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Published in:
Molecular Ecology

DOI:
10.1046/j.1365-294X.2002.01629.x

2002

Molecular Ecology (2002) **11**, 2359–2366

The use of AFLP to find an informative SNP: genetic differences across a migratory divide in willow warblers

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Abstract

We used the amplified fragment length polymorphism (AFLP) method to obtain genetic markers distinguishing two subspecies of willow warblers *Phylloscopus trochilus* that have different migratory behaviours but are not differentiated in mitochondrial DNA or at several microsatellite loci. With the inverse-polymerase chain reaction (PCR) approach we converted a dominant AFLP-marker to a codominant single nucleotide polymorphism (SNP). Across Scandinavia we typed 621 birds at the SNP locus AFLP-WW1 and we found a sigmoid change in allele frequencies centred around 62 degrees latitude. North of the latitudinal cline was a west-east cline. Both clines are narrower than one would expect from dispersal distances in willow warblers, which suggests that these are maintained by selection. The latitudinal cline at the locus AFLP-WW1 is paralleled by changes in several other traits, all of which might be maintained by a single selective force. The most plausible selection factor that we have identified is selection against hybrids because of inferior migratory behaviour. The selective force maintaining the east–west cline is less obvious. We discuss alternatives to the selection scenario, involving colonization history and asymmetric gene flow.

Keywords: AFLP, hybrid zone, inverse PCR, microsatellites, migratory divide, *Phylloscopus trochilus*

Received 9 May 2002; revision received 13 August 2002; accepted 13 August 2002

Introduction

The retreat of the ice after the last glaciation in Eurasia and North America necessitated novel adaptations among plants and animals to meet the rapidly changing environment (Hewitt 2000). These new adaptations must have happened regardless of whether the species stayed in former refugia that progressively became warmer, with associated changes of fauna and flora, or if they expanded north to encounter new conditions, e.g. different day lengths, competitors or habitats. In Fennoscandia, it has been demonstrated that the Scots pine *Pinus sylvestris* has adapted locally to the length of the vegetative growth period, but changes in neutral genetic markers have not been detected (Karhu *et al*. 1996). Striking examples of recent adaptive changes are found in migratory birds that breed in temperate and arctic regions and winter in the tropics. The dramatic postglacial habitat changes affected not only the formerly glaciated areas but also the distribution of deserts and tropical forests (Pielou 1991). To meet these habitat alterations, migratory routes must have undergone major changes over the last 10 000 years in order to take the birds between favourable summer and winter habitats. For many species, in particular the long distance migratory songbirds, the changes of the migratory routes are genetically controlled (Berthold 1996).

The willow warbler (*Phylloscopus trochilus*) is a long distance migratory passerine, breeding in large parts of the Palaearctic and wintering in most of sub-Saharan Africa. Across Scandinavia, there is a narrow migratory divide where birds in the south (*P. t. trochilus*) migrate southwest to West Africa and birds to the north (*P. t. acredula*) migrate south of southeast to Central, East and South Africa (Salomonsen 1928; Hedenström & Pettersson 1987; Chamberlain *et al*. 2000). This migratory divide appears to be a hybrid zone, apparently maintained by selection, resulting from secondary contact between the two subspecies that have differentiated during colonization of Sweden from two directions following expansion from a common Pleistocene refuge (Bensch *et al*. 1999).

Apart from the migratory strategies, the two subspecies differ in morphology and colouration, but overlap is

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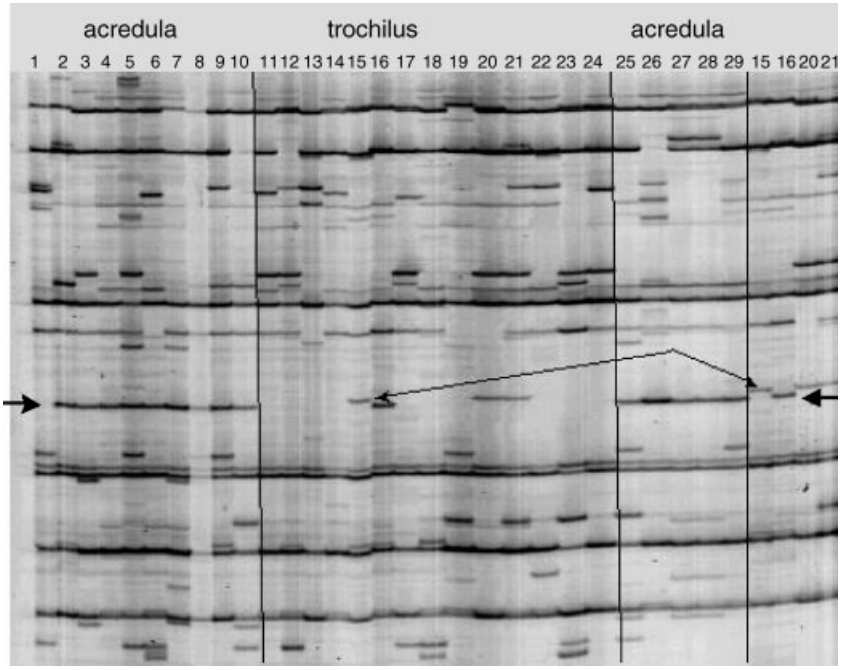

Fig. 1 Amplified fragment length polymorphism profile in willow warblers showing 15 *acredula* and 14 *trochilus*. The fragments were obtained with the primer combination $E_{TGA}xM_{CGT}$. The short thick arrows to left and right indicate the AFLPww1 fragment. The thinner connected arrows point to an individual (*trochilus* 15) that was re-loaded on the right panel of the gel together with two more *trochilus* (20 and 21) that showed a fragment slightly longer than the AFLPww1 fragment.

extensive. The subspecies *trochilus* is on average smaller, shorter winged and more green and yellow than *acredula*. Despite differences in phenotypes and colonization routes to Scandinavia, the two subspecies are not different in mtDNA (control region) and at eight microsatellite loci (Bensch *et al*. 1999). The clines in morphology and migratory direction, as inferred from stable isotope ratios in feathers (Chamberlain *et al*. 2000), are narrow and must be maintained by either selection against hybrids or through the process of assortative mating. To study these issues we need independent markers to identify the parental forms and putative hybrids between them. Here we have used the AFLP method (Vos *et al*. 1995; Mueller & Wolfenbarger 1999) which is now widely used to study hybridization in plants (Ritland & Ritland 2000). So far, only a few studies have used the AFLP method in vertebrates to identify hybrids (Liu *et al*. 2000; Bensch *et al*. 2002). In the present study we used AFLP to screen for a genetic marker with substantially different frequencies in *trochilus* and *acredula*. We converted this dominant AFLP marker to a codominant SNP and then typed a large number of breeding birds across the migratory divide in Scandinavia. We predicted that the change in frequency of this marker across Scandinavia should follow the clines observed for morphology and migratory behaviour.

Materials and methods

AFLP analyses

We used the protocol of Vos *et al*. (1995) with only minor modifications as described in Bensch *et al*. (2002). In short, genomic DNA was digested with the endonucleases *Eco*RI and *Tru*I (an isoschizomer of *Mse*I), followed by a preamplification step before the selective amplification. The fragments were separated in 6% polyacrylamid gels and detected by fluorescein labelled E-primers in a FluorImager (Vistra). For screening of diagnostic markers we selected 15 males from the southernmost part of Sweden that showed characters of the subspecies *trochilus* and 15 males from the northernmost part of Sweden that showed characters of *acredula*. These 30 birds were typed for 11 AFLP primer pair combinations. In total, this yielded more than 1000 amplified fragments of which approximately 50% were polymorphic among the 30 test birds. Only one polymorphic AFLP-fragment showed a clear difference in frequencies between *trochilus* and *acredula*. This fragment, named AFLPww1, was amplified by primer combination $E_{TGA}xM_{CGT}$ and was approximately 200 bp long. It was found in 14 of the 15 *acredula* but in only one of 15 *trochilus* (Fig. 1). This dramatically different frequency was confirmed by typing 15 more of each subspecies.

Converting the AFLP fragment to a codominant SNP

All PCRs, if not otherwise stated, were performed in volumes of 25 μL containing 1.5 mM $MgCl_2$, 0.125 mM of each nucleotide, 0.4 μM of each primer and 0.8 units of *Taq* DNA polymerase. The temperature profile included a first step of 2 min at 94 °C, 35 cycles of 94 °C (30 s), 50–58 °C (30 s) and 72 °C (30 s) followed by a final step of 10 min at 72 °C. Sequencing was done using BigDye sequencing reactions loaded on an ABI 310 capillary sequencer (Perkin Elmer).

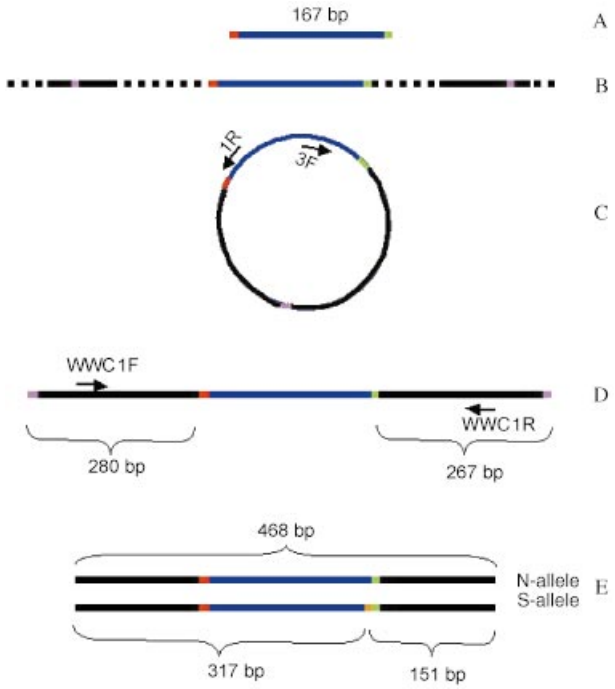

Fig. 2 A schematic illustration of the steps involved in converting the AFLP fragment into a codominant SNP. (A) The AFLPww1 fragment with the cut sites for *Eco*RI (red) and *Mse*I (green) (B) Undigested DNA around the AFLPww1 fragment with cut sites for *Alu*I (pink). (C) DNA around the AFLPww1 fragment digested with *Alu*I and self-ligated. Primers for inverse PCR are indicated. (D) The flanking DNA to the *Alu*I cut sites around the AFLPww1 fragment. Primers used for PCR-RFLP are indicated. (E) The alleles N (+ AFLP) and S (– AFLP) amplified with the WW1CF and WW1CR primers. The *Dra*I cut site on the S allele is indicated (orange).

The location of the AFLPww1 fragment in the gel was determined by marking the glass plates with a black marker pen, which was visualized by the FluorImager. A small piece of polyacrylamide containing the fragment AFLPww1 was excised from the gel, placed in 200 µL of ddH_2O and boiled for 2 min. We used 1 µL of this as a template with the original primers in a PCR to re-amplify the fragment. After ammonium acetate/ethanol precipitation this fragment was sequenced from both ends with the preamplification primers. The length of the sequenced fragment was 167 bp (including the flanking cut site), and this was examined for restriction sites (Fig. 2A) in BIOEDIT (Hall 1999). We identified three common four-cutter enzymes that did not cut the AFLPww1 fragment (*Alu*I, *Taq*I and *Sau*3A). We also designed two primers that were pointing outwards, 1R (5′-ATTCATGTGATTGTTCAACT-3′) and 3F (5′-CCCAAACATATCTCCCTGAAATTC-3′), for the inverse PCR (Fig. 2C). Genomic DNA from one *acredula* with a positive AFLPww1 band was then treated in three separate tubes, each containing one of the above enzymes, for three hours. This was followed by a step of self-ligation by adding one unit of ligase, ligase buffer, ATP (2 mM) and DTT (2 mM) and holding at room temperature for 12 h. We used 1 µL of the self-ligated DNA mix for the inverse PCR with the primers 1R and 3F (annealing temperature 55 °C and extension at 72 °C for 3 min). When checked on a 2% agarose gel, it showed that the template treated with *Alu*I resulted in an amplified fragment of approximately 550 bp. This fragment was sequenced from both ends with the original primers and aligned with the original AFLPww1 sequence. This analysis revealed that, in the original genomic DNA, the *Alu*I cut site was 280 bp 5′ and 267 bp 3′ of the ends of the original AFLPww1 fragment. Thus the total length between the *Alu*I cut sites was 714 bp (Fig. 2D). Two new primers, WW1CF (5′-TCCCATGTCTTTCAAACAGCT-3′) and WW1CR (5′-GCCTTAAATTTATGGCACAGA-3′), were designed (pointing inwards) on each side of the original fragment, to amplify a 468 bp fragment (including primers). These were used in a PCR (annealing temperature 53 °C) on 10 negative AFLPww1 (*trochilus*) and 10 positive AFLPww1 individuals (*acredula*). Direct sequencing demonstrated two different allele classes corresponding to the positive (N, northern) and negative (S, southern) AFLPww1 fragments (accession numbers AY139180 and AY139181 in the GenBank International Nucleotide Sequence Database). The reason the negative AFLPww1 allele did not show up in the AFLP appeared to be a transversion [GT] at the preamplification site on the *Mse*I end of the fragment (Fig. 2). This site could be revealed on agarose gels by first treating the PCR product obtained from the primers WW1CFxWW1CR with the restriction enzyme *Dra*I (Fig. 2E). We used this method to type individual birds.

Individuals, typing and inheritance

The present study includes typing of 621 willow warblers on the codominant marker WW1C/*Dra*I. The birds were captured on breeding grounds in Sweden during May and June, at 32 sites in 1996 or 1997 (Bensch *et al*. 1999) and at 16 sites in 2000 or 2001. Capture methods, morphological measurements and DNA extractions were as in Bensch *et al*. (1999).

After typing the data set at the WW1C/*Dra*I locus we found significant deficiencies of heterozygous individuals in central ($P < 0.001$) and northern Sweden ($P < 0.001$). We therefore checked the inheritance of the alleles in two families in which the male and female were homozygous for alternative alleles, i.e. in broods where we expected all young to be heterozygous. Each of these two families consisted of six chicks for which the genetic paternity and maternity could be confirmed with eight polymorphic microsatellite markers (unpublished). Remarkably, not all of the chicks appeared heterozygous. Sequencing confirmed that there was no trace of a second allele in the apparently

homozygous offspring or their parents. We moved both the forward and reverse primers inwards and matched their locations to identical regions in the S and the N-alleles, but the results remained unchanged. We then actively searched for the S-allele in the individuals apparently homozygous for the N-allele (NN) individuals by using the primer WW1CF in combination with a specific primer WW1BR1 (5′-CCATAAAGTTATCTTCTGTGTTTAAA-3′) targeting-3′ on the variable site matching the S-allele. With this approach we were able to retrieve the S-allele from the apparently homozygous NN mother in family 1, the NN father in family 2 and two young that first appeared as NN homozygous. Both families, then, had corrected genotypes consistent with an expected Mendelian inheritance pattern. The use of a specific primer for the N-allele (WW1BR2, 5′-CCATAAAGTTATCTTCTGTGTTTAAC-3′) resulted in amplification only from individuals typed as NN or heterozygous (NS). Therefore in order to retrieve the S-alleles that might have been hidden by the N-allele when using the WW1C/*Dra*I protocol, we re-typed all putatively homozygous NN individuals with the primers WW1CF and WW1BR1 (using a touch down PCR from 65°–57 °C). This resulted in 43 more heterozygous individuals and no significant departure from the expected frequency of homozygosity. We have no explanation for why the presence of the N-allele, sometimes but not always, inhibits the amplification of the S-allele, but we are confident that use of the allele-specific primers circumvents this problem.

Results

Sequence differences

On average, the S and the N-alleles differed by 1.5% sequence divergence. In the sequenced data set ($n = 20$) we found several variants of both the S and the N-alleles. That these can be defined into two clades based on the nucleotide at the preamplification site on the *Mse*I end of the fragment was supported by phylogenetic tree constructions using the neighbour joining method (Bensch *et al*. in prep.). Both the N and the S-alleles appeared to be noncoding because they contained multiple stop-codons in all reading frames. A nucleotide BLAST search in GenBank failed to find sequences of significant similarity.

Geographic distribution of N and S-alleles

Populations of willow warblers sampled south of 61° N were almost fixed for the S-allele whereas north of 61° N the N-allele increased in frequency (Fig. 3). This increase was steeper along the western transect, through the inland of Sweden, where the N-allele reached a plateau at the frequency of 0.75. Along the coast however, the maximum

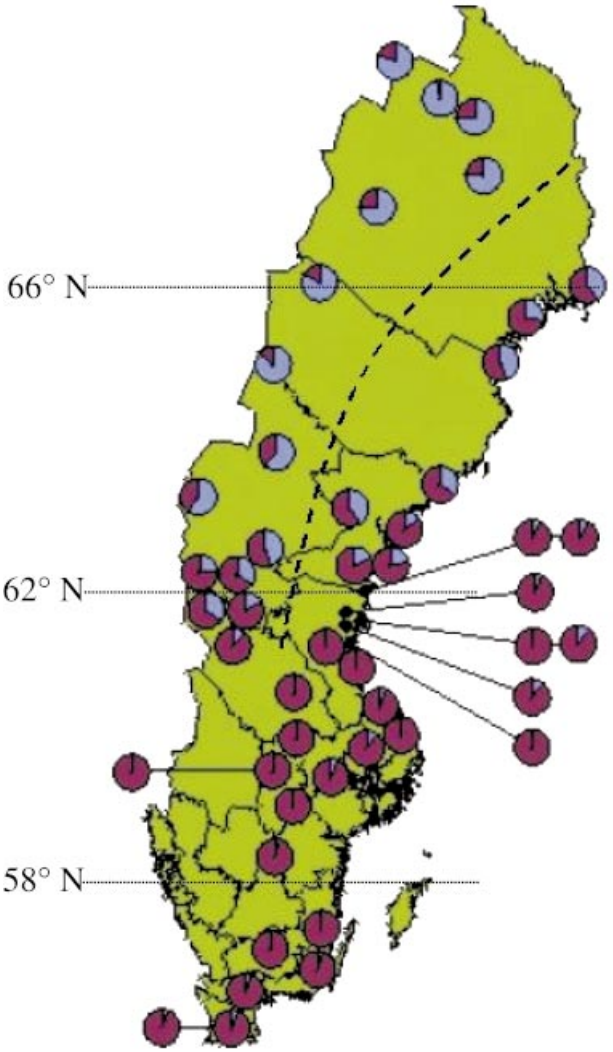

Fig. 3 Map showing the proportion of S (pink) and N (blue) alleles at 44 sampling sites across Sweden. The dashed line indicates the grouping of inland (left) and coastal (sites).

frequency of the N-allele reached just below 0.50 (Fig. 4A). This geographical structure was supported by significant effects of latitude ($t_{1,45} = 10.3$, $P < 0.001$) and longitude ($t_{1,45} = 3.28$, $P = 0.002$) in a multiple regression with the proportion of the N-allele per site as the dependent variable. Going from the south, the N-allele started to increase in frequency in the same area that wing length and body mass started to increase (Fig. 4B,C). However, neither of the morphological traits showed any tendency towards longitudinal variation across the northern half of the Scandinavian Peninsula.

Discussion

Very little is known about how many genes are involved in making species different. In *Drosophila*, the best studied

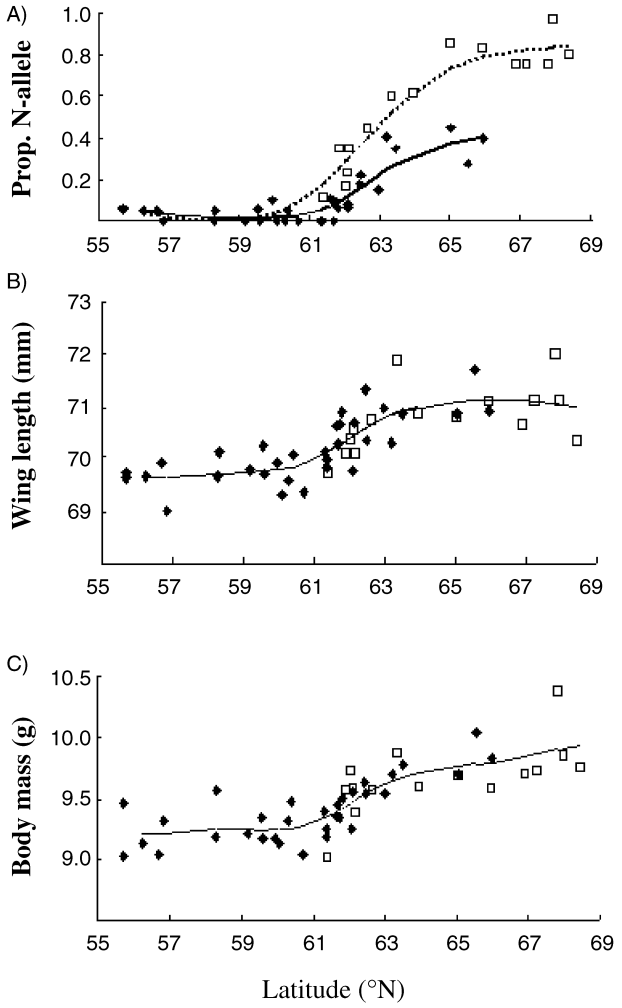

Fig. 4 The relationship between the frequencies of the N-allele (A), wing length (B), body mass (C) and latitude. Open squares denote inland sites. Lines refer to cubic spline functions (Schluter 1998) for inland (stippled) and coastal sites (solid).

group of animals, there is no clear pattern as species pairs have been found to differ from a few to approximately 20 genes (Orr 2001). Studies on crop plants, however, have demonstrated that single genes can have profound effect on morphology (Dorweiler *et al*. 1993). Hence, closely related taxa may only differ by a few coding genes. At the earliest stage of population differentiation, the potentially diverging populations will share most of the genetic variation contained in the ancestral population (Edwards & Beerli 2000). Allele frequencies at coding genes may then diverge rapidly as a result of directional selection, but changes in neutral DNA allele frequencies occur through the much slower process of genetic drift. Hence, when the time of separation between taxa is short, relative to their effective population sizes, neutral genetic markers may not have had time to diverge (Nichols 2001). In such situations, the genetic differences between taxa might only be found in those genes encoding for observable phenotypic differences, e.g. the genes coding for morphology and behaviour, or in closely linked neutral DNA. The subspecies of willow warblers studied here might be such a case of very recently differentiated taxa, as supported by our finding of only one informative marker out of 500 screened polymorphic AFLP markers.

Our approach to convert the dominant AFLP to a codominant SNP enabled a detailed description of allelic change at locus WW1 across Scandinavia. This resolution would not have been achieved with the straightforward AFLP method, because of its inability to distinguish homozygous NN and heterozygous NS individuals. In agreement with our prediction, the allele frequency at the locus WW1 changed around 62 degrees latitude, exactly where we have observed the steepest changes in morphology (Bensch *et al*. 1999) and migratory behaviour (Chamberlain *et al*. 2000). In addition to the south-north variation that paralleled the pattern observed in the phenotypic traits, there was also an east–west cline in northern Scandinavia at the locus WW1. Neither of the morphological traits, nor stable isotopes, showed longitudinal differences in the northern half of the Scandinavian Peninsula. Below, we discuss the history and maintenance of these two clines separately.

The north–south cline around 62° N

The coincident location of clines for size, colouration, migratory behaviour and allele frequency at the WW1 locus supports the notion that this zone of transition is a result of secondary contact between willow warblers colonizing the Scandinavian Peninsula from two directions after the last glaciation. One colonization route apparently came from the south over Denmark and the other from the north via Finland around the Gulf of Bothnia. These two postglacial colonization routes have resulted in a cluster of hybrid zones involving both mammals and plants (Taberlet *et al*. 1998; Jaarola *et al*. 1999) in the same area as the hybrid zone in willow warblers. We argue that the extensive sharing of genetic polymorphism between *trochilus* and *acredula* is a signature from the time before the subspecies diverged, rather than resulting from homogenizing gene flow following secondary contact. In particular, the lack of differentiation in mitochondrial DNA suggests that the species was not structured in subpopulations during the last Pleistocene glaciation and that the subspecies developed as a result of differentiation in adaptive traits during the postglacial colonization of Eurasia starting 10 000 years ago (Bensch *et al*. 1999).

As calculated in Bensch *et al*. (1999), using the equation in Barton & Gale (1993), the latitudinal cline is too narrow to be maintained unless selection is acting against mixing. Bensch *et al*. (1999) used a root-mean-square dispersal

distance (RMS) of 50 km which fits well with an estimated natal dispersal distance of 21 ± 46 km (mean ± SD) based on ringing recoveries of willow warblers on the British Isles (Paradis *et al*. 1998). Without selection, a RMS dispersal distance of 50 km would have resulted in a cline width of 1200 km after 150 years of contact, and this estimate is more than three times as wide as the observed clines in morphology (Bensch *et al*. 1999), migratory direction (Chamberlain *et al*. 2000) and at AFLP-WW1 (this study) which all are less than 300 km wide. Moreover, the number of years in contact might have been substantially longer than the 150 years, and our estimate of the width of a cline without selection is therefore conservative. At present, we do not know the nature of this selection but our working hypothesis is that there is ecological selection (Schluter 2001) against hybrids, presumably because of maladaptive migratory behaviour. In blackcaps *Sylvia atricapilla*, hybrids between southwest and southeast migratory populations take up an intermediate course of migration towards the south (Helbig 1991). This is similar to the migratory direction we predict for willow warbler hybrids between *trochilus* and *acredula*, assuming that they have a similar genetic migratory program as the blackcap. Migrating directly south in autumn would bring hybrids across wider areas of unfavourable habitat (i.e. the Mediterranean Sea and the Saharan desert) than if they had migrated towards the southwest or the southeast. If not associated with a migratory program that enables larger fuel stores and longer nonstop flights, which is not likely to arise *de novo* in hybrids, such a route should result in higher mortality. Although some viable offspring probably result from mixed matings between *trochilus* and *acredula*, we believe hybrids will be less likely to successfully complete migration, thus resulting in some degree of postmating isolation between the two gene pools. Support for this hypothesis is provided by the stable isotope cline, which probably reflects migratory direction, and is at least as sharp as the clines for the morphological traits (Chamberlain *et al*. 2000).

The apparently very similar genomes of *trochilus* and *acredula* suggest that postmating isolation in the form of genetic incompatibility is very unlikely. A possibility is that hybrids suffer more from parasites if they have less fit phenotypes (Sage *et al*. 1986). In line with this is a recent observation of a high incidence of infestation by the blow fly *Trypocalliphora bruceri* in willow warbler nests located in the hybrid zone, a parasite not encountered elsewhere in nestlings of Scandinavian willow warblers (Åkesson *et al*. 2002). Alternative explanations for the maintenance of the cline, which are not mutually exclusive, involve premating isolation in the form of assortative mating. Assortative mating could possibly work, either indirectly through habitat preferences or through differential arrival dates and hence mating dates, or directly on some of the traits that, on average, differ between the subspecies. At present, we have no data supporting or refuting whether willow warblers in the hybrid zone engage in assortative mating.

The west-east cline at the AFLP-WW1 locus

Particularly in northern Scandinavia, very few nestlings and juvenile willow warblers return to breed in study plots in subsequent years (Cuadrado & Hasselquist 1994; Neergaard & Arvidson 1995). With such high natal dispersal, one would expect gene flow to homogenize allele frequencies at selectively neutral loci. However, the N-allele is about twice as common in the inland area compared with the same latitude along the coast, a clearly discernable pattern in northern Scandinavia for about 700 km north of 61° N. At least three different scenarios could have caused this east–west cline: (i) selection; (ii) asymmetric gene flow; or (iii) historical range expansion.

The preferred habitat for willow warblers in the mountainous region of western Sweden is subalpine birch forest, which is quite different from the coniferous or mixed forests along the coast. This is associated with higher elevation in the west and a wetter climate with lower summer temperatures. Therefore one possibility is that the N-allele is closely linked to an allele that has a selective advantage in the birch-forest and the cline is then maintained through a balance between selection and dispersal (Barton & Hewitt 1989; Lenormand 2002).

Another possibility is that asymmetric gene flow has caused allele frequencies of neutral loci to infiltrate beyond a contact zone maintained by selection (Lenormand 2002). The habitat along the coast is more similar to the habitat of south Central Sweden than are the forests and mountains in the inland of northern Sweden. Many species of birds with a mainly southern Scandinavian distribution also have ranges extending north along the coast (Svensson *et al*. 1999), where the onset of spring is several weeks earlier than in the western mountains. The southern subspecies *trochilus* arrives at its breeding quarters earlier than *acredula* (Hedenström & Pettersson 1984). Therefore, the coastal area is more likely to receive an influx of *trochilus*, and because the southern birds are almost fixed for the S-allele, this would push higher frequencies of the S-allele further into the range of *acredula* along the coast than in the inland. However, whereas this could explain the slower increase of the N-allele along the coast, it is less obvious how this could result in a lower asymptotic frequency.

Mismatches between locations of phenotypic hybrid zones and clines for genetic markers have been interpreted as signs of historical hybridization (Avise 1994) or recent spread of adaptive traits (Parsons *et al*. 1993). One of the best documented cases with a mismatch between a genetic marker and a phenotypic hybrid zone is between Townsend's warbler *Dendroica townsendi* and hermit warbler *D. occidentalis* in western North America. The former

species has expanded its range at the expense of the latter during the past 5000 years (Rohwer *et al*. 2001). However, the spread of the Townsend's warbler phenotype has not been matched by a concordant spread of its mitochondrial haplotypes; the hermit warbler haplotype is frequent in Alaska as far as 2000 km north of the narrow phenotypic hybrid zone located in Washington. We do not know whether the contact zone between *trochilus* and *acredula* has always been located around 62° N or if it has been moving. Possibly, *trochilus* might have had its range extending north along the coast until it was more recently pushed south by *acredula*. If so, the higher frequency of the S-allele along the coast could be a genetic footprint of the historical movement of the hybrid zone. However, we believe that the lower frequency of the N-allele along the coast than in the inland speaks against the historical scenario.

In conclusion, we cannot rule out the idea that a balance between selection and dispersal maintains the east–west cline at the AFLP-WW1 locus in northern Scandinavia, favouring the N-allele in the subalpine birch forest in the mountains and the S-allele in the mixed forests along the coast. However this question needs to be addressed in direct studies in the two habitats by comparing fitness of birds with different genotypes. The two hypotheses involving asymmetric gene flow or a historical range expansion might be sufficient to explain the slower increase of the N-allele along the coast than in the inland but neither of the hypotheses can explain the different levels of the asymptotic frequencies reached north of the cline. Both these hypotheses assume that the S-allele was originally confined to *trochilus* and the N-allele to *acredula*. However, in a sample of six willow warblers from western Siberia (Yekaterinburg), about 2500 km into the range of *acredula*, all were homozygous for the S-allele (unpublished). Hence, it seems as if the N-allele is confined to the northwest part of the range of *acredula*. The observed sequence divergence between the two alleles S and N of about 1.5% suggests that the alleles arose long before the postglacial colonization of northwest Europe and did not result from novel mutations during the postglacial colonization of Fennoscandia.

Conclusion

The observed clines at the AFLP-WW1 locus are narrow relative to the dispersal distance of willow warblers and could therefore be maintained by selection. However, we do not know what this selection is or what genes are linked to this marker. We believe that the differences observed in wing length, body mass, colouration and migratory direction between willow warblers breeding south (*trochilus*) and north (*acredula*) of 62° N are genetic, despite the fact that they appear panmictic in mitochondrial DNA, microsatellites (Bensch *et al*. 1999) and at the larger number of AFLP markers that we encountered in the search for the AFLP-WW1 locus. By screening many more AFLP primer combinations, we hope to find additional informative markers that can be used for identification of hybrids and backcrosses between the two subspecies, and possibly marker loci for some of the expressed phenotypic differences.

Acknowledgements

We are indebted to T. Andersson, A. Hedenström and J. Irwin for invaluable help in the field and to O. Hellgren for assistance in the lab. D. Richardson and T. Price provided many valuable suggestions on earlier drafts. Funding was provided by the Swedish Science Research Council, Uddenberg Nordingska Stiftelsen, Crafoordska Stiftelsen, Stiftelsen Olle Engkvist Byggmästare, and the National Science Foundation.

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