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Between-year variation of MHC allele frequencies in great reed warblers: selection or drift?

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Abstract

The major histocompatibility complex (MHC) genes are extremely polymorphic and this variation is assumed to be maintained by balancing selection. Cyclic interactions between pathogens and their hosts could generate such selection, and specific MHC alleles or heterozygosity at certain MHC loci have been shown to confer resistance against particular pathogens. Here we compare the temporal variation in allele frequencies of 23 MHC class I alleles with that of 23 neutral microsatellite markers in adult great reed warblers (a passerine bird) in nine successive cohorts. Overall, the MHC alleles showed a significantly higher variation in allele frequencies between cohorts than the microsatellite alleles, using a multi-variate genetic analysis (AMOVA). The frequency of two specific MHC alleles, A3e ($P = 0.046$) and B4b ($P = 0.0018$), varied more between cohorts than expected from random, whereas none of the microsatellite alleles showed fluctuations exceeding the expectation from stochastic variation. These results imply that the variation in MHC allele frequencies between cohorts is not a result of demographic events, but rather an effect of selection favouring different MHC alleles in different years.

Introduction

The major histocompatibility complex (MHC) plays an important role in the vertebrate immune system. MHC molecules present 'self' and 'non-self' peptides to T-cells, and when a foreign peptide is bound it triggers an immune reaction (Klein, 1986). The MHC genes are extremely polymorphic and this variation is believed to be maintained by balancing selection either through heterozygous advantage or negative frequency dependent selection (Hughes & Nei, 1988, 1989; Potts & Wakeland, 1990; Satta, 1993). The theory behind heterozygous advantage is that each MHC molecule can bind a limited number of peptides. An individual possessing two different MHC alleles at one locus can therefore bind twice the number of foreign peptides compared to a homozygous individual (Nei & Hughes, 1991; Hughes & Hughes, 1995). In contrast, negative frequency dependent selection assumes that it is advantageous to carry rare alleles to which pathogens are not adapted (Bodmer, 1972). Both these hypotheses have gained some support in experiments and studies of natural populations where specific MHC alleles have been shown to confer resistance against particular pathogens (Briles *et al.*, 1977; Schat, 1987; Hill *et al.*, 1991; Plachy *et al.*, 1992; Paterson *et al.*, 1998; Carrington *et al.*, 1999; Langefors *et al.*, 2001), and from studies in humans where heterozygosity has been shown to confer disease resistance (Thursz *et al.*, 1997; Carrington *et al.*, 1999).

In natural populations, it has been suggested that parasites maintain genetic variation in the population through cyclic interactions with their hosts caused by negative frequency dependent selection (Hamilton & Zuk, 1982). An important assumption of the Hamilton–Zuk model was recently supported in a theoretical model by Hedrick (2002) showing that the selective force from pathogens, which vary in time and space, could maintain the genetic polymorphism in MHC genes. In accordance with this, a study of avian blood parasites (*Haemoproteus* spp.) in willow warblers (*Phylloscopus trochilus*) showed

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that different parasite strains fluctuated in frequency both between years and between large geographical areas (Bensch & Åkesson, 2003). This suggests that breeding birds will encounter different parasites in different years even within the same breeding region. Moreover, migratory birds are likely to be affected by spatial and temporal fluctuations in parasite exposure both at their breeding grounds and wintering quarters and will therefore be challenged by a particularly diverse parasite fauna (Møller & Erritzøe, 1998; Waldenström *et al.*, 2002).

The great reed warbler (*Acrocephalus arundinaceus*) is a long-distance migrant that winters in tropical Africa and breeds in Europe and Asia (Cramp, 1992). In the present study we investigate the variation in allele frequencies of MHC class I genes in great reed warblers breeding at Lake Kvismaren, southern central Sweden. At this locality the breeding ecology of great reed warblers has been studied in each year since 1984 (e.g. Bensch & Hasselquist, 1991; Bensch, 1996; Hasselquist, 1998). Approximately 50% of the breeding birds in the study area are locally hatched (Bensch *et al.*, 1998; Hansson *et al.*, 2002). MHC class I genes have been characterized in detail in great reed warblers from this population (Westerdahl *et al.*, 1999, 2004; Westerdahl, 2003), which provides us with a unique possibility to examine how MHC allele frequencies vary over time in a natural population. We chose to analyse variation between nine successive cohorts of adult birds (that have survived until at least 1 year old) since mortality is especially high during the birds' first year of life (Bensch *et al.*, 1998; Hansson *et al.*, 2002). Variation in MHC allele frequencies between cohorts may be the result of changes in selection pressures favouring different alleles in different years. However, variation in allele frequencies between cohorts could also result from demographic events, in particular through variation in annual rate of dispersal between genetically differentiated populations. To control for the latter scenarios, we also examined the variation in allele frequencies of supposedly selectively neutral microsatellite loci (Queller *et al.*, 1993; Jarne & Lagoda, 1996; Hansson *et al.*, 2000). If the between-cohort variation of both MHC and microsatellite allele frequencies vary more than expected from random, the genetic variation at MHC is likely to result mainly from demographic processes. However, if genetic variation is higher than expected from random in MHC alleles, but not in the microsatellite alleles, then selection is likely to be acting on MHC genes in great reed warblers.

Material and methods

Study species and general field methods

Great reed warblers breed in the reed–water interface of patchily distributed lakes and marshes in Europe and Asia (Cramp, 1992). The breeding ecology of the great reed warbler population at Lake Kvismaren in southern central Sweden has been investigated in detail since 1984 and the majority (95–100%) of the breeding birds and their nestlings have been ringed, each with an aluminium ring and an individual-specific combination of colour rings (Bensch, 1996; Hasselquist, 1998). The breeding population increased in size until the late 1980s when a fairly stable level of about 60 breeding adults was reached (Hansson *et al.*, 2000). Blood samples from almost all breeding females, territorial males and nestlings have been collected since 1987.

Extensive capture–recaptures over the species' whole breeding range in Sweden have shown that the dispersal pattern is highly skewed geographically, and that most hatchlings return to breed either at the locality where they hatched or disperse to nearby breeding localities (Hasselquist, 1995; Bensch *et al.*, 1998; Hansson *et al.*, 2002). Therefore, the majority of great reed warblers breeding at our main study site are hatched in the local vicinity, within approximately a 30-km radius (Hansson *et al.*, 2002).

In the present study, we investigated great reed warblers breeding in the Lake Kvismaren area belonging to cohorts hatched between 1985 and 1993, thus including birds that had survived until at least 1 year old. Each cohort included locally hatched previously ringed birds and also previously unringed immigrants to Lake Kvismaren whose age (and thus cohort) was unambiguously determined using eye and tarsus coloration, and the presence/absence of tongue spots (Bensch *et al.*, 1998). Of the individuals included in the analyses 51% were ringed as nestlings in the study area.

Molecular methods

Restriction fragment length polymorphism (RFLP) studies of class I genes from genomic DNA from 55 great reed warblers, using the restriction enzyme Pvu II and the probe 21P, have shown that each individual have 21–25 RFLP bands (Westerdahl *et al.*, 1999). Because there is so little variation in the number of RFLP bands between individuals it is unlikely that the number of class I genes varies between individuals. The exact number of class I genes cannot be determined on the basis of RFLP patterns, but since we have used a short class I probe (261 bp) and know that the length of a single class I allele is about 4 kb we can make an estimate of the number of class I genes (Westerdahl *et al.*, 1999; Westerdahl 2003). The RFLP bands are in the size range 1–9 kb and one RFLP band may approximately correspond to one MHC allele. Depending on homo-/heterozygosity at the MHC loci there are more than ten and possibly as many as 25 MHC class I genes, including pseudo-genes, in great reed warblers.

The class I genes of great reed warblers, including both exons and introns, are very similar and we have not been able to assign alleles to specific loci (Westerdahl *et al.*, 1999; Westerdahl, 2003). Therefore, we have studied the

polymorphism in MHC class I using two different sequence-specific primer combinations that specifically amplify exon 3 sequences (encoding the peptide binding region of the MHC molecule) from one or several genes in the class I gene complex. These primers were designed to amplify MHC alleles with certain motifs, and these motifs have so far only been found in transcribed MHC alleles (cDNA) and not in non-transcribed (pseudogenes) MHC alleles (Westerdahl *et al.*, 1999; Westerdahl, 2003). The two primer combinations amplify two different subsets of MHC alleles that we estimate constitute 60–70% of all transcribed MHC alleles (Westerdahl, 2003). There are positive polymerase chain reactions (PCRs) for both primer combinations in all individuals tested (Westerdahl, 2003). The PCR products are separated by the denaturant gradient gel electrophoresis (DGGE) method (Myers *et al.*, 1987) following the methods described in Westerdahl (2003). The screening method amplifies 2–12 MHC sequences, MHC alleles, per individual (for simplicity, we name these sequences MHC alleles although we are aware that they stem from several different loci rather than a single specific locus). A similar procedure has been used when studying MHC polymorphisms in sticklebacks, *Gasterosteus aculeatus* (Binz *et al.*, 2001; Reusch *et al.*, 2001). In order to confirm that what we scored as different DGGE bands represent unique MHC alleles, we identified eight DGGE bands that had migrated different distances in a DGGE gel and that were present in at least two unrelated individuals. We excised these 2 × 8 DGGE bands from the DGGE gel and then cloned and sequenced these bands as in Westerdahl *et al.* (2004). Seven of eight DGGE bands had identical DNA sequences in the bands, whereas in the remaining case the presumably identical DGGE bands showed five positions with nucleotide differences. Furthermore, the separation of these eight DGGE bands showed that DGGE bands situated more closely on the DGGE gel had a higher sequence similarity than DGGE bands situated more distantly apart (Westerdahl, 2003; Westerdahl *et al.*, 2004). The screening method using motif-specific PCR and DGGE is repeatable, and independent PCRs run on the same DNA sample give the same number and pattern of bands when run on DGGE gels.

In total, 235 adult individuals were screened for MHC alleles, and the average sample size was 26 individuals per cohort (cohort, number of individuals: 1985, 18; 1986, 23; 1987, 18; 1988, 36; 1989, 22; 1990, 34; 1991, 28; 1992, 32; 1993, 24). Using sequence specific amplification and DGGE we encountered 67 different MHC alleles in the study population, and we estimate that there are an additional 40 MHC alleles that we do not pick up using this screening technique (Westerdahl, 2003). Of the 67 alleles we amplified we chose the 23 MHC alleles that occurred in 20–215 individuals when investigating how the MHC alleles varied in frequency over time. We excluded 43 alleles that occurred in fewer than 20 individuals and the most common allele occurring in almost all individuals (Table 1). The average number of MHC alleles per individual was 6.4 ± 1.8 (amplification from several loci; see above).

To construct a comparable data set for a neutral marker, we randomly chose 23 microsatellite alleles that were also present in 20–215 individuals, at microsatellite loci for which the birds were already typed. Microsatellite alleles were amplified with PCR following Hansson *et al.* (2000). Primer sequences and locus-specific amplification conditions are given in Hansson *et al.* (2000) and Richardson *et al.* (2000). Amplified microsatellite alleles were separated by electrophoresis in 6% acrylamide gels. In every gel and for every locus, the alleles of two reference individuals were run as size standards (Hansson *et al.* 2000). Microsatellite alleles from five different loci were investigated and in total 61 alleles were found at these loci (Hansson *et al.*, 2000; Hansson, 2003) (Table 2). We used 20 microsatellite alleles at loci presented in Hansson *et al.* (2000) and three at a locus presented in Richardson *et al.* (2000). We did not choose alleles at loci located on the Z-chromosome (Hansson *et al.*, 2000), or at the Ppi2 locus, which segregates with some survival-associated loci in our population (Hansson *et al.*, 2001).

Table 1 Results of logistic regression for presence/absence of MHC alleles in nine cohorts of great reed warblers (in total 235 individuals).

MHC alleles	*n**	χ2†	*P*‡
A3e	25	15.75	0.046
A4	73	5.68	0.685
A4c	24	14.64	0.066
A5	100	11.28	0.192
A5a	20	14.71	0.065
A5b	47	6.88	0.553
A6	82	10.32	0.247
A6a	37	10.67	0.221
A7	52	9.6	0.298
A7b	44	8.41	0.394
A7c	28	6.98	0.539
A10	25	4.68	0.791
B3	47	3.44	0.902
B3g	31	14.55	0.069
B4b	38	24.61	0.0018§
B4c	34	9.63	0.292
B5	50	2.64	0.953
B5b	39	11.06	0.198
B5c	30	7.94	0.440
B6	24	3.48	0.900
B7c	60	10.08	0.267
B7e	56	7.84	0.454
B8	26	12.92	0.115

*Number of individuals with the allele.

†When the model was over-dispersed, the significance of parameters was tested with an *F* test, whereas a χ^2 test was used otherwise (see Material and methods section).

‡d.f. = 8 in all cases.

§Significant also after Bonferroni correction ($k = 23$, $P_{crit} = 0.0022$).

Statistics

First, we analysed the overall genetic differentiation between cohorts of recruiting great reed warblers based on all the 67 MHC alleles. This was done by a genetic structure analysis (AMOVA) implemented in the program ARLEQUIN version 2.000 (Schneider *et al.*, 2000), treating each allele as either present (1) or absent (0) and examining whether the overall variation was larger between cohorts than expected from random. Then we did the same analysis also for the 23 MHC alleles (those occurring in 20–215 individuals) and 23 microsatellite alleles. Second, we aimed at investigating if the magnitude of variation in allele frequencies between cohorts was different for MHC and microsatellite alleles. To do this analysis, we compared the distributions of the χ^2 values obtained for the MHC and microsatellite alleles in the logistic regressions (see analyses below), considering these χ^2 values as quantitative measures of the variation in allele frequencies between cohorts, using a *t*-test in SYSTAT 9.0 (Wilkinson, 1998). Third, we did separate analyses for each of the 23 MHC and microsatellite alleles (it was not possible to perform this analysis on alleles that occurred less frequently than in 20 individuals) among the 235 great reed warblers (dependent variable) with cohort as the independent variable (categorical) using logistic regressions [SAS 6.12; Genmod module, logit link function, binomial error distribution, type 3 option (SAS, 1990)]. When the scale parameter (deviance/d.f.) of the models was >1, which indicates that the data are over-dispersed, we kept the scale parameter constant at a value of 1 in the logistic regression procedure (by using the DSCALE option in SAS), and the significance of alleles was tested with an *F* test. This procedure is necessary when over-dispersed data with binomial errors are analysed (as is the case in logistic regression models), otherwise too low and thus erroneous *P*-values will follow (Crawley, 1993). When the scale parameter (deviance/d.f.) of the models was <1 we used the default scaling option in SAS and the significance of alleles was tested with a χ^2 test (Crawley, 1993). Finally, the χ^2 test for association between the MHC alleles B4b and A3e was also run in SYSTAT 9.0 (Wilkinson, 1998). All tests are two-tailed.

Results

We investigated whether frequencies of MHC and microsatellite alleles varied more than expected by chance in nine cohorts of adult great reed warblers breeding in Lake Kvismaren. Using an overall approach, where all MHC/microsatellite alleles were included in a genetic structure analysis, the MHC alleles showed slight but significantly different frequencies between cohorts (67 MHC alleles, $\text{AMOVA}_{8,234}$, $F_{st} = 0.0088$, P (F_{st}) < 0.05; 23 MHC alleles, $\text{AMOVA}_{8,234}$, $F_{st} = 0.0062$, P (F_{st}) < 0.05) while the microsatellite alleles did not (23 microsatellite alleles, $\text{AMOVA}_{8,234}$, $F_{st} = -0.0022$, P (F_{st}) = n.s.). To be able to directly compare the variation in allele frequencies between the MHC and microsatellite alleles in a single analysis we used the χ^2 values from the logistic regressions (see below) as a measure of the variation in allele frequencies between cohorts. The 23 MHC alleles had on average a slightly higher variation in allele frequencies (Tables 1 and 2) between cohorts than the microsatellite alleles ($t\text{-test}_{23} = 2.18$, $P < 0.05$).

When analysing each allele separately the frequency of two MHC alleles, A3e ($P = 0.046$) and B4b ($P < 0.01$), varied more between cohorts than expected from random (Fig. 1; Table 1). However, after a Bonferroni correction this variation was significant for B4b only [$k = 23$, $P_{\text{crit}} < 0.01$ (Sokal & Rohlf, 1995)]. In contrast, none of the microsatellite alleles showed more between-year variation than expected by chance (n.s.; Fig. 2; Table 2). The frequency of the MHC allele B4b increased from 1989 until 1991, after which it decreased in frequency again, hence, it reached a peak in 1991 (Fig. 1). According to the microsatellite data, 1991 was not exceptional concerning immigration or drift; since

Table 2 Results of logistic regression for presence/absence of microsatellite alleles in nine cohorts of great reed warblers (in total 235 individuals). Number of microsatellite alleles and expected heterozygosity (H_E) in the study population are also shown (Hansson, 2003).

Microsatellite locus	Number of alleles	H_E	Allele	n^*	$\chi 2$†	P‡
Aar3	32	0.79	194	145	5.44	0.714
			196	93	7.2	0.513
			200	24	3.20	0.921
			218	47	11.54	0.173
			238	22	10.63	0.223
Aar4	6	0.56	112	33	9.27	0.320
			116	45	6.47	0.595
			118	205	11.01	0.201
			120	33	3.38	0.908
			122	26	8.60	0.377
			124	20	9.74	0.284
Aar5	11	0.80	75	68	3.12	0.929
			77	27	9.03	0.340
			85	33	7.45	0.489
			87	89	6.08	0.636
			89	126	8.32	0.399
			93	47	10.80	0.213
Aar8	6	0.49	115	211	8.41	0.395
			117	75	9.52	0.300
			119	47	2.72	0.950
Ase7	6	0.53	9	49	4.08	0.846
			10	202	6.45	0.597
			11	46	6.05	0.642

*Number of individuals with the allele.
†When the model was over-dispersed the significance of parameters was tested with an *F* test, whereas a χ^2 test was used otherwise (see Materials and methods section).
‡d.f. = 8 in all cases.

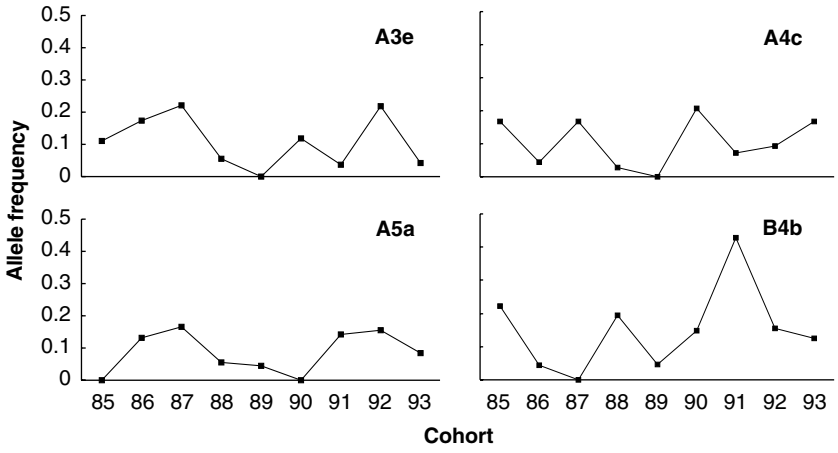

Fig. 1 The frequency of four MHC alleles (with the lowest *P*-values from the logistic regression; A3e, A4c, A5a, B4b; Table 1) in nine successive cohorts of great reed warblers breeding at Lake Kvismaren (cohort, number of individuals: 1985, 18; 1986, 23; 1987, 18; 1988, 36; 1989, 22; 1990, 34; 1991, 28; 1992, 32; 1993, 24).

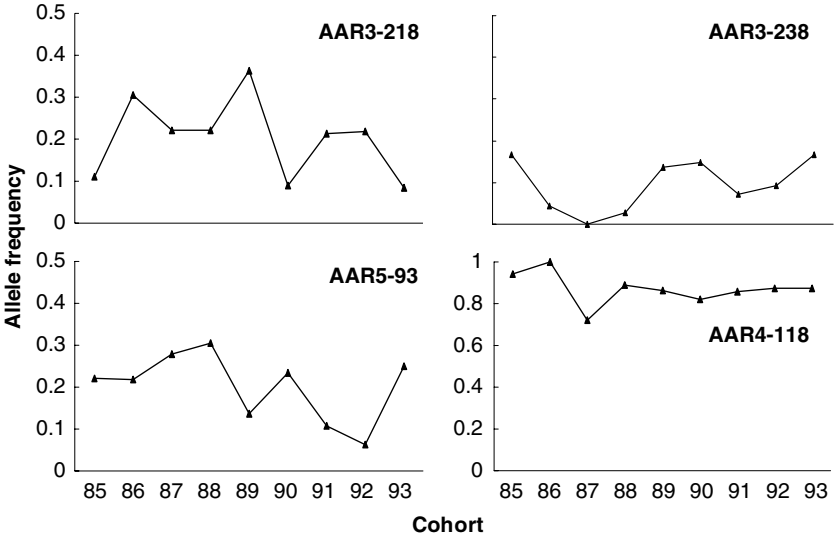

Fig. 2 The frequency of four microsatellite alleles (with the lowest *P*-values from the logistic regression; AAR3-218, AAR3-238, AAR4-118, AAR5-93; Table 2) in nine successive cohorts of great reed warblers breeding at Lake Kvismaren (cohort, number of individuals: 1985, 18; 1986, 23; 1987, 18; 1988, 36; 1989, 22; 1990, 34; 1991, 28; 1992, 32; 1993, 24).

the number of immigrants, the number of novel microsatellite alleles, and the number of immigrants with novel alleles was not higher than expected (Hansson *et al.*, 2003). There was no significant association between the MHC alleles A3e and B4b in the investigated individuals (χ^2 test, $n = 235$; $\chi^2 = 1.38$, d.f. $= 1$, n.s.), which suggests that these alleles represent different MHC-haplotypes.

Discussion

The MHC genes are expected to be under balancing selection, which would maintain the high level of polymorphism found in the MHC genes. In the present study, we found that the MHC alleles had on average a slightly higher variation in allele frequencies between nine successive cohorts of adult great reed warblers than the microsatellite alleles. As many as six MHC alleles had lower *P*-values in the logistic regression analyses ($P = 0.0018$–0.11) than the most variable microsatellite allele (AAR3-218, $P = 0.173$; Tables 1 and 2). In particular, the MHC allele B4b varied significantly in frequency between cohorts (Fig. 1). A similar tendency was also observed for the MHC allele A3e. For the same set of individuals there was no or very little variation in frequencies of selectively neutral microsatellite alleles (Table 2).

Because it was impossible to assign the different MHC alleles to specific loci, we could not compare the temporal variation under exactly the same conditions as for the studied microsatellite alleles. A problem with this approach is that some MHC alleles might exist at more than one locus. We do not believe that this could explain why the MHC alleles fluctuated more between years than microsatellite alleles. If the same MHC allele exists at more than one locus, this would hamper rather than

increase the temporal variation; if identical alleles are inherited independently from two loci, the offspring is more likely to inherit at least one copy of the allele from its parent. Another potential problem with the way we have analysed our data is that MHC alleles within a haplotype are linked, and this could cause one allele to follow the fluctuations of another linked allele. Clearly, this was not the case in our study as the two alleles showing significant variation (B4b and A3e) showed no association and thus represent different MHC haplotypes. Moreover, a previous study showed no linkage between the MHC class I alleles in our study population (even though the alleles from a single MHC haplotype are tightly linked within families, as shown earlier by a segregation analysis in families; Westerdahl, 2003).

We believe the most plausible explanation for the significant variation in MHC allele frequency between cohorts is that it is a consequence of fluctuating selection pressures from pathogens and parasites. Early in life, infections are likely to be more severe and costly than later in life. This is because most pathogens are novel to the immune system of juvenile birds (e.g. Atkinson & van Riper, 1991) and a larger proportion of juvenile birds are in poor condition with suppressed immunity caused by either malnutrition or heritable genetic variation (e.g. Saino *et al.*, 1997, Brinkhof *et al.*, 1999). As a consequence, parasite- and pathogen-induced mortality is likely to be an important natural selection factor in first year birds. In addition, certain MHC alleles may become especially important for parasite resistance in juveniles that generally have immune systems that are less efficient and experienced compared with adults. Preliminary data give further evidence that the MHC allele B4b (the allele that varied significantly in frequency between cohorts) is subject to selection, because allele B4b seems to be different from other previously known great reed warbler MHC alleles in that it has a 'novel' nonsynonymous substitution (causing an amino acid change) in the putative peptide-binding region (own unpublished data).

In a long-distance migrant bird such as the great reed warbler, fluctuating selection patterns, caused by parasite- and pathogen-induced mortality, can occur both at their Swedish breeding grounds and in their African wintering quarters. Assuming that pathogens are spatially structured (e.g. blood parasites; Freeman-Gallant *et al.*, 2001), it is likely that allele frequencies of genes under selection (i.e. MHC genes) differentiate faster than allele frequencies of genes under drift (i.e. microsatellite alleles; Lynch *et al.*, 1999). This would then create more variation in MHC alleles than in microsatellite alleles at the same geographical scale. Such a scenario, however, is unlikely for pathogens transmitted in the Swedish breeding areas, because a majority of the great reed warblers return to breed at or nearby (<30 km) the hatching site (Hansson *et al.*, 2002). We do not find it likely that the selection pressures from pathogens would differ considerably on this very restricted spatial scale. Moreover, a study of willow warblers has shown that each of two blood parasites were distributed over a large geographical area covering most of the host's Swedish breeding range (Bensch & Åkesson, 2003). At the Swedish breeding grounds, we therefore find it more likely that temporal variation in pathogen abundance between years could have contributed to the observed pattern.

Parasite and pathogen abundance at the African wintering grounds are also likely to generate important selection pressures acting on the great reed warblers. Juveniles only stay at, or close to, their hatching site for 1–2 months after fledging (Stervander, 1999) before they migrate to Africa where they spend more than 5 months until spring migration (Cramp, 1992). In great reed warblers, all blood parasites detected so far seem to be transmitted in Africa (Bensch *et al.*, 2000; Waldenström *et al.*, 2002; D. Hasselquist, Ö. Östman & S. Bensch, unpublished). Varying selective regimes between cohorts could be caused by pathogens differing in prevalence between years in Africa, exposing birds from different cohorts to different pathogens despite staying in the same geographical area. However, we cannot exclude that birds from different cohorts stay at different wintering areas with different parasite faunas, causing selection to favour different MHC alleles in different years.

To our knowledge, this is the first study of variation in MHC allele frequencies over time in a natural population. We found that substantial variation in frequencies of MHC alleles occurred over a remarkably short timescale, 9 years (which is about three times the generation time of great reed warblers). Furthermore, allele frequencies even changed dramatically between adjacent years. We find it most plausible that selection pressures from parasites and pathogens have generated the observed variation in MHC allele frequencies between cohorts of great reed warblers. Although we know only little about the relative impact of summer/winter-transmitted diseases and the importance of temporal/spatial variation of pathogens for explaining this pattern, we believe that variations at most of these levels are likely to promote the observed MHC allele fluctuations. Such rapid fluctuations in selection pressure could be generally important for maintaining a high genetic polymorphism at MHC genes also in other populations and species.

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