Tracing the evolutionary history of the Atlas flycatcher (*Ficedula speculigera*)
- A molecular genetic approach

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Forord

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Da var tiden ute og oppgaven ferdig. Men det er selvsagt mange som bør takkes.

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Abstract

The Atlas flycatcher (*Ficedula speculigera*) is a poorly studied species in an otherwise thoroughly studied species complex. In this study I attempt to reconstruct the evolutionary history of this species, I look for possible traces of introgression, I test whether there is contrasting patterns of polymorphism and divergence at autosomal and Z-linked genes as has been found in other flycatcher species, and finally whether the Atlas flycatcher show reduced genetic variation which would be expected due to recent habitat fragmentation. To address these problems I used multilocus sequence analysis, with loci from both autosomes and Z-chromosomes, and compared these with previously published sequences from two other species, the pied flycatcher (*F. hypoleuca*) and the collared flycatcher (*F. albicollis*). Finally, phenotypic measures of the Atlas flycatcher were compared with measures of these other two species. The results appear consistent with a scenario in which an ancestral flycatcher species became isolated in different refugia, presumably around the Mediterranean Sea, at the onset of the Pleistocene glaciations and diverged into the present species. I found no traces of introgression between the Atlas flycatcher and any of the two other species. Further, the Atlas flycatcher showed reduced variation at Z-linked loci compared to autosomal loci, which may indicate a complex demographic history or possibly selection. The Atlas flycatcher also showed high variation compared to the other two species, and therefore seems to have a rather large effective population size.

Introduction

Allopatric speciation, the evolution of reproductive isolation between populations that are separated by a geographic barrier, is by many considered to be the "default" mode of speciation (Coyne and Orr 2004). The simplest and most intuitive mode of allopatric speciation is vicariant speciation, in which previously interbreeding populations get geographically separated (Mayr 1942). This spatial separation can be caused by climatic or geographic events. The isolated population would then diverge through genetic drift and divergent natural selection. Consequently such isolated populations would accumulate genetic differences over time that eventually would lead to reproductive isolation due to behavioural and/or developmental incompatibilities and thus speciation. This process would tend to be speeded up by divergent natural selection and countered or at least slowed down by gene flow.

A recent source of vicariance for many organisms, was the climate cycles of the Pleistocene (1,808,000 to 11,550 years before present) (Hewitt 1996; Avise and Walker 1998; Avise et al. 1998). For instance, with recurrent glaciation in Europe many forest-dwelling species were pushed back to isolated refuge areas located to the peninsulas bordering the Mediterranean Sea (i.e. Iberia, Italy and the Balkans) as well as North Africa, where the forest habitat remained during the cold periods (Hewitt 1996; Avise and Walker 1998; Avise et al. 1998). After the retraction of the ice, the continent was re-colonised, sometimes resulting in populations from different refugia experiencing secondary contact. Consistent with this scenario, we find higher genetic diversity in many organisms in the area around the Mediterranean Sea than in other parts of their breeding ranges, and also hybrid zones further north reflecting the edge of range of dispersal after the retraction of the ice (Hewitt 1996).

The black-and-white *Ficedula* flycatcher species complex appears to have been strongly affected by vicariance episodes during the Pleistocene glaciations (Sætre et al. 2001a). The taxonomic relations and evolutionary history of the species have been much debated (Lundberg and Alatalo 1992). The species complex was previously assumed to consist only of two species, the pied flycatcher (*Ficedula hypoleuca*) and the collared flycatcher (*F. albicollis*). However, sequence comparisons of mitochondrial genes from different flycatcher populations have shown that there are four highly divergent groups (Sætre et al. 2001a; Sætre et al. 2001b). Accordingly, it has been argued that the semicollared flycatcher (*F.*

semitorquata), earlier assumed to be a subspecies of the collared flycatcher, and the Atlas flycatcher (*F. speculigera*), earlier assumed to be a subspecies of the pied flycatcher, should also be regarded as distinct species (Sætre et al. 2001a; Sætre et al. 2001b). These species have been argued to originate from an ancestral population that became isolated in different refuge areas around the Mediterranean Sea approximately 2 million years ago, at the onset of the Pleistocene glaciations. Following the retraction of the ice, the pied and the collared flycatcher expanded northwards from their respective refugia on the Iberian and Italian peninsulas and eventually came into secondary contact in Central and Eastern Europe, whereas the semicollared and the Atlas flycatcher remained close to their assumed refuge areas respectively in Balkan/Caucasus and the Atlas mountains of North Africa (Sætre et al. 2001a).

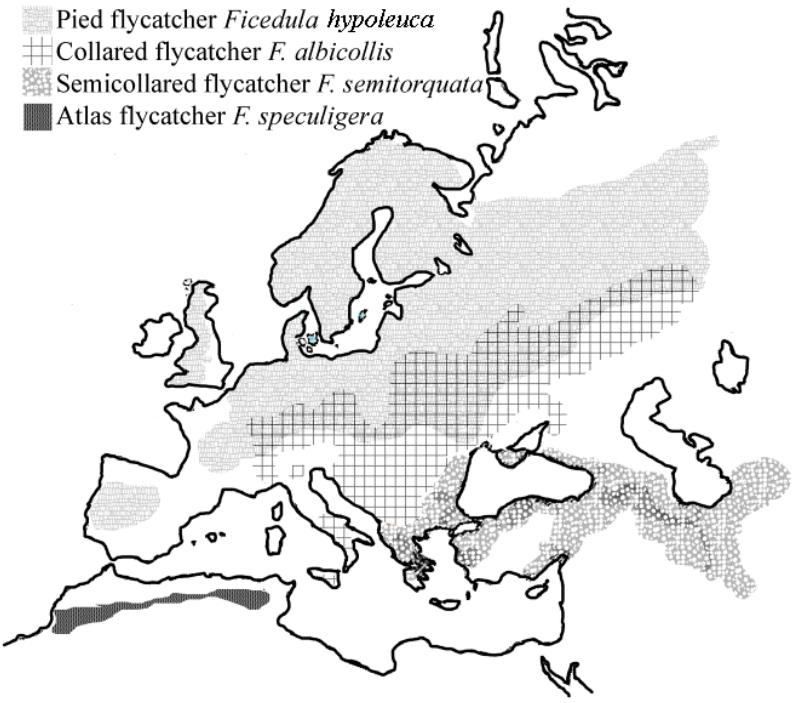


Figure 1 – The present geographical breeding distributions of the four species of Ficedula flycatchers.

The *Ficedula* flycatchers are forest dwelling passerine birds in the *Muscicapidae* family. They are all sexually dimorphic and the four species share many features phenotypically and behaviourally. They are long distance migrants with breeding grounds in Europe and north-western Africa (figure 1) and wintering grounds in tropical Africa (Lundberg and Alatalo 1992). In the areas where pied and collared flycatchers live in sympatry (central and eastern Europe and the Baltic Isles) hybridisation occurs. They breed in nest holes in trees, which limit them to some extent to older forest. *Ficedula* flycatchers are easy to study, since they prefer nest boxes to natural nest holes, and are fairly tame and easily handled for blood samples and phenotypic measures. Consequently the pied and collared flycatchers have been popular study objects for behavioural, ecological and in later years also evolutionary genetic studies. However the two other species are poorly studied.

This study focuses on the Atlas flycatcher specifically. The Atlas flycatcher has its breeding grounds in the Atlas Mountains of Morocco, Algeria and Tunisia, where it is found primarily in old deciduous forest. The study consists of a multilocus sequence analysis of Atlas flycatchers, which are compared with previously published sequences of pied and collared flycatchers, and of the red-breasted flycatcher (*Ficedula parva*) which serve as an outgroup (Borge et al. 2005). I also present descriptive statistics of various phenotypic measures of the species to compare with data from pied and collared flycatchers.

The few previous studies of the Atlas flycatcher are based on few individuals, and mainly mitochondrial DNA has been analysed. The aim of this study is to do a more thorough genetic investigation of the species, using a larger sample size and to focus on nuclear genes. The nuclear and mitochondrial genomes may provide different signals from a species' evolutionary history (Shaw 2002; Bensch et al. 2006). For instance following Haldane's rule, since in birds females are the heterogametic sex, gene flow between incipient species may be brought to a halt at an earlier stage at the mitochondrial than at the nuclear genome. This is because mitochondria are inherited through the maternal line and since females are expected to be the sex first experiencing hybrid incompatibilities in species with female heterogamy (Coyne and Orr 2004). Accordingly, historical contact (gene flow) between the Atlas flycatcher and other flycatcher species may be more likely to be detected using nuclear genetic markers than mtDNA. So far, the taxonomic status of the Atlas flycatcher is only based on a rather limited comparison of mtDNA. It is therefore of interest to investigate other genetic markers to check whether or not one finds concordant results.

In a previous study of pied and collared flycatchers Borge et al. (2005) found contrasting patterns of polymorphism and divergence at autosomal and Z-linked genes. They suggested that selective sweeps on the Z-chromosome could explain this pattern and that such selection events could have been important during their speciation. Here, I compare sequences both from autosomal and Z-linked genes to investigate whether or not I find similar patterns in the Atlas flycatcher. Moreover, since the effective population size of a Z-linked gene is ¾ of that of an autosomal gene, demographic processes, such as changes in population size, may affect Z-linked and autosomal genes differently and thus provide additional information for reconstructions of the demographic history of the species. At present, the Atlas flycatcher appears to have a very patchy and fragmented distribution due to strong deforestation in its breeding range. Hence, one may expect reduced genetic variation if its population structure has been negatively affected by this habitat fragmentation. As such, this study may also have applications for conservation issues of the species.

Materials and methods

Fieldwork

The fieldwork was conducted from 3rd to 13th of May 2005 near the towns of Asrou and Ifrane (33°N, 5°W) located in the Middle Atlas Mountains of Morocco, at around 1500AMSL (above main sea level). The breeding localities here are scattered in patches of old deciduous forest, mainly oak (*Quercus spp)*, with ample supply of breeding holes. 33 birds (30 males and 3 females) were caught using mist nets and male song playback, and all birds were measured for weight, size and plumage characteristics. In addition, data on 5 males caught in the same area during May 1998 were available. 25 µl of blood were collected in capillary tubes after puncturing the brachial vein and suspended in Queen's Lysis Buffer (Seutin et al. 1991).

Phenotypic measures

All birds caught were measured for plumage characteristics. The characteristics measured include the colour score according to the seven-point scale of Drost (1936) where score 1 = completely black head and score 7 = completely brown, height of the white forehead patch, the number of white-edged tail feathers, the maximum wing patch width, further I noticed at which primary the wing patch started, the rump colour and whether a neck collar was found or not. All length, height and width estimates were measured with a dial calliper with an accuracy of 0.1 mm.

DNA extraction

40 µl blood and buffer mix were mixed with 500 µl of 0.1 M NaCl, 10 mM Tris-Cl (pH 8.0), 5% SDS and 50 µl proteinase K (10 mg/ml) and incubated overnight on a thermoshaker. The samples were treated twice with phenol/chloroform/isoamylalcohol (Sambrook et al. 1989) and the DNA was precipitated with 96% ethanol and cleansed with 70% ethanol twice, before the pellets were dried and redissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.5). 9 male individuals were selected for further analysis. Since comparing autosomal with Z-linked loci is an essential part of this study, only males were used. This is because males are the homogametic sex, and therefore possess two Z-chromosomes, and the comparison will be more balanced.

PCR

Several primer pairs that has been extensively tested on *Ficedula* flycatchers were available (Borge et al. 2005). These were designed from GenBank entries on chicken (*Gallus gallus*). In short, primers were designed in exon sequence flanking introns of appropriate size from both autosomal and Z chromosome-linked genes. In cases of variable amplification success or for long introns, new flycatcher-specific primers were designed (Borge et al. 2005). For the present study I tested most primer pairs that had high amplification success in the earlier study on the pied and collared flycatcher (Borge et al. 2005), and chose the ones in which readable sequences were obtained in all individuals for further analysis. These loci are listed in Table 1.

The amplification reaction had a total volume of 10 µl and contained 1X PCR Buffer II (Applied Biosystems), 0.2 mM of each dNTP, 0.32 µM of each primer, 1 µg of bovine serum albumin (BSA), 0.3 units of AmpliTaq DNA polymerase (Applied Biosystems), 1 mM $MgCl_2$ and 10 ng DNA. The amplifications were run on a PTC-200 or a DNA Engine Tetrad 2 (MJ Research, Waterton, MA, USA) with the following profile: 95°C for 1 minute and then 35-40 cycles of 94°C for 30 seconds, 50-62°C for 30 seconds, 72°C for 1 minute. Finally an extension step of 10 minutes at 72°C was used.

Sequencing

Excess nucleotides and primers were removed from the PCR-products using ExoSap-IT (United States Biochemical, Cleveland) and then cycle sequenced using BigDye Terminator sequencing buffer and v3.1 Cycle Sequencing kit (Applied Biosystems) and analyzed on an ABI 3730 DNA Analyzer (Applied Biosystems). Sequences were aligned and edited with MEGA 3.1 (Kumar et al. 2001) and manually adjusted. To get reliable sequences, bases were called on the basis of both strands in all instances, except short regions where one direction was of low quality, due to short insertion/deletions (indels) or sequence repeats. In such instances the sequence was called based on primarily multiple sequences of one strand.

Data analysis

The diploid sequences from the 9 Atlas flycatchers were transformed into "pseudo-haplotypes" together with sequences from 9 collared flycatchers, 9 pied flycatchers and one redbreasted flycatcher. This was done by assigning the two alleles at each polymorphic site to

one of the two sequences, on the basis of the ambiguity codes produced. The sequences were aligned using ClustalW (Thompson et al. 1994) and manually adjusted.

Analysis of polymorphism and divergence were done using the program DnaSP 4.0 (Rozas and Rozas 1999). This program gives basic descriptive statistics of the sequences, estimates of the population mutation rate (nucleotide diversity), π (Nei 1987), and θ (Watterson 1975), and their standard deviation. It also performs tests of neutrality based on the allele frequency spectrum, such as Tajima's D (Tajima 1989b) and Fu and Li's D and F (Fu and Li 1993). Multilocus Hudson-Kreitman-Aguadé (HKA) tests (Hudson et al. 1987) were done using the HKA program (http://lifesci.rutgers.edu/~heylab/). This test investigates if interspecific and intraspecific variation is positively correlated as expected under neutral evolution. To assess whether the different population pairs had originated from an ancestral larger population without later gene flow, isolation model fitting was done as described in Wakeley and Hey (1997) and Wang et al. (1997) using the WH program (http://lifesci.rutgers.edu/~heylab/). Neighbour-joining trees were constructed using the program MEGA 3.1 (Kumar et al. 2001), to see if a larger sample size of nuclear gene gives the same topography as mtDNA and fewer nuclear genes has done earlier (Sætre et al. 2001a; Sætre et al. 2001b).

Results

Data

The sequences generated from the 9 Atlas flycatchers were analysed together with sequences generated in a previous study (Borge et al. 2005), namely 9 collared flycatchers from Italy, 9 pied flycatchers from Spain and one red-breasted flycatcher. Since pseudohaplotypes were constructed, and since only males were used, the sample size per locus is 18 for both Z-linked genes and autosomal genes. In the present study 16 loci were used, 10 of which were Z-linked genes (4189 bp in total) and 6 of which were autosomal genes (3335 bp in total). Some of the sequences possessed short insertion-deletions (indels) across the three species. These short regions were excluded from the analysis to get the same sequence length across the species and individuals for easier comparisons. Because all sequences of one locus had to be the same length, the sequences were all cut down to the same length as the shortest sequence in the sample, resulting in a loss of sequence data. The sequences are mostly intronic, though some coding sequences at the ends are featured in the analysis. Some further details of these loci are presented in table 1.

Table 1 - Sample sequences

	Cytological position	Length		
		Total	Exon	Intron
ALDOB-6	Z	460	23	437
ALDOB-7	Z	153	8	145
BRM-12	Z	1572	0	1572
CHDZ-15	Z	495	34	461
CHDZ-18	Z	209	0	209
GHR-5.1	Z	112	0	112
GHR-5.2	Z	450	0	450
VLDLR-8	Z	127	59	68
VLDLR-9	Z	409	33	376
VLDLR-12	Z	196	72	124
ACLY-16	A	358	3	355
ALAS1-8	A	291	0	291
FAS-Y	A	901	0	901
RHO-1	A	375	0	375
RPL30-3	A	1008	0	1008
TGFB2-5	A	402	0	402

Polymorphism and divergence

Data on polymorphism are summarised in table 2 and figure 2. Loci from the same gene are combined and analysed as one gene. Several measures of polymorphism were used, including π (Nei and Li 1979), the number of nucleotide differences per site between pairs of randomly chosen sequences, and Watterson's estimate of the population mutation rate parameter θ_W (Watterson 1975). The two measures of polymorphism showed similar patterns, with the pied flycatcher exhibiting the lowest level of variation, and rather similar and higher variation in the collared and Atlas flycatchers (Table 2). Divergence from the outgroup was very similar for all species and at all loci, indicating a similar rate of mutation in all three species. The above-mentioned differences in variation are therefore likely to reflect differences in effective population size, N_e.

The divergence from the outgroup was somewhat higher at autosomal loci compared to Z-linked loci in both the Atlas flycatcher (0.0189 at Z-linked loci and 0.0195 at autosomal loci) and the pied flycatcher (0.0189 at Z-linked loci and 0.0195 at autosomal loci), but not in the collared flycatcher (0.0193 at Z-linked loci and 0.0192 at autosomal loci).

Levels of polymorphism were found to be distinctly lower on the Z-linked loci compared to the autosomal loci (Table 2). The levels of variation at the Z-linked were 60%, 48% and 51% that of the autosomal variation in Atlas flycatcher, collared flycatcher and pied flycatcher, respectively. One would expect higher mutation rate at the Z chromosome to lead to higher variation at this chromosome than at autosomal ones. This would on the other hand be counteracted by ¾ because of the lower effective population size of the Z-chromosome since females only have one copy of the chromosome. Note, however, that these expectations only apply under the assumptions of a standard neutral model, i.e. assuming constant population size, random mating, no selection and no migration (Watterson 1975).

The expected variation at Z-linked loci based on the divergence in autosomal loci was calculated as suggested by Borge et al. (2005), using the following equation:

$$S_Z(\exp) = \frac{3D_Z S_A}{4D_A}$$

where $S_Z(\exp)$ is the expected number of segregating sites at a Z-linked locus, D_Z is the divergence between the focal species (i.e. Atlas, collared or pied flycatcher) and the outgroup

(red-breasted flycatcher), S_A is the total number of segregating sites at autosomal loci, and D_A is the total divergence between the three species and the outgroup at autosomal loci. A corresponding formula was used to calculate the expected number of segregating sites at a specific autosomal locus, $S_A(\text{exp})$:

$$S_A(\text{exp}) = \frac{4D_A S_Z}{3D_Z}$$

These equations were used to calculate the expected S-values, and the results are presented in table 2. At Z-linked loci the observed S-values are lower than the ones predicted from polymorphism and divergence at autosomal loci. This was seen in 12 of 15 cases (signs test, P=0.0176). At autosomal loci on the other hand, the observed number of segregating sites were higher than the predicted in 11 of 18 cases (signs test, P=0.2403). These data therefore suggest that variation at Z-linked loci is reduced compared to autosomal loci under a standard neutral model, although the analysis of the autosomal loci was not significant (see also figure 2).

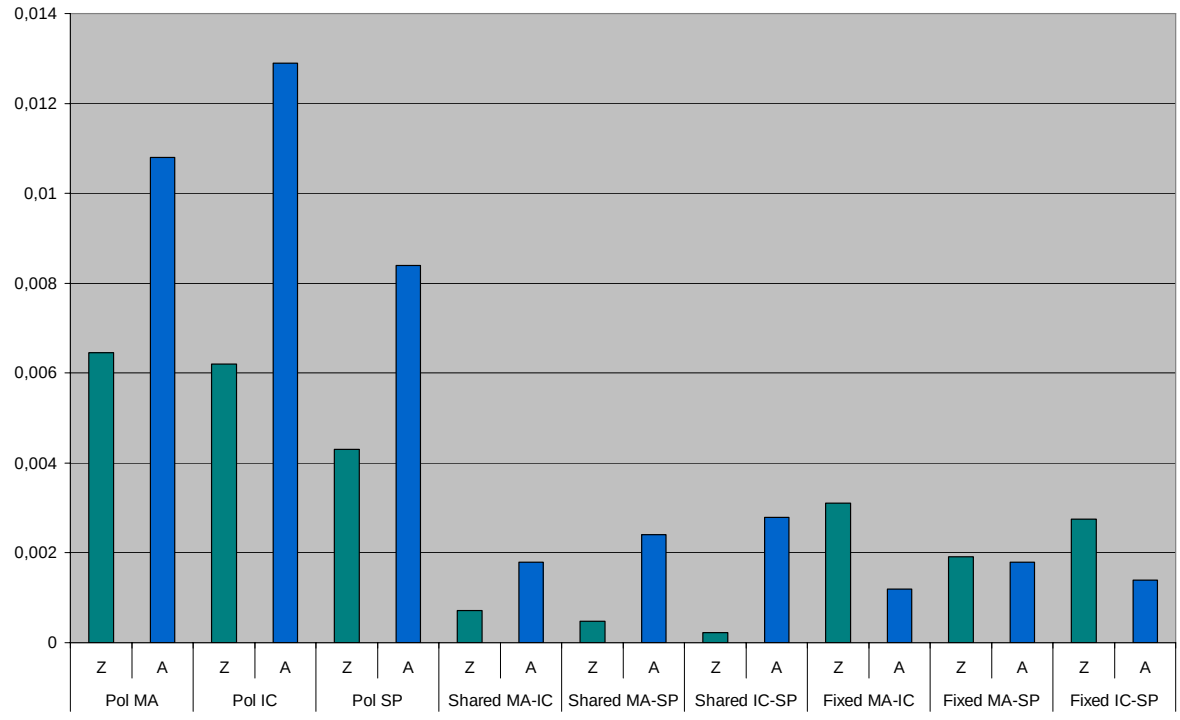


Figure 2 – Descriptive statistics of polymorphism and divergence for Atlas flycatcher (MA), collared flycatcher (IC) and pied flycatcher (SP) at Z-linked loci (4182 bp) and autosomal loci (3334 bp). Z-linked loci are shown in green and autosomal loci in blue. The number of within species polymorphisms (abbreviated Pol), between species shared polymorphisms (Shared) and fixed differences between species (Fixed) are all shown as per base pair.

Table 2 – Polymorphism summary

Gene	Species[a]	L[b]	K[c]	S[d]	S_{exp}[e]	s[f]	π[g]	SD	θ_w[h]	SD	D_T[i]	D_{FL}[j]	F_{FL}[k]
Aldob	MA	620	14	0	5.8154	0	0.0000	0.0000	0.0000	0.0000	0.00	0.00	0.00
	IC	620	14	1	7.0547	0	0.0003	0.0002	0.0005	0.0005	-0.53	0.67	0.40
	SP	620	13	3	4.2000	1	0.0013	0.0003	0.0014	0.0009	-0.26	-0.08	-0.15
Brm	MA	1572	37	15	15.3692	8	0.0017	0.0004	0.0028	0.0012	-1.52	-1.11	-1.42
	IC	1572	41	9	20.6602	5	0.0013	0.0002	0.0017	0.0008	-0.72	-1.11	-1.16
	SP	1571	38	10	12.2769	0	0.0028	0.0002	0.0019	0.0008	1.79	1.41*	1.75*
Chdz	MA	704	14	1	5.8154	0	0.0006	0.0001	0.0004	0.0004	0.87	0.67	0.82
	IC	704	13	1	6.5508	1	0.0002	0.0001	0.0004	0.0004	-1.16	-1.50	-1.61
	SP	704	14	2	4.5231	0	0.0007	0.0002	0.0008	0.0006	-0.33	0.88	0.64
Ghr	MA	555	9	3	3.7385	0	0.0022	0.0003	0.0016	0.0010	1.18	1.02	1.22
	IC	562	9	2	4.5352	1	0.0011	0.0002	0.0010	0.0008	0.25	-0.55	-0.39
	SP	562	10	0	3.2308	0	0.0000	0.0000	0.0000	0.0000	0.00	0.00	0.00
Vldlr	MA	731	5	8	2.0769	4	0.0022	0.0005	0.0032	0.0015	-1.07	-0.84	-1.05
	IC	731	4	13	2.0156	3	0.0058	0.0006	0.0052	0.0022	0.47	0.37	0.46
	SP	731	4	3	1.2923	0	0.0009	0.0005	0.0012	0.0008	-0.78	1.02	0.62
Total	MA	4182	79	27	32.8154	12	0.0014	0.0002	0.0019	0.0007	-1.00	-0.72	-0.93
	IC	4189	81	26	40.8164	10	0.0017	0.0002	0.0018	0.0007	-0.14	-0.40	-0.38
	SP	4188	79	18	25.5231	1	0.0015	0.0001	0.0013	0.0005	0.77	1.27	1.31
Acly-16	MA	358	5	0	2.2785	0	0.0000	0.0000	0.0000	0.0000	0.00	0.00	0.00
	IC	358	5	1	2.1399	1	0.0003	0.0003	0.0008	0.0008	-1.16	-1.50	-1.61
	SP	358	5	2	1.5190	2	0.0006	0.0004	0.0016	0.0012	-1.51	-1.99	-2.13
Alas1-8	MA	291	12	5	5.4684	1	0.0047	0.0006	0.0050	0.0027	-0.22	0.42	0.28
	IC	291	14	7	5.9918	3	0.0055	0.0009	0.0070	0.0035	-0.75	-0.51	-0.67
	SP	291	15	4	4.5570	2	0.0022	0.0008	0.0040	0.0023	-1.35	-0.70	-1.01
Fas-y	MA	900	17	2	7.7468	1	0.0004	0.0002	0.0007	0.0005	-1.10	-0.55	-0.80
	IC	901	17	2	7.2757	0	0.0006	0.0002	0.0007	0.0005	-0.33	0.88	0.64
	SP	900	16	0	4.8608	0	0.0000	0.0000	0.0000	0.0000	0.00	0.00	0.00
Rho-1	MA	375	9	4	4.1013	1	0.0032	0.0006	0.0031	0.0018	0.09	0.21	0.20
	IC	375	7	7	2.9959	1	0.0062	0.0008	0.0054	0.0027	0.51	0.70	0.75
	SP	375	7	7	2.1266	1	0.0067	0.0009	0.0054	0.0027	0.81	0.70	0.84
Rpl30-3	MA	1008	17	19	7.7468	9	0.0055	0.0004	0.0055	0.0022	-0.01	-0.84	-0.70
	IC	1008	16	20	6.8477	4	0.0053	0.0008	0.0058	0.0023	-0.29	0.55	0.36
	SP	1008	17	12	5.1646	6	0.0027	0.0008	0.0035	0.0015	-0.86	-0.91	-1.04
Tgfb2-5	MA	402	5	6	2.2785	2	0.0040	0.0006	0.0043	0.0022	-0.25	-0.10	-0.17
	IC	402	5	6	2.1399	4	0.0028	0.0007	0.0043	0.0022	-1.21	-1.47	-1.61
	SP	402	5	3	1.5190	1	0.0023	0.0004	0.0022	0.0014	0.12	-0.08	-0.04
Total	MA	3334	65	36	29.6203	14	0.0030	0.0002	0.0031	0.0012	-0.19	-0.44	-0.42
	IC	3335	64	43	27.3909	13	0.0033	0.0004	0.0038	0.0014	-0.48	0.03	-0.14
	SP	3334	65	28	19.7468	12	0.0021	0.0003	0.0024	0.0010	-0.57	-0.64	-0.72

* P<0.05

[a] MA, Moroccan Atlas flycatcher; IC, Italian collared flycatcher; SP, Spanish pied flycatcher.

[b] Number of sites surveyed.

[c] Divergence with outgroup

[d] Number of segregating sites

[e] The expected number of segregating sites based on Ne estimated from autosomal or Z-linked loci respectively.

[f] Number of singleton sites.

[g] Average pairwise sequence difference per nucleotide (Nei 1987).

[h] Expected heterozygosity per nucleotide (Watterson 1975).

[i] (Tajima 1989b).

[j] (Fu and Li 1993).

[k] (Fu and Li 1993).

Allele frequency spectra

Three different tests of neutrality were used (Table 2); Tajima's D (1989b) and Fu and Li's D and F (1993). In most cases the tests yielded slightly negative test statistics albeit not significantly different from zero. Negative test statistics means that there is an excess of rare variants compared to neutral expectation. The pied flycatcher, however, showed positive test statistics at several Z-linked loci, and significantly so at the locus BRM at Fu and Li's D and F, and a near significant at Tajima's D. The overall test statistics for the Z-linked loci were also positive, and borderline significant at Fu and Li's D value. This indicates a deficit of rare variants compared to neutral expectation.

HKA-test

Multilocus HKA-tests were run for all species pairs for both Z-linked and autosomal loci (Table 3). In all tests the polymorphism and divergence data from table 2 and 4 were used. The HKA-test tests whether variation within and between species are positively correlated (Hudson et al. 1987), as expected under neutrality. 10,000 coalescent simulations were run to assess significance. None of the species pair combinations yielded significant deviations from the neutral expectation at neither autosomal nor Z-linked loci. In a previous study of pied and collared flycatcher using a large number of loci, significant deviations from neutrality was found at Z-linked loci (Borge et al. 2005). Consistent with this result I did find slightly higher deviations from neutrality at the Z-linked loci than at the autosomal ones, although neither comparison was significant.

Table 3 - HKA-results

	Segregating sites				Divergence	
	Atlas flycatcher		Collared flycatcher			
Gene	Obs[a]	Exp[b]	Obs	Exp	Obs	Exp
ALDOB	0	1.97	1	1.9	4.89	2.02
BRM	15	12.13	9	11,68	12.2	12.4
CHDZ	1	1.12	1	1.08	1.33	1.14
GHR	3	2.29	2	2.2	1.83	2.34
VLDLR	8	9.49	13	9.14	7.33	9.7
ACLY	0	0.37	1	0.44	0.06	0.25
ALAS1	5	5.33	7	6.37	3.33	3.64
FAS	2	2.93	2	3.51	4.44	2
RHO	4	4.86	7	5.81	2.98	3.31
RPL30	19	17.79	20	21.25	12.18	12.13
TGFB2	6	4.71	6	5.63	1.56	3.21

	Segregating sites				Divergence	
	Atlas flycatcher		Pied flycatcher			
Gene	Obs	Exp	Obs	Exp	Obs	Exp
ALDOB	0	1.96	3	1.31	1.83	1.56
BRM	15	14.54	10	9.68	10.82	11.6
CHDZ	1	1.44	2	0.96	0.56	1.15
GHR	3	2.34	0	1.58	2.78	1.86
VLDLR	8	6.72	3	4.48	5.56	5.36
ACLY	0	0.86	2	0.67	0.11	0.58
ALAS1	5	4.97	4	3.87	3.17	3.32
FAS	2	1.29	0	1.01	1.17	0.86
RHO	4	7.11	7	5.53	6.39	4.75
RPL30	19	17.35	12	13.49	11.43	11.59
TGFB2	6	4.41	3	3.43	1.78	2.94

	Segregating sites				Divergence	
	Collared flycatcher		Pied flycatcher			
Gene	Obs	Exp	Obs	Exp	Obs	Exp
ALDOB	1	2.31	3	1.6	1.83	1.92
BRM	9	11.83	10	8.19	10.82	9.8
CHDZ	1	1.41	2	0.98	0.56	1.17
GHR	2	1.9	0	1.31	2.78	1.57
VLDLR	13	8.55	3	5.92	5.56	7.08
ACLY	1	1.91	2	1.25	1.17	1.01
ALAS1	7	7.34	4	4.78	4.97	3.85
FAS	2	2.43	0	1.58	3.28	1.27
RHO	7	8.6	7	5.6	4.72	4.52
RPL30	20	17.95	12	11.69	7.07	9.43
TGFB2	6	4.77	3	3.1	1.38	2.5

[a] Observed; [b] Expected.

Shared and fixed polymorphisms

The polymorphisms were sorted into four different categories in each of the three species pair combinations; polymorphisms exclusive to species 1, polymorphisms exclusive to species 2, fixed substitutions between species 1 and 2, and shared polymorphisms between species 1 and 2 (Table 4). I found an interesting difference between the shared polymorphisms and the fixed substitutions at the Z-linked and the autosomal loci. Z-linked genes exhibit almost no shared polymorphisms but a high number of fixed substitutions. Autosomal genes show the opposite pattern, although less clear cut, with a higher number of fixed substitutions than shared polymorphisms.

Isolation model fitting was performed on the data, with all autosomal and all Z-linked genes grouped together as two "loci". The isolation model is a simple speciation model (Wakeley and Hey 1997) that assumes that two populations arose from one ancestral population some time, t generations in the past. The model further assumes neutral mutations, no gene flow and constant population sizes, except at the separation time. Four parameters describe the isolation model, three values for the population mutation parameter θ: θ_A for the ancestral population, θ_1 for population 1 and θ_2 for population 2. In addition there is the time of the speciation event T, measured in units of $2N_1$ generations (Wakeley and Hey 1997; Wang et al. 1997). With these assumptions random drift would cause shared polymorphisms to be lost and become fixed differences with time. The tests were performed between all three species pair, with 10,000 coalescent simulations to assess significance, and the results can be found in table 5. None of the tests showed significant deviations from the isolation model (WH-values of 12, 8 and 16 and P-values of 0.65, 0.79 and 0.61 respectively), and thus the model with no selection and gene flow is not rejected.

Table 4 - Fixed and shard polymorphisms

Fixed and shared polymorphism between MA and IC

	$S_{x\text{-}MA}$	$S_{x\text{-}IC}$	S_{fixed}	S_{shared}	$S_{average}$	length
ALDOB	0	1	4	0	4.889	620
BRM	14	8	8	1	12.204	1572
CHDZ	1	1	1	0	1.333	704
GHR	2	1	0	1	1.833	555
VLDLR	7	12	0	1	7.333	731
Total-Z	24	23	13	3	27.593	4182
ACLY	0	1	0	0	0.056	358
ALAS1	5	7	0	0	3.333	291
FAS	2	2	4	0	4.444	900
RHO	2	5	0	2	2.981	375
RPL30	15	16	0	4	12.179	1008
TGFB2	6	6	0	0	1.556	402
Total-A	30	37	4	6	24.549	3334

Fixed and shared polymorphism between MA and SP

	$S_{x\text{-}MA}$	$S_{x\text{-}SP}$	S_{fixed}	S_{shared}	$S_{average}$	length
ALDOB	0	3	1	0	1.833	620
BRM	13	8	5	2	10.815	1571
CHDZ	1	2	0	0	0.556	704
GHR	3	0	1	0	2.778	555
VLDLR	8	3	1	0	5.556	731
Total-Z	25	16	8	2	21.538	4181
ACLY	0	2	0	0	0.111	358
ALAS1	5	4	1	0	3.167	291
FAS	2	0	1	0	1.167	900
RHO	4	7	3	0	6.389	375
RPL30	11	4	1	8	11.432	1008
TGFB2	6	3	0	0	1.778	402
Total-A	28	20	6	8	24.044	3334

Fixed and shared polymorphism between IC and SP

	$S_{x\text{-}IC}$	$S_{x\text{-}SP}$	S_{fixed}	S_{shared}	$S_{average}$	length
ALDOB	1	3	3	0	4.722	620
BRM	8	9	5	1	11.247	1603
CHDZ	1	2	1	0	1.333	804
GHR	2	0	2	0	2.556	598
VLDLR	13	3	1	0	4.611	732
Total-Z	25	17	12	1	24.469	4357
ACLY	1	2	1	0	1.167	417
ALAS1	13	5	0	2	4.969	403
FAS	2	0	3	0	3.278	900
RHO	6	6	1	1	4.716	404
RPL30	15	7	0	6	7.074	1065
TGFB2	5	2	0	1	1.377	402
Total-A	42	22	5	10	22.581	3591

S-values are the number of variable sites, where the subscripts indicate those exclusive (x) to the Atlas flycatcher ($S_{x\text{-}MA}$), the collared flycatcher ($S_{x\text{-}IC}$) or the pied flycatcher ($S_{x\text{-}SP}$), fixed differences (S_{fixed}) or shared polymorphisms between the species (S_{shared}), and the average pairwise differences ($S_{average}$) between the species.

Table 5 - Isolation model fitting

	θ_1	θ_2	θ_A	T	WH	P
MA/IC	13.225	14.564	50.244	0.573	12.000	0.660
MA/SP	9.752	7.056	48.822	0.432	8.000	0.793
IC/SP	12.253	7.795	56.870	0.417	16.000	0.615

θ_1 and θ_2 are the estimates of θ for population 1 and 2 of the three species pairs, and θ_A is the estimate for their common ancestral population. **T** is the estimate of time since the speciation event. **WH** is a test statistic designed for this model (Wang et al. 1997) and **P** is the probability calculated from this test statistic.

Phylogenetic reconstruction

On the basis of all the Z-linked genes, all the autosomal genes and finally all loci combined, three neighbour-joining trees were constructed (figure 3-5). The trees were constructed from the above mentioned pseudo-haplotype data. The three different consensus trees showed a slightly different topography for the branching events of the three species. For the Z-linked genes the collared flycatcher branch off before the other two, whereas for the autosomal genes as well as all loci combined the Atlas flycatcher is the first to branch off. However, in all cases, the bootstrap support for the branching events of the three species is low, and the branch lengths short, suggesting that the three species diverged from each other approximately simultaneously. A similar result was found in previous studies using mtDNA sequence data (Sætre et al. 2001a; Sætre et al. 2001b).

Figure 3 – Neighbour-joining consensus tree with bootstrap values of all Z-linked loci

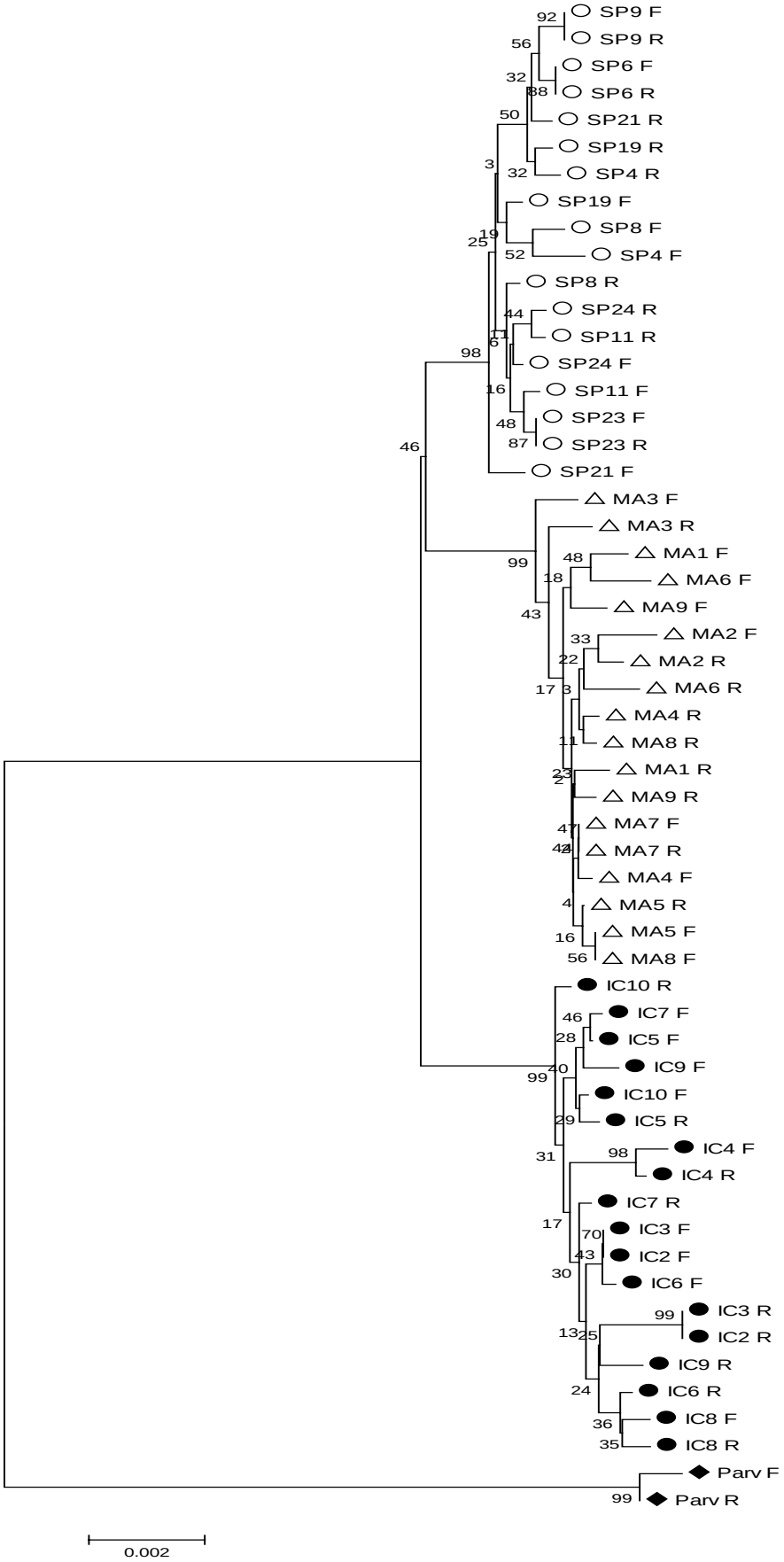


Figure 4 – Neighbour-joining consensus tree with bootstrap values of all autosomal loci

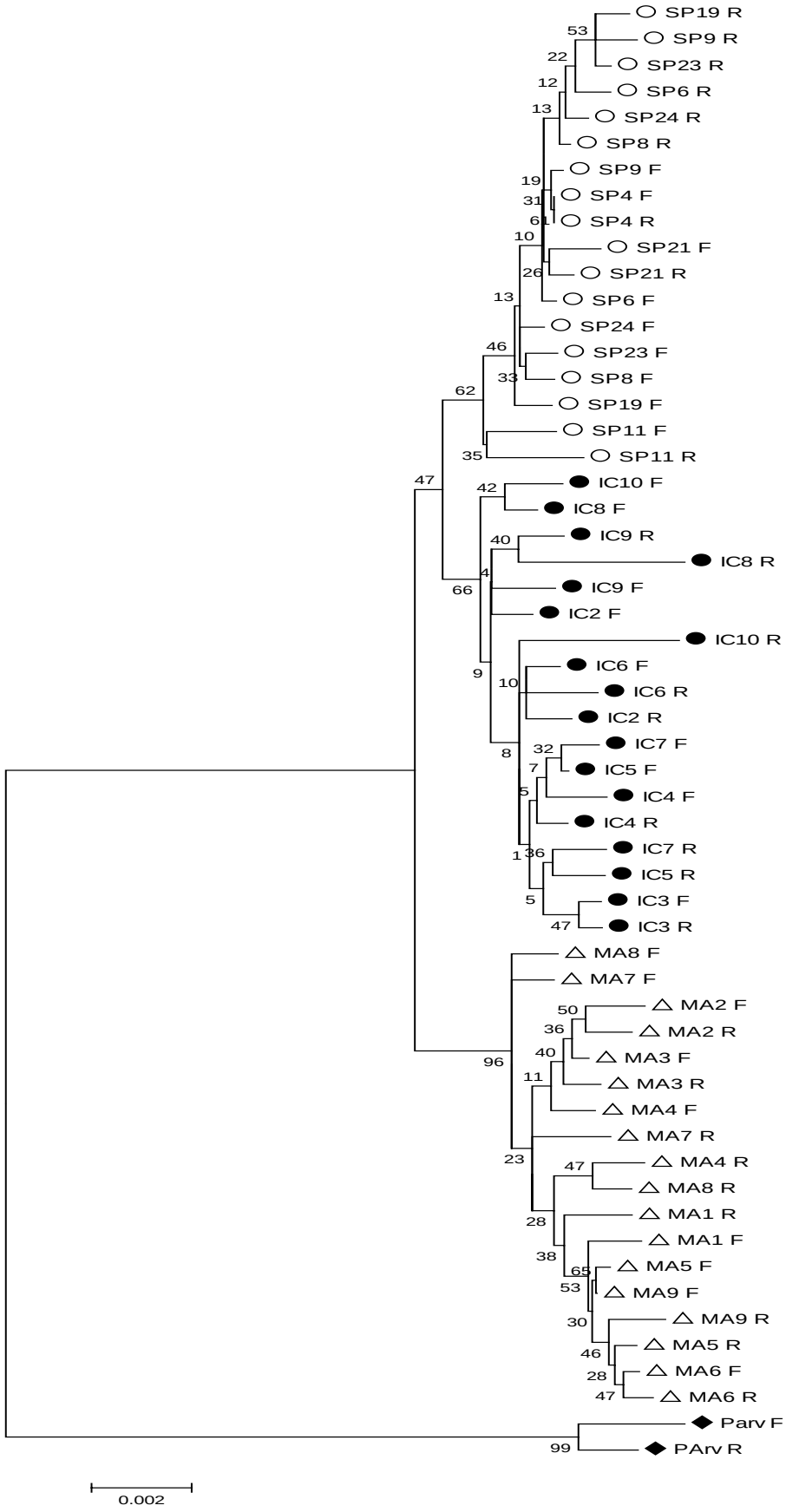
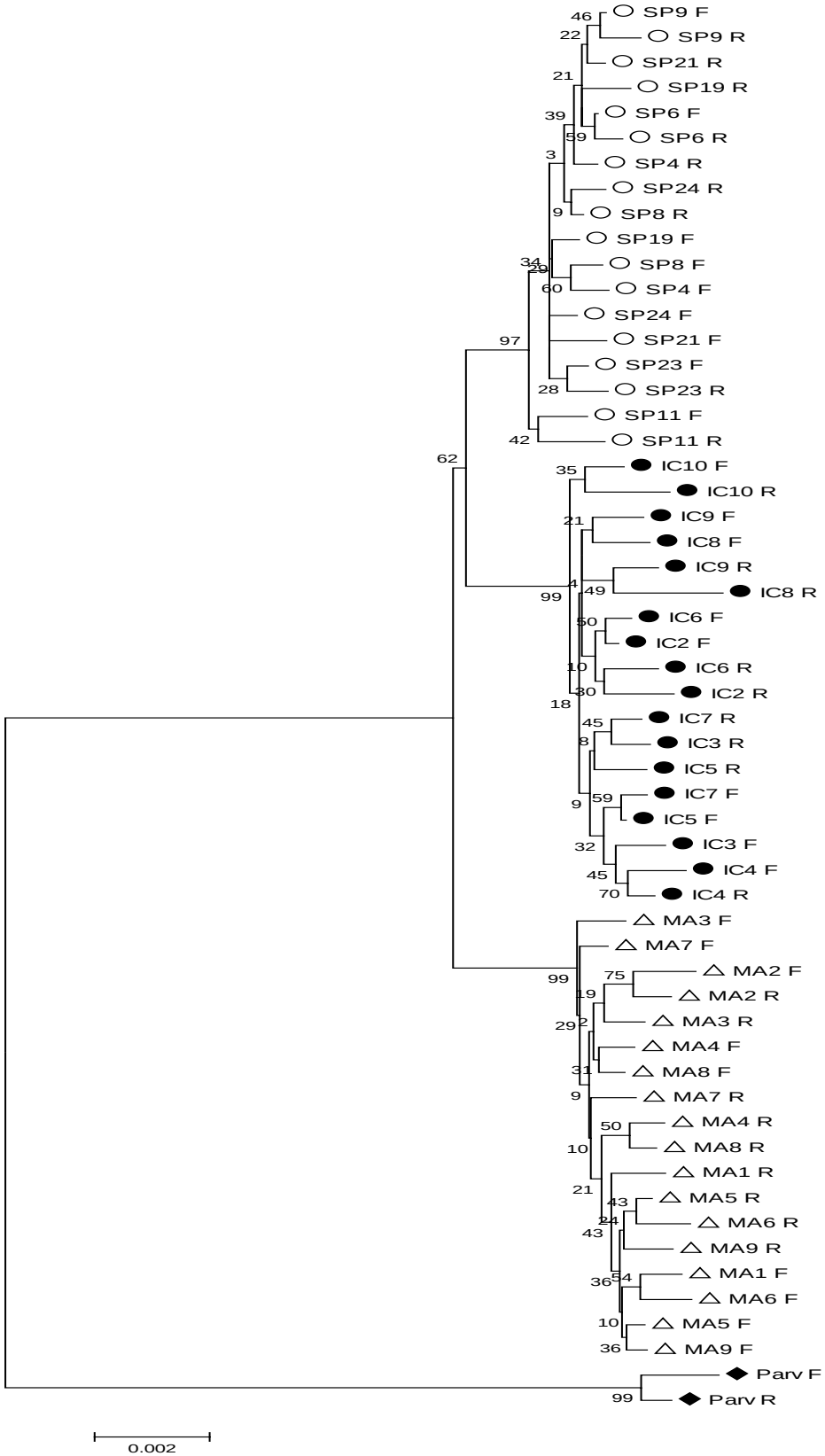


Figure 5 – Neighbour-joining consensus tree with bootstrap values of all loci combined



Phenotypic measures

The phenotypic measures were compared with similar measures from collared and pied flycatchers published by Sætre et al. (2001b). For most of the plumage characteristics the Atlas flycatcher is more similar to the collared flycatcher (Table 6) than to the pied flycatcher. Both the Atlas and the collared flycatcher are significantly darker than the pied flycatchers and they have larger patches of white on the wing and forehead. Moreover, the two former species have black tails and a greyish white rump, whereas the pied flycatcher has a white-edged tail and brownish rump. The Atlas flycatcher has a very large forehead patch that is even significantly larger than that of the collared flycatcher. The maximum wing patch width is intermediate between that of the collared and the pied flycatcher and significantly different from both of them. One interesting feature is that some Atlas flycatchers exhibit a partial neck collar, similar to that found in semicollared flycatchers and hybrids between the pied and collared flycatcher.

Table 6 – **Plumage characteristics**

Character	Atlas flycatcher n=35	Collared flycatcher n=72	Pied flycatcher a n=41	Pied flycatcher b n=487
Colour score [a]	1.3 (1.1-1.5)	1.2 (1.1-1.3)	6.3 (6.1-6.6)	3.4 (3.3-3.5)
Forehead patch height (mm)	9 (8.6-9.4)	7.7 (7.4-8.0)	1.5 (1.0-2.0)	3.6 (3.5-3.7)
Median no. of white-edged tail feathers	0	0	6	(no data)
Max. wing patch width (mm)	7.3 (6.7-7.9)	10 (9.5-10.6)	3.8 (3.3-4.2)	(no data)
Median start of wing patch (primary no.)	4	3	6	(no data)
Rump colour	Greyish white	Greyish white	Brown	Brown
Neck collar	No/Partial	Yes	No	No

[a] Scored according to the seven-point scale of (Drost 1936) where 1=100% black, and 7=100% brown upper parts.

Plumage characteristics of Atlas flycatcher males (mean and 95% confidence intervals, unless otherwise stated). Measures from collared flycatchers from Czech Republic and pied flycatchers from Czech Republic (a) and Norway (b) published in (Sætre et al. 2001b).

Discussion

The evolutionary history of Atlas flycatcher

Both phylogenetic reconstructions and the isolation model fitting suggest that the Atlas flycatcher originated from a larger ancestral population at approximately the same time as the collared and the pied flycatcher. This is likely to be in connection with the onset of the glaciations of the Pleistocene (Sætre et al. 2001a; Sætre et al. 2001b). Since these species of flycatchers all breed in mixed or deciduous forests (Lundberg and Alatalo 1992), and breed in nest holes, they are all confined to old forest. They are therefore vulnerable to changes in the distribution of such forests. During the Pleistocene, the distribution of such forests varied extensively, but certain areas, so-called refugia, were relatively stable. The main refugia were Iberia, Italy, the Balkans and Caucasus, and this has been affirmed by several phylogenetic studies (e.g. DumolinLapegue et al. 1997; King and Ferris 1998). North-western Africa has also been pointed out as a refugium (Helbig and Seibold 1999). It has been suggested that the ancestral flycatcher was isolated in four of these refugia during the Pleistocene (Sætre et al. 2001a), and from this isolation diverged into the four present species. This is also consistent with where they now have their most southern (or only, in the case of the Atlas flycatcher) distribution, with the semicollared flycatcher in the Balkans or Caucasus, the pied flycatcher in Italy, the collared flycatcher in Iberia and the Atlas flycatcher in the Atlas Mountains of north-western Africa.

The D-tests and F-test are slightly negative in two of the species (the Atlas and the collared flycatcher) although not significantly different from zero. Negative test statistics can be a result of a bottlenecked expansion, and this might coincide with the same isolation during the glaciations in the Pleistocene. Negative test statistics is also expected under purifying or positive selection (e.g. Li 1997). As mentioned above, a selective sweep would show the same pattern. An earlier study suggested that recurrent selective sweeps have occurred on the Z-chromosome in collared and pied flycatcher (Borge et al. 2005). They found loss of genetic variation at Z-linked loci that exceeded what could be ascribed to demographic processes, as also found significant deviations from neutral expectation at Z-linked loci according to an HKA-test. In the present study I found reduced variation at the Z-linked loci compared to the autosomal loci also in the Atlas flycatcher, which is consistent with selective sweeps. On the other hand, test of neutrality, including HKA-tests, were not significant. Hence my data

apparently do not suggest the action of selective sweeps. Note, however, that the HKA-test was not significant for the pied flycatcher and collared flycatcher in the present study. One likely explanation for this apparent discrepancy between my study and that of Borge et al. (2005) is that I analysed fewer gene fragments than they did. Hence, further studies of the Atlas flycatcher using more genes may be advisory.

The morphology of the Atlas flycatcher

Very few studies of the plumage characteristics of the Atlas flycatchers have been done. Lundberg and Alatalo (1992) measured a few museum specimens caught in North Africa. However, such museum collections might contain transmigrants in addition to local breeders, and these measures are therefore uncertain. Sætre et al. (2001b) included statistics of plumage characteristics of 5 males caught on the breeding grounds in their study, but with the addition of 30 new males in the present study, it is possible to give a more thorough comparison of the phenotypic variation of the species. By comparing the Atlas flycatcher with other allopatric populations it is possible to assess which features are ancestral. It therefore seems like dark colour and a partial neck colour could be regarded as ancestral traits since they can be found in collared flycatchers, Atlas flycatchers, semicollared flycatchers and also in pied flycatchers from Spain (Potti and Merino 1995). The very large forehead patch of the Atlas flycatcher might be a result of sexual selection as has been found for the forehead patch of the collared flycatcher (Sheldon and Ellegren 1999).

Traces of introgression?

Phylogenetic reconstructions based on sequences from different parts of the genome can be used to infer previous episodes of introgression (Sætre et al. 2003). According to Haldane's rule (Haldane 1922) hybrids of the heterogametic sex are more often sterile or inviable than those of the homogametic sex. Moreover, genes affecting hybrid fitness and sexually selected traits are often sex linked (Reinhold 1998; Noor et al. 2001; Sætre et al. 2003). Since females are the heterogametic sex in birds, they are the ones to suffer the most lowered fitness in the case of hybridisation. Thus introgression through fertile males may continue to occur at autosomal loci after it has stopped at maternally inherited mtDNA and at sex-linked loci. In hybrids between pied and collared flycatchers, females have greatly reduced fitness owing to almost complete sterility, whereas male hybrids are sometimes fertile (Veen et al. 2001).

Accordingly, in hybrid zones between these two species, there is basically no introgression occurring at Z-linked loci or mtDNA, whereas autosomal introgression occurs at a low rate (Sætre et al. 2003). At present, the breeding range of the Atlas flycatcher is separated from those of the pied and collared flycatcher only by small bodies of water at Gibraltar and between Tunisia and Sicily. Hence interbreeding and introgression could occur or could have done so in the past. If so, one would expect potential introgression between Atlas flycatcher and other flycatchers to express the same pattern as found in current hybrid zones of pied and collared flycatchers, which would have resulted in a greater genetic similarity between the species at autosomal loci than at Z-linked loci. This was found not to be the case. In all phylogenetic reconstructions the Atlas flycatcher formed a clade equally distinct as those of the pied and collared flycatcher, and there was no indication of a relative greater genetic resemblance at autosomal loci than at Z-linked loci. Finally, higher levels of introgression at autosomal loci than at Z-linked loci would result in more variation being accumulated at the former loci. However, the Atlas flycatcher was found to possess a high level of variation at the Z-linked loci compared to that found in the other two species and compared to the variation found at autosomal loci. It is therefore no indications in my data set that there has been any historic or recent introgression between the Atlas flycatcher and any of the two other species.

My results are consistent with earlier studies using mtDNA (Sætre et al. 2001a; Sætre et al. 2001b). In all trees the Atlas flycatchers appear as one distinct clade with bootstrap values higher than 96, indicating that the Atlas flycatcher is a monophyletic group. Since no traces of gene flow could be found and since the genetic distance to its closest relatives is as high as that between the pied and the collared flycatcher which evidently exhibit strong reproductive isolation in sympatry, I suggest that the Atlas flycatcher should remain being classified as a distinct species.

Reduced variation at Z-linked loci

The various tests performed showed a reduced level of variation at the Z-chromosome compared to autosomal loci. This might just reflect that the Z-chromosome has an effective population size, N_e, which is ¾ of the N_e of autosomal loci since females only have one copy of the chromosome. One would expect genetic drift to reduce variation and fix shared polymorphisms between two diverging populations faster in small populations than in large

populations (Wakeley and Hey 1997). Another explanation for the lowered level of variation at Z-linked loci is a smaller male population size. Due to female heterogamy, a female-biased operational sex ratio would lower N_e at Z-linked loci more than at autosomal. A female-biased operational sex ratio has been found in the pied and the collared flycatcher due to greater variance in male than in female reproductive success. Male flycatchers are often polygynous, attracting a secondary or sometimes even a tertiary female after their first mate have initiated egg laying. The proportion of secondary females in collared flycatchers has been found to be around 4% (Qvarnstrom et al. 2003) and the equivalent number in pied is on average 10-15% (Lundberg and Alatalo 1992). The mean rate of extra pair paternity (EPP), another measure of the operational sex ratio, was found to be 14,5% in collared flycatcher (Veen et al. 2001), and slightly lower in pied flycatcher. Unfortunately, similar data are not available for the Atlas flycatcher. Yet, due to its close resemblance and genetic relatedness to the other two species it seems likely that also the Atlas flycatcher may exhibit similar features and thus have a somewhat female-biased sex ratio that could contribute to the observed loss of genetic variation at Z-linked loci.

Under a neutral model, assuming random mating, constant population size and no migration, and with the mutation rate at Z-linked and autosomal loci being the same, the ratio of neutral variation between Z-linked and autosomal loci is predicted to be 0.75. With the most extreme form of female-biased operational sex ratio, where one male fathers all the chicks, this ratio would be 0.5. The predicted male-biased mutation rate caused by different numbers of cell divisions in the germ line, however, would most likely result in this ratio being higher. In this study I found the ratio of Z-linked to autosomal variation to be 0.60, 0.48 and 0.51 in the Atlas, collared and pied flycatchers respectively (based on θ_W). These values are slightly higher for collared and pied flycatcher than what has previously been found (Borge et al. 2005), but still close to the expected minimum under the most extreme female-biased operational sex ratio. These low values of variation may indicate that processes such as selective sweeps has contributed to the loss of variation after all, even though I could not detect it statistically in the neutrality tests. Further studies with a higher statistical power (more markers and/or larger sample size) are needed to test this possibility thoroughly.

When two populations become isolated from each other we assume that there will, by random genetic drift, be a reduction in the amount of shared polymorphism and an increase in the amount of fixed differences over time. This process occurs faster when N_e is low, and

therefore we expect it to occur faster at Z-linked loci since they have a N_e that is ¾ that of autosomal loci because of the mode of inheritance of the Z chromosome. I found such a pattern, with a high amount of fixed differences at Z-linked loci and a rather high amount of shared polymorphisms at autosomal loci (figure 2). The isolation model fitting still indicated that these values are consistent with the isolation model of speciation, meaning that these patterns of polymorphism and divergence could be explained without invoking selection and/or gene flow. Historical changes in θ could generate a greater degree of stochastic variance in these patterns (Borge et al. 2005). These historical changes in θ might be a result of changes in N_e.

With a higher mutation rate in males, because of more cell divisions in the male germ line per generation compared to the female germ line, one would expect a higher divergence at Z-linked loci compared to autosomal loci (Haldane 1935). Since the Z chromosome spends 67 % of its time in the male germ line (where the mutation rate is assumed to be high) and 33 % of its time in the female germ line, compared to the autosomal chromosomes that spend 50 % in each germ line, a correspondingly large different mutation rate should be expected. In several bird species a higher mutation rate at the Z-chromosome than at autosomes consistent with a male biased mutation rate has been found (Ellegren and Fridolfsson 1997; Kahn and Quinn 1999; Carmichael et al. 2000). But even though this difference in the number of germ line cell divisions may be the predominant cause of the difference in mutation rate across the genome, other factors might be of importance at a more local scale, such as regional variation in mutation rate, adaptive variation in mutation rate, or gene specific deviations from neutrality (Ellegren 2007). In the present study quite closely related species were compared. In species that diverged recently, much of the observed polymorphisms would be mutations accumulated before the species diverged. We would expect these levels of polymorphisms to differ between different genomic regions, e.g. between autosomal loci and Z-linked loci (Ellegren 2007). This difference in levels of ancestral polymorphisms would then make it difficult to obtain an accurate estimate of the male-to-female mutation rate ratio, as seen between humans and closely related apes (Makova and Li 2002).

Borge et al. (2005) found a higher divergence at Z-linked loci than at autosomal loci of collared and pied flycatcher. This was not confirmed in the present study. The reason for this discrepancy may partly be because Borge et al. (2005) analysed a larger data set than I have and thus more accurate estimates. Moreover they restricted the analysis to intronic data only.

In my analysis I have included flanking exonic sequence. Exons tend to evolve more slowly than introns, and might therefore contribute to explain why I did not get the expected result. Indeed the Z-linked loci I used contained more exon sequences than did the autosomal ones.

Deviations from neutrality

As mentioned above, the test statistics from Tajima's D and Fu and Li's D and F are for Atlas and collared flycatcher overall slightly negative, and for pied slightly negative in autosomal loci. Negative values mean an excess of rare variants, which can result from purifying or positive selection or a population expansion (Tajima 1989a; Braverman et al. 1995; Simonsen et al. 1995). We are to expect more frequent selective sweeps at the Z-chromosome, due to lower recombination rate and because recessive alleles are not masked by dominance in females (Charlesworth et al. 1987; Begun and Whitley 2000; Sætre et al. 2003). The present study found no significant deviation from neutrality, but Borge et al. (2005) found traces of recurrent selective sweeps in their study. At Z-linked loci in the pied flycatcher, however, the test statistics are mainly positive. Positive test statistics, an excess of intermediate-frequency variants, could be a result of balancing selection or certain demographic processes such as recent establishment of secondary contact of differentiated population (Tajima 1989a; Simonsen et al. 1995). Fu and Li's tests are strongly dependent on the number of singleton polymorphisms and the almost complete lack of singleton sites at the Z-linked loci in the pied flycatcher samples might be causing the significant deviation from neutral expectations. The positive values might be caused by a recent reduction in population size, that have affected the Z-linked loci differently from the autosomal, as seen between human mtDNA and nuclear loci (Fay and Wu 1999).

Conservation issues

Since the Atlas flycatcher lives in a fairly limited geographic area, mainly consisting of small fragmented patches of forest, it may be a species needing special attention for conservation. A study focusing on conservation issues of the barbary macaques (*Macaca sylvanus*) from the same forest these Atlas flycatchers were sampled from, reported a significant increase in degradation of oak and cedar forest attributed to a significant increase in grazing animals since 1994 (Ciani et al. 2005). This degradation of oak forest would represent fragmentation and a loss of habitat, and is a potential threat to the Atlas flycatcher. Further, not much is

known about the status of the wintering grounds of the Atlas flycatcher in tropical Africa. The Atlas flycatcher is not present in the IUCN Red list (IUCN 2006). However, its status as distinct species is of new date (Sætre et al. 2001b) and it is likely that it is classified as a subspecies of pied flycatcher in the mentioned report, a species that is assigned to the Least Concern category. If the Atlas flycatcher is severely affected by habitat loss and fragmentation one might expect it to show low genetic variation due to a reduced effective population size. The Atlas flycatcher, however, shows relatively high levels of variation, almost as high as the collared flycatchers analysed here, which were sampled from a large population in Italy in apparently non-fragmented habitat (Abruzzo National Park). The pied flycatcher from Spain has lower variation, and therefore presumably a smaller effective population size, Ne, than the two other species. Indeed the pied flycatcher population analysed here if from fragmented mountain forests of Central Spain (Potti and Montalvo 1991). In short, my data on genetic variation of the Atlas flycatcher appears to be consistent with a genetically healthy population of relatively large size. However, with further degradation of the forest habitats in the Atlas Mountains this situation could change rapidly.

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