Population genetic structure in the paddyfield warbler

(Acrocephalus agricola Jerd.)

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Abstract

Population genetic structure was studied in paddyfield warblers Acrocephalus agricola breeding in NE Bulgaria, SE Russia and S Kazakhstan. We were particularly interested in the degree of genetic differentiation and gene flow of the Bulgarian population due to its geographical isolation, recent origin and unique migratory strategy. Analyses of mitochondrial DNA (mtDNA) showed that there was no divergence between Bulgarian and Russian populations ($F_{ST} = 0.007$), whereas those in Kazakhstan differed significantly from European breeding populations (Russia: $F_{ST} = 0.058$; Bulgaria: $F_{ST} = 0.114$). The degree of differentiation between populations at nuclear markers (five microsatellite loci; $F_{ST} \approx 0$) was weaker than for mtDNA. We suggest that this relatively weak differentiation over the range of this species reflects a recent postglacial expansion, and results from mismatch distribution analyses and Fu’s $F_{S}$ tests are in agreement. Preservation of small and geographically isolated populations which may contain individuals with unique adaptive traits, such as the studied Bulgarian population of paddyfield warbler, is valuable for the long-term conservation of expanding migratory bird species [Current Zoology 57 (1): 63–71, 2011].

Key words

Phylogeography, Post-glacial expansion, Population structure, Mitochondria, Microsatellite

Climatic fluctuations during the Pleistocene caused repeated range expansions and contractions with consequences for temperate and boreal populations of species in the Northern Hemisphere (Hewitt, 2000; Schluter, 2000; Coyne and Orr, 2004). Data of genetic distances between sister taxa in birds translate to divergence times between 0.1 and 2.0 Myr and a common view is that these speciation events took place in isolated refugia over one to several full glacial cycles (Klicka and Zink, 1997; Johnson and Cicero, 2004; Weir and Schluter, 2007). An alternative mode of speciation is that diversification takes place during expansion rather than during isolation (Seutin et al., 1995; Ødeen and Björklund, 2003; Mila et al., 2007b; Mila et al., 2007a). During rapid postglacial range expansions advancing populations encounter a wide variety of unoccupied habitats with varying selection regimes which could drive diversification in comparatively short periods of time. Recent divergence with extensive morphological variation has been documented in several bird species, e.g. yellow wagtails (Motacilla flava; Ødeen and Björklund, 2003), dark-eyed juncos (Junco hyemalis; Mila et al., 2007a), long-tailed tit (Aegithalos caudatus, Zink et al., 2008) and willow tit (Parus montanus; Salzburger et al., 2002; Kvist et al., 2001).

Phylogeographic studies on Palearctic birds are of interest to reveal general patterns of range expansion and population structure. Less information exists on post-glacial range expansion and migratory strategies of species wintering in India and SE Asia (but see Irwin et al., 2005; Zink et al., 2008; Saitoh et al., 2010). Nevertheless, we may expect phylogeographical differences between migratory species wintering in Africa and India since Europe was extensively glaciated during the last glacial advance, whereas Asia was not.

Genetic distances in mtDNA within species in the genus Acrocephalus indicate high intraspecific variation, which is often related to geographic separation but needs further investigation (Leisler et al., 1997). The existence of distinct mtDNA haplotypes between populations of a species may be explained by population history (e.g. drift in separated postglacial refugia), or
adaptive differences selected for during the expansion, which, so far, have simply been neglected (Hansson et al., 2008).

The paddyfield warbler *Acrocephalus agricola* has a mosaic distribution in the Palearctic from NE Bulgaria in the west to NW Mongolia in the east (Voistvenskii, 1960; Paspaleva and Talpeanu, 1980). The breeding range of the paddyfield warbler has recently expanded westwards (Cramp, 1992) and was first recorded in Bulgaria in 1968 (Dontschev, 1970; Nadler and Ihle, 1988) (Fig.1). Interestingly, the birds breeding in the most western point of the breeding area (i.e. Durankulak Lake, NE Bulgaria) winter in the Indian peninsula; thus, they must fly much longer distances to the wintering grounds than more eastern populations. In autumn, Bulgarian paddyfield warblers circumvent the Black Sea along its northern coast following the historical expansion route of the species to this region (Zehtindjiev et al., 2010). To enable this seasonal journey the paddyfield warbler has evolved a unique migratory program during the recent westward expansion along the Black Sea to Europe (Zehtindjiev et al., 2010).

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**Fig. 1** Sampling localities overlaid on the breeding distribution (horizontal lines) and overwintering range (vertical lines) of the species
Here, we evaluated the degree of population genetic differentiation using mtDNA and a set of nuclear microsatellite markers in Paddyfield warbler populations in NE Bulgaria, SE Russia, and S Kazakhstan. We aimed to understand whether phenotypically differentiated populations (in this case a population with differing migratory behavior) show divergence in neutral markers, and whether this differentiation occurred recently (post-glacially) or is older (Pleistocene). Assessments of population genetic structure are currently lacking for the Paddyfield Warbler, and our study can provide data of importance for the conservation of populations in Europe and elsewhere. We were particularly interested in the degree of genetic differentiation for the Bulgarian population due to its geographical isolation, recent origin and recently evolved migratory program (Zehtindjiev et al., 2010).

1 Material and Methods

1.1 Population samples

We mist-netted paddyfield warblers and collected a blood sample (5–20 µl) from the wing or tarsus veins, that was stored in SET-buffer (0.015 M NaCl, 0.05 M Tris, 0.001 M EDTA, pH 8.0) at ambient temperature in the field and later at -20°C. We collected 129 samples at one site in Bulgaria, four sites in Russia, and two sites in Kazakhstan (Fig. 1, Table 1). The Bulgarian and Russian birds were sampled during the breeding seasons of 2005 and 2006. Six samples from Kazakhstan (from Spring 2001) in the collection of Molecular Ecology Lab in Lund were used and three other samples of breeding birds from Kazakhstan were provided by Petr Procházka (Institute of Vertebrate Biology, Brno, Czech Republic).

1.2 DNA analyses

For all individuals, except the three samples from Balkhash Lake in Kazakhstan, total DNA was extracted from whole blood (5–20 µl) stored in SET-buffer (500 µl) by a standard proteinase k Ammonium Acetate protocol (Richardson et al., 2001). The three samples from Balkhash Lake consisted of blood in ethanol and were first dried and then resolved in SET-buffer and extracted according to the protocol used for the rest of the samples. Genomic DNA extracts were diluted to a working concentration of 25 ng/µl.

1.3 Bird mitochondrial DNA and microsatellites

Mitochondrial control region II was amplified and sequenced by primers BCML4 (5’-TTCACAGATACAAATGCTTGGG-3’) and 12SH2 (5’-AGCAAAACACCAACGTAAG-3’) (Bensch and Hasselquist, 1999). Polymerase chain reactions (PCRs) were performed in volumes of 25 µL and included 10–50 ng of total genomic DNA, 0.125 mM of each nucleotide, 1.5 mM MgCl₂, 0.6 µM of each primer and 0.5 U AmpliTaq polymerase. The PCRs were run using the following conditions: 30s at 94°C, 30s at 50°C, 30s at 72°C (35 cycles). Before the cyclic reactions the samples were incubated at 94°C for 2 min, and after completion at 72°C for 10 min. The PCR product was precipitated (NH₄Ac and ethanol) and then dissolved in 20 µL of water; 2–4 µL was then used for sequencing (BigDye sequencing kit; Applied Biosystems) in an ABI Prism 3100 capillary sequencer (Applied Biosystems).

For the microsatellite study five pairs of primers originally designed for seychelles warbler Acrocephalus sechellensis were used: Ase8, Ase9, Ase12, Ase19 and Ase56 (Richardson et al., 2000). The genotyping protocol that we used has been described in Hansson et al. (2000) and Richardson et al. (2000). Microsatellite alleles were amplified with PCR in GeneAmp 9700 thermal cyclers (Applied Biosystems). The PCR-mix contained 4 pmol of each primer, 1×NH₄-buffer, 15 nmol MgCl₂, 2 nmol dNTP, 0.5 U AmpliTaq Polymerase (Perkin Elmer) and 25 ng template in 10 µl reaction volume. One of the primers in a pair was labeled with a

Table 1  Ringing site of paddyfield warblers and number of samples per analysis

| Ringing site               | Latitude/Longitude  | Number of samples analysed: |
|----------------------------|---------------------|-----------------------------|
| Solyanka (Russia)          | 50°49’N/47°05’E     | 30 33                       |
| Algay (Russia)             | 50°03’N/48°14’E     | 13 14                       |
| Step liman (Russia)        | 50°43’N/46°27’E     | 8 11                        |
| Bobrovka (Russia)          | 51°31’N/49°15’E     | 8 12                        |
| Durankulak (Bulgaria)      | 43°40’N/28°32’E     | 29 24                       |
| Stone Lake (Kazakhstan)    | 42°49’N/70°56’E     | 6 6                         |
| Balkhash (Kazakhstan)      | 46°51’N/74°56’E     | 3 3                         |
fluorescent dye (6-FAM or HEX). PCR-conditions were as follows: 94 °C for 2 min, then 35 cycles at 94 °C for 30 s/$T_A$ for 30 s/72 °C for 30 s, followed by 72 °C for 10 min; where $T_A$ is the locus-specific annealing temperature (locus/$T_A$; G61/56, Aar1/62, Aar2/60, Aar4/53, Ase7/60, Ase18/60, Ase34/60, Ase44/60, Ase50/60). The fluorescent-labeled PCR products were separated and alleles were detected in an ABI PRISM 3730 capillary sequencer (Applied Biosystems). Genotypes were analyzed in GENEMAPPER 3.0 (Applied Biosystems).

1.4 Statistical analyses

The genetic relationship between mtDNA sequences was investigated using the neighbor-joining method with a Kimura 2-parameter distance matrix, in the program MEGA version 2.1 (Kumar et al., 2004). Haplotype diversity (Nei, 1987), $F$-statistics (Hudson et al., 1992), Tajima’s D (Tajima, 1989) and Fu’s $F_S$ (Fu, 1997) were calculated using DnaSP 4.10.9 (Rozas et al., 2003). The degree of mtDNA genetic differentiation was measured as $F_{ST}$ (Hudson et al., 1992, equation 3) and statistically evaluated with permutation tests (10,000 replicates) between pairs of populations. The observed Tajima’s D and Fu’s $F_S$ were compared statistically against simulated expectations for a neutral infinite-sites model and assuming a large constant population size using coalescent simulations implemented in DnaSP (Rozas et al., 2002; 10,000 replicates). In DNAsp, we also calculated mismatch distributions. The observed mismatch distribution was tested against expected values in a stable population (i.e. population with constant population size large population; following Watterson, 1975), and in a growing population (following Rogers and Harpending, 1992, equation 4), with $\chi^2$-tests (pooling classes to achieve a minimum of 5 expected counts in each class).

Descriptive statistics (number of alleles, allele frequencies, allelic richness and Nei’s unbiased estimator of gene diversity) for the microsatellite data was calculated in FSTAT 2.9.3 (Goudet, 2001). Expected heterozygosities based on observed allele frequencies were also calculated. Global population differentiation in the three populations was evaluated with Weir and Cockerham’s (1984) $F_{ST}$-statistics implemented in FSTAT. Confidence intervals were generated by bootstrapping over loci (1000 resamplings). In the pair-wise tests of population differentiation in FSTAT we used a nominal value of 1/1000 when accounting for multiple testing. We analysed population structure at the microsatellites also with the Bayesian statistical framework provided by the program STRUCTURE 2.1 (Prichard et al., 2000). A main difference to the $F_{ST}$-approach is that STRUCTURE evaluates the most likely structure without prior information of population membership and therefore provides unbiased estimate of the structure with respect to the sampled populations. In STRUCTURE we used a ‘burn-in’ period of 20,000 replicates and a sampling period of 50,000 replicates in admixture models with correlated allele frequencies (we also used models with uncorrelated allele frequencies with very similar outcome). We conducted runs for a number of clusters ($K$) ranging from 1 to 4 (i.e. the true number of sampling localities plus one).

2 Results

2.1 Mitochondrial control region sequences

We sequenced 456 bp of control region II in 97 samples. The sequenced region contained 28 variable sites which defined 29 haplotypes (accession numbers HM439056-HM439084) with sequence divergence ranging from 0.2 to 1.6% (Table 2). None of the individuals appeared to be heteroplasmatic (carrying two different haplotypes). All substitutions except four were transitions. The most common haplotype (N1) was detected in 23 (24%) of the samples and was also the most widespread and recorded at all sites.

In the Bulgarian population there were 12 different haplotypes, a haplotype diversity ($h$) of 0.88 and a nucleotide diversity ($\pi$) of 0.0038. Tajima’s D was negative ($D = -1.12; P = 0.14$) as was Fu’s $F_S$ ($F_S = -5.61, P = 0.002$). In the Russian population, 30 different haplotypes were observed, $h$ was 0.94, and $\pi$ 0.0045. Both Tajima’s D and Fu’s $F_S$ were strongly negative ($D = -1.99, P = 0.005; F_S = -30.34, P < 0.001$). In the Kazakhstani population, we observed nine different haplotypes which gave a $h$ of 1.00 and a $\pi$ of 0.0049. Tajima’s D was close to zero ($D = 0.06; P = 0.54$), whereas Fu’s $F_S$ was negative ($F_S = -7.55, P < 0.001$). In contrast to the two European populations, the control region haplotypes of the Kazak population had a deeper root, which can explain the comparatively higher Tajima’s D-value in that population.

There was no evidence of divergence between Bulgarian and Russian populations ($F_{ST} = 0.007, Ks = 2.27, P = 0.22$), whereas the samples from Kazakhstan differed significantly from the European breeding populations (Bulgaria vs. Kazakhstan: $F_{ST} = 0.114, Ks = 2.16, P = 0.009$; Russian vs. Kazakhstan: $F_{ST} = 0.058, Ks = 2.43, P = 0.037$).

Comparing the phylogenetic topology of the haplotypes with their geographical occurrence did not reveal any apparent trends in origin and distribution of haplotypes (Fig. 2).
Table 2  Haplotype frequencies per locality for each of the 29 mt control region II haplotypes of paddyfield warblers

| Code | Haplotype (variable sites) | Russia | Bulgaria | Kazakhstan | Total |
|------|-----------------|--------|----------|------------|-------|
| 1    | GCCCGGGCAACGGTCTCCGCACGTATT | 13     | 8        | 2          | 23    |
| 2    | .A..................... | 9      | 7        | 2          | 18    |
| 3    | .A.....................C. | 6      | 3        |            | 9     |
| 4    | ..T................... | 6      | 1        | 2          | 9     |
| 5    | ..T...................T   | 1      | 1        |            | 2     |
| 6    | . ..................C.C. | 1      | 4        |            | 5     |
| 7    | .A......G...T...........G. | 1      |          |            | 1     |
| 8    | .A.T................... | 3      |          |            | 3     |
| 9    | ........A................. | 3      |          |            | 3     |
| 10   | .A.....................T. | 1      |          |            | 1     |
| 11   | .A........T.............C. | 1      |          |            | 1     |
| 12   | .................A......... | 1      |          |            | 1     |
| 13   | .........................A | 1      |          |            | 1     |
| 14   | .........................C. | 3      |          |            | 3     |
| 15   | .A........A.........C. | 2      |          |            | 2     |
| 16   | .......T................ | 1      | 1        |            | 2     |
| 17   | .A..A............T....... | 1      |          |            | 1     |
| 18   | .........................C... | 1      |          |            | 1     |
| 19   | .A........A.G............ | 1      |          |            | 1     |
| 20   | .......T.............C.... | 1      |          |            | 1     |
| 21   | .........................C... | 1      |          |            | 1     |
| 22   | .A.T.C............G....C. | 1      |          |            | 1     |
| 23   | ..............A........... | 1      |          |            | 1     |
| 24   | .................C.......A... | 1      |          |            | 1     |
| 25   | C.G.................     | 1      |          |            | 1     |
| 26   | .......T............T..... | 1      |          |            | 1     |
| 27   | .A.T.................A.C... | 1      |          |            | 1     |
| 28   | .........................A......C. | 1      |          |            | 1     |
| 29   | .A.....................T. | 1      |          |            | 1     |

The observed mismatch distribution among haplotypes (data from all populations) had a clear peak at approximately 2 bp (Fig. 3). This distribution did not correspond to what would be expected in a stable population ($\chi^2 = 69.1$, $df = 6$, $P < 0.001$), but did not deviate from what would be expected in an expanding population ($\chi^2 = 3.57$, $df = 5$, $P = 0.61$).

2.2 Microsatellites

We genotyped 103 samples at 5 polymorphic microsatellite markers from the three populations (Table 3).
Fig. 2  Phylogenetic relationships among Paddyfield warbler mtDNA haplotypes based on 456 bases of control region II. Bootstrap values are given at the nodes only if the value is greater than 50. Haplotype codes as in Table 2.

A similar degree of genetic variation was found in different populations (e.g. expected heterozygosity over loci was 0.80 in Bulgaria, 0.81 in Russia, and 0.82 in Kazakhstan; more data given in Table 3). $F_{ST}$-tests did not reveal any differentiation at these markers between pairs of populations (Bulgaria vs. Russia: $F_{ST} = 0.0081$; Bulgaria vs. Kazakhstan: $F_{ST} = 0.0004$; Russia vs. Kazakhstan: $F_{ST} = -0.0070$) and no global differentiation ($F_{ST} = 0.004$). No significant sub-structuring was found using the program STRUCTURE: $K = 3$ was the most likely number of clusters with $\ln P(D) = -2228.1$, but the $\ln P(D)$-values were very similar for $K = 1$ to 3 (range: -2232.6 to -2228.1) and, for all $K$s the probability of each individual to belong to one of the suggested clusters was very similar for individuals in different geographical populations.

Fig. 3  Mismatch distributions for mtDNA haplotypes in all three populations
Shown are the observed distribution (solid lines), the expected distribution of a population with constant size (dotted) and the expected distribution of an expanding population (dashed).
Table 3  Number of alleles, allelic richness and expected heterozygosity at five microsatellite loci in the three study localities

| Locality     | Locus | Number of alleles | Allelic richness | Expected heterozygosity | Gene diversity |
|--------------|-------|-------------------|------------------|-------------------------|---------------|
| Bulgaria (n = 24) | Ase8  | 8                 | 5.7              | 0.80                    | 0.80          |
|               | Ase9  | 4                 | 3.3              | 0.58                    | 0.60          |
|               | Ase12 | 14                | 9.4              | 0.88                    | 0.90          |
|               | Ase19 | 12                | 8.1              | 0.83                    | 0.85          |
|               | Ase56 | 18                | 10.8             | 0.91                    | 0.93          |
| Mean         | 11.2  | 7.6               | 0.80             | 0.81                    | 0.81          |
| Russia (n = 70) | Ase8  | 9                 | 5.2              | 0.77                    | 0.78          |
|               | Ase9  | 6                 | 3.7              | 0.58                    | 0.58          |
|               | Ase12 | 19                | 9.3              | 0.88                    | 0.88          |
|               | Ase19 | 15                | 8.0              | 0.87                    | 0.88          |
|               | Ase56 | 30                | 12.5             | 0.94                    | 0.95          |
| Mean         | 15.8  | 7.8               | 0.81             | 0.81                    | 0.81          |
| Kazakhstan (n = 9) | Ase8  | 6                 | 6                | 0.75                    | 0.83          |
|               | Ase9  | 5                 | 5                | 0.67                    | 0.71          |
|               | Ase12 | 14                | 14               | 0.91                    | 0.95          |
|               | Ase19 | 10                | 10               | 0.86                    | 0.92          |
|               | Ase56 | 14                | 14               | 0.92                    | 0.98          |
| Mean         | 9.8   | 9.8               | 0.82             | 0.88                    | 0.88          |

3 Discussion

We found only weak population differentiation between the three separated breeding populations of the paddyfield warbler. There was no mtDNA divergence between Bulgarian and Russian populations whereas Kazakhstan differed significantly from the European breeding populations. We suggest that this relatively weak differentiation over the species’ wide distribution range reflects a recent postglacial expansion from the glacial refugium and the results from the mismatch distribution analyses and the Fu’s $F_{ST}$ tests confirmed an expansion scenario (cf. Ramos-Onsins and Rozas, 2002). The mitochondrial haplotype diversity in the breeding populations of paddyfield warblers was intermediate compared to other species breeding in the Palearctic, for example Acrocephalus arundinaceus (Bensch and HasSELquist, 1999; Hansson et al., 2008), Ficedula parva, Emberiza schoeniclus, Aegithalos caudatus and Alauda arvensis (Zink et al., 2008). The degree of differentiation at nuclear markers (five microsatellite loci) was weaker than at the mtDNA, which may be explained by differences in effective population size and/or in the mutation-drift equilibrium for these two types of markers (Zink and Barrowclough, 2008; Edwards and Bensch, 2009).

The strong divergence between Russia and Kazakhstan, and Bulgaria and Kazakhstan, suggests an isolation-by-distance pattern where populations have become gradually differentiated over time or became differentiated during expansion. This reasoning is in line with the observation that the breeding range of the Paddyfield warbler has recently expanded westwards and that these populations are geographically isolated (Dontschev, 1970; Nadler and Ihle, 1988). Interestingly, there was no evidence of differentiation on neutral markers between Bulgaria and Russia despite the apparent differences in the orientation behavior of these populations concerning seasonal migration (Zehtindjiev et al, 2010). This suggests that differences in migratory behavior are of recent origin, and it has been shown that migratory behavior can evolve too quickly to be reflected in mtDNA variation (Pérez-Tris et al., 2004; Bensch et al., 2009).

The only previous study of the genetic variability of the paddyfield warbler was based on mtDNA cyt b sequences (1068 bp). Interestingly, two haplotypes that differed by as much as 4.5% were detected in only two samples. One sample originated from Crimea, Ukraine (A. a. septirnus) and the other from Lake Alakol, Kazakhstan (A. a. agricola) (Leisler et al., 1997). Although based on two samples, this result may indicate population differentiation. Moreover, we sequenced 456 bp of the control region II in 91Paddyfield Warblers. The sequenced region contained 28 variable sites which defined 29 haplotypes with a divergence of between 0.2 % and 1.6%. The most common haplotype (N1) was de-
ected in 23 (24%) of the samples and was also the most widespread and recorded at all sites. The phylogenetic topology of these haplotypes does not correspond to their geographical distribution.

Geographical barriers may result in limits to present-day distributions and failures to colonize seemingly suitable habitat (Hewitt, 2000; Slatkin, 1987). Thus, glacial range oscillation is one of several factors that contribute to the evolution of species ranges (Hewitt, 2000; Slatkin, 1987; Garcia-Ramos and Kirkpatrick, 1997; Kirkpatrick and Barton, 1997). The presence of the Black Sea seems to provide a plausible (and at least partial) explanation for the delayed expansion of the Paddyfield warbler to the western part of its current breeding range.

Different features of migratory strategies such as timing, orientation and flight distance have been observed to evolve rapidly in nature (Berthold et al., 1992; Able and Belthoff, 1998). Rapid adaptive evolution of complex characteristics forming migratory strategies has been demonstrated in Sylvia atricapilla (Pérez-Tris et al., 2004). Similarly, the paddyfield warbler has evolved a unique migratory program during its recent westward expansion along the Black Sea to Europe (Zehtindjiev et al., 2010). Considerable changes in morphology, behavior and physiology may take place in just a few generations (Losos et al., 1997; Orr and Smith, 1998). It remains to be investigated to what extent the rapid changes in the orientation behavior of the paddyfield warbler may have been paralleled by other life-history adaptations, such as resistance to locally occurring blood parasites, forming highly divergent populations across the breeding range. Our results indicate that heterogeneous selection pressures can cause intraspecific variation in important life-history traits, such as migration strategies, without divergence at neutral genetic markers (cf. Nagel and Schluter, 1998; Pérez-Tris et al., 2004). A study focusing on candidate genes underlying the traits under selection, or on variable markers (preferably sequence-based markers; cf. Brito and Edwards, 2008) closely linked to such genes, would be necessary to find molecular evidences for this, but unfortunately no candidate genes are yet available for migratory traits. Nevertheless, our study highlights that small and geographically isolated populations, like the Bulgarian population of paddyfield warblers, are of particular interest for conservation since they may contain individuals with unique adaptations, despite showing very weak neutral population differentiation.

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