the contact zone (48 *collybita*, 48 *brehmii*, nine mixed singers, containing all 103 birds whose mitochondrial haplotype was known) and 25 individuals from allopatric populations (11 *collybita* and 14 *brehnmii*) were typed for allelic length variation at four heterologous microsatellite loci. These markers were developed for three other passerine birds: Pocc1 and Pocc6 were isolated from *Phylloscopus occipitalis* (Bensch *et al*., 1997), Phtr1 from *Phylloscopus trochilus* (Fridolfsson *et al*., 1997) and LS2 from *Lanius ludovicianus* (Mundy & Woodruff, 1996). The PCR amplifications were performed under the following conditions: Precycling incubation at 94 °C for 3 min, then 28 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C and finally a further 5 min at 72 °C. Reactions of 10 μL included 20–50 ng of total genomic DNA, 0.2 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.4 μM of reverse primer, 0.2 μM of unlabelled forward primer, 0.2 μM of ^{32}P-ATP labelled forward primer and 0.3 units of Taq DNA polymerase. The PCR products were resolved on 8% denaturing polyacrylamide gels. Gels were dried and exposed for 3–48 h. The R_{st} and N_m values between populations were calculated using the program RstCalc (version 2.2; Goodman, 1997) and significance levels determined with permutation tests. Deviation from Hardy–Weinberg equilibrium was tested in GenePop 3.1 (Raymond & Rousset, 1995). To examine the congruence between the nuclear, mitochondrial and phenotypic data, we employed an assignment test based on the microsatellite allele frequencies (Paetkau *et al*., 1995; 1997), using a test calculator available at the web site http://gause.biology.ualberta.ca/jbrzusto/.

Results

Mating pattern

Both partners of a total of 260 pairs ($n = 520$ individuals) were identified in the zone of sympatry. We distinguished nine pair combinations according to sex and phenotype (*collybita, brehmii*, mixed singer). The observed and expected frequencies of pairs across these nine combinations are listed in Table 1. Within the sympatry zone, the observed frequencies of pairings differed significantly from the expected values for the following combinations:

1 In homospecific pairs, we found strong positive assortative mating among *brehmii*, but not among *collybita* individuals.

2 Mixed matings were strongly asymmetric with *brehmii* females discriminating against *collybita* males, whereas *collybita* females were paired to *brehmii* males about as often as expected under a random mating hypothesis.

3 Overall we found 45 (8.65%) mixed singers among the 520 mated individuals identified in the sympatry zone. There was a strong sex bias among mixed singers with females being significantly underrepresented (12 females vs. 33 males; $\chi_1^2 = 4.9$; d.f. = 1; $P < 0.05$).

4 Male mixed singers were overrepresented as partners of *collybita* females, but not of *brehmii* females.

Chiffchaff nests can be located in the field by following adults carrying food for nestlings, but are very difficult to find. We located only 19 nests with young (without extensive searching). Twelve of these nests belonged to pairs of the same phenotype (containing, on average, 4.1 young; range 2–6), two nests were of mixed parentage

($\bar{x} = 1.5$ young, range 1–2) and five involved one mixed singer ($\bar{x} = 4.0$ young; range 3–5). Mixed pairs, therefore, can produce offspring viable at least to an advanced nestling stage. The numbers are too low for statistical analysis, but, if anything, they indicate lower reproductive success in mixed compared with pure pairs.

Mitochondrial genetics

Out of a total of 94 pure phenotypes investigated from the contact zone, 89 (94.7%) had the 'correct' mitochondrial haplotype, i.e. the one predominating within their respective population. In five individuals there was a mismatch between acoustic phenotype and mitochondrial haplotype (Table 2). The null hypothesis of random distribution of haplotypes with respect to acoustic phenotype was rejected with high confidence ($\chi_2^2 = 73.5$; d.f. = 2; $P < 0.001$). Among the nine mixed singers, four had a *brehmii* and five had a *collybita* haplotype.

An analysis of molecular variance (Excoffier *et al.*, 1992) for the 94 birds of pure phenotype showed that 88.49% of the variance was distributed between phenotypes ($\Phi_{st} = 0.885$) and haplotype frequencies differed significantly between the two phenotypic categories ($P < 0.001$; 1000 permutations). Assuming an island model of population structure, this corresponds to 0.065 female migrants per generation between *brehmii* and *collybita* populations.

Sequencing of up to 1041 nucleotides (cytochrome *b* gene) per individual revealed seven haplotypes among 18 *collybita* and seven haplotypes among 21 *brehmii* from the contact zone. There was, therefore, no obvious difference in mitochondrial genetic diversity between the two taxa. Within each taxon, haplotypes differed only by one or two nucleotides, whereas between taxa there were 48 fixed differences (4.6% sequence divergence, see above). All birds from the allopatry zones (19 *collybita* from northern France and Germany, 13 *brehmii* from southern Spain and one from Tunisia) carried haplotypes expected for the respective taxon. Except for single nucleotide differences, *collybita* haplotypes from the contact zone were identical with those found in eight birds breeding near Radolfzell, SW Germany, i.e. 1500 km further NE in *collybita's* range. *Brehmii* haplotypes in the contact zone were identical to those found in five individuals (caught on breeding territory) in Andalusia, southern Spain (600 km SW of contact zone), although they differed by four nucleotides from a *brehmii* individual (singing male) caught in Tunisia. These data show that mitochondrial genotypes are uniform across large areas within each taxon's range, but change abruptly at the contact zone.

Table 2 Frequencies of mitochondrial haplotypes in Chiffchaffs *Phylloscopus collybita* and *P. brehmii* (n = 102).

	Mitochondrial haplotype	
Phenotype	*collybita*	*brehmii*
collybita	41	4
brehmii	1	48
Mixed singer	5	4

Microsatellite data

The frequencies of allelic length variants at the four loci are shown in Table 3 for each of the parental phenotypes and the mixed singers in the contact zone (sympatry) and from allopatric populations of *brehmii* and *collybita.* There were no private alleles at substantial frequencies in either the *collybita* or *brehmii* population. The observed levels of heterozygosity did not differ significantly from the expected values for any of the phenotypic groups or loci ($P > 0.1$, Hardy–Weinberg test, P-values estimated by the Markov chain method in GenePop 3.1). However, the expected heterozygosity tended to be lower in *brehmii* than in *collybita* (pairwise t-test on sympatric samples: $t = 2.96$; d.f. = 3; $P = 0.059$). Permutation tests using individuals as replicates and considering each locus separately (with Bonferroni correction across loci) showed that heterozygosity was significantly lower in *brehmii* than in *collybita* at loci Pocc6 and LS2.

There was no evidence of genetic differences at the four microsatellite loci between the allopatric and sympatric populations in either *collybita* ($R_{st} = 0.026$; $P = 0.11$) or *brehmii* ($R_{st} = 0.012$; $P = 0.25$). This is in agreement with the mitochondrial DNA results, which showed a uniform distribution of haplotypes across large geographical areas within each taxon. Hence for the following analyses, we pooled the samples of allopatric and sympatric populations for each taxon. For all four loci combined, we detected a significant difference in allele frequencies between *brehmii* and *collybita* ($R_{st} = 0.048$; $P = 0.001$). However, a closer inspection of the data (Table 4) showed that *collybita* and *brehmii* were significantly divergent at one locus (LS2) only, and in fact appeared panmictic at the other three loci. At locus LS2, *brehmii* is almost fixed for the allele 188 bp whereas a longer allele of 189 bp is present at >20% in the two samples of *collybita* (Table 3). The overall estimate of gene flow from the four microsatellite markers is $N_m = 4.9$ (95% confidence interval 2.4–11.7). If calculated on the most differentiated locus (LS2), our estimate of the nuclear gene flow is 1.3 migrants per generation (95% confidence interval 0.6–3.4). Thus, our analyses of microsatellites suggest that the nuclear gene flow between *brehmii* and *collybita* is well over one order of magnitude larger than the estimated mitochondrial gene flow.

Numerically, the mixed singers were more similar to *collybita* ($R_{st} = -0.015$; $N_m \gg 100$) than to *brehmii* ($R_{st} = 0.009$; $N_m = 28$), but none of these R_{st} estimates was significantly different from zero. It should be noted

Table 3 Allele frequencies, expected heterozygosity (H_E) and observed heterozygosity (H_O) of three phenotypic groups of chiffchaffs at four microsatellite loci. Sample sizes in parentheses. Allele sizes are given in number of base pairs (bold).

Locus	Population†	Alleles (size in bp) and their proportions											H_E*	H_O
Pocc1		**203**	**205**	**207**	**209**	**211**	**213**	**215**						
(14)	*brehmii* A		0.57	0.18	0.25								0.58	0.57
(48)	*brehmii* S	0.05	0.75	0.10	0.08	0.02							0.42	0.38
(9)	Mixed singers		0.67	0.17	0.11			0.05					0.51	0.56
(48)	*collybita* S	0.03	0.65	0.21	0.05	0.05	0.01						0.53	0.56
(11)	*collybita* A		0.73	0.23		0.04							0.41	0.27
Pocc6	**174**	**176**	**178**	**180**	**182**	**184**	**186**	**188**	**190**	**192**	**194**	**200**		
(14)	*brehmii* A		0.10		0.68	0.04	0.14	0.04					0.53	0.57
(48)	*brehmii* S	0.01	0.04	0.02	0.68	0.15	0.07	0.02				0.01	0.51	0.48
(9)	Mixed singers	0.06	0.06	0.06	0.43	0.16	0.06	0.06		0.11			0.75	0.78
47)	*collybita* S	0.03	0.16	0.03	0.25	0.18	0.14	0.13	0.02	0.03	0.01	0.01	0.84	0.89
(11)	*collybita* A	0.09	0.36		0.23	0.14		0.14		0.04			0.77	0.82
Phtr1		**88**	**90**	**92**	**94**	**96**	**100**	**102**	**104**					
(14)	*brehmii* A		0.14	0.54	0.32								0.59	0.50
(48)	*brehmii* S		0.14	0.61	0.24				0.01				0.55	0.54
(9)	Mixed singers			0.72	0.28								0.40	0.56
(48)	*collybita* S	0.03	0.07	0.53	0.31	0.04	0.01	0.01					0.61	0.75
(11)	*collybita* A			0.86	0.14								0.24	0.27
LS2		**188**	**189**											
(14)	*brehmii* A	0.96	0.04										0.08	0.07
(48)	*brehmii* S	0.95	0.05										0.10	0.10
(9)	Mixed singers	0.83	0.17										0.28	0.33
(48)	*collybita* S	0.72	0.28										0.40	0.48
(11)	*collybita* A	0.77	0.23										0.35	0.45

* = Under the assumption of Hardy–Weinberg equilibrium , † = Samples from outside (A = allopatric) or within (S = sympatric) the contact zone.

that these tests have low statistical power because the data set contains only nine mixed singers.

To investigate the match between phenotype, mitochondrial haplotype and microsatellite allelic profiles of individuals, we carried out an assignment test (Paetkau *et al.*, 1995; Paetkau *et al.*, 1997). We grouped the birds according to their mitochondrial haplotype and used the allelic distributions at the four microsatellite loci to calculate for each individual the log-likelihood of origin from either the *collybita* or the *brehmii* population (Fig. 2). Overall, the two groups were only slightly divergent with few likelihood estimates differing by more than two orders of magnitude, suggesting that the risk of misassignment caused by sampling error is relatively high. Despite this, the assignment test correctly classified 78% of the birds with a song type matching their mitochondrial haplotype. In contrast, four of the nine mixed singers were misassigned (Fig. 2). Two of these had a *brehmii* mt haplotype but were assigned to *collybita* on their microsatellites, and two had a *collybita* mt haplotype but were assigned to *brehmii*. Of the five birds with a mismatch between song type and mitochondrial haplotype, three were misassigned (Fig. 2). Thus, the success rate of the assigment test was significantly higher (Fisher's Exact test, two-tailed, $P = 0.045$) for the group of birds with a song type matching their mitochondrial haplotype (78%) than for the pooled group of mixed singers and mismatched birds (50%).

Table 4 Population differentiation (R_{st}) and gene flow (N_m) between *collybita* (n = 59) and *brehmii* (n = 62) calculated from allele frequencies at four microsatellite loci.

Locus	R_{st}	N_m (95% CI)*	P
Pocc1	–0.0021	»100	0.4
Pocc6	0.01508	16.3	0.12
Phtr1	0.00740	33.5	0.21
LS2	0.16219	1.3 (0.6–3.4)	<0.0001
Total	0.04827	4.9 (2.3–11.7)	0.001

*Confidence interval (CI) given for significant N_m estimates.

Frequency of hybrids

An interesting question is whether the observed proportion of hybrids in the reproductive population is as large as would be expected from the observed mating pattern. To be conservative, we restrict our discussion to the frequency of F_1-hybrids, although back-cross hybrids may also occur. These would be expected to further increase the number of birds with a mtDNA/phenotype mismatch. Assuming that fitness is equal

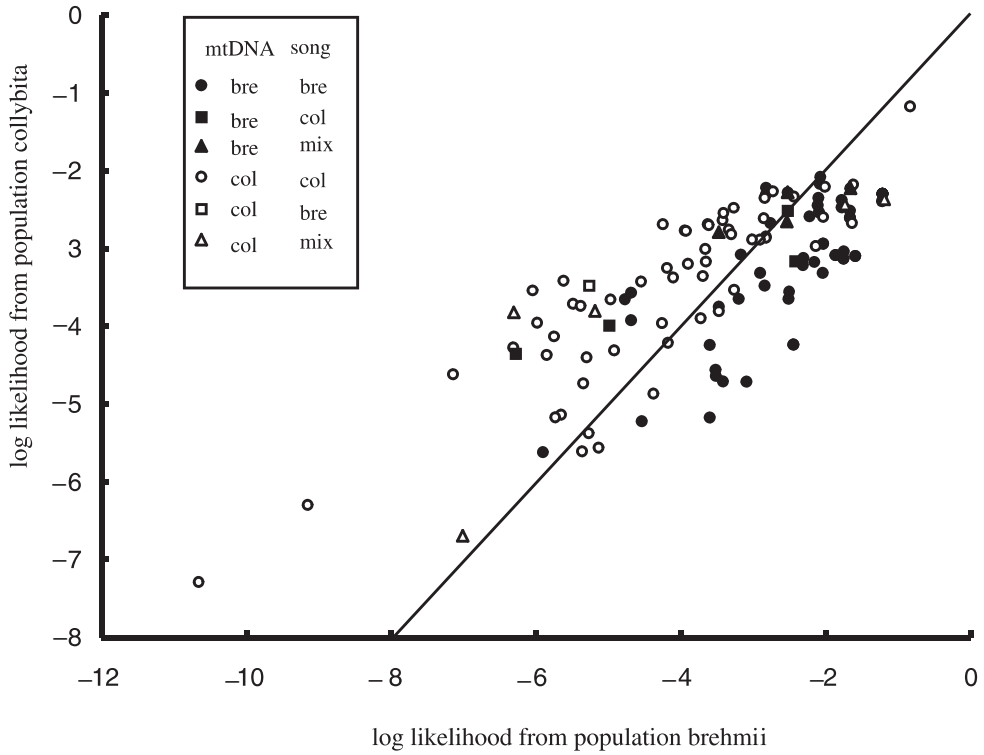

Fig. 2 Results of assignment test for origin of individuals from *brehmii* and *collybita* populations, respectively, based on allelic frequencies at four microsatellite loci. Mitochondrial haplotypes are indicated by filled symbols for *brehmii* and open symbols for *collybita*. Circles denote individuals with a song type matching their mitochondrial haplotype; squares, individuals with a mismatch between song type and mitochondrial haplotype; triangles show individuals with mixed vocalizations.

between pure and mixed pairs and that the contact zone is a closed population, the expected proportion of F_1-hybrids is 23.7% (i.e. equal to the proportion of mixed pairs among all pairs involving pure phenotypes [$n = 219$; see Table 1]). One indication of the actual proportion of hybrids in the reproductive population is the number of mixed singers, although we do not know whether they are all hybrids. Mixed singers make up 8.65% of the population ($n = 45$ out of 520 individuals), i.e. much lower than the expected frequency of hybrids. However, this could be an underestimate, because some hybrids may only produce vocalizations of one taxon. The number of F_1-hybrids not detected acoustically can be estimated from the frequency of individuals with a mismatch between song type and mitochondrial haplotype (of 94 genotyped birds five were 'mismatched' = 5.32%). The underlying assumption is that male fledglings learn the song of their father. The total number of F_1-hybrids can then be estimated as the proportion of mtDNA/phenotype mismatches plus the proportion of mixed singers, which together make up 13.97%. This is significantly lower than the expected 23.7% (tested with absolute numbers of individuals: $\chi_2^2 = 22.3$; d.f. = 1; $P < 0.001$).

We conclude that within the area of sympatry there are fewer F_1-hybrids in the reproductive population than expected from the observed mating pattern.

Discussion

Among the 10 taxa of the Chiffchaff complex, there are at least three zones of secondary contact across which gene flow seems to be restricted: between *abietinus* and *tristis* in western Siberia, between *caucasicus* and *lorenzii* in the Caucasus Mts. and between *collybita* and *brehmii* in SW France/N Spain (see map in Helbig *et al*., 1996). A fourth contact zone is about to form in the near future when *collybita*, which is spreading northward in Scandinavia, will meet *abietinus* in central Sweden (Hansson *et al*. 2000). The *collybita/brehmii* contact zone is the first to be studied in any detail (Salomon, 1987; Salomon & Hemim, 1992; Salomon *et al*., 1997). It conforms to Short's (1969) definition of a 'zone of overlap and hybridization', where the two 'pure' phenotypes predominate, but hybridization does occur. It is worth noting that *brehmii* represents the most divergent (i.e. presumably most ancient) lineage within the Chiffchaff complex based on mtDNA (Helbig *et al*., 1996). Thus

collybita and *brehmii* are not sister taxa. The width of a hybrid zone can only be defined with reference to particular characters (Barton & Gale, 1993), changes of which may vary both in steepness and geographical location (Parson *et al.*, 1993). In the case of the contact zone between *collybita* and *brehmii*, the change in morphological characters and in mtDNA coincides geographically along a transect of 20 km. Whether such a zone is 'narrow' can be evaluated in relation to dispersal distances (Barton & Gale, 1993). To our knowledge, root mean square dispersal distances (σ) in this or other Chiffchaff populations are not available, but are likely several tens of km in migratory warblers (Paradis *et al.*, 1998; Rohwer & Wood, 1998). According to the model in Barton & Gale (1993), the contact zone between *brehmii* and *collybita* should be 240 km wide after 10 generations of contact, assuming no selection against hybrids and a root mean square dispersal distance of 30 km. Clearly, the present contact zone of 20 km in width is relatively narrow and must be maintained by selection against mixing. In agreement with this observation, all three lines of evidence, mating patterns, mitochondrial DNA and microsatellite markers, indicate significantly reduced gene flow across the contact zone between the two chiffchaff taxa.

Mating pattern

Although the rate of heterospecific pairs is quite high (23.7%) in the zone of sympatry, mating is still assortative. However, individuals of the two taxa did not seem to be equally selective: assuming that female mate choice determines pair composition, we found that *brehmii* females preferred *brehmii* males, whereas they avoided *collybita* and mixed singer males. In contrast, *collybita* females did not avoid *brehmii* males and even seemed to prefer mixed-singer males. It is unclear why *collybita* females are less discriminating in favour of males of their own phenotype. In this context it is of interest that mixed singers are indistinguishable from *collybita*, but differ from *brehmii*, in morphology (Salomon *et al.*, 1997). Moreover, the allele frequencies at the analysed microsatellite loci suggest closer resemblance of mixed singers to *collybita* than to *brehmii*. Perhaps male mixed singers are more likely to be genetically pure *collybita* which learned the *brehmii* song than vice versa. This would mean that the fitness cost of choosing a mixed singer male is less for *collybita* females than it is for *brehmii* females.

Among mixed singers, there was a significant sex bias with females being under-represented (12 females, 33 males). This could reflect poorer survival or mating success of female vs. male F_1-hybrids (assuming that mixed singers are mostly hybrids). However, the deviation from a 1 : 1 sex ratio may also be influenced by the way birds were classified: mixed vocalizations may be less likely to be detected in females than in males, because females produce only contact calls, whereas males also produce prolonged territorial song.

Frequency of hybrids and barriers to gene flow

The assignment test demonstrated a relatively good match between the microsatellite allele profiles of individuals and their mitochondrial haplotypes, suggesting that the majority of the birds within the contact zone consist of pure genotypes. For mixed singers and birds with a mismatch between song type and mitochondrial haplotype, a higher proportion of individuals was misassigned. Thus, these two groups appear to contain more individuals with a mismatch between their mtDNA and nuclear genome, which suggests they are F_1 or backcross hybrids.

We found a significantly lower proportion of potential hybrids in the reproductive population than would be expected under the assumption that pure and mixed pairs have the same fitness. Our estimate of the number of potential F_1-hybrids in the reproductive population was based on three assumptions: (1) all mixed singers are indeed hybrids; (2) all birds with a mismatch between acoustic phenotype and mitochondrial haplotype are hybrids; (3) young males copy the song type of their father rather than the song type predominant in the population. The first two assumptions are conservative, the third may lead to an underestimation of the hybrid frequency. Nonetheless, there does appear to be a discrepancy between the observed and expected number of hybrids, which may be because of the fact that one or several of the following fitness components are negatively affected in mixed matings: (1) reproductive output may be lower in mixed compared with pure pairs (2) survival of hybrids may be lower compared with nonhybrids (3) pairing success of hybrids may be lower than that of nonhybrids and (4) F_1-hybrids may show reduced fertility. An alternative, nonexclusive explanation is that our assumption of the sympatric population being closed is not fulfilled. If a substantial fraction of the birds that are hatched in the relatively narrow sympatric zone disperse and settle as breeders well into the allopatric range of either *collybita* or *brehmii*, many hybrids will escape detection.

Our field data on reproductive output are too limited to test whether mixed pairs experience reduced reproductive success relative to pure pairs. The restricted gene flow we observed is perhaps more likely because of some post-fledging selective disadvantage of the hybrids. Apart from the observation of a lower than expected proportion of hybrids, no data are available on survival, pairing success or fecundity of hybrids.

In our opinion, the strong association (lack of mixing) between song phenotype and mitochondrial haplotype cannot be explained by lowered hybrid survival alone. Even if only 5.4% of the entire reproductive population (i.e. the proportion of 'mismatched' birds observed) were

hybrids, there should still be considerable mitochondrial introgression. The fact that this has not occurred, although the hybrid zone is certainly not of very recent origin, indicates that the reproductive performance of female F_1-hybrids must be significantly reduced compared with nonhybrid females.

The microsatellite data indicate that *collybita* and *brehmii* are much less differentiated in nuclear than in mitochondrial DNA. In fact, for three of the microsatellite loci, the two taxa seemed panmictic. Using all four microsatellite loci, the nuclear gene flow is estimated to be about five migrants per generation, i.e. 75 times higher than the mitochondrial gene flow. This estimate is still low compared with the high proportion (23.7%) of mixed pairs actually observed (considering only pairs in which both partners were pure phenotypes). If we assume that it takes 10 hybridization events to produce one successful migrant, the microsatellite data let us expect only 50 mixed pairs per year in the entire zone of symaptry (containing several thousand breeding pairs). This contrasts with our finding of 52 mixed pairs among 260 studied. The proportion of successful migrants transmitting nuclear genes from one taxon to the other is thus much lower than expected from the frequency of hybridization, indicating a significant hybrid fitness reduction.

In hybridization, nuclear genes can be transmitted from one taxon to the other via both males and females, whereas mitochondrial gene transfer requires reproductively viable female hybrids. Thus, the discrepancy between nuclear and mitochondrial gene flow indicates that male hybrids either survive better or have higher fecundity than female hybrids. The latter would be in agreement with predictions from Haldane's rule (Haldane, 1922). Field data are needed to measure the relative fitness of hybrids directly, but this would require a long-term ringing study which has not yet been conducted in the contact zone between these *Phylloscopus* taxa.

The lower heterozygosity of *brehmii* compared with *collybita* indicates a smaller effective population size of the former or perhaps a relatively recent bottleneck. The first prediction is consistent with available information on current population sizes: *collybita* has a very large range and is one of the commonest insectivorous passerines in Western Europe (Tiainen & Wesolowski, 1997). *Phylloscopus brehmii*, on the other hand, is restricted to the western and southern parts of the Iberian Peninsula and a small area in NW Africa. Within its range it is far less common than is *collybita* in Western Europe (Purroy Iraizoz, 1997).

The role of pre- and post-mating isolation in avian hybrid zones

Our results suggest that both pre-zygotic and post-zygotic isolating mechanisms are contributing to the maintenance of the sharp hybrid zone between *collybita* and *brehmii*. Similar cases of narrow hybrid zones have been described for pairs of taxa in both Europe (Becker, 1995; Sætre *et al*., 1997a; Faivre *et al*., 1999) and North America (e.g. Moore & Price, 1993). Hybrids between well differentiated species might suffer reduced fitness resulting from genetic incompatibility (Coyne & Orr, 1998), Haldane's rule being a special case in which a fitness reduction occurs mainly in the heterogametic sex. The much higher gene flow we estimated for nuclear vs. mitochondrial DNA, suggests that reduced fitness of female hybrids is contributing to the gene flow barrier between *collybita* and *brehmii*. However, well defined contact zones also occur between taxa less differentiated in mt DNA than in the chiffchaff case (Saino & Villa, 1992; Rohwer & Wood, 1998; Bensch *et al*., 1999). It is unclear whether some degree of genetic incompatibility is a necessary prerequisite for the formation of hybrid zones and whether there is a minimum level of overall genetic divergence that coincides with incompatibility (Coyne & Orr, 1998). For example, the Willow Warbler subspecies *Phylloscopus trochilus trochilus* and *P. t. acredula* are identical in mt DNA and show an intergradation zone of 300 km in width across Scandinavia (Bensch *et al*., 1999). This zone coincides with a migratory divide (Chamberlain *et al*. 2000) and is apparently maintained by selection against hybrids on their migratory direction (Bensch *et al*., 1999). The contact zone between Carrion *Corvus corone corone* and Hooded Crows *C. c. cornix* in Central Europe is maintained both by hybrid fitness reduction (Saino & Villa, 1992) and assortative mating (Risch & Andersen, 1998). In Hermit and Townsend's Warblers (*Dendroica occidentalis* and *D. townsendi*), with a mitochondrial DNA sequence distance of less than 1% (Lovette *et al*., 1999), the width of the contact zone is presumably maintained by a balance between dispersal and selection against hybrids (Rohwer & Wood, 1998). There is evidence that this contact zone has been moving, with dominant Townsend's Warblers excluding Hermit Warblers from much of their former range (Rohwer & Wood, 1998).

Further work is required to better understand whether avian hybrid zones are primarily maintained by pre-zygotic or post-zygotic isolating mechanisms and to what extent the latter consist of genetic incompatibility or natural selection on some ecological or behavioural trait that may confer hybrid disadvantage. Apart from estimates of mitochondrial and nuclear gene flow as we provided them here, a more direct assessment of hybrid fitness would be desirable. Ideally, this should be based on measures of life-time reproductive success in hybrids vs. parental phenotypes. However, in relatively long-lived birds, obtaining such data requires a considerable effort.

Acknowledgments

This work was in part supported by a research fellowship by the Deutsche Forschungsgemeinschaft (He 1792/2–1) to the first author. Financial support from the French Ministry of the Environment to MS and from the

Swedish Natural Science Research Council and the Royal Physiographic Society to SB is gratefully acknowledged. MS is grateful to Joel Bried and Didier Vangeduwe for their valuable technical support, to the CRBPO for the capture and ringing material.

References

Barton, N.H. & Gale, K.S. 1993. Genetic analysis of hybrid zones. In: *Hybrid Zones and the Evolutionary Process* (R. G. Harrison, ed.), pp. 13–45. Oxford University Press, New York.

Becker, J. 1995. Sympatrisches Vorkommen und Hybridisierung von Sprosser *Luscinia luscinia* und Nachtigall *L. megarhynchos* bei Frankfurt (Oder), Brandenburg. *Vogelwelt* **116**: 109–118.

Bensch, S., Andersson, T. & Åkesson, S. 1999. Morphological and molecular variation across a migratory divide in willow warblers, *Phylloscopus trochilus*. *Evolution* **53**: 1925–1935.

Bensch, S., Price, T. & Kohn, J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. *Mol. Ecol.* **6**: 91–92.

Catchpole, C.K. & Slater, P.J.B. 1995. *Bird Song. Biological Themes and Variations.* Cambridge University Press. Cambridge.

Chamberlain, C.P., Bensch, S., Feng, X., Åkesson, S. & Andersson, T. 2000. Stable isotopes examined across a migratory divide in Scandinavian willow warblers (*Phylloscopus trochilus trochilus* and *Phylloscopus trochilus acredula*) reflect their African winter quarters. *Proc. R. Soc. Lond. B* **267**: 43–48.

Coyne, J.A. & Orr, H.A. 1998. The evolutionary genetics of speciation. *Phil. Trans. R. Soc. Lond. B* **353**: 287–305.

Excoffier, L., Smouse, P.E. & Quattro, J.M. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to mitochondrial DNA restriction data. *Genetics* **131**: 479–491.

Faivre, B., Secondi, J., Ferry, C., Chastragnat, L. & Cézilly, F. 1999. Morphological variation and the recent evolution of wing length in the icterine warbler: a case of unidirectional introgression? *J. Avian Biol.* **30**: 152–158.

Fridolfsson, A.-K., Gyllensten, U. & Jakobsson, S. 1997. Microsatellite markers for paternity testing in the willow warbler *Phylloscopus trochilus*. *Hereditas* **126**: 127–132.

Gelter, H., Tegelström, H. & Gustafsson, L. 1992. Evidence from hatching success and DNA fingerprinting for the fertility of hybrid Pied x Collared Flycatchers *Ficedula hypoleuca* x *albicollis*. *Ibis* **134**: 62–68.

Goodman, S.J. 1997. Rst Calc: a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. *Mol. Ecol.* **6**: 881–885.

Haffer, J. 1989. Parapatrische Vogelarten der paläarktischen Region. *J. Orn.* **130**: 475–512.

Haffer, J. 1992. Parapatric species of birds. *Bull. Brit. Orn. Club* **112**: 250–261.

Haffer, J. 1993. Gattung *Ficedula* Brisson 1760. In: *Handbuch der Vögel Mitteleuropas* (U. N. Glutz von Blotzheim & K. M. Bauer, eds), Vol. 13, pp. 80–83. Aula-Verlag, Wiesbaden.

Haldane, J.B.S. 1922. Sex ratio and unisexual sterility in hybrid animals. *J. Genet.* **12**: 101–109.

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

Helb, H.-W., Dowsett-Lemaire, F., Bergmann, H.-H. & Conrads, K. 1985. Mixed singing in European songbirds – a review. *Z. Tierpsychol.* **69**: 27–41.

Helbig, A.J., Salomon, M., Wink, M. & Bried, J. 1993. Absence de flux génique mitochondrial entre les Pouillots 'véloces' médio-européen et ibérique (Aves: *Phylloscopus collybita collybita, P.* (*c.*) *brehmii*). *Comptes Rendues Acad. Sci. Paris* **316:** 205–210.

Helbig, A.J., Seibold, I., Martens, J. & Wink, M. 1995. Genetic differentiation and phylogenetic relationships of Bonelli's Warbler *Phylloscopus bonelli* and Green Warbler *P. nitidus*. *J. Avian Biol.* **26**: 139–153.

Helbig, A.J., Martens, J., Henning, F., Schottler, B., Seibold, I. & Wink, M. 1996. Phylogeny and species limits in the Palaearctic Chiffchaff *Phylloscopus collybita* complex: mitochondrial genetic differentiation and bioacoustic evidence. *Ibis* **138**: 650–666.

Hewitt, G.M. 1996. Some genetic consequences of ice ages, and their role in divergence and speciation. *Biol. J. Linn. Soc.* **58**: 247–276.

Lovette, I.J., Bermingham, E., Rohwer, S. & Wood, C. 1999. Mitochondrial RFLP and sequence variation among closely related avian species and the genetic characterization of hybrid *Dendroica* warblers. *Mol. Ecol.* **8**: 1431–1441.

Martens, J. 1996. Vocalizations and speciation of Palearctic birds. In: *Ecology and Evolution of Acoustic Communication in Birds* (D. E. Kroodsma & E. H. Miller, eds), pp. 221–240. Cornell University Press, Ithaca.

Moore, W.S. & Price, J.T. 1993. Nature of selection in the northern flicker hybrid zone and its implications for speciation theory. In: *Hybrid Zones and the Evolutionary Process* (R. G. Harrison, ed.), pp. 196–225. Oxford University Press, New York.

Mundy, N.I. & Woodruff, D.S. 1996. Polymorphic microsatellite markers in the loggerhead shrike *Lanius ludovicianus* isolated from a library enriched for CA repeats. *Mol. Ecol.* **5**: 811–813.

Paetkau, D., Calvert, W., Sterling, I. & Strobeck, C. 1995. Microsatellite analysis of population structure in Canadian polar bears. *Mol. Ecol.* **4**: 347–354.

Paetkau, D., Waits, L.P., Clarkson, P.L., Craighead, L. & Strobeck, C. 1997. An empirical evaluation of genetic distance statistics using microsatellite data from bear (Ursidae) populations. *Genetics* **147**: 1943–1957.

Paradis, E., Baillie, S.R., Sutherland, W.J. & Gregory, R.D. 1998. Patterns of natal and breeding dispersal in birds. *J. Anim. Ecol.* **67**: 518–536.

Parsons, T.J., Olson, S.L. & Braun, M.J. 1993. Unidirectional spread of secondary sexual plumage traits across an avian hybrid zone. *Science* **260**: 1643–1646.

Purroy Iraizoz, F.J. 1997. Mosquitero Común *Phylloscopus collybita*. In: *Atlas de Las Aves de España (1975–95)* (Sociedad Español de Ornithologia, ed.), pp. 410–411. Madrid.

Raymond, M. & Rousset, F. 1995. Genepop (Version 2.2): population genetics software for exact tests and ecumenicism. *J. Heredity* **86**: 248–249.

Risch, M. & Andersen, L. 1998. Non-random mating of crows (*Corvus corone*) in a hybrid zone of carrion crow (*C. c. corone*) and hooded crow (*C. c. cornix*). *J. Ornithol.* **139**: 173–177.

Rohwer, S. & Wood, C. 1998. Three hybrid zones between hermit and Townsend's warblers in Washington and Oregon. *Auk* **115**: 284–310.

Sætre, G.-P., Král, M. & Bures, S. 1997a. Differential species recognition abilities of males and females in a flycatcher hybrid zone. *J. Avian Biol.* **28**: 259–263.

Sætre, G.-P., Moum, T., Bures, S., Král, M., Adamjan, M. & Moreno, J. 1997b. A sexually selected character displacement in flycatchers reinforces premating isolation. *Nature* **387**: 589–592.

Sætre, G.-P., Král, M., Bures, S. & Ims, R.A. 1999. Dynamics of a clinal hybrid zone and a comparison with island hybrid zones of flycatchers (*Ficedula hypoleuca* and *F. albicollis*). *J. Zool. Lond.* **247**: 53–64.

Saino, N. & Villa, S. 1992. Pair composition and reproductive success across a hybrid zone of Carrion Crows and Hooded Crows. *Auk* **109**: 543–555.

Salomon, M. 1987. Analyse d'une zone de contact entre deux formes parapatriques: Le cas des Pouillots véloces *Phylloscopus c. collybita* et *P. c. brehmii*. *Rev. Ecol.* **42**: 377–420.

Salomon, M. 1989. Song as a possible reproductive isolating mechanism between two parapatric forms. The case of the Chiffchaffs *Phylloscopus c. collybita* and *P. c. brehmii* in the Western Pyrenees. *Behaviour* **111**: 270–290.

Salomon, M., Bried, J., Helbig, A.J. & Riofrio, J. 1997. Morphometric differentiation between male Common Chiffchaffs, *Phylloscopus [c.] collybita* Vieillot, 1817, and Iberian Chiffchaffs, *P. [c.] brehmii* Homeyer, 1871, in a secondary contact zone (Aves: Sylviidae). *Zool. Anzeiger* **236**: 25–36.

Salomon, M. & Hemim, Y. 1992. Song variation in the Chiffchaffs (*Phylloscopus collybita*) of the Western Pyrenees – the contact zone between the *collybita* and *brehmii* forms. *Ethology* **92**: 265–282.

Schneider, S., Kueffer, J.M., Roessli, D. & Excoffier, L. 1998. ARLEQUIN: A software for population genetic data analysis, Version 1.1. University of Geneva, Switzerland.

Schwartz, D. 1963. *Méthodes statistiques à l'usage des médicins et des biologists.* Flammarion, Paris.

Short, L.L. 1969. Taxonomic aspects of avian hybridization. *Auk* **86**: 84–105.

Sorjonen, J. 1986. Mixed singing and interspecific territoriality – consequences of secondary contact of two ecologically and morphologically similar nightingale species in Europe. *Ornis Scand.* **17**: 53–67.

Sperlich, F.A.H. 1993. Mitochondrial DNA variation and Haldane's rule in the *Papilio glaucus* and *P. troilus* species groups. *Heredity* **71**: 227–233.

Tegelström, H. & Gelter, H.P. 1990. Haldane's rule and sex-biased gene flow between two hybridizing flycatcher species (*Ficedula albicollis* and *F. hypoleuca*, Aves: Muscicapidae). *Evolution* **44**: 2012–2021.

Tiainen, J. & Wesolowski, T. 1997. *Phylloscopus collybita* Chiffchaff. In: *The EBCC Atlas of European Breeding Birds: Their Distribution and Abundance* (W. J. M. Hagemeijer & M. J. Blair, eds), pp. 610–611. T & AD Poyser, London.

Wu, C.-I.N., Johnson, A. & Palopoli, M.F. 1996. Haldane's rule and its legacy: why are there so many sterile males? *Trends Ecol. Evol.* **11**: 281–284.

Received 20 October 2000; accepted 25 October 2000