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A temporal analysis shows major histocompatibility complex loci in the Scandinavian wolf population are consistent with neutral evolution

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The major histocompatibility complex (MHC) has an integral role in the immune system, and hence diversity at its genes may be of particular importance for the health of populations. In large populations, balancing selection maintains diversity in MHC genes, but theoretical expectations indicate that this form of selection is absent or inefficient in small populations. We examine the level of diversity at three MHC class II loci in the wolf population of Scandinavia, a population naturally recolonized with a genetic contribution from as few as three founders, and in four neighbouring wolf populations. In the Scandinavian wolf population, two alleles were found for each locus and the distribution of alleles is compatible with their linkage into two haplotypes. Changes in the level of heterozygosity over time since recolonization demonstrate the effects of the proposed arrival of an immigrant wolf. The maintenance of diversity is shown to be compatible with a neutral, random allocation of alleles, in conjunction with crossing between packs. A total of 15 *DRB1*, seven *DQA* and 10 *DQB1* alleles are found in four neighbouring wolf populations, with substantial sharing across populations. Even in these larger populations, bottlenecks and fragmentation with consequent genetic drift are likely to have resulted in few indicators for balancing selection and significant differentiation of populations.

Keywords: *DRB1*; *DQA*; *DQB1*; balancing selection; wolf; *Canis lupus*

1. INTRODUCTION

Encoded within the major histocompatibility complex (MHC) are gene regions responsible for the presentation of peptides for immune recognition, a role that is integral to the normal functioning of the immune system and hence potentially important for the long-term survival of populations. The level of genetic diversity at these MHC genes has been studied in many vertebrate species and has been shown to be under the influence of balancing selection (Hughes & Yeager 1998). In large, outbred populations, this form of selection maintains high levels of variability in terms of numbers of alleles, amino acid divergence and heterozygosity levels beyond neutral expectations (Hedrick & Thomson 1983; Klein *et al.* 1993; Garrigan & Hedrick 2003).

However, in small populations, the dynamics of MHC variability may differ from that in large, outbred populations in two ways. First, demographic effects such as bottlenecks will result in the stochastic loss of MHC alleles as a part of a genome-wide loss of variability. The presence of no, or low levels of, variability at MHC loci in several species has been attributed primarily to such demographic effects (e.g. O'Brien *et al.* 1985; Ellegren *et al.* 1996*a*). Second, theoretical expectations suggest balancing selection will not be effective in maintaining diversity at MHC loci in small populations. Neutrality is expected when $N_e < 1/(2s)$ (Kimura 1983), which is equivalent to an effective population size (N_e) of 25 individuals when using the expected long-term selection intensity (s) of $s < 0.02$ (Klein *et al.* 1993), although this will vary with the selection intensity which can fluctuate over time or space (Edwards & Hedrick 1998).

In this study, we explore the changing levels of MHC variability over time in the wolf population of Scandinavia, a population influenced by both its small size and population history. The extant wolf population of Sweden and Norway provides a rare example of a natural recolonization of a mammalian species and demographic (Wabakken *et al.* 2001) and genetic (Ellegren *et al.* 1996*b*; Sundqvist *et al.* 2001; Vilà *et al.* 2003) analyses give a detailed account of its formation. Through hunting and persecution, the wolf population in Scandinavia declined to become functionally extinct by the late 1960s (Wabakken *et al.* 2001). However, successful reproduction in southern Sweden occurred in 1983, with a population subsequently establishing in this region (Wabakken *et al.* 2001). Two founders in the early 1980s from the neighbouring eastern population was inferred from genetic data, with incestuous inbreeding maintaining the population at low numbers until a male immigrant, also from the eastern population, arrived in the early 1990s (Vilà *et al.* 2003). Following the arrival of this immigrant and its integration with the breeding population, there has been a subsequent expansion of the population, currently numbering 84–100 wolves in eight packs (Wabakken *et al.* 2004). No further successful immigration events have been recorded (Vilà *et al.* 2003), hence this population has been through an extreme bottleneck and continues to have an effective population size well below the level at which balancing selection is expected to act.

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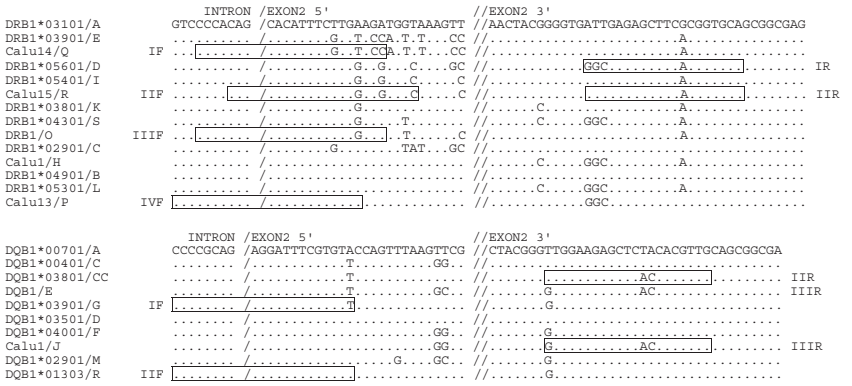

Figure 1. The non-contiguous 5′ and 3′ regions of sequences are shown. Primers are boxed and primer names given alongside. Identity with the first sequence is indicated by dots. The intron–exon boundary is indicated as a forward slash; a break in the sequence is indicated by two forward slashes.

Wolf populations in neighbouring countries maintain larger effective population sizes than the Scandinavian population and so are more likely to show the effects of balancing selection on MHC loci. Populations sizes are currently estimated at 109–114 in Finland (Wabakken *et al.* 2004), 250 in Estonia and 600 in Latvia (Kojola 2002). Estimates of 10 000 wolves west of the Urals suggest the Russian wolf population is large overall but substantially smaller counts are given regionally, for example only 750 in the Leningrad region (Kojola 2002). These wolf populations have also been affected by hunting in recent centuries, when wolves were extirpated from much of Europe, leaving the remaining populations fragmented (Mech 1981; Randi *et al.* 2000). Hence, MHC diversity in these populations will be influenced by the antagonistic action of the effects of higher effective population sizes and the historical effects of bottlenecks and fragmentation.

2. MATERIAL AND METHODS

Genomic DNA was extracted using a standard phenol–chloroform method (Sambrook *et al.* 1989) from tissue or blood samples of wolves from Scandinavia ($n = 90$), Finland ($n = 22$), Latvia ($n = 15$) and northwest Russia ($n = 51$). The Scandinavian wolf samples correspond in the main to those presented in Vilà *et al.* (2003) and also include those presented in a previous analysis of MHC sequence evolution in wolves (Seddon & Ellegren 2002). The variable second exon was amplified for *DQA*, *DQB1* and *DRB1* using primers and conditions outlined elsewhere (Seddon & Ellegren 2002). Amplified products from each population were screened using single-stranded conformation polymorphism (SSCP) (conditions described in Seddon & Ellegren 2002) and the variants sequenced, as summarized below.

Allelic sequences were confirmed by multiple sequencing from homozygotes where possible. When cloning alleles from heterozygotes, we found several examples of recombinants among cloned sequences, presumably from recombination in heterozygotes during PCR. This phenomenon has been noted by others (Langefors *et al.* 2001; Kennedy *et al.* 2002). To avoid such problems, we designed group-specific primers (figure 1) to amplify single alleles from heterozygotes. The primer sequences are given in figure 3 in Appendix A. Using the heterozygous sequence and SSCP banding pattern, two appropriate primer pairs were selected to amplify the predicted alleles. The PCR reactions contained 0.1 μM primers, 0.2 mM dNTPs, 1.5 mM (*DQB1*) or 1.25 mM (*DRB1*) $MgCl_2$ and 0.025 U μl^{-1} AmpliTaq (Applied Biosystems) *Taq* polymerase in 1× AmpliTaq buffer. The PCR programme had an intial denaturation of 95 °C for 7 min, 20 cycles of 95 °C for 30 s, 70 °C for 30 s (decreasing 0.5 °C per cycle) and 72 °C for 1 min, followed by 20 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min and a final extension of 72 °C for 10 min. All sequencing was performed in both directions with the ABI Big Dye Terminator Kit. In addition to MHC alleles previously presented in wolves (Seddon & Ellegren 2002), the following alleles are presented for the first time in wolves: *DRB1*E*, *DRB1*L*, *DRB1*O* and *DQB1*03801*. A newly identified allele (*DRB1*O*) has been deposited in GenBank (accession number AY694183).

Polymorphism calculations were performed in DnaSP (Rozas & Rozas 1999) and Mega v. 2.1 (Kumar *et al.* 2001). Estimates of Wright's *F*-statistics and the calculation of Hardy–Weinberg equilibrium were performed in Arlequin (Schneider *et al.* 2000) and Genetix (Belkhir *et al.* 1996) with significance levels estimated by permutation. Fu and Li's F^*-test of neutrality was calculated in DnaSP (Rozas & Rozas 1999). Synonymous and non-synonymous distances (d_S and d_N, respectively) were calculated using the Nei–Gojobori method with a Jukes–Cantor correction in Mega v. 2.1 (Kumar *et al.* 2001). Peptide binding regions (PBR) and non-PBR sites were identified assuming homology with predictions made for human MHC molecules (Brown *et al.* 1988, 1993).

To determine the neutral expectations for the level of heterozygosity in the recolonized Scandinavian wolf population, simulations were performed in Excel, using the Poptools v. 2.5.3 plug-ins (available at www.cse.csiro.au/Poptools). The simulations attempt to mimic the proposed changes in population structure over time in this population (Wabakken *et al.* 2001; Vilà *et al.* 2003). The alleles of two founders, which have a total of two alleles and an average heterozygosity of 0.5, were randomly allocated to give rise to five offspring. Two generations of inbreeding followed, using randomly picked individuals from the previous generation. A third founder, homozygous for the allele in low frequency in the third generation, was introduced and two packs formed. In the following two generations, two, then three packs were formed using obligatory crosses between packs. The proportion of heterozygotes among the offspring at each generation was calculated and the simulation repeated 1000 times.

3. RESULTS

(a) *History of the Scandinavian wolf population*

There are two alleles at each MHC locus in the resident Scandinavian wolf population (table 1). Furthermore, the distribution of these alleles among samples is consistent

Table 1. Distribution of alleles and genetic diversity of *DRB1*, *DQA* and *DQB1* loci by wolf population.
(*N*, the number of samples; *n*, the observed number of alleles; n_{sim}, the unbiased number of alleles calculated by resampling $2N = 30$ alleles with 1000 replicates. Observed and expected heterozygosity, F_{IS} and the statistic F^* in Fu and Li's test of neutrality are shown. Significant departures from Hardy–Weinberg or from neutrality are indicated as $^*p < 0.1$, $^{**}p < 0.05$.)

	allele	Scandinavia	Finland	Russia	Estonia	Latvia	DLA allele or GenBank
	$2n$	180	44	102	50	30	
DRB1	A	104	10	4	4	3	*03101
	B	73	6	3		2	*04901
	C	1[a]					*02901
	D		4	23		1	*05601
	E		1				U58685
	G	1[a]	9	27	26	7	*03601
	H			1		1	AY126657
	I	1[a]	8	26	14	12	*05401
	K		1	1		3	*03801
	L		2	13			*05301
	O				4	1	new
	P			3			AY126659
	Q		3				AY126660
	R			1	1		AY126661
	S				1		*04301
	n	5	9	10	6	8	
	n_{sim}	2.0	8.3	7.0	5.2	8.0	
	H_{obs}	0.62	0.91	0.78	0.72	0.87	
	H_{exp}	0.49**	0.87	0.80	0.67	0.78	
	F_{IS}	−0.274	−0.061	0.022	−0.109	−0.110	
	F^*	3.47**	1.71**	1.73**	−0.57 ns	1.45 ns	
DQA	B		4	23		1	*014012
	D	104	13	4	4	3	*01101
	E	1[a]	9	26	25	7	*01201
	F	73	7	9		5	*005011
	G	2[a]	10	39	21	13	*00301
	M			1		1	*01001
	O		1				*00201
	n	4	6	6	3	6	
	n_{sim}	2.0	5.7	5.0	3.0	6.0	
	H_{obs}	0.62	0.91	0.73	0.64	0.80	
	H_{exp}	0.49**	0.81	0.74	0.60	0.74	
	F_{IS}	−0.274	−0.135	0.014	−0.108	−0.080	
	F^*	1.45 ns	1.76**	2.20**	1.71**	1.26 ns	
DQB1	A			2			*00701
	C	2[a]	10	39	20	13	*00401
	CC				1		*03801
	D	1[a]	9	26	25	7	*03501
	E		4	23		1	*04401
	F	104	10	4	4	3	*04001
	G	73	6	6		2	*03901
	J			1		1	AY126652
	M		4				*02901
	R		1	1		3	*01303
	n	4	7	8	4	7	
	n_{sim}	2.0	6.7	5.7	3.6	7.0	
	H_{obs}	0.62	0.91	0.73	0.68	0.87	
	H_{exp}	0.49**	0.84	0.74	0.61	0.76	
	F_{IS}	−0.274	−0.087	0.019	−0.146	−0.152	
	F^*	3.53**	2.35**	2.73**	2.21**	1.09 ns	

[a] Three samples that are known to be from non-successful immigrants to the Scandinavian population introduce two to three alleles per locus, and have been removed for diversity and neutrality calculations.

with the existence of only two haplotypes, identified as *DRB1**A–*DQA**D–*DQB1**F and *DRB1**B–*DQA**F–*DQB1**G. We will refer to the haplotypes as A and B, named by their *DRB1* allele.

The Scandinavian wolf population has been sampled since the founding of the population in the early 1980s, which gives us the ability to trace genetic changes in the population over time and, more unusually, to record or infer

Table 2. Haplotype distribution and heterozygosity in the Scandinavian wolf population divided by estimated year of birth. (Haplotypes are named according to the *DRB1* allele and comprise *DRB1**A–*DQA**D–*DQB1**F and *DRB1**B–*DQA**F–*DQB1**G. **$p < 0.05$.)

		heterozygosity		haplotype and genotype frequencies				
Scandinavia	N	H_{obs}	H_{exp}	A	B	AA	AB	BB
prior to 1991	12	0.50	0.38	0.75	0.25	0.50	0.50	0
F_1 generation	4	0.50	0.38	0.25	0.75	0	0.50	0.50
after 1991	71	0.65	0.49**	0.58	0.42	0.25	0.65	0.10

the genetic composition of the founders. The founding female has been sampled and is heterozygous for the two haplotypes, A and B. The allelic composition of the founding male is unknown, but the presence of the same two haplotypes and AA homozygotes among their offspring implies that he carried at least one copy of the A haplotype also. In the following generations, in which inbreeding is thought to have occurred, the B haplotype falls to a low frequency and no BB homozygotes are seen (table 2). The distribution of alleles in the neighbouring wolf populations does not identify the origin of the two founders but the *DRB1***A* and *DRB1***B* alleles are found at frequencies of 0.22 and 0.14, respectively, in the Finnish population (see below).

An increase in heterozygosity and the presence of new alleles at microsatellite loci in the early 1990s were attributed to the arrival of an immigrant wolf, first breeding in 1991 (Vilà *et al.* 2003). This male immigrant was also identified by the sudden appearance of a new Y chromosome haplotype (Sundqvist *et al.* 2001; Vilà *et al.* 2003). For the MHC loci, the allele frequencies for each time period (table 2) show that the immigrant did not contribute any new MHC alleles, but reintroduced the haplotype (B) that had drifted to low levels in the initial generations.

There is an overall significant excess of heterozygotes at the MHC loci (table 1) and, dividing the samples by estimated year of birth, the departure from Hardy–Weinberg equilibrium is limited to those samples that post-date the successful immigration event in the early 1990s (table 2). Graphing the proportion of heterozygotes over time, this can be further attributed to the high values observed between 1995 and 1999 (figure 2). This differs somewhat from the increase in microsatellite heterozygosity, which was observed in 1991, immediately following the arrival of the immigrant (Vilà *et al.* 2003). An explanation for this difference in timing is indicated by the distribution of MHC alleles over time (table 2). Two of the four presumed offspring of the immigrant are BB homozygotes, while among the wolves born from 1988 to 1990, that is, prior to the arrival of the immigrant, four out of five wolves are AA homozygotes. Hence, the arrival of the immigrant led to an increase in BB homozygotes, but it is only with the crossing of the immigrant-derived and the original founder-derived packs that MHC heterozygosity will increase substantially. This is likely to have occurred in 1995 or 1996 when 8 out of 10 wolves are AB heterozygotes. The peak in heterozygosity seems to have passed and MHC heterozygosity values are seen to fall in 2000 and 2001.

Although the overall trend in changes in heterozygosity is similar in MHC and microsatellite loci, there is no apparent correlation among Scandinavian wolf samples between heterozygosity at MHC loci and genome-wide heterozygosity indicated by 19 microsatellite loci. The mean individual heterozygosity based on microsatellites is 0.603 ± 0.129 among the 56 MHC heterozygotes and 0.577 ± 0.130 among the 33 MHC homozygotes ($t = -0.916$, $p = 0.3624$). Note that a correlation analysis of individual heterozygosities is not possible because of the presence of only two MHC haplotypes.

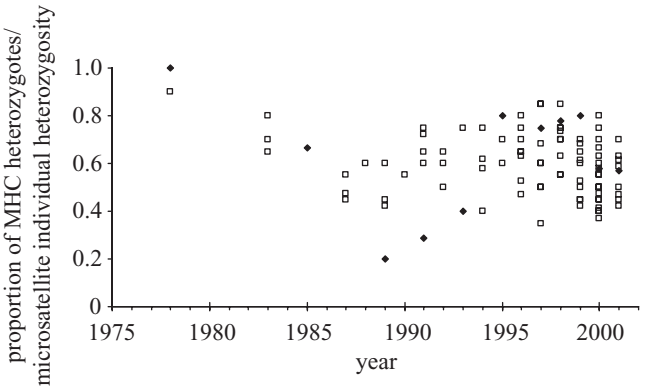

Figure 2. Changes in heterozygosity over time. Proportion of MHC heterozygotes (diamonds) are shown for estimated year of birth. The data point for 1978 represents the founder female, which was heterozygous, and was included for comparative purposes. To avoid low sample sizes, values are pooled into the following bins: 1983–1987 ($n = 6$), 1988–1990 ($n = 5$), 1991–1992 ($n = 7$), 1993–1994 ($n = 5$) and 1995–1996 ($n = 10$) and given as single-year bins from 1997 to 2001 ($n = 7$–19). Individual heterozygosities based on 19 microsatellite loci (squares) are taken from Vilà *et al.* (2003). Note that MHC values are population averages and microsatellite heterozygosities are individual values.

A further three *DRB1* alleles, two *DQA* alleles and two *DQB1* alleles, each identified only once or twice, are observed among wolves sampled in Scandinavia (table 1). However, these occur in two wolves found in northern Sweden in 1977 and 2002 that autosomal and Y chromosome microsatellites and mitochondrial DNA indicate are immigrants (Sundqvist *et al.* 2001; Flagstad *et al.* 2003; H. Ellegren, unpublished data). Finding alleles that are unusual in the Scandinavian population only among these immigrant wolves confirms that they have not made a genetic contribution to the extant population of wolves and also provides a means for the future identification of immigrants.

Table 3. Pairwise population F_{ST} estimates based on haplotype frequencies of *DRB1*.
(Lower left, F_{ST}; upper right, probabilities based on 1000 permutations. Significance values where $p < 0.05$ after sequential Bonferroni correction are shown in bold. Note that unsuccessful immigrants to the Scandinavian population have not been removed. n.s., not significant)

	Scandinavia	Finland	Russia	Estonia	Latvia
1. Scandinavia	—	0.000	0.000	0.000	0.000
2. Finland	0.200	—	0.011	0.000	0.090 n.s.
3. Russia	0.347	0.036	—	0.000	0.020 n.s.
4. Estonia	0.424	0.086	0.074	—	0.026 n.s.
5. Latvia	0.347	0.025	0.034	0.054	—

(b) *Major histocompatibility complex variability in neighbouring wolf populations*

A total of 15 *DRB1*, 7 *DQA* and 10 *DQB1* alleles were found in 203 samples from five north European wolf populations (table 1). Out of these, only one allele, *DRB1*O*, has not been previously published for wolves or dogs. This allele differs from other alleles identified in wolves or dogs by one non-synonymous substitution from *DRB1*03601*. As expected, the variable beta genes, *DQB1* and *DRB1* have much greater sequence diversity than *DQA* at both the nucleotide level (mean *p*-distance *DRB1* 0.073, *DQA* 0.018, *DQB1* 0.064) and amino acid level (mean *p*-distance *DRB1* 0.148, *DQA* 0.043, *DQB1* 0.121).

The distribution of alleles among populations and the genetic diversity at *DRB1*, *DQA* and *DQB1* in each population is summarized in table 1. Both the Scandinavian and, to a lesser degree, the Estonian wolf populations show low levels of diversity, in terms of the corrected number of alleles and expected heterozygosity. Interestingly, Finland, which has a relatively low population size, shows the greatest amount of variation, with consistently the highest heterozygosity and number of alleles in all loci.

It is noticeable that there is substantial sharing of alleles among all populations (table 1). Out of the nine alleles with a distribution restricted to a single population, five alleles are found in the most variable locus, *DRB1*, and seven alleles have a frequency of less than 5%, suggesting greater sampling effort will show these alleles also to be more widespread. A consequence of this sharing of alleles is the lack of any systematic differences in the nucleotide and amino acid diversity among populations (data not shown) and of motifs specific for a population.

Despite the substantial sharing of alleles among populations, analysis of estimators of Wright's *F*-statistics indicates that there is significant population differentiation. Using a frequency-based estimator, the overall F_{ST} is similar in the three loci: 0.251 (*DRB1*, $p = 0.000$), 0.264 (*DQA*, $p = 0.000$) and 0.269 (*DQB1*, $p = 0.000$). Pairwise F_{ST}-values are presented for *DRB1* (table 3) but similar results are found for the other two loci. After a sequential Bonferroni correction of significance values, all populations are differentiated except Latvia to Finland, Russia and Estonia and this may reflect the lower sample size for the Latvian population. Scandinavia is strongly differentiated from all other populations, presumably owing to genetic drift. To assess the significance of the high F_{ST}-value between Scandinavia and Finland, we made 1000 random draws of three individuals (equivalent to the number of confirmed founders or immigrants) from Finland using the observed *DRB1* allele frequencies. Calculating F_{ST} between the random 'founders' and the Finnish population, none showed values greater than the observed Scandinavia–Finland F_{ST}-value. Hence, the observed differentiation is not a result of the founder event alone, but to drift since the founder event in the early 1980s.

(c) *Indications of selection*

Because MHC loci are under balancing selection, it is expected that there will be evidence of departures from neutrality in the wolf populations of northern Europe. Selection will act over long evolutionary periods of time to maintain diversity at the functionally important PBR in the MHC sequence, leaving an excess of non-synonymous over synonymous substitutions. For each population and locus (table 4), there is a trend for increased non-synonymous over synonymous substitutions at the PBR. Five of the 15 comparisons reach significance, suggesting selection has acted on these alleles, despite the low power in the statistical analysis owing to the small numbers of alleles. Furthermore, using Fu and Li's test of neutrality, all populations except Latvia showed significant deviations from neutrality in at least one locus (table 1).

At a population level, balancing selection is expected to result in high levels of heterozygosity, leading to departures from Hardy–Weinberg equilibrium. Although the observed heterozygosity exceeds neutral expectations for most populations and loci (table 1), only the Scandinavian population shows a statistically significant excess of heterozygotes. Furthermore, the F_{IS} values of the MHC loci are not more extreme than for presumably neutral microsatellite loci (figure 3). It is difficult to determine if the observed heterozygosity excess in the Scandinavian population is a result of admixture following the arrival of the immigrant in the early 1990s or to a selective effect. A simple simulation was performed in which alleles are selected at random under conditions that mimic the initial inbreeding, immigration and subsequent expected outbreeding among wolf packs. Out of the 1000 replicates, 12% show final values of the proportion of heterozygotes greater than the observed value of 0.648 (the proportion of heterozygotes found after 1991), consistent with non-selective events, suggesting that selection is not necessary to explain the observed heterozygosity values. The simulations show the expected increase in heterozygosity following the arrival of the immigrant, and 7% of the simulations show an increase equal to or greater than 40% (the observed increase from 1993 to 1995; figure 2) in the two generations following the immigrant F_1 generation, suggesting that this pattern is compatible with the neutrality of

Table 4. Synonymous and non-synonymous substitutions for *DRB1*, *DQA* and *DQB1* wolf alleles.
(Distances were calculated separately for PBR sites and non-PBR sites. Standard errors, calculated using 500 bootstrap replicates, are shown in brackets. Significant excess of non-synonymous substitutions was determined using a one-tailed *t*-test: $^{*}p < 0.1$; $^{**}p < 0.05$; n.s., not significant; n.d., not determined.)

		PBR			non-PBR		
locus		d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
DRB1	Scandinavia	0.228 (0.169)	0.000 (0.000)	n.d.*	0.045 (0.034)	0.012 (0.013)	3.60 n.s.
	Finland	0.167 (0.077)	0.095 (0.152)	1.75 n.s.	0.022 (0.015)	0.049 (0.019)	0.46 n.s.
	Russia	0.153 (0.069)	0.049 (0.088)	3.13 n.s.	0.021 (0.016)	0.056 (0.024)	0.37 n.s.
	Estonia	0.136 (0.064)	0.000 (0.000)	n.d.**	0.013 (0.014)	0.042 (0.023)	0.31 n.s.
	Latvia	0.140 (0.069)	0.000 (0.000)	n.d.**	0.019 (0.014)	0.058 (0.025)	0.32 n.s.
DQA	Scandinavia	0.020 (0.021)	0.000 (0.000)	n.d., n.s.	0.007 (0.007)	0.000 (0.000)	n.d. n.s.
	Finland	0.020 (0.012)	0.000 (0.000)	n.d**	0.019 (0.010)	0.015 (0.010)	1.34 n.s.
	Russia	0.024 (0.014)	0.000 (0.000)	n.d.**	0.022 (0.012)	0.014 (0.011)	1.55 n.s.
	Estonia	0.027 (0.020)	0.000 (0.000)	n.d.*	0.024 (0.012)	0.014 (0.015)	1.70 n.s.
	Latvia	0.024 (0.014)	0.000 (0.000)	n.d.**	0.022 (0.011)	0.014 (0.010)	1.55 n.s.
DQB1	Scandinavia	0.187 (0.082)	0.119 (0.094)	1.57 n.s.	0.055 (0.028)	0.003 (0.004)	15.94**
	Finland	0.185 (0.052)	0.070 (0.054)	2.64*	0.049 (0.020)	0.013 (0.008)	3.69**
	Russia	0.167 (0.051)	0.083 (0.052)	2.02 n.s.	0.044 (0.018)	0.007 (0.005)	6.78**
	Estonia	0.169 (0.055)	0.080 (0.063)	2.12 n.s.	0.047 (0.021)	0.007 (0.005)	6.54**
	Latvia	0.170 (0.055)	0.069 (0.052)	2.46*	0.046 (0.020)	0.006 (0.005)	7.44**

alleles within this mating pattern. Hence, there is little evidence for selection acting at a population level in these wolf populations.

It has been postulated in studies of the Arabian oryx (Hedrick *et al.* 2000*b*) and red wolves (Hedrick *et al.* 2002) that the MHC alleles passing through a bottleneck are more different than random alleles, suggesting that selection has favoured the maintenance of functionally divergent alleles. The two alleles present among resident Scandinavian wolves differ by eight (*DRB1*), two (*DQA*) and 11 (*DQB1*) amino acid changes. To assess if these differences are greater than expected by chance, two alleles were randomly drawn from the Finnish population 1000 times. Greater values of amino acid differences were found in 32.2% (*DRB1*), 58.9% (*DQA*) and 48.6% (*DQB1*) of allele pairs, showing the alleles maintained in the Scandinavian wolf population are not influenced by balancing selection.

4. DISCUSSION

Both the level and patterns of diversity at MHC loci observed in natural populations are influenced by an interaction of balancing selection and the demographic history of the population. Balancing selection has been shown to act on most MHC loci in vertebrates (Bernatchez & Landry 2003) and there is an expectation that balancing selection will be a predominant force in the larger wolf populations.

Sequence-based changes, particularly amino acid diversity at functionally important sites, indicate the strength of selection over evolutionary time-scales. In this study, the d_N/d_S ratio for PBR sites across all *DRB1* alleles is 2.57, a value that is similar to that in Mexican wolves (2.46, Hedrick *et al.* 2000*a*) but lower than in red wolves (3.81, Hedrick *et al.* 2002). Although the trend of the excess of non-synonymous over synonymous substitutions at PBR sites and the high nucleotide diversity among alleles suggests the action of balancing selection, significant departures from neutrality in Fu and Li's test provide a clear indication that balancing selection has maintained mutational diversity in these wolf alleles over long periods of time (Hughes & Yeager 1998; Garrigan & Hedrick 2003).

However, the evidence for selection is weak at the population level, where the expected effects of balancing selection include the maintenance of many alleles and high levels of heterozygosity. The number of alleles in wolves in this study is less than that of the coyote, *Canis latrans* (15 *DRB1* alleles found among 29 samples; Hedrick *et al.* 2002), a closely related species that also shows a high level of genetic diversity in mitochondrial DNA (Vilà *et al.* 1999). Although in historical times wolves have had large population sizes across Europe (Mech 1981), and hence have had the ability to support high levels of MHC diversity, lower numbers of alleles suggest that all the studied wolf populations have been affected by bottlenecks and the potential interference in migration caused by fragmentation.

The number of alleles generally corresponds with the size of the population but, unexpectedly, the Finnish wolf population shows comparatively many alleles at the MHC loci, with nine *DRB1* alleles maintained in a small population of *ca.* 110 wolves (Kojola 2002). It seems probable that this observed level of diversity is a result of high levels of current or past immigration from the neighbouring Russian wolf population, which has been observed in conjunction with wolf population expansion in Russian Karelia during the 1940s, 1950s and 1970s (Pulliainen 1965, 1980). The ability of dispersal to mask the effects of bottlenecks on MHC diversity has been demonstrated in other species, such as the African buffalo (Wenink *et al.* 1998). Furthermore, the wolf population may be continuous across the Finnish–Russian border and hence the Finnish population will have a much larger effective population size than suggested by national census counts.

The influence of bottlenecks in the wolf populations is further supported by a failure of heterozygosity levels to

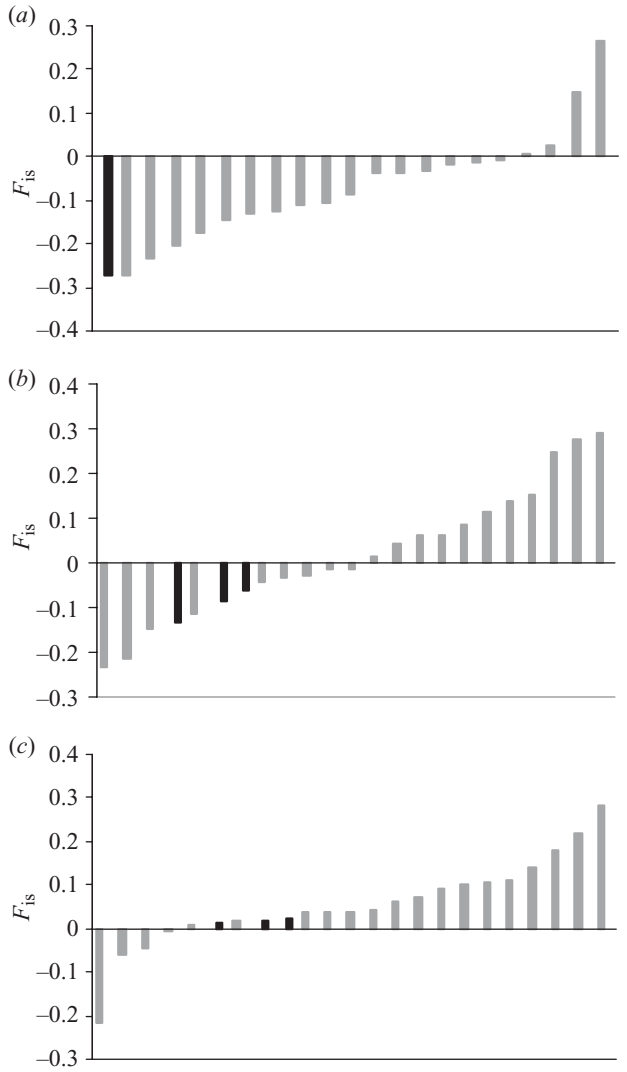

Figure 3. F_{IS} values for MHC and microsatellite loci for three wolf populations: (*a*) Scandinavia; (*b*) Finland; and (*c*) Russia. Values are shown for each MHC locus (this study (black bars)) and for microsatellite loci (Vilà *et al.* 2003 (grey bars)).

extend beyond Hardy–Weinberg expectations in all but one population. Lowered heterozygosity levels could be attributed to an increase in assortative mating following reductions in population size or population fragmentation or a Wahlund effect. The size of the wolf population in Russia makes it possible that our limited sampling has introduced a Wahlund effect, as has the designation of wolf populations by political borders (Hartl & Clark 1989). Furthermore, fragmentation of wolf populations (Mech 1981; Randi *et al.* 2000) has influenced the observed *patterns* of MHC diversity. Although the substantial sharing of alleles indicates past gene flow in this mobile carnivore, F_{ST} estimators based on allele frequencies show significant and high levels of differentiation among most of the wolf populations. This is in contrast to expectations of limited differentiation with balancing selection (Schierup *et al.* 2000) and suggests that genetic drift in these small or fragmented populations in more recent times is strong.

Unlike the other wolf populations, the Scandinavian wolf population shows heterozygosity levels that exceed neutral expectations; however, the small effective size of this population suggests that MHC loci will behave according to neutral expectations (Robertson 1962) under realistic long-term estimates of the selection coefficient. Selection intensity is thought to vary spatially and temporally and levels as high as 0.5 have been postulated to account for high MHC diversity in the San Nicolas island fox (Aguilar *et al.* 2004). However, even in the other larger wolf populations recent effects such as bottlenecks and fragmentation have overwritten patterns of selection. In this study, simulations confirm that the distribution of MHC alleles in the Scandinavian population is compatible with a random selection of alleles, that is, without heterozygote advantage or a frequency-dependent selection and, hence, such tests of neutrality should be interpreted with caution.

Although simulations based on random selection of alleles are sufficient to explain high heterozygosity levels in the Scandinavian wolf population, several other explanations should be considered. First, the simulations introduce outbreeding by enforcing matings between immigrant-derived and resident packs. This structure is a form of inbreeding avoidance and, although usual among wolf packs (Mech 1981), it violates the assumptions of Hardy–Weinberg equilibrium. A similar explanation of kin avoidance in a managed captive breeding programme of the red wolf may have led to a high heterozygosity (Hedrick *et al.* 2002). Second, MHC dependent dissassortative mating, suggested for mice (Potts *et al.* 1991) and deer (Ditchkoff *et al.* 2001) but not Soay sheep (Paterson & Pemberton 1997), can potentially occur in wolves. Third, when the number of breeders is small, a heterozygote excess can result from allelic frequency differences in male and female parents (Pudovkin *et al.* 1996). Fourth, associative overdominance, fitness differences between heterozygotes and homozygotes because of linkage with deleterious mutations, may be generated by population bottlenecks (Bierne *et al.* 2000). Fifth, heterozygote advantage has been suggested for MHC loci, although this would require a very high selection coefficient for the observed population size. There is a heterozygote excess among more recent samples in the microsatellite analysis of the Scandinavian population (Vilà *et al.* 2003) and similar F_{IS} values across MHC and microsatellite loci are noted (figure 3), which suggests that inbreeding avoidance plays a contributing role and argues against a MHC-specific heterozygote advantage or dissortative mating. However, if associative overdominance or inbreeding avoidance has led to an increase in MHC heterozygosity, it is likely that there would be a correlation between MHC heterozygosity and genome-wide heterozygosity (Thelen & Allendorf 2001), although the variances in effect across markers can be large (Pàlsson & Pamilo 1999).

In contrast to larger populations that have become fixed for MHC alleles (Seddon & Baverstock 1999), the maintenance of two alleles in the Scandinavian population despite its extreme bottleneck is probably because (i) the inbreeding in the population occurred for only a few generations, and (ii) the immigrant in the 1990s reintroduced the allele that had drifted to low frequency. Furthermore, the reintroduction of this allele may be responsible for the difference in timing of the observed rise in heterozygosity between microsatellite loci (proposed first generation after immigrant arrival) and MHC loci (proposed second generation). Nonetheless, the Scandinavian wolf population shows an overall low MHC diversity and the response of MHC evolution to

pathogen-driven selection (Paterson *et al.* 1998; Wegner *et al.* 2003) suggests that MHC variability is potentially important for the long-term survival of populations. For example, one of the two *DRB1* alleles common in the Scandinavian wolf population (allele A) contains a motif that has been linked to polyarthritis in dogs (Ollier *et al.* 2001). Increasing the population size to reduce the effects of genetic drift together with immigration to bring new alleles, as in the Finnish wolf population, will maintain both MHC and genome-wide genetic diversity and are important strategies in the conservation of populations, including the wolf population of Scandinavia.

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