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SHORT COMMUNICATION

MHC diversity in two Acrocephalus species: the outbred Great reed warbler and the inbred Seychelles warbler

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Abstract

The Great reed warbler (GRW) and the Seychelles warbler (SW) are congeners with markedly different demographic histories. The GRW is a normal outbred bird species while the SW population remains isolated and inbred after undergoing a severe population bottleneck. We examined variation at Major Histocompatibility Complex (MHC) class I exon 3 using restriction fragment length polymorphism, denaturing gradient gel electrophoresis and DNA sequencing. Although genetic variation was higher in the GRW, considerable variation has been maintained in the SW. The ten exon 3 sequences found in the SW were as diverged from each other as were a random sub-sample of the 67 sequences from the GRW. There was evidence for balancing selection in both species, and the phylogenetic analysis showing that the exon 3 sequences did not separate according to species, was consistent with transspecies evolution of the MHC.

Keywords: Balancing selection, Genetic variation; MHC; Passerines; Population bottleneck; Transspecies evolution

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Introduction

The major histocompatibility complex (MHC) is an important component of the vertebrate immune system where it determines which antigens trigger an immune response (Hughes & Yeager 1998). Extraordinary levels of genetic variation occur within the MHC, and how this polymorphism is maintained is still debated, but the selection pressure exerted by pathogens is considered a major factor (Jeffery & Bangham 2000; Hess & Edwards 2002). The MHC is thought to play a role in aspects such as disease resistance, kin recognition, inbreeding avoidance and mate choice (Grob *et al*. 1998; Penn & Potts 1999).

Studies have characterized and investigated the ecological consequences of MHC variation in mammal and fish species (e.g. Paterson *et al*. 1998; Langefors *et al*. 2000; Ditchkoff *et al*. 2001; Reusch *et al*. 2001). But in birds, used extensively in studies of natural and sexual selection, little work has focused on the role of the MHC so far (von Schantz *et al*. 1996, 1997). Recent developments in molecular methods have facilitated the study of the avian MHC and various studies on birds are now underway (Zelano & Edwards 2002). However, before attempting to investigate the ecological consequences of MHC variation in a wild population, it is important to assess MHC variation to determine the sense and feasibility of such a study.

The Great reed warbler (GRW, *Acrocephalus arundinaceus*) and the Seychelles warbler (SW, *Acrocephalus sechellensis*), two of the worlds most intensively studied wild bird species, are congeners (Leisler *et al*. 1997) with markedly different demographic histories. Founded in 1978, the population of GRWs at Lake Kvismaren now consists of approximately 60 breeding individuals (Bensch & Hasselquist 1991; Hasselquist *et al*. 1995) and, with 12–25 immigrants each year, can be considered part of the normal distribution of this outbred migrant species (Hansson *et al*. 2000, 2003). In contrast, the SW, a rare endemic of the Seychelles islands that was pushed to the verge of extinction, is an inbred, isolated species. Between 1959 and 68, this species went through a severe genetic bottleneck with only 26–29 birds remaining on the island of Cousin (Crook 1960). The population has since recovered and now remains stable at a carrying capacity of 320–350 birds (Komdeur 1992; Richardson *et al*. 2002).

In the SW the low levels of variation seen at neutral microsatellite loci (Richardson *et al*. 2000) are probably the result of the bottleneck this species has been through.

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However, balancing selection is thought to be able to maintain variation in MHC genes even in very restricted populations (Hughes & Yeager 1998). Here we measure the MHC class I exon 3 variation in the inbred SW compared to the GRW. We assess both the number of, and genetic variability within, MHC class I Glleles and determine if balancing selection has been able to maintain MHC variation in the SW. Finally, we construct a phylogenetic tree to investigate the relationship among MHC alleles between the GRW and the SW.

Materials and methods

Study populations and samples

The SW is a small, sedentary, passerine endemic to the Seychelles Islands. The isolated Cousin Island population has been studied intensively since 1985 (Komdeur 1992; Komdeur *et al*. 1997; Richardson *et al*. 2003). In contrast, the GRW is a medium sized passerine bird which breeds in reed beds in the central and northern Palaearctic and migrates to overwinter in sub-Saharan Africa (Cramp 1992). In 1978 the first GRWs were breeding at the study site, 15 km east of Örebro (59°10′ N, 15°25′ E) in Sweden, and since 1983 the population has been studied intensively (e.g. Bensch & Hasselquist 1991; Hasselquist *et al*. 1995). During the years of study, nearly all birds within both populations have been individually colour-ringed, monitored, and blood sampled (Hasselquist *et al*. 1995; Richardson *et al*. 2001). The present study includes samples from the 485 SWs (*c*. 96%), present on Cousin Island between 2000 and 2002, and the 354 GRW's (> 96%), breeding in Lake Kvismaren between 1985 and 1996.

Restriction fragment length polymorphism (RFLP) analysis

Total genomic DNA was extracted using either a phenol extraction technique (following Bruford *et al*. 1998), or a salt extraction method (Richardson *et al*. 2001). Ten μg of DNA was digested with the restriction enzyme *Pvu*II (Boehringer Mannheim), separated on an agarose gel, transferred to a nylon membrane and hybridized overnight with the probe 21P labelled with [α-^{32}P] dCTP (Westerdahl *et al*. 1999). The membranes were then washed and exposed to an X-ray film. For the SW, RFLP analysis followed the methods outlined in Westerdahl *et al*. (1999) and the GRW results are from Westerdahl *et al*. (1999).

Motif specific polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

The protocol for motif specific amplification using PCR, followed by the separation of exon 3 sequence using DGGE developed by (Westerdahl *et al*. 2003) was used to screen efficiently MHC class I exon 3 sequence variation. Exon 3 encodes the peptide-binding region (PBR) of the MHC molecule; hence the region that is critical for the binding of peptides and for generating specific immune responses when the peptide bound is nonself (Hughes & Yeager 1998).

In the GRW, we have evidence of at least four transcribed MHC class I (cDNA) genes (Westerdahl *et al*. 1999) and an additional four genomic MHC class I genes (Westerdahl *et al*. 2003). Our sequence specific amplification detects a limited number of alleles from several class I loci, and gives a broad estimate of the MHC class I exon 3 variation within each individual. It is a repeatable method that is more sensitive in detecting genetic variation than the RFLP method and enables us to avoid screening pseudogenes.

Two primer combinations (HN36-GC46 and HN38-GC46) were used in motif specific PCRs; one motif specific primer within exon 3 and one general primer in intron 3 (Westerdahl *et al*. 2003). The two primer sets amplify different sets of sequences since the primers HN36 and HN38 have different motifs in the 3′-end (AGT and ACG, respectively). Each primer combination amplified 260 bp from the variable exon 3 (the complete exon 3 is 274 bp). Sequences were separated using gels containing 7% 19:1 acrylamide/bisacrylamide, 1 × TAE buffer and a denaturing gradient of urea and formamide (Myers *et al*. 1987). Gels were run at 60 °C in 1 × TAE buffer (C.B.C. Scientific Company, Inc.) with PCR products from both primer combinations run together in 40–70% (or 40–65% for the GRW primer combination HN36-GC46) denaturant at 190 V for 16.5 h. Standardized markers were used to enable comparisons between gels. Gels were stained using SYBR gold (Molecular Probes), and the DNA was visualized in a FluorImage SI (Molecular Dynamics Inc.).

Sequencing

Ten different DGGE bands occurred in the SW, while 67 bands were detected in the GRW population. Ten, randomly chosen, MHC class I exon 3 sequences amplified with the DGGE primers, were selected in the GRW's to constitute an equal subsample of DGGE bands for between species comparisons. DGGE bands were excised from gels and dissolved in 150 μL of ddH_2O. This solution was frozen (−80 °C) and melted (4 °C) repeatedly, diluted 1:50 and then reamplified with the original primers. The PCR product was purified and directly sequenced on an ABI PRISM 310 Genetic Analyser (Perkin Elmer). Each band was sequenced in 2–5 unrelated individuals. There was good evidence that none of the sequences were from pseudo-genes; there were no deletions in the sequences, particular features for functional MHC genes were found,

e.g. conserved cysteine codons, and several motives were identical to chicken, *Gallus domesticus*, class I genes.

When running DGGE's based on a PCR where several alleles have been amplified, heteroduplexes may be formed. In this study, all the DGGE bands used gave clear, unambiguous sequences and were not the result of heteroduplexes.

Phylogenetic analysis

All 10 SW warbler exon 3 sequences, and a total of 32 GRW exon 3 sequences, including cDNA-sequences (Westerdahl *et al*. 1999), genomic sequences and sequences likely to be from pseudogenes (Westerdahl *et al*. Submitted) were used in the phylogenetic tree. The tree was constructed in MEGA 2.1 software (Nei & Gojobori 1986) using the minimum evolutionary method and the entire exon 3 sequences (273 bp). Bootstrap tests verified the probability of the branches. All sequences have been submitted to GenBank (accession numbers; Acar-UA, AY306008–AY306009; Ase-UA, AJ557874–AJ557883).

Statistical analysis

The evolutionary distance (p) between exon 3 sequences was computed using the KIMURA 2-parameter model, while the number of synonymous and nonsynonymous substitutions per site in the PBR of exon 3 was calculated by Nei and Gojobori's method of pair-wise comparisons, using MEGA version 2.1 (Nei & Gojobori 1986). The PBR was superimposed from the human sequence (Bjorkman *et al*. 1987). All tests are two-tailed and means are given ± one standard deviation.

Results and Discussion

The level of variation at the MHC class I exon 3 loci is high in the GRW but low in the SW. Both species have roughly an equal number of RFLP fragments (21–25 GRW vs. 23–25 SW), which suggests they have an equal number of MHC class I genes. However, 89% (49/55) of GRW's had unique class I RFLP genotypes — similar to the 88% (42/48) of Savannah sparrow (*Passerculus sandwichensis*) with unique MHC class II, RFLP genotypes (Freeman-Gallant *et al*. 2002) — while only 61% (37/61) of SW's had unique class I RFLP genotypes. Furthermore, the number of new RFLP genotypes observed in the SW (37) had reached a plateau, but was still increasing in the GRW (49). The total number of both DGGE-alleles (10 SW vs. 67 GRW) and DGGE genotypes (87 out of 485 SWs vs. 339 out of 354 GRWs) was considerably lower in the SW compared with the GRW. Finally, the mean number of DGGE-alleles per individual was significantly lower in the SW than in the GRW (3.97 ± 1.27 vs. 6.54 ± 1.85; t-test, $t_{837} = 22.55$, $P < 0.001$).

The patterns of genetic variation found in the GRW and the SW are consistent with their demographic history. The GRW population is relatively outbred, with individuals immigrating in from the pan-European population (Hansson *et al*. 2000; Hansson *et al*. 2003), while the SW has recently been through a bottleneck and remains totally isolated (Komdeur 1994). The number of MHC alleles in the SW does, however, appear to be high considering the recent population bottleneck, especially when compared to the extremely low levels of variation found with neutral markers (Richardson *et al*. 2000). For example, significantly more microsatellites characterized in the SW were monomorphic (32/63 = 51%, Richardson *et al*. 2000) than were microsatellites characterized in the GRW (1/11 = 9%, Hansson *et al*. 2000) (Fisher exact $P = 0.018$).

Balancing selection appears to play a determinant role in MHC evolution (Bernatchez & Landry 2003) and one indication of this is a higher number of nonsynonymous (d_n) than synonymous (d_s) substitutions in the PBR. In the present study the ratio of d_n to d_s tended to be greater than one in the PBR, but less than one in the non-PBR, for both the SW [PBR; $d_n/d_s = (0.34 \pm 0.109)/(0.20 \pm 0.11) = 1.65$; Non-PBR $d_n/d_s = (0.05 \pm 0.01)/(0.11 \pm 0.03) = 0.43$] and the GRW [PBR; $d_n/d_s = (0.38 \pm 0.12)/(0.33 \pm 0.17) = 1.17$; Non-PBR $d_n/d_s = (0.05 \pm 0.01)/(0.09 \pm 0.03) = 0.52$]. Although these differences were not significant (SW, t-test = 0.28, $P > 0.05$; GRW, t-test = 0.28, $P > 0.05$), this may be because we are comparing exon 3 sequences across loci, in which case the numbers of synonymous substitutions are likely to be higher than when comparing alleles within a locus (Hughes & Nei 1989; Westerdahl *et al*. 1999). Evidence for balancing selection also comes from the fact that both species have significantly higher than average amino acid variation per site in the PBR than in the non-PBR (Table 1).

Theory suggests that heterozygous individuals with diverged MHC alleles will be at an advantage, since they will be able to respond to a wider range of pathogens compared with homozygous individuals, or even heterozygous individuals with two similar alleles (Hughes & Yeager 1998). Within a population, a high level of divergence between MHC alleles would provide further evidence that selection is acting to maintain MHC variation. In both the SW and the GRW populations, a high level of divergence was apparent within exon 3 sequences. The average number of nucleotide differences between any two exon 3 sequences was high (Table 1), and only a few exon 3 sequences were closely related. There was no significant difference between the SW and the GRW in the number of nucleotide differences, overall amino acid variation, or average amino acid variation per site in the PBR or in the non-PBR (Table 1). Furthermore, within species variation was not significantly different from between species variation for each measure (Table 1). Interestingly,

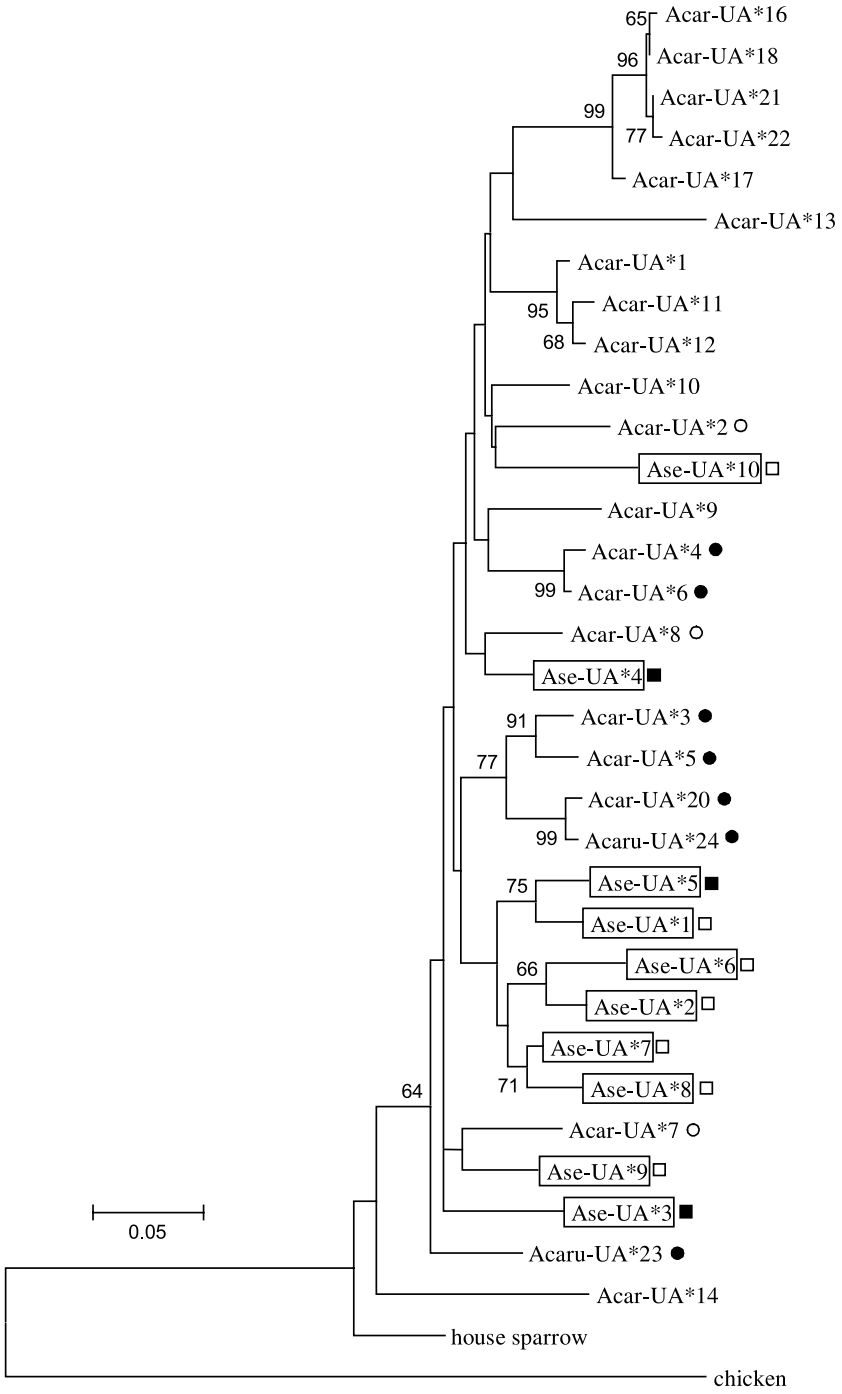

Fig. 1 Phylogenetic tree comparing great reed warbler (GRW) and Seychelles warbler (SW) MHC class I exon 3 sequences. GRW sequences amplified with DGGE primers are denoted with circles and SW sequences are within boxes and denoted with squares (primer combinations; filled symbols = HN36/GC46, open symbols = HN38/GC46). The evolutionary distance was computed with the Kimura 2-parameter model, the tree was constructed with the ME-method and the probability of the branches are bootstrap values for 2000 replications (bootstrap values > 50 are shown in the tree, the scale bar indicates genetic distance in units of nucleotide substitutions per site). Exon 3 sequences from the chicken, *Gallus gallus*, and the house sparrow, *Passer domesticus* (C. Bonneaud, unpublished data) were used as outgroups.

although fewer DGGE-alleles (exon 3 sequences) were observed in the SW (10 vs. 67), there was an equally high level of divergence between exon 3 sequences in the SW as there was in the GRW population. This indicates that individuals with diverged MHC alleles have been selected for during and\or after the severe bottleneck the SW population went through. Such a pattern of a low number of MHC alleles, with a high degree of divergence between alleles, has been found in studies of other bottlenecked species (e.g. Mikko *et al*. 1997; Hedrick *et al*. 1999; Hoelzel *et al*. 1999; Hedrick *et al*. 2000).

In the present study, we have investigated divergence between sequences from several different (unspecified) loci. This could lead to higher estimations of divergence than in intra locus comparisons, since synonymous substitutions are likely to accumulate between loci over time. However, the GRW and SW seem to have an equal number of class I loci and therefore the between species

Table 1 Nucleotide and amino acid (AA) divergence, and the proportion of variable AAs per site, within and between, the two *Acrocephalus* species

Species	*N*	Nucleotide differences	AA variation	PBR, AA p-distance	Non-PBR, AA p-distance	(*t*-test) PBR vs. Non-PBR
Great reed warbler	10	25.11 ± 2.77†	14.11 ± 2.14†	0.44 ± 0.07†	0.10 ± 0.02†	4.67***
Seychelles warbler	10	22.71 ± 2.78†	13.09 ± 2.14†	0.39 ± 0.07†	0.10 ± 0.02†	3.92**
Between species	20	25.45 ± 2.73‡	14.96 ± 2.15‡	0.44 ± 0.07‡	0.12 ± 0.02‡	4.28***

Statistical tests; † vs. †, *t*-tests between GRW and SW of nucleotide differences, AA variation and the proportion of variable AA per site (p-distance) were all ns. † vs. ‡, *t*-tests for the between species variation compared with the mean value of the within species variation were all ns.
***$P < 0.005$.
**$P < 0.01$.

comparison is not affected. Furthermore, there is evidence of concerted evolution in birds, and hence selection for homogenization of alleles across loci (Edwards *et al*. 1995b; Wittzell *et al*. 1999)

The phylogenetic tree suggests that some GRW exon 3 sequences are more similar to SW sequences than to other GRW sequences, and that the sequences are not separated according to species, but are intermixed with only a few forming significantly supported clusters (Fig. 1). This intermixing suggests a transspecies persistence of MHC class I exon 3 sequences, with the origin of some allelic lineages predating the phylogenetic split between the species. The prolonged maintenance of MHC alleles is contrary to what is predicted for neutral loci, and also supports the idea that long-term balancing selection on the MHC alleles has occurred (Figueroa *et al*. 1988). Our results are consistent with the transspecies evolution of MHC alleles (Klein 1987), which has previously been supported by many studies of mammal and fish species (reviewed in Hedrick 2001). In passerines, other studies have shown that MHC class II exon 2 sequences do not always cluster by species (Edwards *et al*. 1995a; Freeman-Gallant *et al*. 2002; Hess & Edwards 2002), but the present study is, to our knowledge, the first to study MHC class I in passerines in this context.

The MHC system in passerine birds is more complex than that observed in chickens (Kaufman *et al*. 1999; Westerdahl *et al*. 2000; Freeman-Gallant *et al*. 2002). This, combined with the extensive MHC class I polymorphism seen in the GRW, has made it difficult to identify the number of class I genes present, and thus to characterize the MHC in detail (Westerdahl *et al*. 1999). The SW may be an excellent model organism in which to do this, as its limited genetic variation means that the loci involved are more likely to be homozygous, thereby making characterization simpler. The high levels of polymorphism in the GRW have also complicated attempts to investigate the ecological consequences of MHC characteristics in a wild avian population. The SW provides a simplified system in which to investigate such questions: the population is isolated and, while it contains limited genetic variation at the MHC (thus making statistical analysis more tractable), this appears to be maintained by selection. The SW has also been the focus of extensive study and many important factors required for an in-depth investigation are already available or known. On the other hand, the GRW population is more likely to be representative of a normal passerine system than is the SW. Ultimately, using both species in complimentary and comparative studies should provide the most productive research strategy.

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