MHC Variability in an Isolated Wolf Population in Italy

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Abstract

Small, isolated populations may experience increased extinction risk due to reduced genetic variability at important functional genes, thus decreasing the population’s adaptive potential. The major histocompatibility complex (MHC), a key immunological gene cluster, usually shows high variability maintained by positive or balancing selection in response to challenges by pathogens. Here we investigated for the first time, the variability of 3 MHC class II genes (DRB1, DQA1, and DQB1) in 94 samples collected from Italian wolves. The Italian wolf population has been long isolated south of the Alps and is presently recovering from a recent bottleneck that decreased the population to less than 100 individuals. Despite the bottleneck, Italian wolves show remarkable MHC variability with 6–9 alleles per locus, including 2 recently described alleles at DRB1. MHC sequences show signatures of historical selective pressures (high $\delta_{c}/\delta_{a}$ ratio, $\omega > 1.74$) but no evidence of ongoing selection. Variation at the MHC genes and 12 background microsatellite loci were not apparently affected by the recent bottleneck. Although MHC alleles of domestic dog origin were detected in 8 genetically admixed individuals, these alleles were rare or absent in nonadmixed wolves. Thus, despite known hybridization events between domestic dogs and Italian wolves, the Italian wolf population does not appear affected by deep introgression of domestic dog MHC alleles.

Key words: bottleneck, Canis lupus, hybridization, major histocompatibility complex, MHC phylogenetics, natural selection

During the past few centuries, wolves (Canis lupus) in Italy were threatened by direct human persecution and decline of their natural prey (Breitenmoser 1998). By the late 1960s, less than 100 individuals survived in remote areas of central and southern Apennine (Zimen and Boitani 1975). Reduced wolf genetic diversity at the mitochondrial (mtDNA) and nuclear DNA levels has been attributed to the recent population bottleneck or to historical isolation south of the Alps (Lucchini et al. 2004). Recent studies have found that heterozygosity at autosomal microsatellites (short tandem repeats or STRs) and single-nucleotide polymorphism (SNP) markers in Italian wolves was approximately 25% lower than that in other wolf populations (Fabbri et al. 2007, Sastre et al. 2011) and a unique mtDNA control region (CR) haplotype has also been detected (W14; Randi et al. 2000). Italian wolves are thus genetically distinct from all other C. lupus populations worldwide (vonHoldt et al. 2011; their Figure 3). However, erosion of genetic diversity may be offset by hybridization with free-ranging domestic dogs (Canis lupus familiaris; Boitani 1984, Randi 2008). Admixture analyses of neutral molecular markers (autosomal STR and mtDNA CR) identified approximately 4–7% hybrid genotypes among wolves in Italy (Randi 2008), some of which also showed anomalous phenotypic traits characteristic of domestic dogs (e.g., vestigial first toes on the hind legs, white nails, black coats; Ciucci et al. 2003; Caniglia et al. 2013a). Similarly, a melanistic $\beta$-defensin deletion causing black coats could have been introduced in North American and Italian wolf populations via hybridization with dogs (Anderson et al. 2009; Caniglia et al. 2013a). In contrast, black individuals were rarely observed elsewhere in Europe (Godinbo et al. 2011).

Previous studies have examined the genetic structure of wolves in Italy by genotyping putatively neutral molecular markers (Randi et al. 2000; Fabbri et al. 2007; Iacolina et al. 2010; Scandura et al. 2011). However, it is well known that the dynamics of functional genes may be very different and that patterns of genetic diversity at neutral or quantitative trait loci in small populations vary according to the interplay between...
Some small isolated populations host more genetic diversity than expected from neutral models because of avoidance of active inbreeding (Vila et al. 2003; Geffen et al. 2011) or due to positive natural selection pressures on functional genes (Bernatchez and Landry 2003, Spurgin and Richardson 2010). In an exemplary case, the San Nicolas Island fox (Urocyon littoralis dickeyi) has been identified as an extreme case of genetic monomorphism, measured using neutral markers (Goldstein et al. 1999), but it shows a high degree of variation at the DRB1 and DQB1 loci, suggesting that strong balancing selection can maintain variability at functional regions despite strong bottleneck events (Aguilar et al. 2004). Alternatively, for populations with small effective size, genetic drift can overwhelm selective pressures, leading to decreased genetic diversity in functional regions (Bollner et al. 2011). For highly fragmented African wild dog populations (Lycaon pictus), past declines have led to extremely low variability at 2 major histocompatibility complex (MHC) loci (Marsden et al. 2009), thus exposing this species to increased risk of extinction.

The MHC multigene cluster controls a variety of immune response functions (Klein 1986, Ploegh and Watts 1998). MHC genes are among the most variable in vertebrate genomes, often showing exceptionally high heterozygosity compared with neutral markers. Multiple selection models have been proposed to explain the observed polymorphisms and evolutionary dynamics of the MHC in vertebrates (Bernatchez and Landry 2003; van Oosterhout 2009). However, identifying the processes of pathogen-mediated selection that have shaped the MHC structure in populations is never trivial (Spurgin and Richardson 2010).

Canid MHC studies have primarily focused on domestic dogs. The dog leukocyte antigens cluster (DLA; Angles et al. 2005, Yuhki et al. 2007) includes more than 100 genes, grouped into 3 major subfamilies (classes I, II, and III) according to their structure and function (Wagner et al. 1999; Yuhki et al. 2007). DLA class II genes DQA1, DQB1, and DRB1 were found to be highly polymorphic in multiple canid species (i.e., Wagner et al. 1996; Francisco et al. 1997; Kennedy et al. 1998, 1999a, 1999b, 2005; Angles et al. 2005; Runstadler et al. 2006; Fliegner et al. 2008). MHC variability is sometimes limited in inbred dog breeds (Angles et al. 2005), whereas village dogs maintain high variability (Runstadler et al. 2006). Numerous diseases in dogs and in the endangered Ethiopian wolf (C. simensis, Kennedy et al. 2011) were associated with specific MHC alleles or with lack of heterozygosity (Quinnell et al. 2003; Kennedy et al. 2006; It et al. 2010; Barber et al. 2011; Jokinen et al. 2011). Correlations between MHC heterozygosity and resistance to pathogens are also described in the highly endangered Mexican wolf (C. lupus baileyi; Hedrick et al. 2003). Wild-living gray wolves often display high MHC variation (Kennedy et al. 2007; Arbanasic et al. 2013), with some alleles shared with dogs and coyotes (C. latrans). Signatures of balancing selection are observed in wild populations of red (C. rufus, Hedrick et al. 2002) and gray wolves (C. lupus, Berggren and Seddon 2005, 2008). These findings indicate that even limited variation at the MHC loci can be essential for the survival of species threatened by small population numbers. Nonetheless, bottlenecks, fragmentation, and genetic drift can mask evidence of selection (Seddon and Ellegren 2004).

In this study, we explored the influences of historical bottlenecks and gene introgression on the genetic variability of the Italian wolf population, comparing patterns of polymorphism at the MHC with background STR variation. We predicted several outcomes for this study. In a case of shared demographic history and absence of selection, putatively neutral STR loci and functional MHC class II genes should display equivalent levels of genetic diversity. Alternatively, positive natural selection may have acted to maintain genetic variation at functional MHC loci. Finally, genetic diversity could be introduced into the Italian wolf population via hybridization and introgression with domestic dogs, as may have occurred in the case of the causal mutation of the black coat color (Anderson et al. 2009) in North American wolves. Therefore, we sought for dog-derived MHC alleles in a sample of wild-living Italian wolves composed of apparently purebred wolves, wolf × dog admixed individuals, and putative wolves showing the melanistic β-defensin deletion or anomalous phenotypic traits (e.g., vestigial first toes on the hind legs, black coats) but without any detectable signal of admixture at the autosomal STR loci.

**Methods**

**Samples**

In this study, we used 94 DNA samples obtained from wild-living wolves or putative wolf × dog hybrids of both genders sampled in Italy. Samples were collected from 3 categories of individuals that included the following: 1) genetically and phenotypically pure wolves (n = 65); 2) wolf × dog admixed individuals (n = 16), as detected by admixture analyses of their multilocus STR genotypes (Verardi et al. 2006; Caniglia et al. 2013a); and 3) wolves (or hybrids) showing the melanistic β-defensin deletion or anomalous phenotypic traits (dewclaws, white nails, or black coats; n = 13) but that did not show any detectable signal of admixture at their multilocus genotypes (Randi and Lucchini 2002; Ciucci et al. 2003; Caniglia et al. 2013a). Muscle tissue was collected from opportunistically found mortalities, primarily roadkill or poached animals (Caniglia et al. 2013a). Tissue was stored at −20 °C in 10 volumes of 95% ethanol. Additionally, blood was obtained from wounded or live-trapped individuals (Ciucci et al. 2009, Galaverni et al. 2012). DNA was extracted using the Qiagen DNeasy Blood and Tissue Kits (QIAGEN). Phenotypic information was recorded, including the presence of morphological abnormalities (dewclaw, black or darker-than-usual coat color, and white nails). Samples were from 4 geographic regions encompassing the wolf distribution across Italy, including the Alps (A) and northern (nAp), central (cAp) and southern Apennine (sAp).

**MtdNA Sequencing and Microsatellite Genotyping**

DNA was amplified and sequenced at the 350-bp region of the mtDNA CR, which contains diagnostic mutations for the identification of the Italian wolf haplotype W14 (Randi et al. 2000).
Samples were genotyped at 12 canine autosome STRs that were selected for their high polymorphism in the Italian wolf population (FH2004, FH2079, FH2088, FH2096, FH2137, CPH2, CPH4, CPH5, CPH8, CPH12, C09.250, and C09.253). These loci yield unique individual genotypes with a probability of identity (PID) equaling 3.2 × 10⁻¹⁰, and an expected PID among full-sib dyads, PID_sibs = 1.1 × 10⁻⁴ in the Italian wolf population (Caniglia et al. 2013a). When unknown, the gender was determined by restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)–amplified fragments (PCR–RFLP) of diagnostic ZFX/ZFY sequences. Paternal haplotypes in males were identified by genotyping 4 Y-linked microsatellites: MS34A, MS34B, MS41A, and MS41B (Iacolina et al. 2010). The samples were assayed for a β2-defensin melanistic mutation (a 3-bp deletion at the CBD103 gene, also named the Saksen gene) that induces a black coat color in wolves and dogs (Candille et al. 2007). Negative and positive controls were used in each PCR. Locus-specific PCR conditions and additional details on the analyzed loci can be found in Caniglia et al. (2013a). PCR products were analyzed in an automated sequencer ABI 3130XL (Foster City, CA), using the software SEQUENCE version 2.5 for sequences and GENEMAPPER version 4.0 for microsatellites.

Assignment Tests

The software STRUCTURE version 2.2 (Falush et al. 2003) was used to assign the 94 samples to reference wolves (n = 154) or village dogs (n = 116) selected from a large database of Italian wolf and dog genotypes (the Canis database at the Instituto Superiore per la Protezione e la Ricerca Ambientale; Caniglia et al. 2013b). Reference wolves did not show any detectable phenotypic or genetic signal of hybridization. Reference village dog samples were collected from rural areas in Italy (Caniglia et al. 2013a). We ran STRUCTURE with a burn-in period of 10⁸ iterations, followed by 5 repetitions of 10⁸ iterations, independent of any prior nongenetic information, selecting the “admixture” (each individual may have ancestry in more than 1 parental population) and the “F” models (independent allele frequencies) with the population flag option activated. The optimal number of populations was set at K = 2, the value that maximized the posterior probability of the data (according to Randi and Lucchini 2002, Verardi et al. 2006). We then assessed the average proportion of membership (q) of the sampled populations to the inferred clusters. The threshold for the individual assignment was set at q = 0.95, as determined from the minimum values observed in the reference wolves. Wide credibility interval (CI) of the q values could indicate admixture (in absence of missing data; Falush et al. 2003); thus, we also set a threshold for the lower limit of 90% CI as 0.79. Individuals that showed a proportion of membership higher than the threshold were entirely assigned to the wolf cluster as pure wild-type wolves (W); individuals showing values of q or CI less than 0.95 and 0.79, respectively, were considered admixed (H). Independent of microsatellite-based assignments, samples that showed mtDNA haplotypes different from W14 (Randi et al. 2000) or Y chromosome haplotypes different from those described in the Italian wolf population (Iacolina et al. 2010, Caniglia et al. 2013a) were also considered as hybrid origin and assigned to group H. Individuals genetically assigned to the wolf cluster but showing anomalous phenotypic features were assigned to a third group (Ph).

DLA Genotyping

We amplified the second exon of the MHC class II genes DRB1, DQA1, and DQB1 in the 94 Italian wolf or admixed samples, using primers DRB1F (5'- ccc ttc cca cag cac att t - 3') and DRB1R (5'- ttg gtc cca cac ctc aga a - 3'); Hedrick et al. 2002, after Kennedy et al. 1998); DQA1F1 (5'- taa ggt tct ttt ctc cct ct - 3') and DQA1F2 (5'- gga cag att cag tga aga ga - 3'); DQB1F (5'- ctc act gtc ggc gca ttc - 3') and DQB1R2 (5'- ctc ctc gcc gca aag tgc gt - 3'; Kennedy et al. 2006, after Wagner et al. 1996). Each of these primers is intronic and locus specific. Amplifications were carried out in a 10-μl mix, including 2 μl genomic DNA solution, 1 μl bovine serum albumin (2%), and 0.2 μl of each primer (10 μM) plus 0.25 units Taq, at conditions specific for each primer pair. PCR products were purified with Exo/SAP and sequenced in both directions using BigDye Terminator 1.1, according to the manufacturer’s protocol. Sequences were analyzed in an automated sequencer ABI 3130XL with the software SEQUENCE version 2.5, using the sequences DLA-DRB1*03101 (AF336108.1), DLA-DQA1*014012 (AJ316220.1) and DLA-DQB1*05601 (FM246843.1) as references.

Genetic Variability and Phylogenetics

MHC genotypes were phased in DNAsP version 5.10 (Librado and Rozas 2009) using PHASE (Stephens and Donnelly 2003), with the “recombination” model (-RM0) and 1000 iterations after 100 burn-ins. Unlike similar software, PHASE is able to cope with triallelic sites that are commonly found in MHC sequences. When the probability of reconstruction of the alleles was lower than 0.9 and with multiple combinations of alleles being possible, the sample was discarded. The alleles were then matched via BLASTn at the National Center for Biotechnology Information (Johnson et al. 2008) with those available in GenBank for all the species of the genus Canis, which were downloaded and aligned in GENEIOUS version 5 (Drummond et al. 2011). We also included the sequences available in the Immuno Polymorphism-MHC Database (IPD; http://www.ebi.ac.uk/ipd/mhc/dla/index.html; Robinson et al. 2010). Sequences that matched along the analyzed regions but showed different accession numbers were grouped and a single name was used, respecting the rules defined in the official International Society for Animal Genetics (ISAG) reports (Kennedy et al. 1999a, 1999b, 2001; Robinson et al. 2003; Ellis et al. 2006). Alleles were accepted if they matched previously described ones; otherwise, if they were observed in homozygosis and in at least 2 different samples, they were considered as potential new alleles and were submitted to the DLA Nomenclature Committee, then to GenBank. Multilocus haplotypes were also reconstructed, following the subtractive method described...
in Kennedy et al. (2007). Haplotype reconstruction was then confirmed computationally in Phase (Berggren and Seddon 2008) by concatenating the gene sequences prior to the phasing step and applying the recombination model with 2 hotspots (MR2) corresponding to the boundaries between adjacent genes (DRB1/DQA1/DQB1).

MEGA version 5 (Tamura et al. 2011) was used to reconstruct the phylogenetic relationships of all the sequences available for each gene, using a Neighbor-Joining method, with 5000 bootstrap replicates based on the Kimura 2-parameter substitution model (Berggren and Seddon 2008). As outgroups for each gene, we included 2 corresponding MHC sequences of macaque (1 from Macaca fascicularis and 1 from M. mulatta). When present, gaps were excluded from pairwise comparisons. Similarly, we reconstructed single-locus haplotype networks in NETWORK version 4.6.1, using the median-joining method with values of ε = 10.

For both microsatellites and MHC genes, the number of alleles, allele frequencies (AF) by population and by locus, and the observed (H_o) and expected (H_e) heterozygosities were assessed in GENALEX version 6.4 (Peakall and Smouse 2005). F-statistics and departures from Hardy–Weinberg equilibrium (HWE) were evaluated after 1000 permutations in GENALEX and verified in ARLEQUIN version 3.5 (Excoffier et al. 2005).

In order to identify the specific sites that were responsible for the largest effects on the latter metrics, we also tested the comparison was tested by a Likelihood Ratio Test (LRT) corresponding to the boundaries between adjacent genes (DRB1/DQA1/DQB1). As outgroups for each gene, we included 2 corresponding MHC sequences of macaque (1 from Macaca fascicularis and 1 from M. mulatta). When present, gaps were excluded from pairwise comparisons. Similarly, we reconstructed single-locus haplotype networks in NETWORK version 4.6.1, using the median-joining method with values of ε = 10.

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In order to identify the specific sites that were responsible for the largest effects on the latter metrics, we also tested the departure from HWE at every SNP, considering each variable site as a single marker. Linkage disequilibrium (LD) between markers was assessed in GENEPop version 4.2 (Rousset 2008), Web version (http://genepop.curtin.edu.au), after 1000 iterations. DNASP version 5 was used to compute the number of segregating sites, haplotype (H_t), and nucleotide diversity (π) for each MHC gene. Rather than F_ST, we used Jost’s D (Jost 2008), calculated in SMOOG (Crawford 2010), to overcome the differences in marker types and variability when comparing the levels of differentiation between pure and admixed individuals at MHC and background microsatellites.

Selection and Neutrality Analyses
The average pairwise ratio (d_S/d_N) was calculated in DNASP. In MEGA, we calculated a codon-based test of neutrality with the Nei–Gojobori counting method, in which the significance of the difference between d_S and d_N was assessed for each gene through a 1-tailed t-test after 500 bootstrap replicates. However, different selective pressures probably act on specific portions of a gene. Therefore, we evaluated the d_S/d_N ratio on a single-codon basis, as implemented by the software CODEML in PAML (Yang 2007). CODEML was run under the M2a, M3, and M8 models, to identify the codons showing d_S/d_N (ω) values significantly higher than 1—suggesting positive selection—and also for comparison under the models M1a, M0, and M7, respectively. Significance of the model comparison was tested by a Likelihood Ratio Test (Anisimova et al. 2003); single codons were considered to be under positive selection when this probability was higher than 0.95 under all models (M2a, M3, and M8) by both the Bayes Empirical Bayes and the Naïve Empirical Bayes tests (for models M2a and M8).

Average observed heterozygosity at both STR loci and MHC genes was compared with that expected by an Ewens–Watterson statistics of heterozygosity, implemented in BOTTLETNECK version 1.2.02 (Cornuet and Luikart 1996), under the following assumptions: 1) an infinite allele mutation model (IAM); and 2) a 2-phase mutation model (TPM) with 90% single-step mutations. In populations where a recent bottleneck occurred, as for the Italian wolf population (Lucchini et al. 2004; Fabbri et al. 2007), allele number (k) decreases faster than gene diversity (H_e or Hardy–Weinberg heterozygosity) at polymorphic loci. This discrepancy leads to an observed gene diversity that is higher than the expected equilibrium gene diversity (H_eo), which can be computed from the observed number of alleles under the assumption of a constant-sized population (Cornuet and Luikart 1996). Thus, the test calculates the difference (DH) between the observed and expected heterozygosity values and divides it by the standard deviation (SD) of gene diversity, retrieving the corresponding P values after simulating 1000 iterations per locus. The significance of the results was evaluated by a Wilcoxon test and a mode-shift test was also computed.

Results
Genotyping and Sequencing Success
We obtained complete and reliable genotypes at the 12 autosomal STR in all 94 samples. However, 37 samples provided low-quality sequences at 1 or more MHC loci due to poor DNA isolation and/or storage conditions and so were discarded. We therefore obtained reliable sequences at the 3 MHC loci in 74 out of 94 samples (79%), which were retained for subsequent analyses.

Sample Assignment to the Italian Wolf Population
Results from STRUCTURE analyses led to the assignment of 48 (65%) of these samples to the reference wolf cluster, with q1 greater than 0.95 and the lower limit of the 90% CI greater than 0.79. These samples also showed the Italian wolf mtDNA W14 haplotype and the most frequent Y chromosome haplotypes in the Italian wolf population (haplotypes H1 and H2, Jaconina et al. 2010). Although these samples were genetically identified as Italian wolves, they showed either the typical Italian wolf Wt phenotype (σ = 38) or unusual phenotypic traits, such as black coat, white nails, or dewclaw (σ = 10, named “Ph”). The other 26 samples (35%) showed both q1 values less than 0.95 and lower limit of 90% CI less than 0.79 and thus they were identified as admixed (and labeled “H,” independent of their phenotypes).

MHC Genetic Variability
The 3 MHC loci were polymorphic with 9 (DRB1), 6 (DQA1), and 8 (DQB1) alleles across the 74 genotyped samples (Table 1). Both H_o and H_e were highest at DQB1 and lowest at DQA1. The effective number of alleles (N_e) was largest at DRB1 (Supplementary Table S1). The number of segregating sites was 43 in DRB1, 8 in DQA1,
Table 1  Official names and frequencies (f) of alleles found at each locus in the Italian wolf population, with corresponding GenBank names and accession numbers (AN), and the canid populations where they were described to date:

<table>
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<th>Gene</th>
<th>Nomenclature</th>
<th>Taxa</th>
<th>GenBank Name</th>
<th>AN</th>
<th>Total (n = 74)</th>
<th>Wt (n = 38)</th>
<th>Ph (n = 10)</th>
<th>H (n = 26)</th>
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<td></td>
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<tr>
<td></td>
<td>DLA-DQB1*02901</td>
<td>We</td>
<td>02901</td>
<td>AY126648.1</td>
<td>6</td>
<td>0.04</td>
<td>3</td>
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</tr>
<tr>
<td></td>
<td>DLA-DQB1*00301</td>
<td>D</td>
<td>00301</td>
<td>AF043151.1</td>
<td>5</td>
<td>0.03</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DLA-DQB1*02001</td>
<td>D</td>
<td>02001</td>
<td>AF043148.1</td>
<td>3</td>
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<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>DLA-DQB1*02002</td>
<td>D,Wa,D</td>
<td>02002</td>
<td>AF043164.1</td>
<td>2</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

We = European wolf; Wa = North American wolf; Wm = Mexican wolf; Rw = red wolf; D = dog; C = coyote; Wit = Italian wolf (from present study only). n indicates the number of individuals, 2r the number of chromosomes carrying a given allele, all over the population and by group (Wt = wild type; Ph = atypical phenotype; H = admixed wolves). The H group also includes wild × dog first-generation hybrids with a known origin. Alleles that are private to a group are highlighted in bold in the corresponding column.

and 39 in DQB1. The average nucleotide diversity was \[ \pi = 0.049 \] in DRB1, 0.008 in DQA1, and 0.048 in DQB1. All alleles matched previously published MHC sequences, with the exception of 2 DRB1 alleles, which were the most frequent in the Italian wolf population. Because each allele was found in homozygosity across multiple individuals, they met the ISAG and DLA nomenclature committee criteria (Kennedy et al. 2001; Ellis et al. 2006). These sequences also matched recently proposed alleles and thus received the official names DLA-DRB1*092013 and DLA-DRB1*12801 (Kennedy, I., personal communication) and were submitted to GenBank (accession numbers JX206798, JX206799). DLA-DRB1*092013 differed at a single nucleotide site (a C/A mutation at nucleotide 60 in our alignment) from the already known allele DRB1*092011 found in North American wolves (Kennedy et al. 2007). DLA-DRB1*12801 also showed a single nucleotide difference in relation to Calu-DRB1*13, already described in European wolves (Seddon and Ellegren 2002), with a G/A mutation at site 255 in our alignment. DRB1*092011 was found in 40 wolves from the southern and central Apennine, whereas Calu-DRB1*13 was never found in the Italian wolf samples.

Values of observed heterozygosity were lower than expected at all loci across groups. The average fixation index was not significantly different from 0 (\[ F_{IS} = 0.033, P = 0.19 \]; exact test in Arlequin). The genotype frequencies were significantly different from that based on HWE at locus DRB1 (\[ P = 0.03 \], Exact Test in Arlequin; \[ P = 0.004 \], chi-square test in GenAlEx). The nucleotides mostly responsible for HWE departures were in positions 60, 96, 156, and 158 (\[ P < 0.05 \], chi-square test in GenAlEx), which also showed significant LD (\[ P < 0.05 \] for nucleotide 60 vs. 96, \( P < 0.01 \) for all other combinations). Most of them (60, 156, and 158) also had significantly high \( F_{Is} \) values (\[ F_{Is} > 0.2, P < 0.05 \]), similar to 2 additional DRB1 nucleotides (12 and 65). A single nucleotide was out of HWE at DQB1 (Site 155, \( P = 0.03 \)). Allele frequencies at the MHC were variable among the 3 groups (Table 1) and significantly different between H and Wt (\( F_{ST} = 0.046, P = 0.003 \)), as well as between H and Ph (\( F_{ST} = 0.088, P = 0.001 \)). Moreover, 5 low-frequency alleles were detected only in admixed individuals (Group H). Within groups, departures from HWE were significant only in Wt, mainly at Site 60 of the DRB1 sequence (\( P < 0.01 \)), which is the private mutation in the DRB1*092013 allele.
Reconstruction of the multilocus MHC haplotypes revealed the presence of 13 combinations of alleles, confirmed both by the subtractive approach and by PHASE (Table 2). The frequency of the 3 most common haplotypes (Nos. 1, 2, and 3 in Table 2) accounted for approximately 80% of the total. Further, 3 (private) haplotypes were present at low frequency only in the H group and 2 in the Wt group (Table 2). Haplotype frequencies were variable among the 3 groups and significantly different between H and Wt (\( F_{ST} = 0.036, P = 0.008 \)), as well as between H and Ph (\( F_{ST} = 0.076, P = 0.012 \)). The number of haplotypes was higher in the Apennine \( (n = 10 \) in nAp, \( n = 10 \) in cAp, and \( n = 7 \) in sAp) than in the Alps \( (n = 3 \) Figure 1, Supplementary Table S2). However, the observed frequency distribution partially reflects the uneven sample size, as confirmed by the significant correlation between \( \log_{10}(\text{haplotype number}) \) and sample size \( (R^2 = 0.97) \). Moreover, wolves sampled in the Apennine also carried haplotypes of presumed dog derivation (particularly in cAp). The 2 newly described alleles at DRB1 were, respectively, basal (DRB1*12801) and terminal (DRB1*092013) to the closest ones described in previous studies (Supplementary Figure S1a). The network reconstruction confirmed the dispersion of the wolf alleles throughout a relatively unstructured topology (data not shown).

Selection and Neutrality Tests

The average \( d_{S}/d_{K} \) values were higher than 1 at each locus (Table 3). The \( d_{S} - d_{K} \) statistics computed in MEGA were significant at all loci \( (P < 0.05) \) and highest at DQB1. CODEML results indicate that the models accounting for positive selection (M2a, M3, and M8; \( \omega > 1 \)) explained the \( d_{S}/d_{K} \) values significantly better than the corresponding ones (M1a, M0, and M7) assuming neutral \( (\omega = 1) \) or negative \( (\omega = 0) \) selection (Table 4). Model M2a, which includes 3 classes of \( \omega \) values \( (0, 1, \) and estimated from the data), suggested that sites under positive selection \( (\omega > 1) \) are 20% at DRB1, 11% at DQA1, and 18% at DQB1, whereas the majority of sites \( (67\%, 89\%, \) and 64%, respectively) are under negative selection. M2a fits the data significantly better than M1a \( (\omega = 0) \) at all loci. Model M3 (discrete), which assumes 3 site classes \( (\omega \) values inferred from the data), also suggested that the majority of sites \( (64–89\%) \) are under negative selection \( (\omega \leq 0.01) \) at all loci but that the remaining sites are under positive selection \( (\omega = 1) \). M3 fits the data significantly better than M0 \( (\omega = 1) \) which only assumes a single \( \omega \) value. Model M8 \( (\omega \) assumed to follow a beta distribution) also suggests that 20%, 11%, and 18% of sites at DRB1, DQA1, and DQB1, respectively, are under diversifying selection and fits the data significantly better than model M7 \( (\omega \) values inferred from the data). Among the loci, DQB1 showed the highest number of sites \( (n = 11) \) possibly affected by positive selection, compared with 8 in the more variable DRB1 and 5 at DQA1 (Table 4). These codons included all the sites that have been observed to significantly depart from HWE at DRB1 and DBQ1, except for the synonymous variant differentiating DRB1*092013 from DRB1*092011.

### Table 2 Haplotype counts \( (2n) \) and frequencies \( (f) \) across the population and by group

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Nomenclature (DRB1 / DQA1 / DQB1)</th>
<th>Total ( (n = 74) )</th>
<th>Wt ( (n = 38) )</th>
<th>Ph ( (n = 10) )</th>
<th>H ( (n = 26) )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( 2n )</td>
<td>( f )</td>
<td>( 2n )</td>
<td>( f )</td>
</tr>
<tr>
<td>1</td>
<td>DRB1<em>12801/DQA1</em>005011/DQB1*03901</td>
<td>54</td>
<td>0.36</td>
<td>27</td>
<td>0.36</td>
</tr>
<tr>
<td>2</td>
<td>DRB1<em>092013/DQA1</em>005011/DQB1*00701</td>
<td>39</td>
<td>0.26</td>
<td>27</td>
<td>0.36</td>
</tr>
<tr>
<td>3</td>
<td>DRB1<em>03601/DQA1</em>012011/DQB1*03501</td>
<td>21</td>
<td>0.14</td>
<td>11</td>
<td>0.14</td>
</tr>
<tr>
<td>4</td>
<td>DRB1<em>02001/DQA1</em>00401/DQB1*01303</td>
<td>9</td>
<td>0.06</td>
<td>2</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>DRB1<em>03202/DQA1</em>00201/DQB1*02002</td>
<td>6</td>
<td>0.04</td>
<td>3</td>
<td>0.04</td>
</tr>
<tr>
<td>6</td>
<td>DRB1<em>03701/DQA1</em>005011/DQB1*00701</td>
<td>5</td>
<td>0.03</td>
<td>2</td>
<td>0.03</td>
</tr>
<tr>
<td>7</td>
<td>DRB1<em>01501/DQA1</em>00601/DQB1*00301</td>
<td>4</td>
<td>0.03</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>8</td>
<td>DRB1<em>00101/DQA1</em>00101/DQB1*00201</td>
<td>3</td>
<td>0.02</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>9</td>
<td>DRB1<em>092011/DQA1</em>00601/DQB1*02002</td>
<td>2</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>10</td>
<td>DRB1<em>12801/DQA1</em>005011/DQB1*00701</td>
<td>2</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>11</td>
<td>DRB1<em>01501/DQA1</em>00401/DQB1*00301</td>
<td>1</td>
<td>0.01</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>12</td>
<td>DRB1<em>02001/DQA1</em>00401/DQB1*03901</td>
<td>1</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>13</td>
<td>DRB1<em>092013/DQA1</em>005011/DQB1*03501</td>
<td>1</td>
<td>0.01</td>
<td>1</td>
<td>0.01</td>
</tr>
</tbody>
</table>

The 2 most common haplotypes in the Italian wolf population include the 2 newfound alleles at DRB1; one of them is also present in a low-frequency combination but is associated with the most common DQA1 allele. Haplotypes that are private to a group are highlighted in bold in the corresponding column. Haplotypes 3 and 6 have also been reported in North American wolves (Kennedy et al. 2007). Haplotype 8, private to the H group, has been identified as the most common in purebred dogs (Kennedy et al. 2002). Haplotypes 9–13 should be treated with caution, because they have been observed in this study less than twice and never previously reported.
The mean values of observed and expected heterozygositites and the number of alleles per locus at the MHC were higher, although not significantly ($P > 0.05$, $t$-test), than the ones averaged for the 12 STR markers across groups (Supplementary Table S3). The average fixation index was lower at the MHC than at the STR loci. Both expected and observed heterozygositites was higher at the MHC than at the STRs, and this difference was more marked in the admixed individuals ($P = 0.04$ for $H_e$; $P = 0.06$ for $H_o$, 1-tailed $t$-test) than in the Wt and Ph groups (Supplementary Table S4). The differentiation between genetically pure (Wt + Ph) and admixed wolves (H), as calculated from Jost’s $D$ statistics across loci, was higher at the MHC ($D_{est} = 0.123$) than at the STRs ($D_{est} = 0.025$). The Ewens–Watterson statistics (Supplementary Table S5) showed that the heterozygosity levels at the STRs in the population were higher than expected under the IAM (Wilcoxon test, 1 tail for $H_o$ excess $P = 0.01$) but not under the TPM (Wilcoxon test, 1 tail for $H_o$ excess $P = 0.36$), which is the most appropriate model to describe STR mutations (Luikart et al. 1998). Conversely, at the MHC, we did not find any trace of significant excess under both models (Wilcoxon test, 1 tail for $H_o$ excess, $P = 0.12$ under the IAM, and $P = 1.00$ under the TPM). When looking at the allele frequency spectrum, we found a higher proportion of rare alleles (frequency $< 0.1$) at the MHC than at STRs (0.65 vs. 0.48 across the population, and 0.56 vs. 0.35 in Wt wolves), but once again, these allele frequencies were not significantly different from a normal L-shaped distribution.

**Discussion**

In this study, we investigated the variability of 3 MHC class II loci (DRB1, DQA1, and DQB1) in the Italian wolf population, which has been affected by long-term isolation and a recent population decline (Lucchini et al. 2004). Wolves in Italy are now expanding at a fast-growing pace, recolonizing many areas of their former distribution range (Fabbri et al. 2007). However, hybridization with feral dogs has been repeatedly documented (Randi and Lucchini 2002, Verardi et al. 2006; Caniglia et al. 2013a). Although the use of putatively neutral markers (mtDNA control region and autosomal microsatellites) revealed apparently limited frequency of hybridization and backcrossings (Randi and Lucchini 2002, Verardi et al. 2006), past-generation introgression of functional genetic variants could be underestimated (Anderson et al. 2009). Our findings show that, despite the demographic declines, the Italian wolf population retained high levels of MHC variability, both in number of alleles (from 6 to 9 at DRB1, DQA1, and DQB1 loci) and multilocus haplotypes ($n = 13$), comparable with more abundant populations in stable mutation-drift equilibrium (Seddon and Ellegren 2002). The alleles retained in the Italian wolf population correspond to more than 50% of the alleles described in the overall European or North American wolf populations (Seddon and Ellegren 2002; Kennedy et al. 2007). As a comparison, the highly endangered Mexican wolf population only shows 5 DRB1 alleles (Hedrick et al. 2000), whereas the Swedish population, which probably

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**Table 3** Distribution of Synonymous (SynDif) and Nonsynonymous differences (NsynDif), their proportions ($d_S/d_N$), relative to the total number of Synonymous (SynPos) and Nonsynonymous sites (NsynPos), and their ratios ($d_S/d_N$), as calculated in DNASP.

<table>
<thead>
<tr>
<th>Locus</th>
<th>SynDif</th>
<th>SynPos</th>
<th>$d_S$</th>
<th>NsynDif</th>
<th>NsynPos</th>
<th>$d_S$</th>
<th>$d_S/d_N$</th>
<th>$d_N - d_S$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRB1</td>
<td>2.74</td>
<td>62.50</td>
<td>0.05</td>
<td>15.34</td>
<td>204.50</td>
<td>0.08</td>
<td>1.74</td>
<td>1.823</td>
<td>0.035</td>
</tr>
<tr>
<td>DQA1</td>
<td>0.00</td>
<td>56.50</td>
<td>0.00</td>
<td>3.53</td>
<td>189.50</td>
<td>0.02</td>
<td>N/A</td>
<td>2.441</td>
<td>0.008</td>
</tr>
<tr>
<td>DQB1</td>
<td>2.24</td>
<td>64.98</td>
<td>0.04</td>
<td>16.61</td>
<td>202.02</td>
<td>0.09</td>
<td>2.45</td>
<td>3.039</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The probability values ($P$) of the difference $d_N - d_S$, based on the Nei and Gojobori method implemented in MEGA (Tamura et al. 2011), have been reconstructed after 500 bootstrap replicates.
originated from a very limited (<5) number of founders (Seddon and Ellegren 2004), shows 5, 4, and 4 alleles at the same loci, respectively (which decrease to 2 at each locus when excluding nonbreeding immigrants). The neighboring Croatian wolf population hosts an even higher number of alleles per locus (13, 7, and 11, respectively; Arbanasic et al. 2013). Croatian wolves are connected with larger populations in the Balkans, but gene flow with the Italian Apennine wolves during the past century is very unlikely. Wolves in the Italian Alps show approximately half of the observed or expected MHC haplotypes compared with wolves in the Apennine. This is explained by the recent colonization of the Alps by a small (<10) number of founders (Fabbri et al. 2007). Five alleles were detected only in admixed individuals (group H). All of these have previously been described only in dogs (Sarmiento et al. 1990; Sarmiento et al. 1993; Wagner et al. 1996; Kennedy et al. 2005), with the exception of DLA-DQA1*00101, which has been reported in other wolf populations and is shared with dogs and coyotes (Sarmiento et al. 1993; Hedrick et al. 2002). On the other hand, 1 allele (DLA-DRB1*02001) previously only described in dogs (Wagner et al. 1996) was found in the admixed H group and also in 3 Italian Wt wolves. It should be noted that the samples used in this study were not selected randomly and so the number of admixed genotypes is not proportional to their frequency in the Italian wolf population.

The phylogenetic trees did not show any clustering of the alleles found in the Italian wolf population, suggesting ancient coalescence of the MHC haplotypes. This is concordant with the trans-species polymorphisms described for all class II MHC loci (Seddon and Ellegren 2002; van Oosterhout 2009). Long-term consequences of positive selection left detectable molecular signatures at the MHC in the Italian wolves. The high values of the $d_{S}/d_{k}$ ratio, significantly better explained by models that allow for positive selection, are clear signatures of strong historical selective pressure driving the MHC variation. Such traces can be observed at both gene-wide and codon-specific levels, with the highest number of codons found to be under selection at the DQB1 locus. Ongoing natural selection might generate deviations from HWE, as observed at the locus DRB1. In particular, 4 nucleotides were responsible for this skew, 3 of which belong to codons under positive selection (CODEML model analyses). All of these are included within 2 of the DRB1 Hyper Variable Domains (Marsden et al. 2009), and 2 nucleotides specifically matched the same putative PBR site (codon 52). The remaining nucleotide corresponds to the single synonymous mutation differentiating 1 of the 2 newly described alleles (DRB1*092013) from its closest sequence, possibly suggesting a recent mutation in the derived state that has not yet reached HWE. However, in the case of ongoing selection, heterozygosity excess should be expected, whereas we observe both an overall heterozygosity deficit and high $F_{IS}$ values at departing nucleotides. Moreover, deviations from HWE could be explained by a Wahlund effect, because at background loci, the pairwise $F_{ST}$ is significant among all geographic locations ($P < 0.05$), except for cAp versus sAp (see also Fabbri et al. 2007). Therefore, the results do not allow us to clearly document traces of ongoing selection.

Balancing selection, leading to an excess of alleles with similar frequencies (i.e., for symmetrical overdominance), and bottlenecks, leading to a loss of rare alleles, are expected to skew allele frequencies toward a uniform distribution and an excess of heterozygosity. Moreover, balancing selection can maintain more variability at functional loci than at neutral markers (Aguilar et al. 2004), although, in small populations, the effects of selection to maintain variability may be overwhelmed by genetic drift (Ejsmond and Radwan 2011).

---

**Table 4** Likelihood of the selection models tested in CODEML

<table>
<thead>
<tr>
<th>Models’ likelihood</th>
<th>DRB1</th>
<th>DQA1</th>
<th>DQB1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 0</td>
<td>−728.755</td>
<td>−390.862</td>
<td>−707.094</td>
</tr>
<tr>
<td>Model 1a</td>
<td>−694.025</td>
<td>−392.665</td>
<td>−673.630</td>
</tr>
<tr>
<td>Model 2a</td>
<td>−682.410</td>
<td>−385.461</td>
<td>−656.114</td>
</tr>
<tr>
<td>Model 3</td>
<td>−670.534</td>
<td>−385.459</td>
<td>−652.942</td>
</tr>
<tr>
<td>Model 7</td>
<td>−694.143</td>
<td>−392.999</td>
<td>−673.637</td>
</tr>
<tr>
<td>Model 8</td>
<td>−682.517</td>
<td>−385.461</td>
<td>−656.135</td>
</tr>
</tbody>
</table>

Likelihood-ratio test

<table>
<thead>
<tr>
<th>D</th>
<th>P</th>
<th>D</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>M2a</td>
<td>M1a</td>
<td>M3</td>
<td>M0</td>
</tr>
<tr>
<td>23.230</td>
<td>14.409</td>
<td>116.441</td>
<td>10.804</td>
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<tr>
<td>8.9E−06</td>
<td>5.3E−04</td>
<td>10.804</td>
<td>2.5E−08</td>
</tr>
</tbody>
</table>

Codons under positive selection

| 6, 8, 27, 32, 52, 58, 62, 81 | 5, 20, 50, 64, 71, 77 | 6, 8, 23, 24, 25, 32, 52, 62, 66, 80, 84 |

All the values are indicated as log-likelihood; for each gene, the 3 models that better fit the data are shown in bold. The models have been compared also by a pairwise comparison based on a likelihood-ratio test (rows 9–11), with $D$ being twice the log-likelihood difference between the methods; its probability value is based on the chi-square distribution expected with the number of degrees of freedom (df) indicated, corresponding to the differences in free parameters among the models. Codons (row 12) were assumed to be under positive selection only when the probability ($P$) of $\omega$ being higher than 1 was greater than 0.95, by both the Bayes Empirical Bayes (for the models M2a and M8) and the Naïve Empirical Bayes (for M2a, M3, and M8) tests. The codons that are likely to correspond to the peptide-binding regions (PBRs) are indicated in bold.
In our case, the Ewens–Watterson test could not clearly document any deviation from neutrality, either at STRs or at MHC. However, computer simulations carried out by Garrigan and Hedrick (2003) showed that in a population of approximately 100 individuals—the number of individuals estimated to have survived the Italian wolf population bottleneck—the time needed to gain statistical significance in similar tests can be as long as 30 generations. Assuming a generation time of 3 years in wolves, this corresponds to approximately 90 years, thus preceding the population bottleneck that occurred in the 1960s. Therefore, we do not have enough statistical power to detect traces of a bottleneck at the neutral loci nor balancing selection at the MHC. Similar conclusions were drawn by Seddon and Ellegren (2004) in a study on northern European wolf populations. Moreover, the mating schemes of the species could also bias the observed heterozygosity patterns. In wolves, mate choice has only been studied in relation to inbreeding avoidance at STRs (Geffen et al. 2011). Thus, the possible existence of reproductive schemes different from disassortative mating (Galaverni et al. in preparation) could also result in values of heterozygosity lower than those expected under either drift or balancing selection.

Differences in heterozygosity between MHC and STR loci were not significant but more marked in the putatively admixed wolf × dog individuals, suggesting that genetic differentiation among parental populations is higher at the MHC than at STRs, as confirmed by estimates of Jost’s D between genetically pure and admixed individuals. Similarly, private MHC alleles have been found in the admixed group, whereas wolves showing atypical phenotypic traits, such as dark coat color (Anderson et al. 2009, Randi 2011), only possessed alleles also found in the Wt wolves. These findings confirm the assignment based on the STRs, although a limited number of neutral markers can be inefficient in detecting past hybridization events or gene introgression (Randi 2011). Typically, these events can be reliably identified only up to the second-generation backcrosses using the same panel of STRs (Caniglia et al. 2013a). A single DRB1 allele (DRB1*02001), described only in dogs thus far, was found in 3 Wt wolves and in admixed individuals. Its distribution could be explained in 2 ways: 1) the allele is present both in wolves and dogs but has not been described so far in the former; 2) the allele is dog specific and has been retained in the wolf population after gene introgression not detected at other neutral markers, such as mtDNA CR or STRs. In this study, Y chromosome haplotypes could not be investigated because all 3 Wt individuals were female wolves. Therefore, a greater number of markers will be needed to better discriminate the 2 hypotheses.

Conclusions

Although thoroughly studied, the role of natural selection in shaping MHC variation is still controversial (Bernatchez and Landry 2003, Sutton et al. 2011). When decreased MHC variation is compounded by population isolation, past bottlenecks, and loss of genetic diversity or inbreeding, the long-term population viability for these species is questionable (Radwan et al. 2010). However, direct correlations between MHC variability and fitness traits (e.g., parasite load) have been seldom demonstrated (Wegner et al. 2003; but see Hedrick et al. 2003, Kennedy et al. 2011). Our study describes the variability at important functional genes in the isolated Italian wolf population, dispelling doubts about a dramatic loss of variation that could threaten its future survival. We found traces of historical selection, but we could not detect clear signals of ongoing selection. Results also showed that the assessment of wolf or dog private MHC class II alleles and haplotypes should be used in addition to traditional neutral markers to improve the identification of past-generation hybrids.

Supplementary Material

Supplementary material can be found at http://www.jhered.oxfordjournals.org/

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