Molecular Ecology (2006) **15**, 161–171 doi: 10.1111/j.1365-294X.2005.02766.x

Conflicting patterns of mitochondrial and nuclear DNA diversity in *Phylloscopus* warblers

STAFFAN BENSCH,* DARREN E. IRWIN,*† JESSICA H. IRWIN,*† LAURA KVIST*‡ and SUSANNE ÅKESSON*
Department of Animal Ecology, Ecology Building, S-223 62 Lund, Sweden

Abstract

Molecular variation is often used to infer the demographic history of species, but sometimes the complexity of species history can make such inference difficult. The willow warbler, *Phylloscopus trochilus*, shows substantially less geographical variation than the chiffchaff, *Phylloscopus collybita*, both in morphology and in mitochondrial DNA (mtDNA) divergence. We therefore predicted that the willow warbler should harbour less nuclear DNA diversity than the chiffchaff. We analysed sequence data obtained from multiple samples of willow warblers and chiffchaffs for the mtDNA cytochrome *b* gene and four nuclear genes. We confirmed that the mtDNA diversity among willow warblers is low ($\pi = 0.0021$). Sequence data from three nuclear genes (CHD-Z, AFLP-WW1 and MC1R) not linked to the mitochondria demonstrated unexpectedly high nucleotide diversity (π values of 0.0172, 0.0141 and 0.0038) in the willow warbler, on average higher than the nucleotide diversity for the chiffchaff (π values of 0.0025, 0.0017 and 0.0139). In willow warblers, Tajima's *D* analyses showed that the mtDNA diversity, but not the nuclear DNA diversity, has been reduced relative to the neutral expectation of molecular evolution, suggesting the action of a selective sweep affecting the maternally inherited genes. The large nuclear diversity seen within willow warblers is not compatible with processes of neutral evolution occurring in a population with a constant population size, unless the long-term effective population size has been very large ($N_e > 10^6$). We suggest that the contrasting patterns of genetic diversity in the willow warbler may reflect a more complex evolutionary history, possibly including historical demographic fluctuations or historical male-biased introgression of nuclear genes from a differentiated population of *Phylloscopus* warblers.

Keywords: coalescence time, introgression, mitochondrial DNA, nuclear genes, *Phylloscopus*

Received 16 May 2005; revision accepted 1 September 2005

Introduction

In animals, mitochondrial DNA (mtDNA) has become the standard molecule used for reconstructing species' phylogenies, obtaining indirect measures of effective population sizes, and detecting historical population bottlenecks. For any study dealing with questions about population differentiation and speciation, sequencing mtDNA is a natural starting point for many reasons (Avise 2000). For example, mtDNA protocols have been available for most kinds of animals for more than a decade (Kocher *et al*. 1989) resulting in a huge body of sequences with which the data can be compared (Johns & Avise 1998; Hebert *et al*. 2004). Also, mtDNA is maternally inherited, haploid and in most species nonrecombining, characteristics that make analysis of mtDNA sequence data more straightforward than analysis of nuclear genes from diploid organisms. That the mitochondrion may not accurately reflect patterns of variation in the nuclear genome and sometimes could be seriously biased is emphasized by the fact that it exhibits a different mutation rate, mode of inheritance and effective population size than the nuclear genes (Ballard & Whitlock 2004).

Correspondence: Staffan Bensch, Fax: +46-46-2224716; E-mail: staffan.bensch@zooekol.lu.se.
†Present address: Department of Zoology, University of British Columbia, 6270 University Blvd, Vancouver, BC V6T 1Z4, Canada.
‡Present address: Department of Biology, FIN-90014 University of Oulu, Finland.

© 2006 Blackwell Publishing Ltd

Measuring the pattern of genetic diversity within and between closely related species can contribute to an understanding of the evolutionary history of those species. The amount and distribution of genetic diversity may reveal information about historical population sizes and structure (Sabeti *et al*. 2002; Wang *et al*. 2002; Sivasundar & Hey 2003) as well as population divergence and speciation (Kliman *et al*. 2002; Shaw 2002). Measuring intraspecific genetic diversity is complicated by the fact that the pattern of diversity may vary substantially across the genome (Sachidanandam *et al*. 2001). Most studies of intraspecific genetic diversity have examined information from only one locus. Although the problem of inferring a species evolutionary history based on one gene has been addressed both empirically (Wise *et al*. 1997; Ting *et al*. 2000; Tosi *et al*. 2000; Ballard *et al*. 2002; Irwin *et al*. 2005) and theoretically (Hudson & Coyne 2002; Irwin 2002), researchers still often make conclusions regarding evolutionary history based solely on mitochondrial sequence data (Roman & Palumbi 2003), relying on the assumption that variation in mtDNA is representative of variation in the whole genome. There are many reasons why this assumption might be violated (Ballard *et al*. 2002; Ballard & Whitlock 2004). For example, demographic fluctuations (Takahata & Satta 1997), selective sweeps (Mishmar *et al*. 2003) or hybridization (Grant & Grant 1992; Cohen *et al*. 1997; Barton 2001) can cause different loci to show dramatically different patterns of diversity.

The aim of the study was to examine multiple nuclear genes to investigate whether the evolutionary history of species can be predicted from geographical patterns of morphological variation and mtDNA diversity. We studied the willow warbler, *Phylloscopus trochilus,* and the chiffchaff, *Phylloscopus collybita,* that both are common species in large parts of the Palaearctic, but they show different patterns of intraspecific variation. The chiffchaff is divided into nine morphologically and behaviourally well-differentiated subspecies showing diagnostic mtDNA cytochome *b* lineages (Helbig *et al*. 1996). In contrast, the willow warbler has three morphologically similar subspecies (Cramp 1992), and a study on the mitochondrial control region of willow warblers (Bensch *et al*. 1999) did not detect any differentiation between the subspecies *P. t. trochilus* and *P. t. acredula*. The low amount of variation suggested that the species had passed through a recent population bottleneck (Bensch *et al*. 1999). Hence, prior to this study all information at hand suggested that the willow warbler should harbour less nuclear variation than the chiffchaff.

We studied sequence variation at four nuclear genes. The willow warbler and the chiffchaff are sister species in a genus containing about 40 species (Richman & Price 1992; Price 1996; Irwin *et al*. 2001a), and to evaluate the level of diversity in the two focal species, we also investigated one of their closest relatives, the dusky warbler, *Phylloscopus fuscatus*. We also investigated a few representatives from three other more distantly related species of *Phylloscopus* warblers. Two of the loci were located on sex chromosomes and two on autosomes. The two sex-linked regions were introns in the genes encoding for the chromo-helicase-DNA-binding protein located on both avian sex chromosomes (CHD-Z and CHD-W) (Griffiths & Tiwari 1993; Ellegren 1996). In birds, females are the heterogametic sex and their genomes thus contain one copy each of the Z- and the W-chromosome, whereas males have two sets of Z-chromosomes per genome. As examples of autosomal loci we studied the melanocortin-1-receptor gene (MC1R), which is expressed in melanocytes of developing feathers and is involved in melanin production (Theron *et al*. 2001), and the AFLP-WW1 locus, which is an anonymous non-coding autosomal region (Bensch *et al*. 2002a).

In the present study, we examine the pattern of mitochondrial and nuclear DNA variation in willow warblers and chiffchaffs, with the prediction of finding less nuclear variation in the willow warbler than in the chiffchaff because it exhibits substantially less variation both in mtDNA and in morphology than the other species. With the failure of this prediction, our study emphasizes the complexity of gene and species evolution. We discuss how different processes, such as population size changes, selective sweeps and introgression, might have contributed to the conflicting patterns of genetic diversity seen across loci and species.

Methods

Molecular work

For each of the studied genes we selected willow warblers collected from Britain, Sweden and western Siberia, hence covering almost the full range of the observed geographical variation. For the chiffchaff, we analysed representatives from four subspecies (one *Phylloscopus collybita collybita*, 10 *abietinus*, one *tristis* and one *brehmii*). Because we included a specimen from the most divergent subspecies *P.* (*c.*) *brehmii* (Helbig *et al*. 1996) we could expect to recover the deepest node in each of the gene trees. We also analysed 10 dusky warblers (*Phylloscopus fuscatus*) from one population in eastern Siberia (Forstmeier *et al*. 2002). For rooting phylogenetic trees and to obtain relative estimates of gene-specific mutation rates we also analysed a few specimens from more distantly related *Phylloscopus* warblers; two wood warblers (*Phylloscopus sibilatrix*) from Sweden, one Bonelli's warbler (*P. b. bonelli*) sampled during migration in Nigeria and two greenish warblers, represented by the two most divergent and apparently reproductively isolated subspecies, *P. trochiloides viridanus* from western Siberia and *P. t. plumbeitarsus* from eastern Siberia (Irwin *et al*. 2001b). We also included sequences from the GenBank database for the cytochrome *b* (*P. c. brehmii*, Z73476; *P. c. abietinus*, Z73479; *P. c. collybita*, Z73487; *P. t. viridanus*, Z73493; *P. fuscatus*, Y10729 and

P. t. plumbeitarsus, Y10740) gene and MC1R (*P. c. collybita*, AY308747; *P. trochilus*, AY308748; *P. t. trochiloides*, AY308749 and *P. fuscatus*, AY308754). We failed to obtain sequences for all the outgroup taxa for all genes because (i) analyses of the CHD-W gene requires samples from females, which we lacked from some of the taxa, (ii) the primers for the AFLP-WW1 gene did not amplify samples of greenish warblers and (iii) the primers for the MC1R gene did not amplify samples of Bonelli's and wood warblers.

DNA was extracted from blood samples using a standard phenol–chloroform procedure. Polymerase chain reactions (PCR) were performed for 35 cycles (30 s at 94 °C, 30 s at 50–55 °C, and 30–120 s at 72 °C) in volumes of 25 μL that included 25 ng of template DNA, 0.125 mM of each nucleotide, 1.5 mM $MgCl_2$, 0.6 μM of each primer and 0.5 U of *Taq* DNA polymerase. The cytochrome *b* gene was amplified with the primers L-14995 and H-16065 (Helbig *et al*. 1995). An intron from the chromo-helicase-DNA-binding protein (CHD) gene on the W chromosome was amplified from female birds using the primers 3112 and 2987 (Ellegren & Fridolfsson 1997). We had samples of female chiffchaffs only from the subspecies *abietinus* and *tristis*. In order to avoid obtaining sequences containing two different alleles we primarily used female birds also for the amplification of the CHD-Z intron, using the primers 3007 and 3112 (Ellegren & Fridolfsson 1997). The primers for the AFLP-WW1 locus (WW1CF, 5′-TCCCATGTCTTTCAAACAGCT-3′ and WW1CR, 5′-GCCTTAAATTTATGGCACAGA-3′) were obtained from an anonymous AFLP polymorphic fragment in willow warblers that appears to be noncoding, as there are multiple stop codons in all possible reading frames (Bensch *et al*. 2002a). For the melanocortin-1-receptor gene (MC1R) (Theron *et al*. 2001) we used the primers MSH8 (5′-CCTCAAGAACAGGAATCTGCACTC-3′) and MSH9 (5′-CTGGCTCCGGAAGGCATAGAT-3′) to amplify and sequence 451 bp of the 5′ end of the MC1R gene (MacDougall-Shackleton *et al*. 2003).

We used the BigDye Sequencing Kit loaded on ABI 310 or ABI 377 (PerkinElmer) sequencing robots. The PCRs for all loci except MC1R generated products of only the expected length and could hence be directly sequenced after ammonium-acetate ethanol precipitation. Before sequencing the MC1R gene, the PCR product was run on agarose gels and the fragment of correct length was identified and excised from the gel and cleaned (Glenn & Glenn 1994). For several of the samples, sequencing of PCR products revealed that the sample consisted of two alleles that differed at more than one site. We identified the sequences of these alleles by cloning the PCR products (using the TA Cloning Kit from Invitrogen according to the manufacturer's instructions), sequencing multiple clones from each individual, and finally comparing these sequences with the one previously obtained from direct sequencing. Sequences have been deposited in GenBank (Accession nos DQ174550–DQ174677).

Statistical analyses

Sequences were edited and aligned using the software BIOEDIT (Hall 1999) and all alignments were unambiguous. We did maximum likelihood phylogenetic analyses in PAUP* 4.0 (Swofford 1998) for each locus separately. The analyses followed a heuristic search with random addition of sequences, keeping best trees only, and using the tree-bisection–reconnection algorithm for branch swapping. Support to internal branches was based on a heuristic bootstrap analysis with 1000 replicates. For each gene, we used the Akaike information criterion to identify the most appropriate nucleotide substitution model out of 56 models tested, as implemented in MODELTEST 3.6 (Posada & Crandall 1998). For the loci where we had data from *viridanus*/*plumbeitarsus* (Cyt *b*, CHD-W, CHD-Z and MC1R), these were used as outgroups as they appear the most distantly related taxa to the ingroup (Richman & Price 1992; Price *et al*. 1997). The gene tree for AFLP-WW1 was instead rooted with *sibilatrix*/*bonelli*. Trees were visualized using TREEVIEW (Page 1996). The only differences between the CHD-W intron of willow warblers and chiffchaffs were two indels, and we treated these as transitions in the analyses.

We used the likelihood-ratio test to evaluate, for each locus, whether there was a homogenous rate of molecular evolution along all branches (Felsenstein 1981). Trees were constructed in PAUP* 4.0 (Swofford 1998) as described above. Because of computational limitations, we excluded all but the five most divergent willow warbler haplotypes, but included all unique haplotypes from the other species. A constant clock was rejected if, twice the difference in likelihood scores between the tree with constant clocks and the tree without enforced clocks, was larger than critical chi-squares with S-2 degrees of freedom, where S is the number of included sequences.

In order to be able to estimate the expected nucleotide diversity for each locus and species, given an assumed effective population size, we needed to obtain estimates of the gene specific mutation rate. We used the following procedure to calculate the gene specific mutation rates. For each of the genes, we calculated the pairwise divergences (using a gene-specific DNA substitution model, see Table 1) from willow warblers, chiffchaffs and dusky warblers to two more distantly related *Phylloscopus* species groups, the wood warbler/bonelli's warbler and greenish warblers (Table 2). However, some of the comparisons were not possible because we failed to obtain sequences for some of the gene and species combinations (see Fig. 1). Then we compared the pairwise divergence for each of the nuclear genes with the corresponding pairwise divergence in the cytochrome *b* gene. This gave us a divergence rate for each gene relative to the cytochrome *b* gene. Our interpretations only require relative mutation rate estimates, but we have chosen to express the estimates as absolute rates (Table 2)

Table 1 DNA substitution model and parameter setting as obtained by the program MODELTEST (Posada & Crandall 1998)

Parameter	Cytochrome *b*	CHD-W	CHD-Z	MC1R	AFLP-WW1
Fragment length (bp)	1041	353	285	451	424
Best-fit model	TVM + I	HKY	TVM + G	TrN + G	TVM + G
Base composition					
A	0.30	0.33	0.35	0.17	0.35
C	0.40	0.14	0.12	0.36	0.16
G	0.11	0.16	0.16	0.24	0.13
T	0.19	0.37	0.37	0.23	0.36
Ts/tv ratio		8.12			
R-matrix (G↔T = 1)					
A↔C	28.2		0.893	1.00	0.534
A↔G	300.2		4.73	6.46	4.29
A↔T	16.9		0.178	1.00	0.520
C↔G	0.0001		3.59	1.00	2.99
C↔T	300.2		4.73	14.58	4.29
Rates	equal	equal	gamma	gamma	gamma
Shape	—	—	0.233	0.142	0.743
Proportion invariable sites	0.69	0	0	0	0

Table 2 Sequence divergence (in percentage) between *Phylloscopus trochilus/collybita/fuscatus* and two distantly related *Phylloscopus* clades based on gene-specific DNA substitution models (Table 1). Gene-specific mutation rates μ were calculated relative to the cytochrome *b* distance and assuming a mitochondrial mutation rate of 1.0×10^{-8} substitutions per year

Sequence divergence between:	Genes: cytochrome *b*	CHD-W (μ)	CHD-Z (μ)	MC1R (μ)	WW1 (μ)
sibilatrix/bonelli					
and *trochilus*	36.8	1.73 (4.70×10^{-10})	6.72 (1.83×10^{-9})	—	4.08 (1.11×10^{-9})
and *collybita*	37.3	1.76 (4.71×10^{-10})	5.79 (1.55×10^{-9})	—	4.01 (1.07×10^{-9})
and *fuscatus*	24.2	1.76 (7.25×10^{-10})	3.96 (1.64×10^{-9})		4.57 (1.89×10^{-9})
viridanus/plumbeitarsus					
and *trochilus*	44.2	1.15 (2.60×10^{-10})	5.88 (1.33×10^{-9})	2.42 (5.48×10^{-10})	—
and *collybita*	52.9	1.16 (2.20×10^{-10})	5.49 (1.04×10^{-9})	2.95 (5.58×10^{-10})	—
and *fuscatus*	27.4	1.16 (4.25×10^{-10})	3.68 (1.34×10^{-9})	1.90 (6.94×10^{-10})	
Average μ	[1.0×10^{-8}]	4.29×10^{-10}	1.46×10^{-9}	6.00×10^{-10}	1.36×10^{-9}

by assuming that the cytochrome *b* gene has diverged at a rate of 2% per million years, corresponding to a per-lineage mutation rate of 1×10^{-8} per site and year (Shields & Wilson 1987; Tarr & Fleischer 1993). Although the assumed mtDNA mutation rate in birds contains many uncertainties (García-Moreno 2004; Lovette 2004), the qualitative conclusions of our results will remain even with very different rates.

The amount of within-species genetic variation for each locus was estimated as nucleotide diversity (π) and segregating sites (s) in DNASP (Rozas & Rozas 1997). We used Tajima's *D* to test the null hypothesis of neutral evolution of a population at equilibrium, comparing the two measures of genetic variation, π and s, which are differentially affected by processes such as drift and selection (Tajima 1989).

The amount of expected genetic variation at a locus does not only depend on the long-term effective population size of individuals and the mutation rate, but also on the mode of inheritance of the locus. If N_e is the effective population size of individuals considering both males and females and assuming a balanced sex ratio, then the effective population sizes of the sex-linked loci correspond to $0.5 \times N_e$ for CHD-W (and for mtDNA), and $1.5 \times N_e$ for CHD-Z, in contrast to $2 \times N_e$ for autosomal genes. We considered these adjustments of N_e when calculating expected nucleotide diversities.

We used the calculated mutation rates (μ) together with the estimated nucleotide diversity (π) to calculate the long-term effective population size (N_e) from variation at each of the loci, using the relationship $N_e = \pi/\mu$ for cytochrome *b* and CHD-W, $N_e = \pi/(3 \times \mu)$ for CHD-Z and $N_e = \pi/(4 \times \mu)$ for the autosomal genes (Li 1997).

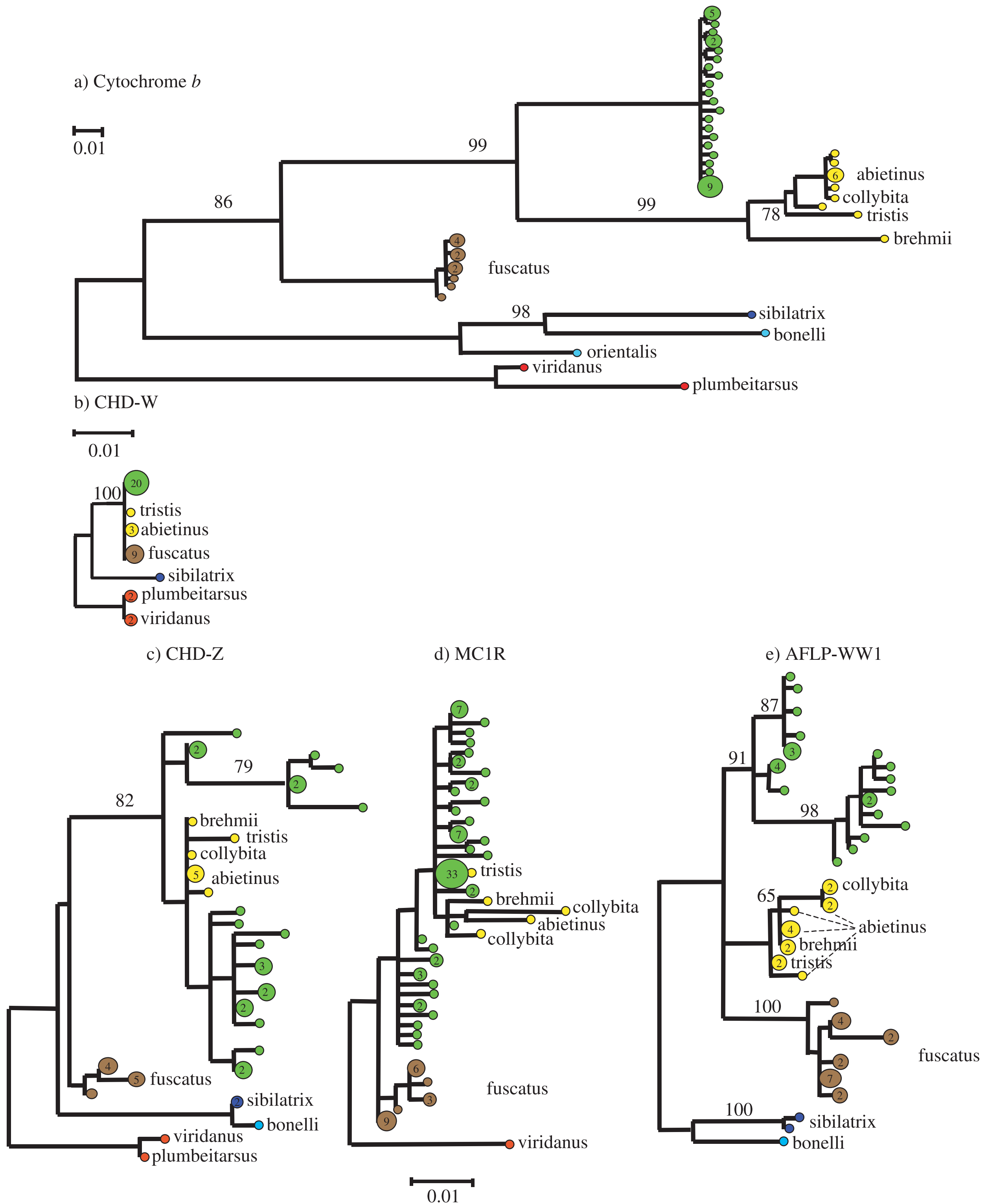

Fig. 1 Maximum-likelihood trees for five genes (a, cytochrome *b*; b, CHD-W; c, CHD-Z; d, MC1R; and e, AFLP-WW1) in six species of *Phylloscopus* warblers. The trees are drawn on the same scale of molecular divergence except the cytochrome *b* gene tree which is reduced by a factor 0.5 (see Methods). Sequenced gene copies refer to willow warblers (*Phylloscopus trochilus*, green circles), chiffchaffs (*Phylloscopus collybita*, yellow), dusky warblers (*Phylloscopus fuscatus*, brown), Bonelli's warblers (*Phylloscopus bonelli*, light blue), wood warblers (*Phylloscopus sibilatrix*, dark blue) and greenish warblers (*Phylloscopus trochiloides*, red). Larger symbols represent multiple identical alleles, the number shown by the figure in the circle. Greenish warblers were used to root the trees except for the WW1 tree, in which we used Bonelli's and wood warblers.

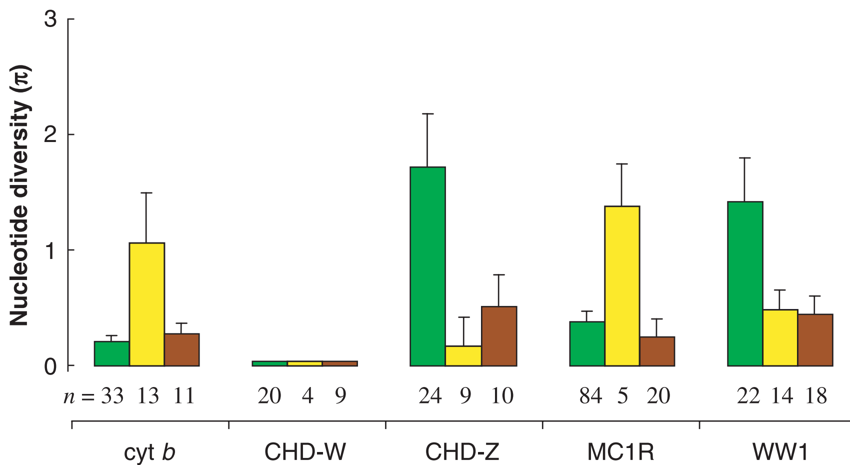

Fig. 2 Nucleotide diversity (+ SE) for five genes (cytochrome *b*, CHD-W, CHD-Z, MC1R and AFLP-WW1) in willow warblers (*Phylloscopus trochilus*, green bars), chiffchaffs (*Phylloscopus collybita*, yellow) and dusky warblers (*Phylloscopus fuscatus*, brown). Sample sizes (sequenced gene copies) are given below each bar.

Results

The deepest mitochondrial divergence within chiffchaffs (*brehmii–abietinus*) corresponds to 5.4% sequence divergence whereas the deepest divergence between willow warbler haplotypes is only 0.58% (Fig. 1a). Although we found somewhat more diversity in the cytochrome *b* gene than was earlier observed for the control region (Bensch *et al*. 1999), our result confirms a pattern of low levels of mtDNA diversity in willow warblers with an average nucleotide diversity, π, of 0.00207 (Fig. 2). This diversity is slightly lower than in dusky warblers (Fig. 2) but higher than within the chiffchaff subspecies *abietinus* (0.0014).

The pattern of diversity in the nuclear loci differed from the pattern in mtDNA. For the CHD-W gene, none of the examined species showed intraspecific variation (Fig. 2). For the other three nuclear loci, the willow warbler showed higher nucleotide diversity than in mtDNA, the chiffchaff showed less nucleotide diversity than in mtDNA and the dusky warbler quite similar level of nucleotide diversity across all loci (Fig. 2).

Figure 1(a–e) shows the gene trees for the five investigated genes, depicted on the same scale of molecular distance relative to the cytochrome *b* gene. The gene trees show some striking characteristics: (i) the well-resolved and long branches separating the species when analysing the cytochrome *b* gene, (ii) the compressed tree obtained for CHD-W, (iii) the unresolved cluster of willow warbler and chiffchaff sequences for the MC1R gene, and (iv) the relatively diverse and deep branches within willow warblers for the CHD-Z and AFLP-WW1 genes. Some aspects of these patterns can be explained by different rates of molecular evolution for the five genes.

Estimated mutation rates (Table 2), relative to an assumed rate for the cytochrome *b* gene, showed that the CHD-W gene exhibits the lowest mutation rate (4% of the cytochrome *b* rate) followed by MC1R (6%), AFLP-WW1 (14%) and CHD-Z (15%). These calculations are complicated by the observation that the rate of molecular change was not homogeneous for all five gene trees. A constant molecular clock was rejected for cytochrome *b* ($\chi^2_{18} = 37.2$, $P < 0.01$) and AFLP-WW1 ($\chi^2_{15} = 27.1$, $P < 0.05$), but not for the other three genes (CHD-Z, $\chi^2_{14} = 19.4$, $P > 0.1$; CHD-W, $\chi^2_{5} = 3.1$, $P > 0.1$; MC1R, $\chi^2_{15} = 22.7$, $P < 0.1$). However, we continued the analyses with an assumption of a constant mutation rate for all genes, evaluating the consequences of variable rates in the discussion.

One of the nuclear genes that we sequenced, the CHD-W intron, is expected to strictly follow the evolution of the mitochondria, as both are inherited together in the female line (Berlin & Ellegren 2001). In general, the shape of the gene trees for the cytochrome *b* and CHD-W appears similar; however, the latter is much more compressed (Fig. 1b), reflecting the slow rate of molecular evolution for this W-chromosome intron (Table 1). The main difference between the gene trees is the position of the dusky warbler, exhibiting identical sequences to chiffchaffs on the CHD-W gene, whereas being clearly outside the species pair willow warbler and chiffchaff when analysed for the cytochrome *b* data. However, this incongruence is not statistically supported. The difference between the willow warbler and chiffchaff/dusky warbler sequences at CHD-W is restricted to two indels, and with so few changes, the relative position of the three species in a gene tree is therefore liable to the stochastic variation of mutations. Hence, we argue that the different positions of the three species [willow warbler (dusky warbler and chiffchaff)] observed in the CHD-W gene tree compared to their statistically well-supported positions in the cytochrome *b* gene tree, merely reflects the low statistical support for the CHD-W gene tree.

The different pattern of variation between willow warblers and chiffchaffs is most striking when comparing the gene tree for mtDNA and the gene trees for the AFLP-WW1 and

Table 3 Nucleotide diversity, long-term effective population size (number of individuals) and Tajima's *D* for five genes in willow warblers and dusky warblers

Gene	Cytochrome *b*	CHD-W	CHD-Z	MC1R	WW1
Willow warbler					
Sequenced gene copies (*n*)	33	20	24	84	22
Long-term effective population size ($1000 \times N_e$)	207	(309)†	3931	1570	2419
N_e ratio (nuclear DNA/mtDNA)	—	(< 1.5)	28.5	11.4	17.5
Tajima's *D*‡	–2.28**	—	–1.03	–2.11*	–0.265
Chiffchaff					
Sequenced gene copies (*n*)	13	4	9	5	14
Long-term effective population size ($1000 \times N_e$)	1059	—	383	5709	884
N_e ratio (nuclear DNA/mtDNA)	—	—	0.36	5.4	0.83
Tajima's *D*‡	–1.95*	—	–1.36	–1.01	–0.67
Dusky warbler					
Sequenced gene copies (*n*)	11	9	10	20	18
Long-term effective population size ($1000 \times N_e$)	269	—	1159	1058	819
N_e ratio (nuclear DNA/mtDNA)	—	—	4.3	3.9	3.0
Tajima's *D*‡	–1.16	—	0.85	0.86	–1.03

† Calculated from a data set in which one substitution had been introduced in one of the 20 studied sequences. This would have resulted in a value of $\pi = 0.028\%$.
‡*$P < 0.05$, **$P < 0.01$.

CHD-Z loci. Chiffchaffs, exhibiting deep divergence in mtDNA, display a low amount of variation at the AFLP-WW1 and CHD-Z loci. On the other hand, willow warblers showed the reversed pattern with a low amount of mtDNA divergence and high amount of divergence at the AFLP-WW1 and CHD-Z loci. At these latter loci, the depth of the gene trees within the willow warbler is deeper (CHD-Z) or similar (AFLP-WW1) as compared with good species within the genus (e.g. *sibilatrix* vs. *bonelli*, Fig. 1). At two loci, CHD-Z and MC1R, the best tree identified willow warblers and chiffchaffs as paraphyletic.

We used the calculated mutation rates (μ) and observed nucleotide diversity to obtain estimates of the long-term effective population sizes (N_e) from each of genes (Table 3). In willow warblers, the lowest N_e was obtained from the cytochrome *b* gene (207 000 individuals) and the highest from CHD-Z (3931 000 individuals). Because we found no intraspecific variation among the 20 sequenced willow warbler CHD-W copies, we cannot calculate N_e from this locus, but the finding of no variation fits well with a mitochondrial-based effective population size (N_e of 207 000 individuals) and a W-chromosome mutation rate of 0.43×10^{-9} (Table 3). The estimates of N_e from the nuclear genes were larger than the N_e estimate from the mitochondria by a factor of 28.5 for CHD-Z, 17.5 for AFLP-WW1 and 11.4 for MC1R. Data from the dusky warbler showed much more even estimates of N_e across loci (Table 3) with the estimates from the nuclear genes being larger than the mtDNA estimate by a factor of 3.0–4.3. The calculated N_e for the nuclear relative to the cytochrome *b* gene in the chiffchaff varied between 0.4 and 5.4; however, these estimates are uncertain because the data set for the chiffchaff is biased in terms of the distribution of samples from the well-differentiated subspecies. This is not a problem for estimating depth in gene trees but will obviously influence estimates of π and hence N_e.

In willow warblers, the calculated Tajima's *D* was significantly negative for the cytochrome *b* gene and MC1R (Table 3), meaning that there were more variable sites in the total data set relative to the number of pairwise differences than would be expected if population sizes were constant and genes were not under selection. In chiffchaffs, Tajima's *D* was significantly negative for the cytochrome *b* gene, whereas in dusky warblers, Tajima's *D* did not differ from expectation for any of the investigated genes (Table 2).

Discussion

Nucleotide diversity

Based on previous information of geographical variation in morphology and mtDNA (Cramp 1992; Helbig *et al*. 1996; Bensch *et al*. 1999), we predicted to find much less nuclear genetic variation in the willow warbler than in the chiffchaff. Our analyses of four nuclear genes clearly demonstrated the contrary; if anything, the willow warbler is harbouring more nuclear diversity than the chiffchaff (Fig. 2). Our measured π in willow warblers should not be sensitive to our sampling scheme, except for the AFLP-WW1 locus which shows phylogeographical structure in

© 2006 Blackwell Publishing Ltd, *Molecular Ecology*, 15, 161–171

Scandinavia and may be linked to a gene under selection (Bensch *et al*. 2002a). However, for the other loci, including the mtDNA, there is no geographical pattern, even when comparing the most distant sample locations (Great Britain and central Siberia). With the exception of π for the AFLP-WW1 locus, our wide sampling scheme should ensure that we have obtained accurate estimates of both π and the deepest nodes for each of the genes. We admit however, that the distribution of samples is not optimal for obtaining unbiased estimates of π in the chiffchaff, but the inclusion of the most divergent subspecies should ensure that we have recovered the deepest nodes within each of the gene trees. Hence, the mismatch between the willow warbler and the chiffchaff in terms of the amount of nuclear DNA variation relative to the amount of mtDNA variation should not be a result of incomplete or biased sampling.

The incongruent diversity seen between willow warbler mtDNA and nuclear DNA might result from either exceptionally low mtDNA diversity or exceptionally high nuclear diversity. The observed cytochrome *b* diversity within willow warblers is slightly below the average in a comparison of mtDNA diversity including 35 species of birds (Moore 1995) and similar to the diversity observed in the closely related dusky warbler. The observed diversity in the dusky warbler is however, likely to be underestimated, because all the samples originated from one single locality.

The finding of a significantly negative Tajima's *D* value for the cytochrome *b* gene of willow warblers indicates however, that the diversity is low relative to the expected diversity found in a constant population without selection (Tajima 1989). The present distribution of mitochondrial haplotypes would therefore underestimate N_e relative to estimates of N_e based on nuclear loci.

There are very few studies estimating the intraspecific diversity for the nuclear genes studied here. For the MC1R gene, only one other species has been studied, the bananaquit *Coereba flaveola*, and it shows a sequence diversity of $\pi = 0.0032$ (Theron *et al*. 2001) which is similar to that observed in the willow warbler. For the CHD-Z gene, however, the sequence diversity within willow warblers is higher by almost one order of magnitude than the most diverse of eight other bird species (Montell *et al*. 2001) including the chiffchaff and the dusky warbler.

The contrasting pattern between the mtDNA and nuclear gene trees might have resulted in part from a selective sweep reducing the diversity of the maternally inherited genes (Li 1997). However, this is unlikely to be the complete explanation. From visual inspections of the phylogenetic trees, the branch depths within willow warblers in the gene trees for CHD-Z and AFLP-WW1 are almost as deep as branches separating species in the genus. Such diversity could have been retained had the effective population size during the last glacial episode being much larger ($\geq 10^6$) than commonly assumed for forest-living passerines (Moore 1995), or by the action of frequency-dependent balancing selection. Although the latter clearly maintains diversity of MHC alleles (Hedrick 1994), it is not thought to be the prevailing selective pattern on the majority of nuclear genes. The overall nuclear DNA diversity in willow warblers ($\pi = 1.17$, average for the three non-maternally inherited genes) is about five times higher than genome-wide nuclear π estimates from SNP data in two species of flycatchers (Primmer *et al*. 2002). It is worth noting that the estimates of π for the flycatchers were considered to be remarkably high compared to the available data for other vertebrates (Primmer *et al*. 2002).

Mutation rates

Overall, the obtained estimates of mutation rates for the nuclear genes are quite consistent with rates assumed for vertebrates (Li 1997). The lowest rate was found for the CHD-W gene (4.29×10^{-10}), slightly lower than a previous estimate for the same locus in other birds (Ellegren & Fridolfsson 1997). A potential problem when using the obtained mutation rates for comparing estimates of effective population sizes for the nuclear genes vs. the cytochrome *b* gene is that that latter showed evidence of a nonhomogenous rate of evolution. Inspecting the gene tree (Fig. 1a) suggests that this pattern is due to a reduced rate of dusky warblers and a somewhat higher rate in chiffchaffs relative to the average rate. Hence, the low level of mtDNA variation in the willow warbler does not appear to be explained by a particularly low mutation rate. The nuclear gene AFLP-WW1 also showed evidence of a nonhomogenous rate of evolution across the gene tree (Fig. 1e), and one clade of willow warbler alleles seems to show a somewhat elevated rate. Hence, if we have underestimated the willow warbler mutation rate at the AFLP-WW1 locus, we have correspondingly overestimated the effective population size for this locus.

The three-time rule

One dramatic difference between the gene trees is the clear reciprocal monophyly for the cytocgrome *b*, whereas the chiffchaff alleles appear mixed with willow warbler alleles at the CHD-Z and MC1R loci. From the within-species diversity (π) in mtDNA and the genetic distance (L) to a sister taxon, one can estimate the coalescence ratio ($C_R = L/\pi$) which has been suggested to be predictive of the proportion of monophyletic nuclear loci in the focal species (Palumbi *et al*. 2001). With a $C_R > 3$, it has been proposed that the majority of nuclear loci should be monophyletic (Palumbi *et al*. 2001). However, this estimate has recently been shown to be misleading because it does not take into account the inherent stochasticity in

mitochondrial coalescence expected under neutral theory (Hudson & Turelli 2003). Taking this stochasticity into account leads to the conclusion that, roughly speaking, a $C_R > 10$ is required for having a 95% expectation under neutral theory that the majority of nuclear loci will be monophyletic, but other factors can cause genetic patterns to deviate widely from this expectation (Hudson & Turelli 2003).

We used our cytochrome *b* data to calculate the C_R of willow warblers relative to the distance to chiffchaffs. The length of the branch leading to the node connecting the willow warbler with the chiffchaff corresponds to 0.068 unit of sequence distance (0.07050 minus 0.00207, which is the intraspecific divergence of willow warblers). This value divided by the sequence diversity within willow warblers (0.00207) gives us a value of C_R of 33, which is substantially higher than the threshold ($C_R > 10$) for expecting the predominance of monophyly at nuclear loci under neutral theory (Hudson & Turelli 2003). The finding of two genes, CHD-Z and MC1R, not being monophyletic in the willow warbler, is therefore in strong conflict with the calculated coalescence ratio (C_R of 33).

A failure of the coalescence ratio to predict the expected proportions of monophyletic loci might be more interesting than its success, because it suggests that more complex historical factors have played a role in the species evolution than is often assumed (Palumbi *et al*. 2001). In the following, we discuss how changes in historical population sizes and hybridization could have affected the observed gene trees in willow warblers.

Complex population histories

Nuclear genes generally have longer expected coalescence times than mitochondrial genes and therefore tend to accumulate genetic diversity over longer periods of history (Takahata & Satta 1997). A striking difference between mtDNA and nuclear diversity has been observed in humans (*Homo sapiens*) and chimpanzees (*Pan troglodytes* and *Pan paniscus*). Humans harbour low mtDNA diversity and high nuclear diversity whereas the opposite is true for chimpanzees (Wise *et al*. 1997), and this discrepancy has been suggested to result from differences in the demographic histories in the two species. For instance, a reduction in population size following the separation between chiffchaffs and willow warblers is more likely to have reduced the coalescence time of mitochondrial than nuclear DNA. This could have exaggerated the difference in diversity between nuclear and mtDNA loci. Population size changes are probably more a rule than an exception (Avise 1992). For example, the present-day world population size of willow warblers is in the range of 100 million pairs (Cramp 1992), hence larger by two orders of magnitude than the long-term effective population size estimated from any of the five genes.

Clearly, introgression of genes from temporarily isolated and differentiated populations would result in higher diversity than would be expected from a balance between mutation and drift in a homogenous population. It has been demonstrated that introgression between species can result in unexpected phylogenies (Degnan 1993; Cohen *et al*. 1997; Tosi *et al*. 2000). The nuclear diversity in willow warblers appears however, not to be explained by recent introgression from an existing taxon, since none of the willow warbler alleles at the investigated loci are shared with another species. We believe we have studied all closely related taxa to willow warblers with which they might be able to hybridize successfully. Hence, if introgression is responsible for the unexpected allelic diversity, the willow warbler genome must have been mixed with chiffchaffs a long time ago or with a now extinct taxon.

Assuming that introgression occurred, an important question is why did it result only in paraphyletic nuclear gene trees and not in paraphyletic mitochondrial gene trees? In birds, females are the heterogametic sex and hybridization between species often results in lower viability and/or fertility among female than male hybrids, a pattern consistent with Haldane's rule (Orr 1997). This would limit the possibility of mitochondrial DNA and W-chromosomes introgressing between species (Tegelström & Gelter 1990; Moore 1995). In *Phylloscopus* warblers, male-biased introgression resulting from inferred reduced fitness of female hybrids has been observed in a contact zone of the chiffchaff subspecies *brehmii* and *collybita* (Bensch *et al*. 2002b). An alternative explanation to the incongruent gene trees of maternally and biparentally inherited genes is that rare mitochondrial introgression easily becomes lost by drift leaving no trace behind (Nordborg 1998) or that genes may face different directions of selection when introgressed in a new genetic background (Martinsen *et al*. 2001).

Conclusions

The low amount of mitochondrial variation compared to nuclear variation in the willow warblers might in part have resulted from a selective sweep on the maternally inherited loci. However, this seems not to be a sufficient explanation to the large nuclear diversity seen within willow warblers unless the long-term effective population size has been very large. The chiffchaff, containing several subspecies highly differentiated both in phenotypic traits and in mtDNA, exhibits less nuclear variation than the willow warbler. Also, the dusky warbler, which appears somewhat more variable in mtDNA than the willow warbler, exhibits less variation at all the nuclear genes. The polyphyletic nuclear gene trees of the willow warbler may rather reflect a more complex evolutionary history. Historical demographic fluctuations or historical male-biased introgression of nuclear

genes from a differentiated population of *Phylloscopus* could have caused a high ratio of nuclear DNA variation to mtDNA variation. These hypotheses can be tested by studying more nuclear genes and evaluating whether the distribution of coalescent times differs from expectation when assuming an evolutionary scenario involving just one homogeneous species (Nichols 2001; Ballard & Whitlock 2004). The results of the present study strongly emphasize that the use of mtDNA alone can give a much too simplified and biased view of the phylogenetic history of a species group (Hudson & Coyne 2002).

Acknowledgements

We are grateful to S. Edwards, A. Janke, N. Mundy, T. Price, A. Richman and M. Turelli for discussions and comments on earlier drafts. L. Gibbs kindly provided primer sequences for the MC1R locus and W. Forstmeier provided samples of dusky warblers. Funding was supported to S.B. by the Swedish Research Council, Crafoordska Stiftelsen and Stiftelsen Olle Engkvist Byggmästare. D.E.I. was supported by an International Research Fellowship from the National Science Foundation, L.K. with a postdoc grant from the Research Council for Biosciences and Environment of the Academy of Finland and S.Å. by the Swedish Research Council.

References

Avise J (1992) Molecular population structure and biogeographic history of regional fauna: a case history with lessons for conservation biology. *Oikos*, **63**, 62–76.

Avise JC (2000) *Phylogeography*. Harvard University Press, Cambridge, Massachusetts.

Ballard JWO, Chernoff B, James AC (2002) Divergence of mitochondrial DNA is not corroborated by nuclear DNA, morphology, or behavior in *Drosphila simulans*. *Evolution*, **56**, 527–545.

Ballard JWO, Whitlock MC (2004) The incomplete natural history of mitochondria. *Molecular Ecology*, **13**, 729–744.

Barton NH (2001) The role of hybridization in evolution. *Molecular Ecology*, **10**, 551–568.

Bensch S, Åkesson S, Irwin DE (2002a) The use of AFLP to find an informative SNP: genetic differences across a migratory divide in willow warblers. *Molecular Ecology*, **11**, 2359–2366.

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, Helbig AJ, Salomon M, Seibold I (2002b) Amplified fragment length polymorphism analysis identifies hybrids between two subspecies of warblers. *Molecular Ecology*, **11**, 473–481.

Berlin S, Ellegren H (2001) Evolutionary genetics — clonal inheritance of avian mitochondrial DNA. *Nature*, **413**, 37–38.

Cohen BL, Baker AJ, Blechschmidt K *et al.* (1997) Enigmatic phylogeny of skuas (Aves: Stercorariidae). *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **264**, 181–190.

Cramp S (1992) *The Birds of the Western Palearctic*. Oxford University Press, Oxford, UK.

Degnan SM (1993) The perils of single gene trees — mitochondrial versus single-copy nuclear DNA variation in white-eyes (Aves: Zosteropidae). *Molecular Ecology*, **2**, 219–225.

Ellegren H (1996) First gene on the avian W chromosome (CHD) provides a tag for universal sexing of non-ratite birds. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **263**, 1635–1641.

Ellegren H, Fridolfsson A-K (1997) Male-driven evolution of DNA sequences in birds. *Nature Genetics*, **17**, 182–184.

Felsenstein J (1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *Journal of Molecular Evolution*, **17**, 368–376.

Forstmeier W, Kempenaers B, Meyer A, Leisler B (2002) A novel song parameter correlates with extra-pair paternity and reflects male longevity. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, **269**, 1479–1485.

García-Moreno J (2004) Is there a universal mtDNA clock for birds? *Journal of Avian Biology*, **35**, 465–468.

Glenn TC, Glenn SJ (1994) Rapid elution of DNA from agarose gels using polyester plug spin inserts (PEPSIs). *Trends in Genetics*, **10**, 344.

Grant PR, Grant BR (1992) Hybridization of bird species. *Science*, **256**, 193–197.

Griffiths R, Tiwari B (1993) The isolation of molecular genetic markers for the identification of sex. *Proceedings of the National Academy of Sciences, USA*, **90**, 8324–8326.

Hall TA (1999) BIOEDIT: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95–98.

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM (2004) Identification of birds through DNA barcodes. *PLoS Biology*, **2**, e312.

Hedrick PW (1994) Evolutionary genetics of the major histocompatibility complex. *American Naturalist*, **143**, 945–964.

Helbig AJ, Martens J, Seibold I *et al.* (1996) Phylogeny and species limits in the Palaearctic chiffchaff *Phylloscopus collybita* complex: mitochondrial genetic differentiation and bioacoustic evidence. *Ibis*, **138**, 650–666.

Helbig AJ, Seibold I, Martens J, Wink M (1995) Genetic differentiation and phylogenetic relationships of Bonelli's warbler *Phylloscopus bonelli* and green warbler *P. nitidus*. *Journal of Avian Biology*, **26**, 139–153.

Hudson RR, Coyne JA (2002) Mathematical consequences of the genealogical species concept. *Evolution*, **56**, 1557–1565.

Hudson RR, Turelli M (2003) Stochasticity overrules the 'three-times rule': genetic drift, genetic draft and coalescence times for nuclear loci vs. mtDNA. *Evolution*, **57**, 182–190.

Irwin DE (2002) Phylogeographic breaks without geographic barriers to gene flow. *Evolution*, **56**, 2383–2394.

Irwin DE, Alström P, Olsson U, Benowitz-Fredericks ZM (2001a) Cryptic species in the genus *Phylloscopus* (old world leaf warblers). *Ibis*, **143**, 233–247.

Irwin DE, Bensch S, Price TD (2001b) Speciation in a ring. *Nature*, **409**, 333–337.

Irwin DE, Bensch S, Irwin JH, Price TD (2005) Speciation by distance in a ring species. *Science*, **307**, 414–416.

Johns GC, Avise JC (1998) A comparative summary of genetic distances in the vertebrates from the mitochondrial cytochrome *b* gene. *Molecular Biology and Evolution*, **15**, 1481–1490.

Kliman RM, Andolfatto P, Coyne JA *et al.* (2002) The population genetics of the origin and divergence of the *Drosophila simulans* complex species. *Genetics*, **156**, 1913–1931.

Kocher TD, Thomas WK, Meyer A *et al.* (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences, USA*, **86**, 6196–6200.

Li W-H (1997) *Molecular Evolution*. Sinauer Associates, Sunderland, Massachusetts.

Lovette IJ (2004) Mitochondrial dating and mixed support for the '2% rule' in birds. *Auk*, **121**, 1–6.

MacDougall-Shackleton EA, Blanchard L, Gibbs HL (2003) Unmelanized plumage patterns in old world leaf warblers do not correspond to sequence variation at the melanocortin-1 receptor locus (MC1R). *Molecular Biology and Evolution*, **20**, 1675–1681.

Martinsen GD, Whitham TG, Turek RJ, Keim P (2001) Hybrid populations selectively filter gene introgression between species. *Evolution*, **55**, 1325–1335.

Mishmar D, Ruiz-Pesini E, Golik P *et al.* (2003) Natural selection shaped regional mtDNA variation in humans. *Proceedings of the National Academy of Sciences, USA*, **100**, 171–176.

Montell H, Fridolfsson A-K, Ellegren H (2001) Contrasting levels of nucleotide diversity on the avian Z and W sex chromosomes. *Molecular Biology and Evolution*, **18**, 2010–2016.

Moore WS (1995) Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution*, **49**, 718–726.

Nichols R (2001) Gene trees and species trees are not the same. *Trends in Ecology & Evolution*, **16**, 358–364.

Nordborg M (1998) On the probability of neanderthal ancestry. *American Journal of Human Genetics*, **63**, 1237–1240.

Orr HA (1997) Haldane's rule. *Annual Review of Ecology and Systematics*, **28**, 195–218.

Page RDM (1996) TREEVIEW: an application to display phylogenetic trees on personal computers. *Computer Applications in the Biosciences*, **12**, 357–358.

Palumbi SR, Cipriano F, Hare MP (2001) Predicting nuclear gene coalescence from mitochondrial data: the three-time rule. *Evolution*, **55**, 859–868.

Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Price T (1996) Exploding species. *Trends in Ecology & Evolution*, **11**, 314–315.

Price T, Helbig AJ, Richman AD (1997) Evolution of breeding distributions in the old world leaf warblers (genus *Phylloscopus*). *Evolution*, **51**, 552–561.

Primmer CR, Borge T, Lindell J, Saetre GP (2002) Single-nucleotide polymorphism characterization in species with limited available sequence information: high nucleotide diversity revealed in the avian genome. *Molecular Ecology*, **11**, 603–612.

Richman AD, Price T (1992) Evolution of ecological differences in the old world leaf warblers. *Nature*, **355**, 817–821.

Roman J, Palumbi SR (2003) Whales before whaling in the north Atlantic. *Science*, **301**, 508–510.

Rozas J, Rozas R (1997) DNASP version 2.0: a novel software package for extensive molecular population genetic analysis. *Computer Applications in the Biosciences*, **13**, 307–311.

Sabeti PC, Reich DE, Higgins JM *et al.* (2002) Detecting recent positive selection in the human genome from haplotype structure. *Nature*, **419**, 832–837.

Sachidanandam R, Weissman D, Schmidt SC *et al.* (2001) A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature*, **409**, 928–933.

Shaw KL (2002) Conflict between nuclear and mitochondrial DNA phylogenies of a recent radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets. *Proceedings of the National Academy of Sciences, USA*, **99**, 16122–16127.

Shields GF, Wilson AC (1987) Calibration of mitochondrial DNA evolution in geese. *Journal of Molecular Evolution*, **24**, 212–217.

Sivasundar A, Hey J (2003) Population genetics of *Caenorhabditis elegans*: the paradox of low polymorphism in a widespread species. *Genetics*, **163**, 147–157.

Swofford DL (1998) PAUP*. *Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Version 4 edn. Sinauer Associates, Sunderland, Massachusetts.

Tajima F (1989) Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics*, **123**, 585–595.

Takahata N, Satta Y (1997) Evolution of the primate lineage leading to modern humans: phylogenetic and demographic inferences from DNA sequences. *Proceedings of the National Academy of Sciences, USA*, **94**, 4811–4815.

Tarr CL, Fleischer RC (1993) Mitochondrial DNA variation and evolutionary relationships in the Amakihi complex. *Auk*, **110**, 825–831.

Tegelström H, Gelter HP (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.

Theron E, Hawkins K, Bermingham E, Ricklefs RE, Mundy NI (2001) The molecular basis of an avian plumage polymorphism in the wild: a melanocortin-1-receptor point mutation is perfectly associated with the melanic plumage morph of the bananaquit, *Coereba flaveola*. *Current Biology*, **11**, 550–557.

Ting C-T, Tsaur S-C, Wu C-I (2000) The phylogeny of closely related species as revealed by the genealogy of a speciation gene, *Odysseus*. *Proceedings of the National Academy, USA*, **97**, 5313–5316.

Tosi AJ, Morales JC, Melnick DJ (2000) Comparison of Y and mtDNA phylogenies leads to unique inference of macaque evolutionary history. *Molecular Phylogenetics and Evolution*, **17**, 133–144.

Wang R-L, Stec A, Hey J, Lukens L, Doebley J (2002) The limits of selection during maize domestication. *Nature*, **398**, 236–239.

Wise CA, Sraml M, Rubinsztein DC, Easteal S (1997) Comparative nuclear and mitochondrial genome diversity in humans and chimpanzees. *Molecular Biology and Evolution*, **14**, 707–716.

Staffan Bensch is Professor in Animal Ecology at Lund University and has a broad interest in molecular ecology with current research projects involving population genetics of migratory birds and avian malaria parasites. Darren Irwin is Assistant Professor of Zoology at the University of British Columbia. Together with Jessica Irwin, he conducts research on speciation, population genetics, behavioral evolution, and biogeography, using birds of Eurasia and North America as model systems. Laura Kvist is currently a docent in molecular ecology and evolution at University of Oulu, with research interests focused on genetic structures, phylogeography and molecular population ecology of birds. Susanne Åkesson is Associate Professor in Animal Ecology at Lund University. Her research interests include sensory adaptations of animal navigation, evolution of compass systems and genetic migration programs.