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Diversification of porcine MHC class II genes: evidence for selective advantage

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Abstract The major histocompatibility complex (MHC) is an immunological gene-dense region of high diversity in mammalian species. *Sus scrofa* was domesticated by at least six independent events over Eurasia during the Holocene period. It has been hypothesized that the level and distribution of MHC variation in pig populations reflect genetic selection and environmental influences. In an effort to define the complexity of MHC polymorphisms and the role of selection in the generation of class II gene diversity (DQB, DRB1, and pseudogene ΨDRB3), DNA from globally distributed unrelated domestic pigs of European and Asian origins and a Suidae out-group was analyzed. The number of pseudogene alleles identified (ΨDRB3 33) was greater than those found in the expressed genes (DQB 20 and DRB1 23) but the level of observed heterozygosity (ΨDRB3 0.452, DQB 0.732, and DRB1 0.767) and sequence diversity (ΨDRB3 0.029, DQB 0.062, and DRB1 0.074) were significantly lower in the pseudogene, respectively. The substitution ratios reflected an excess of d_N (DQB 1.476, DRB1 1.724, and ΨDRB3 0.508) and the persistence of expressed gene alleles suggesting the influence of balancing selection, while the pseudogene was undergoing purifying selection. The lack of a clear MHC phylogeographic tree, coupled with close genetic distances observed between the European and Asian populations (DQB 0.047 and DRB1 0.063) suggested that unlike observations using mtDNA, the MHC diversity lacks phylogeographic structure and appears to be globally uniform. Taken together, these results suggest that, despite regional differences in selective breeding and environments, no skewing of MHC diversity has occurred.

Keywords Porcine major histocompatibility complex · MHC class II genes · MHC diversity · Balancing selection

Introduction

The pig (*Sus scrofa*) was domesticated approximately 9,000 years ago through an estimated six independent global domestication events (Larson 2005). Subspecies of the European wild boar and the Southeast Asian pig were the main genetic contributors of the domestic pig (Okumura et al 2001). Mitochondrial DNA (mtDNA) analyses have provided evidence of independent wild boar domestication events of European and Asian subspecies with significant differentiation between domestic populations in Europe and Asia (Giuffra et al. 2000). This division suggests that the distribution of genetic variation is consistent with differences observed in artificial selection and environmental influences. Genetic diversity and population structure vary among populations with different demographic histories as well as selective pressures, such as artificial and natural selection. Studies of immunological genes, such as the major histocompatibility complex (MHC), are ideal candidates to determine the effects of selection on diversity.

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Maintaining MHC diversity is important since the level of MHC variability may reflect a loss of immune responsiveness (Hosokawa-Kanai et al. 2002; Wimmers et al. 2004).

MHC genetic diversity is marked by an excess of nonsynonymous substitutions (d_N), a high level of heterozygosity, a large number of alleles, and divergence between alleles within a population (Takahata and Nei 1990). MHC genes contain both polymorphic and monomorphic exonic coding sequences, thus making it difficult to determine how gene conversion or interlocus genetic exchange may have contributed to the degree of MHC polymorphism (Widera and Flavell 1984). Consequently, neither gene conversion nor interlocus genetic exchange can explain observed differences between synonymous (d_S) and d_N rates for individual exons of expressed MHC genes or the lower frequency of d_S at non-peptide-binding sites than observed in peptide-binding site codons (Hughes 1991; Jonsson et al. 1989).

MHC polymorphism may be maintained by selective pressures possibly associated with disease rather than random mutations and genetic drift (Hughes and Nei 1992; Figueroa et al. 1988; Clarke and Kirby 1966; Maruyama and Nei 1981). In addition, selective breeding of traits genetically linked to the MHC (SSC7) could have unintentional effects of narrowing genetic diversity and, hence, potentially increase the risk of disease susceptibility (Barbosa et al. 2004). To test the hypothesis that decreased MHC diversity results from inadvertent selective breeding, we examined the extent of MHC diversity in various independent global populations of European and Asian origins and determined which mechanism(s) contributed towards maintaining MHC diversity.

In order to determine the influence of selective pressures, it is necessary to compare MHC variation to a region presumably under neutral selection. Previous studies have used MHC intronic sequences to access neutral selectivity (Aguilar et al. 2005). Pseudogenes can also serve as neutral sites and to control for genetic hitchhiking due to their close proximity to functional gene regions (Li et al. 1981). Porcine MHC haplotypes can encode four DRB pseudogenes and one DQB pseudogene (denoted ΨDRB2, 3, 4, 5 and ΨDQB2; Chardon et al. 1985). These MHC pseudogenes are greater than 90% identical to expressed MHC loci (Gustafsson et al. 1990). In addition to MHC class II pseudogenes, out-groups of Suiformes (*Sus barbatus* and *Pecari tajacu*) were also used to provide insights into phylogenetic divergence of MHC alleles. The *P. tajacu*, the collared peccary, is of the family Tayassuidae and the suborder Suiformes. The *P. tajacu* and the *S. scrofa* diverged 33.7 to 23.8 million years ago. Additionally, the *S. barbatus* (bearded pig) is of the suborder Suiformes and the Suidae family. The *S. barbatus* and *S. scrofa* diverged 1.3 to 6.6 million years ago (Randi et al. 1996).

To test the hypothesis that the unintentional reduction of MHC diversity results from selection of linked economically important production traits, three topics were addressed. First, experiments determined MHC diversity at expressed genes compared to a neutral region. Second, the extent of d_S and d_N substitutions in populations with varying breeding criteria was defined. Finally, analyses were performed to determine whether global selective pressure extends MHC diversity in populations under artificial or environmental pressures.

Materials and methods

Sources and isolation of DNA Genomic DNA obtained from Asian and European pigs was used for targeted MHC sequencing. Out-group DNA from two peccaries and a bearded pig were also included. Animals represented globally distributed germplasm and were selected to (1) increase the likelihood of independent ancestral haplotypes, (2) maximize the detection of MHC polymorphism by using unrelated animals (no shared grandparents), and (3) to include a Suidae out-group (composed of peccaries and bearded pig) for identifying shared ancestral alleles. Pigs representing European origins were from Berkshire, Yorkshire, Hampshire, Duroc, Landrace, and Large White breeds, and pigs representing Asian origins were selected from Meishan, Lanyu, and Erhualian breeds. DNA from whole blood or semen was isolated in accordance with Miller et al. (1988). Differences in the number of observations for each loci reflect two issues. First, the differences of observations between the expressed MHC genes (86 and 93) were due to a lack of adequate DNA available for DQB validation. Only if the samples had enough high-quality DNA for re-analysis were they included in the study. Differences for the ΨDRB3 locus again reflect the lack of adequate DNA availability and absence of the pseudogene locus in these particular animals. The lack of a pseudogene in MHC haplotypes is consistent with previous observations demonstrating that MHC II haplotypes vary in copy number of DRB genes (Chardon et al. 1999; Vaiman et al. 1998).

Sequence primer design Porcine SLA DRB1 (accession # AY303991) and DQB (accession # BX088590) sequences were obtained from the National Center for Biotechnology Information (NCBI) GenBank. Primer pairs were designed using PRIMER 3.0 for conserved sequences of intron 1 and intron 2 (Shia et al. 1995; Ho et al. 2006). Amplicons were verified by amplifying divergent and ancestral species. Each primer pair was tested against a panel of DNA representing 300 domestic pigs and wild boars. Since specific haplotypes may not encode the ΨDRB3 pseudo-

genes (Chardon et al. 1999), DNA from cattle, peccaries, and the bearded pig were included to verify the ability of the primers to amplify divergent ΨDRB3 alleles. The annealing temperature used for each primer pair was 64°C and 68°C for DQB and DRB, respectively. Product sizes for DQB and DRB were 400 and 700 bp, respectively. The primer sequences for DRB1 were 5′-GGGCGAATCCTTGGGGAGC-3′ (forward) and 3′-ACACACACTCTGCCCCCCG-5′ (reverse). The primer sequences for DQB were 5′-CGGGCGGAGGCCT GACTG-3′ (forward) and 3′-CGGCGGGCAAGCACT CAC-5′ (reverse). ΨDRB3 sequences were obtained from NCBI GenBank (accession # BX088590.7) and primers were designed within intron 1 and intron 2 using PRIMER 3.0 to amplify all of exon 2. The ΨDRB3 product size was 300 bp. The sequences for the ΨDRB3 primers were 5′-GCCTTCAGCCTTTTCAGGAG-3′ (forward) and 3′-TTGGATACTTACCTGGTGCC-5′ (reverse).

Polymerase chain reactions (PCR) were performed using 10-ng genomic DNA in a 20-μl volume reaction containing 10× buffer at a final concentration of 1×, 200 μM dNTPs (Invitrogen, Carlsbad, CA, USA), 2.5 units of HotStarTaq Polymerase (Qiagen, Valencia, CA, USA), and 0.5 μM of each primer. PCR conditions had an initial denaturing temperature of 94°C for 15 min followed by 35 cycles of a three-step process of 30 s at 94°C, 30 s at 62°C, and 1 min at 72°C followed by a final step of 7 min at 72°C. In order to minimize the probability of erroneously identifying an artificial sequence as an actual allele, two independent PCR reactions were performed for each animal and were bidirectionally sequenced. PCR products were analyzed by electrophoresis in a 1% agarose 1× TBE gel and visualized by ethidium bromide staining and UV light. Positive PCR products were purified using a Qiagen MinElute 96 UF PCR purification kit (Qiagen, Valencia, CA, USA). Purified DNAs were bidirectionally sequenced with ABI Prism® BigDye™ Terminator Cycle Sequencing Kit (version 3.1; Applied Biosystems, Foster City, CA, USA). All sequencing reactions were analyzed using an ABI 3730 DNA capillary sequencer (Applied Biosystems, Foster, CA, USA.)

Cloning Heterozygous individuals were determined using direct sequencing of PCR products previously mentioned. PCR products were ligated into pCR®2.1-TOPO® vector using the TOPO TA cloning kit and transformed into One Shot® TOP10 chemically competent bacterial cells according to the manufacturer's instructions (Invitrogen). Transformed bacteria were plated onto Luria–Bertani agar containing 50 μg/ml kanamycin, 40 mg/ml X-gal solution, and 100 mM IPTG for clone selection. Initially, 12 positive colonies were picked from each plate; additional clones were sequenced if needed to verify the sequences of both alleles. Clones were cultured overnight in 2 ml Luria–Bertani containing 50 μg/ml kanamycin and plasmid DNA was isolated using Montage Plasmid MiniprepHTS 96 Kit (Millipore). Verification of targeted insert was done by *Eco*RI digestion of plasmid DNA using electrophoresis in a 1% agarose 1× TBE gel and UV visualized by ethidium-bromide-stained gels.

DNA sequencing and analysis Purified plasmid DNA with targeted insert were bidirectionally sequenced using ABI Prism® BigDye™ and standard M13 (−20) 5′-GTAAAAC GACGGCCAGT-3′ forward and reverse primers 5′-AACAGCTATGACCATG-3′. All sequencing reactions were conducted at the W. M. Keck Center for Comparative and Functional Genomics, University of Illinois, using ABI 3730 DNA capillary sequencers (Applied Biosystems, Foster, CA, USA). Sequence comparisons were done using Phrap and Gap4 integration from the Staden Package (Cambridge, UK). Newly identified alleles were submitted to GenBank and were assigned allele designation in accordance with established guidelines (Smith et al. 2005).

Analysis Observed heterozygosity (H_o) and expected heterozygosity (H_e) were estimated using the POPGENE software package (Yeh et al. 1999). Testing for departures from Hardy–Weinberg equilibrium was done using the GENEPOP version 3.1 (Raymond and Rousset 1995). The statistical package, MEGA4, was used to calculate the average rates of synonymous (d_S) and nonsynonymous (d_N) substitutions per site (calculated separately for antigen-binding sites (ABS) and non-antigen-binding sites assuming congruence with human MHC sites (Brown et al. 1988, 1993)) using the Nei and Gojobori (1986) method with the Jukes–Cantor correction for multiple substitutions and polymorphism calculations (Tamura et al. 2007). Standard errors of the estimates were obtained through 1,000 bootstrap replicates. Additionally, genetic distances within exon 2 were estimated using Kimura's two-parameter method (Kimura and Crow 1964) among populations and alleles, calculated in MEGA v. 2.0, and used to draw the neighbor-joining (NJ) phylogenetic trees.

To assess the genetic relationship between European and Asian populations, ARLEQUIN version 4.0 (Excoffier et al. 2005) computer package was utilized to calculate fixation indices (F_{ST}, the level of population differentiation), genetic diversity, and analysis of molecular variation (AMOVA). The AMOVA was utilized in order to quantify the degree of genetic variation at three hierarchical levels, namely, among region, among populations within regions, and within populations. *F* statistics were used to determine population substructure between populations (F_{ST}). F_{ST} estimates are based on the infinite allele model (Kimura and Crow 1964).

Results

Level of MHC genetic variation MHC variation of expressed genes, the number of alleles, the level of heterozygosity, and diversity indices (number of variable sites and nucleotide diversity) were compared to the nonexpressed MHC locus (ΨDRB3). A total of 15 DQB, 21 DRB1, and 31 ΨDRB3 alleles were detected in 86, 93, 61 animals, respectively (Tables 1 and 2 and Supp. Table 1). Eight new DQB and 13 DRB1 alleles were identified in the current study (GenBank accession numbers EU039916 through EU039962; Fig. 1). Each new allele was assigned an accession number in accordance with guidelines established by the SLA Nomenclature Committee of the International Society for Animal Genetics (ISAG; Smith et al. 2005). Alleles DQB*0101 and DRB1*0201 were the most common, occurring at a frequency of 36% and 17% for DQB and DRB1, respectively (Tables 1 and 2). DQB*0101 and DRB1*0201 were identified in both European and Asian populations, and DQB*0101 was also observed in the out-group. Of the alleles identified, 60% and 38% occurred in more than one animal for DQB and DRB1, respectively. The remaining alleles were identified in only one animal, thus with an allele frequency less than 1%. In addition, of the 31 ΨDRB3 alleles, only 20% of the alleles occurred in more than one animal with the most common allele occurring at a frequency of 16% (Supp. Table 1).

The observed levels of heterozygosity for the two expressed MHC loci were consistent with Hardy–Weinberg Expectations (HWE; all significance values $P<0.05$), while at the ΨDRB3 locus both populations significantly deviated from HWE. Observed and expected heterozygosity (Table 3) are closely related measures of heterozygosity, where a significant difference between the two suggests a population experiencing heterozygote deficiency. In this study, heterozygote deficiency provides evidence that DQB and DRB1 lacked heterozygote deficiency, whereas, within ΨDRB3, the populations were experiencing heterozygote deficiency (Table 3). Additionally, the general pattern of higher observed heterozygotes (0.80 and 0.81) than expected (0.73 and 0.77) was consistently observed within both DQB and DRB1, respectively. The opposite was observed for the pseudogene, where the observed heterozygosity (0.49) was lower than expected heterozygosity (0.80), revealing a significant heterozygote deficiency. These heterozygosity results suggest an advantage for maintenance of heterozygosity for the two expressed genes which was not observed at the linked pseudogene.

Since elevated polymorphism and heterozygosity are suggestive of higher population diversity, analyses of nucleotide and amino acid variation were performed to provide insights into diversity for each expressed MHC gene (DQB and DRB1) compared to the ΨDRB3. Exon 2 DNA sequences for DQB and DRB1 (270 bp) of which 55 and 71 nucleotides (20% and 26%) were polymorphic,

Table 1 Allelic frequency of DQB alleles

Allele	European (108)	Asian (58)	Out-group (6)	Total frequency
0101	0.380	0.328	0.333	0.360
0901	0.056	0.000	0.000	0.035
0201	0.278	0.224	0.333	0.262
0801	0.093	0.310	0.000	0.163
0701	0.102	0.000	0.000	0.064
0601	0.023	0.000	0.000	0.017
0202	0.019	0.052	0.000	0.029
0204	0.019	0.000	0.000	0.012
01ha02	0.019	0.000	0.000	0.012
01Lu01	0.000	0.017	0.000	0.006
Lu02	0.000	0.017	0.000	0.006
08Lu03	0.000	0.017	0.000	0.006
02Me01	0.000	0.017	0.000	0.006
01Me03	0.000	0.017	0.000	0.006
01be01	0.009	0.000	0.000	0.006

Allele names listed are in accordance with SLA Nomenclature Committee of the International Society for Animal Genetics. *Bolded* alleles represent new allele designations. Number of independent nonrelated chromosomes in *parentheses*

Table 2 Allelic frequency of DRB1 alleles

Allele	European (116)	Asian (64)	Out-group (6)	Total Frequency
0901	0.103	0.281	0.000	0.156
0201	0.190	0.141	0.167	0.167
0701	0.121	0.234	0.167	0.156
1001	0.164	0.047	0.167	0.120
1401	0.009	0.000	0.000	0.005
0102	0.086	0.188	0.000	0.115
1301	0.069	0.000	0.000	0.042
0101	0.181	0.016	0.000	0.115
La02	0.009	0.000	0.000	0.005
La03	0.009	0.000	0.000	0.005
La04	0.009	0.000	0.000	0.005
La05	0.009	0.000	0.000	0.005
Ha01	0.009	0.000	0.000	0.005
Ha04	0.069	0.000	0.000	0.006
du05	0.009	0.000	0.000	0.005
02Du01	0.009	0.000	0.000	0.005
Er01	0.000	0.016	0.000	0.006
Lu02	0.000	0.047	0.000	0.017
10Lu03	0.000	0.016	0.000	0.006
Me02	0.000	0.016	0.000	0.006
Be01	0.009	0.000	0.000	0.006

Allele names listed are in accordance with SLA Nomenclature Committee of the International Society for Animal Genetics. *Bolded* alleles represent new allele designations. Number of independent nonrelated chromosomes in *parentheses*

DQB

```
                  38                                                                                                  128
                     + + +    +           + + +     ++         +         ++  ++   +   + ++  +    +  ++  + + ++
#SLA-DQB1*0101    DFVFQFKFEC YFFNGTQRVR LVARYIYNQE ELLRFDSDVG EYRAVTPLGR PEADSWNGQK DVLEQTRAEL DTVCKHNYQI EEGTTLQRR
#SLA-DQB*0901     ...V...G.. .......... ..T.F..... .YA....... .......... .D..Y..S.. .I........ .......... .........
#SLA-DQB1*0201     ...Y...... .......... G...WV.... .HV....... .F........ .T..Y..... .....K...V .......... .........
#SLA-DQB1*0801     ...Y...... .......... ..T..... .YA....... .......... .A..Y..S.. .I........ .......... .........
#SLA-DQB1*0701     ...Y...... .......... .LT..... .HV....N.. .......... .D..Y..... .......... .......... .........
#SLA-DQB1*0601     ...Y...G.. .......... H.T..... .HV....... .F........ .D..Y..... .F........ .......... .........
#SLA-DQB1*0202     ...Y...... ..Y....... ....WV..R. .HV....... .F........ .D..Y..... E....K.... .......... .........
#SLA-DQB1*0204     ...V...G.. ..Y....... G...WV.... .YV....... .F........ .D..Y..... E....K.... .......... .........
#SLA-DQB1*01ha02   .......... .....A.... .......... .......... ..Q....... ........R. .......... .......... .........
#SLA-DQB1*01Lu01   .......... .LS....... ..T..... .......... .......... .......... ..M....... .......... .........
#SLA-DQB1*Lu02     ...V...GQ. .......... G.T.F..... .F........ .F........ ....Y..S.. .........A .......... .........
#SLA-DQB1*08Lu03   ...Y...L.. .......... ..T..... .YA....... .......... .A..Y..S.. .I...A.... .......... .........
#SLA-DQB1*02Me01   ...Y...... .......... G...WV.... .HV....... .F........ .T..Y..... .....K...V .......... G........
#SLA-DQB1*01Me03   ...L...... .......... .......... .......... ..Q...T... .......... .......... .......... K........
#SLA-DQB1*01be01   .......L.. .......... .......... .......... .......... .......... .......... .......... ...A.....
```

DRB

```
                  35                                                                                                  115
                     + + +                 + + +     ++         +         ++  ++       + ++  +    +  ++  + + ++
#SLA-DRB1*0901    HFLFLGKFEC HFFNGTEQVR LLERQYYNGE EFVRFDSDVG EYRAVTELGR PDAKNYNSQK DLLEQRRAEV DTYCRHNYRT SDTFLVPRR
#SLA-DRB1*0201     ...H.L.... .......R.. .......... ..L....... .......... ....DW.... .......... .........I L........
#SLA-DRB1*0701     ...L.V.S.. .......R.. FM..H..... .H........ .......... .S..YW.... .I..EK.... .......... .........
#SLA-DRB1*1001     ..F.ME.... .......R.. ..D.H..... .IL....... .......... .E..DW..R. .I........ .........I .........
#SLA-DRB1*1401     ...H.V.S.. R......R.. F.D.YF.... .......... .F.E...... ....YW.... .FM..K.... ..V.....EI .E.......
#SLA-DRB1*0102     .....V.D.. .......R.. ..QK...... .H........ .......... ....YW.... .....M..A. .......... .........
#SLA-DRB1*1301     .....V.D.. .......R.. .......... .H........ .......... ....YW.... .I...K.... ..V......I L........
#SLA-DRB1*0101     .....V.D.. .......R.. ..QK...... .H........ .......... ....YW.... .....M..A. .........I L........
#SLA-DRB1*La02     .......... .......... .......... .......... .......Q.. .......... .......... .......... .........
#SLA-DRB1*La03     ..F.ME.... .......R.. ..D.H..... .IL....... .......... .......... .......... .........I .........
#SLA-DRB1*La04     ..F.ME.... .......... .......... .......... .......... .......... .......... .......... .........
#SLA-DRB1*La05     ..F.ME.... .......R.. ..D.H..... .......... .......... .......... .......... .........I .........
#SLA-DRB1*Ha01     .......... .......R.. ..QK...... .H........ .......... ....YW...R .....M..A. .........I L........
#SLA-DRB1*Ha04     .....V.D.. .......R.. .......... .H........ .......... ....YW.... .....M..A. .........I L........
#SLA-DRB1*du05     .......... ......G... .......... ........A. .......... .........E .......... .......... .........
#SLA-DRB1*02Du01   ...H.L.... ......DRL. .....F.... ..L....... .......... ....DW.... .......... .........I L........
#SLA-DRB1*Er01     ..F.ME.... .......R.. ..D.H..... .IL....... G......... ....YW.... .....M..A. .......... .........
#SLA-DRB1*Lu02     ...L.V.... .......R.. FM..H..... .H........ .......... ....YW.... .....K.GA. .R.......I L........
#SLA-DRB1*10Lu03   ..F.ME.... .......R.. ..DKH..... .IL....... .......... .E..DW..R. .I........ ......D..I .........
#SLA-DRB1*Me02     .....V.D.. .......R.. ..QK...... .H........ .......... .......... .......... .......... .........
#SLA-DRB1*Be01     .....V.D.. .......R.. ..QK...... .H........ .......... ....YW.... .....M..A. ..V......I L........
```

Fig. 1 Amino acid sequences of MHC DQB and DRB1 alleles detected in *S. scrofa domesticus.* A *plus* indicates amino acid positions constituting the antigen-binding sites (Hosokawa 1998, Brown et al. 1993). A *dot* represents identity to consensus sequence. *Bold sequences* are newly identified alleles assigned ISAG SLA accession numbers

respectively (Table 4). Conversely, the proposed pseudogene exon 2 DNA sequences consist of 250 bp of which 50 nucleotides (20%) were polymorphic. Comparisons of nucleotide diversity revealed that both expressed loci contained significantly higher diversity (0.062 and 0.074) compared to the pseudogene (0.029). The relatively comparative number of polymorphic sites between expressed and nonexpressed MHC genes suggests similar mutation rates. Nucleotide diversity was significantly lower at the pseudogene, which implies that despite the high number of alleles and polymorphic sites, diversity among ΨDRB3 alleles was lower than the expressed MHC genes, which was further supported by the number of synonymous exceeding nonsynonymous substitutions.

Table 3 Identification of level of polymorphism between expressed and nonexpressed MHC genes

Animal	*n* DQB1	He	Ho	HWE	*n* DRB1	He	Ho	HWE	*n* ΨDRB3	He	Ho	HWE
All European	108	0.68	0.76	0.06	116	0.82	0.79	0.07	70	0.89	0.49	0.04**
All Asian	58	0.71	0.83	0.90	64	0.74	0.81	0.44	40	0.67	0.40	0.00**
All	172	0.732	0.803	0.75	186	0.767	0.807	0.74	116	0.796	0.486	0.07*

n number of independent nonrelated chromosomes, *He* expected heterozygosity, *Ho* observed heterozygosity, *HWE* Hardy–Weinberg equilibrium probabilities

*$P<0.1$; **$P<0.05$ tested the significance of the presence of heterozygous deficiency

Table 4 Sequence diversity analysis in SLA class II alleles

Locus	Number of alleles	Nucleotide		Amino acid	
		Var sites	Diversity (SE)	Var sites	Diversity (SE)
DQB					
European	10	51	0.047 (0.008)	28	0.093 (0.022)
Asian	9	47	0.047 (0.008)	24	0.093 (0.022)
All	15	64/270	0.062 (0.008)	33/89	0.123 (0.021)
DRB1					
European	16	76	0.062 (0.009)	37	0.125 (0.024)
Asian	9	45	0.059 (0.009)	29	0.121 (0.023)
All	21	83/270	0.074 (0.01)	52/89	0.159 (0.025)
ΨDRB3					
European	26	45	0.023 (0.005)	23	0.040 (0.011)
Asian	9	30	0.017 (0.004)	14	0.026 (0.009)
All	34	50/250	0.029 (0.005)	26/82	0.039 (0.011)

Nucleotide diversity was calculated with Jukes–Cantor correction and standard errors estimated by 1,000 bootstrap replicated

Evidence for balancing selection

Of the 89 exon 2 amino acids for DQB and DRB1, the number of allelic polymorphic sites varied from one to 20 (Fig. 1). The average level of codon heterozygosity was 0.40 with 50% of the polymorphisms residing in the ABS. Additionally, the average ABS codon heterozygosity for both expressed genes was 0.45, compared to 0.10 for non-ABS codons (Fig. 4). DQB amino acid diversity was less divergent than DRB1 with only 33 variable sites with a diversity of 0.123 compared to 52 variable sites with a diversity of 0.159 at DRB1 (Table 4). The higher amino acid than nucleotide diversity for both expressed genes suggests that nonsynonymous substitutions were favored over synonymous substitutions indicating selective maintenance of nonsynonymous polymorphisms.

Additional evidence for selection was provided by comparing the rate of nonsynonymous (d_N) and synonymous (d_S) substitutions within and outside the ABS. The porcine ABS was defined by assuming congruence with human MHC sites (Brown et al. 1988, 1993). Within the ABS codons, a significant excess of d_N was observed with ratios of 2.61 and 2.02 for DQB and DRB1, respectively (Table 5). Conversely, a deficiency in d_N was observed for the pseudogene with a ratio of 0.49. Substitutions outside of the ABS revealed a d_N deficiency with ratios of 0.64, 0.42, and 0.95 for DQB, DRB1, and ΨDRB3, respectively. The substitution results imply that substitutions outside of the

Table 5 Synonymous and nonsynonymous distances for antigen-binding sites (ABS) and non-ABS in SLA class II alleles

Locus	Number of alleles	ABS			Non-ABS		
		d_S	d_N	d_N/d_S	d_S	d_N	d_N/d_S
DQB							
European	10	0.046 (0.034)	0.119 (0.039)	2.59**	0.043 (0.017)	0.023 (0.010)	0.54 ns
Asian	9	0.045 (0.035)	0.120 (0.039)	2.67**	0.044 (0.080)	0.022 (0.010)	0.50 ns
All animals	15	0.046 (0.033)	0.120 (0.040)	2.61**	0.058 (0.024)	0.037 (0.013)	0.64 ns
DRB1							
European	16	0.098 (0.047)	0.206 (0.043)	2.10**	0.044 (0.016)	0.019 (0.006)	0.43 ns
Asian	9	0.107 (0.054)	0.197 (0.043)	1.84*	0.031 (0.016)	0.019 (0.006)	0.61 ns
All animals	21	0.103 (0.052)	0.208 (0.042)	2.02**	0.048 (0.015)	0.019 (0.006)	0.42 ns
ΨDRB3							
European	26	0.032 (0.020)	0.019 (0.006)	0.60 ns	0.020 (0.009)	0.019 (0.006)	0.95 ns
Asian	9	0.037 (0.021)	0.011 (0.006)	0.30 ns	0.016 (0.008)	0.014 (0.007)	0.88 ns
All animals	34	0.041 (0.009)	0.020 (0.009)	0.49 ns	0.020 (0.008)	0.019 (0.006)	0.95 ns

Synonymous (d_S) and nonsynonymous (d_N) distances and their ratio were calculated using the Nei–Gojobori method with a Jukes–Cantor correction. The significance of an excess in nonsynonymous substitutions was tested with a one-tailed z test

ns not significant

*$P<0.1$; **$P<0.05$

Table 6 Percentages of variation through an analysis of molecular variance

Source of variation	DQB	DRB1	DRB3	mtDNA
Among regions	0	0	2	46
Among breeds within regions	29	23	19	29
Within breeds	71	77	79	25

AMOVA statistics were performed using conventional *F* statistics and 1,000 permutations

expressed genes ABS may be under purifying selection (a value of less than 1) while substitutions within the ABS favor d_N and may be under balancing selection (Table 5).

Assessment of global uniformity of MHC polymorphism

Previous mtDNA genetic analyses have revealed significant genetic distances between European and Asian pig populations (Larson 2005). Thus, this study was aimed to

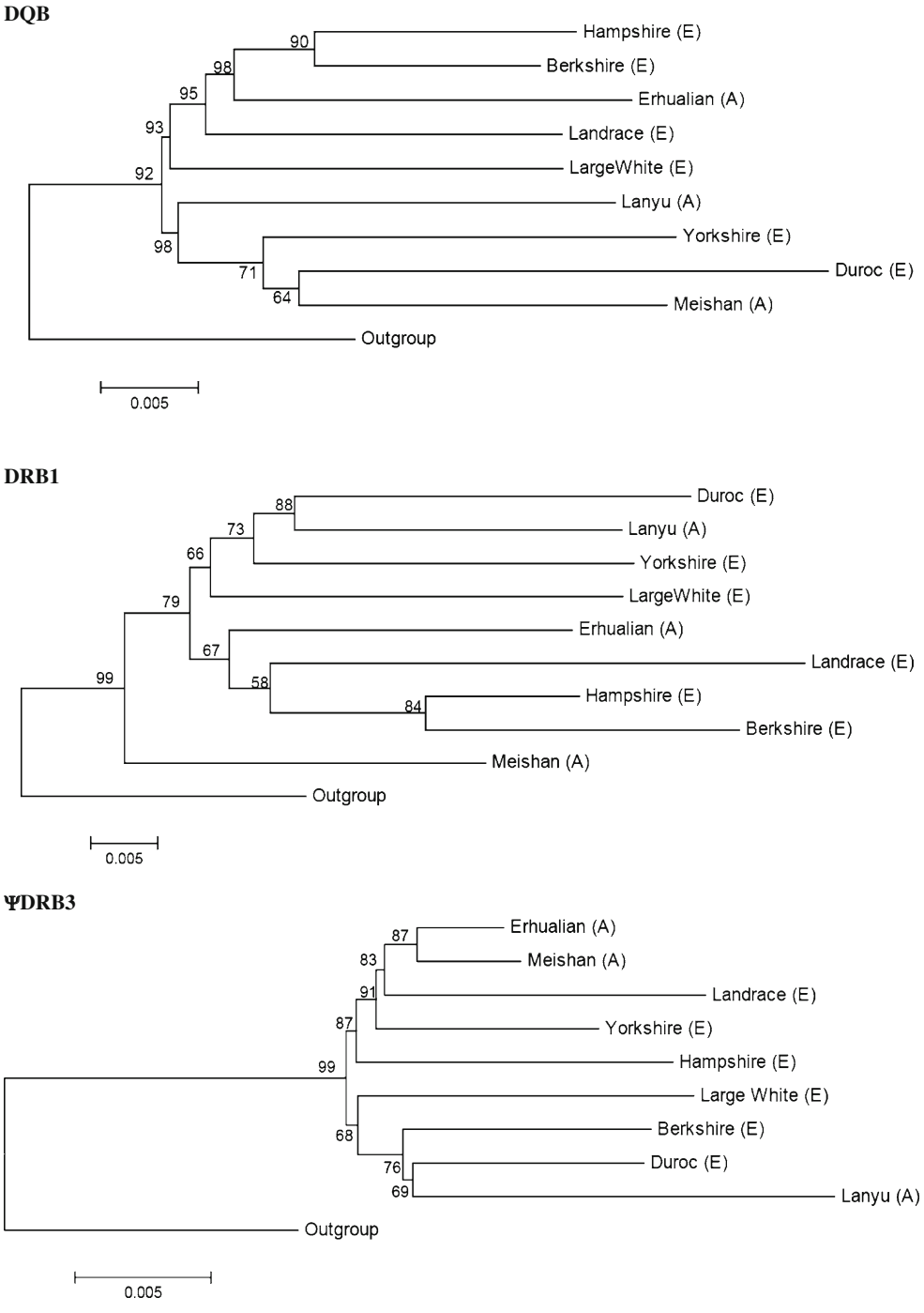

Fig. 2 Neighbor-joining phylogeny of exon 2 in MHC expressed genes and pseudogene. The evolutionary history was inferred using the neighbor-joining method with Kimura two-parameter rooted at the outgroup. The trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. *Numbers* indicate bootstrap percentages (1,000 iterations). Phylogenetic analyses were conducted in MEGA4. Abbreviations: (*E*) populations of European origin and (*A*) populations of Asian origin

determine whether regional selective breeding affected MHC diversity. An AMOVA demonstrated that greater than 70% of DQB and DRB1 variation was due to differences within breeds and not between regions. This pattern of variation contrasts with mtDNA analyses where 25% of the observed variation was attributed to differences within breeds and 46% was due to differences between regions (Table 6). The genetic distances for DQB and DRB1 demonstrated a lack of geographic branching (Fig. 2). Despite the restricted sharing of European and Asian mtDNA haplotypes, both expressed MHC loci contained region-specific alleles which were represented throughout the NJ tree and did not preferentially form single regional clades (Fig. 3). Allele DQB*0101 was most frequent in both European and Asian populations. Allele DRB1*0901 was most frequent in Asian populations, while DRB1*0201 was most frequent in European animals (Fig. 3). The results from the MHC phylogenetic analysis suggest that despite potential regional differences in selective breeding (such as intense selection for linked traits, i.e., back fat) no skewing of MHC diversity was observed.

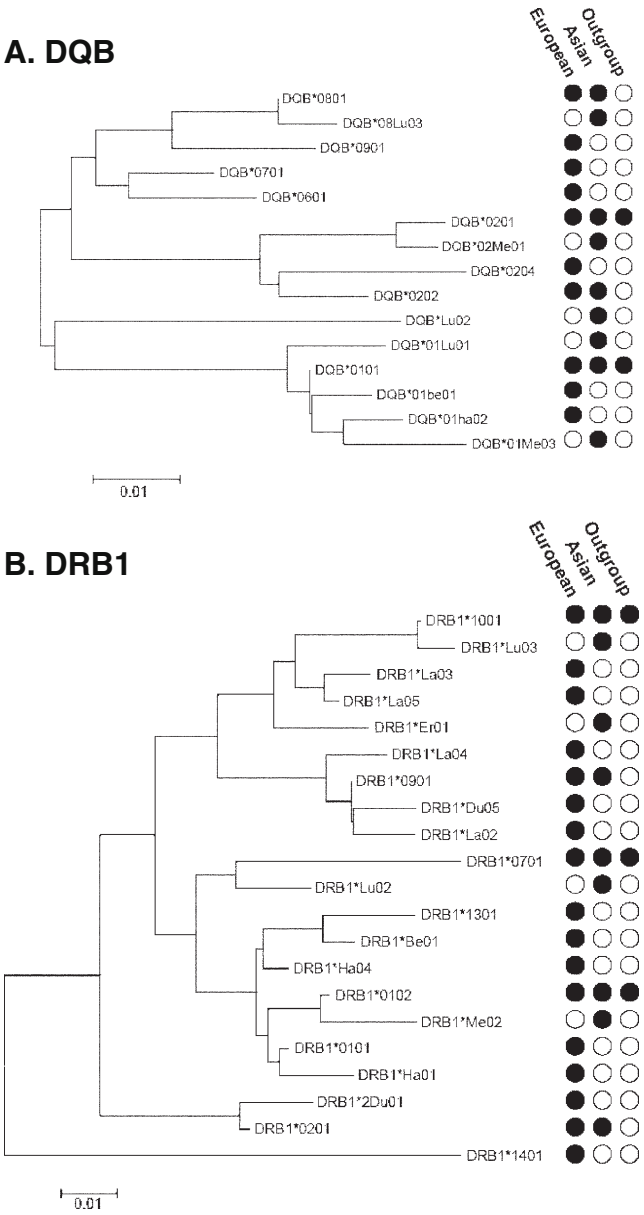

Fig. 3 Phylogenetic relationships of exon 2 of SLA class II alleles. Neighbor-joining tress based on Kimura's two-parameter distances for **a** DQB and **b** DRB1. The population distribution of each allele is indicated by *filled circles*

Discussion

This study was undertaken with the aim of determining the extent which diverse global selective breeding programs affect MHC diversity. An analysis of the second exonic sequences of the porcine MHC class II genes revealed three major findings: (1) the presence of increased sequence variability within expressed MHC genes (compared to a MHC pseudogene), (2) evidence of selective maintenance of MHC diversity, and (3) that selective pressures maintain MHC diversity.

Diversity of MHC genes in global populations

In order to determine the extent of MHC loci diversity, the level of heterozygosity (observed and expected), the number of alleles, and the sequence diversity were examined. MHC class II loci revealed comparable levels of expected heterozygosity ranging from 67% to 93%. These levels of heterozygosity are in accordance with previous findings demonstrating that MHC genes are in linkage disequilibrium (Albert et al. 1977). Conversely, when comparing observed heterozygosity among all loci, the pseudogene revealed significantly lower levels compared to both expressed loci (Table 3). The lack of MHC heterozygote deficiency for the expressed loci suggests a selective advantage for maintenance of heterozygosity which was not observed for the pseudogene (Doherty and Zinkernagel 1975). These data support the overdominance hypothesis or heterozygous advantage characterized as the fitness of the heterozygote being greater than that of a homozygote. Thus, in contrast to the expressed loci, the deficiency in heterozygosity most likely demonstrated reduced pseudogene selective advantage for heterozygotes (Agrawal et al. 2007).

When comparing the number of MHC alleles and level of sequence diversity between the expressed genes and the pseudogene, the pseudogene revealed a greater number of alleles but represented at lower frequencies compared to the

expressed genes. Comparative sequence analyses of these alleles revealed that pseudogene polymorphic sites were randomly distributed throughout the exon leading to the development of many alleles, while polymorphisms within the expressed loci occurred at specific sites (Fig. 1). Additionally, the degree of nucleotide diversity represented at the pseudogene was significantly lower than that observed at the two expressed loci, suggesting that a selective mechanism was maintaining polymorphisms in expressed genes, whereas the pseudogene has no selective advantage (Babik et al. 2005).

Selective maintenance of MHC class II genes

Previous studies using Eurasian beaver (Babik et al. 2005) and Island fox (*Urocyon littoralis dicheyi*; Hedrick 2004; Aguilar et al. 2004) have identified significant levels of MHC diversity which lack genetic diversity across the genome. It was concluded that, even with a lack of genome diversity due to a population bottleneck, these species maintain MHC diversity due to the influence of selection. To address the selective maintenance of porcine MHC diversity, exon 2 synonymous and nonsynonymous substitutions in domestic pigs representing divergent origins were compared to an ancestral Suiformes out-group. The current study revealed evidence of balancing selection at porcine MHC class II genes since (1) significantly higher variability at the amino acid positions in the functionally important ABS than non-ABS positions was observed and (2) an excess of d_N in the ABS was demonstrated.

Within exon 2 of the class II MHC genes, 26 codon positions have been identified in humans as being responsible for direct binding of the antigenic peptide (ABS; Brown et al. 1988). Assuming congruence with the human MHC sequence, of the 29 and 37 porcine polymorphic sites identified for DQB and DRB1, respectively, approximately 50% were in the ABS (Fig. 1). Additionally, the level of ABS codon heterozygosity ranged from 20% to 80% compared to 1–25% heterozygosity at non-ABS codons (Fig. 4). The high level of ABS codon heterozygosity compared to non-ABS suggests that a selective mechanism maintains polymorphisms particularly in the functionally ABS sites (Yuhki et al. 1989). The exon 2 d_N/d_S ratios in the expressed genes demonstrated the influence of selection favoring diversity, whereas the pseudogene ratios suggested the presence of purifying selection (Goldman and Yang 1994). The d_S/d_N ratios provide compelling evidence of past selection operating on the MHC since substitutions were not only favored in the functionally important codons but also substitutions in which the amino acid and potential binding affinity were altered (Jonsson et al. 1989). This is consistent with previous findings that DQB and DRB1 are under diversifying selection due to their role in antigen presentation, whereas pseudogenes such as ΨDRB3 are not influenced by strict selective pressures and appear to be under purifying or neutral selection (Hughes 2007). Furthermore, NJ MHC phylogenetic trees revealed less divergent time between the out-group and the domestic animals for the expressed genes than for the pseudogene, providing further support that selection of MHC expressed genes has contributed towards the maintenance or retention of ancestral alleles (Yang and Bielawski 2000; Fig. 2).

Verification of the maintenance of ancestral expressed MHC alleles was performed by the inclusion of an out-group containing peccaries, Tayassuidae, and bearded pig, *S. barbatus*, which diverged from domestic pigs 33.7 to 23.8 and 1.6 to 6.6 million years ago, respectively (Randi et al. 1996). Five shared alleles were identified in the out-group and in domestic pig populations thus providing evidence of divergence and retention of ancient alleles due to selection within the history of the species (Fig. 3; Chen et al. 2004, 2005).

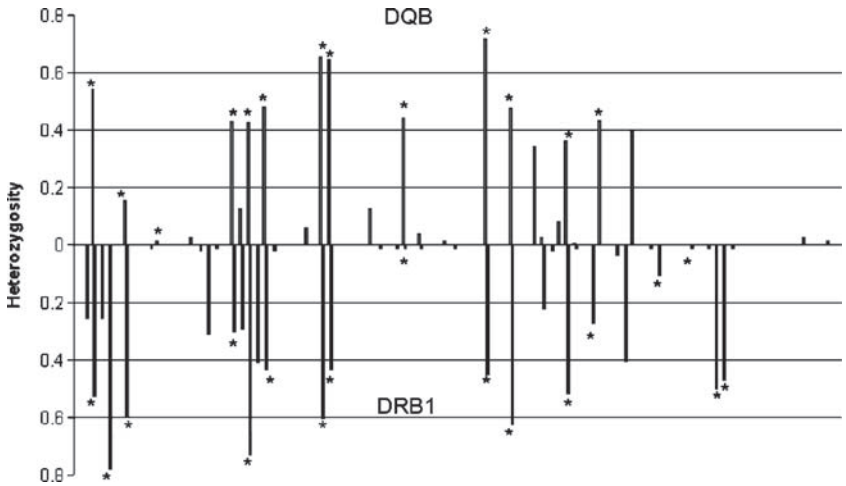

Fig. 4 Amino acid heterozygosity. The heterozygosity at individual amino acid positions. *Asterisks* indicated sites that are putative antigen-binding sites

Global uniformity of MHC variation

The mtDNA and MHC analyses in this study permit an in-depth examination involving demographic changes associated with regions (Kijas and Andersson 2001). The overall phylogenetic distribution of MHC alleles derived from European and Asian origins was inconsistent with patterns observed for mtDNA analyses suggesting that demographic and past population events have not been a predominant force in shaping the pattern of MHC variation (Fig. 2). Consistent with previous studies, mtDNA analyses of this study revealed a division between European- and Asian-derived animals, whereas no such division was observed when analyzing MHC loci diversity (Supp Fig. 1; Fang and Andersson 2006). Distinct division between European and Asian populations observed through mtDNA analyses was most likely the result of shared regional haplotypes, which was additionally supported in this study. Conversely, there were many shared MHC alleles (DQB 4 and DRB1 4) represented in both geographic regions (Fig. 3). The poorly resolved MHC phylogenetic tree may reflect short DNA sequences which were highly polymorphic. Alternatively, the poor MHC phylogenetic resolution represents shared alleles between the European and Asian populations due to long-term balancing selection or hybridization (Aguilar et al. 2004; Schwensow et al. 2008). The possibility of shared alleles being due to hybridization is unlikely in the current study since distinct clades were identified in the mtDNA phylogenetic analysis for those populations originating in Europe and Asia (Supp. Fig. 1). Therefore, the incongruence between the mtDNA and MHC phylogenetic trees suggests that, since MHC alleles were found in both geographic origins and in the out-group, MHC polymorphisms evolved before the split of Suiformes (Klein 1987; Gyllensten et al. 1991). The selective mechanism maintaining the high degree of MHC diversity seems to be relatively similar around the world, most likely the result of balancing selection acting to slow the rate of fixation of alleles causing some to be maintained for millions of years despite geographic separation of populations (Slatkin 2000).

In conclusion, this study revealed that expressed MHC genes exhibit a high degree of diversity especially within the ABS and appear to be selectively maintained. Additionally, analyses of *S. scrofa* and the ancestral Suiformes out-group demonstrated that alleles have been maintained for 20–30 million years. Finally, MHC variation displayed globally uniformity, implying that a selective mechanism(s) maintains high MHC diversity.

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References

Agrawal S, Khan F, Bharadwaj U (2007) Human genetic variation studies and HLA class II loci. Int J Immunogenet 34:247–252, doi:doi:10.1111/j.1744-313X.2007.00683.x

Aguilar A, Roemer G, Debenham S, Binns M, Garcelon D, Wayne RK (2004) High MHC diversity maintained by balancing selection in an otherwise genetically monomorphic animal. Proc Natl Acad Sci U S A 101:3490–3494, doi:doi:10.1073/pnas.0306582101

Aguilar A, Smith TB, Wayne RK (2005) A comparison of variation between a MHC pseudogene and microsatellite loci of the little greenbul (*Andropadus virens*). BMC Evol Biol 5:47, doi:10.1186/1471-2148-5-47

Albert ED, Balner H, Cohen N, Collins NH, David CS, Dorf ME, Duncan WR, Geczy AF, Gotze D, Grosse-Wilde H, Gunther E, Hala K, Ivanyi P, Klein J, Stark O, Streilein JW, Vriesendorp HM, de Weck AL (1977) The major histocompatibility complex system in man and animals. Springer, New York

Babik W, Durka W, Radwan J (2005) Sequence diversity of the MHC-DRB gene in the Eurasian beaver (*Castor fiber*). Mol Ecol 14:4249, doi:10.1111/j.1365-294X.2005.02605.x

Barbosa A, Demeure O, Urien C, Milan D, Chardon P, Renard C et al (2004) A physical map of large segments of pig chromosome 7q11-q14: comparative analysis with human chromosome 6p21. Mamm Genome 15:982–995, doi:10.1007/s00335-004-3008-6

Brown JH, Jardetzky T, Saper MA, Samraoui B, Bjorkman PJ, Wiley DC (1988) A hypothetical model of the foreign antigen-binding site of class-II histocompatibility molecules. Nature 332:845–850, doi:10.1038/332845a0

Brown JH, Jardetzky TS, Gorga JC, Stern LJ, Urban RG, Strominger JL, Wiley DC (1993) Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. Nature 364:33, doi:10.1038/364033a0

Chardon P, Vaiman M, Kirszenbaum M, Geffrotin C, Renard C, Cohen D (1985) Restriction fragment length polymorphism of the major histocompatibility complex of the pig. Immunogenetics 21:161–171, doi:10.1007/BF00364868

Chardon P, Renard C, Vaiman M (1999) The major histocompatibility complex in swine. Immunol Rev 167:179–192, doi:10.1111/j.1600-065X.1999.tb01391.x

Chen F, Xie J, Zhor Y, Li N, Chou K-Y et al (2004) Novel SLA-DR alleles of three Chinese pig strains and the related function in human T cell response. Cell Mol Immunol 1(3):212–218

Chen F, Xie J, Li N, Zhou Y, Xin L, Chou K-Y (2005) Novel SLA-DQ alleles and their recombinant molecules in xenogeneic stimulation of human T cells. Transpl Immunol 14:83–89, doi:10.1016/j.trim.2005.02.002

Clarke B, Kirby DRS (1966) Maintenance of histocompatibility polymorphisms. Nature 211:999–1000, doi:10.1038/211999a0

Doherty PC, Zinkernagel RM (1975) Enhanced immunological surveillance in mice heterozygous at the H-2 gene complex. Nature 256(3):50, doi:10.1038/256050a0

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: an integrated software package for population genetics data analysis. Evol Bioinform Online 1:47–50

Fang M, Andersson L (2006) Mitochondrial diversity in European and Chinese pigs is consistent with population expansions that occurred prior to domestication. Proc R Soc Lond B Biol Sci 273:1803–1810, doi:10.1098/rspb.2006.3514

Figueroa F, Gunther E, Klein J (1988) MHC polymorphism pre-dating speciation. Nature 335(6187):265–267, doi:10.1038/335265a0

Giuffra E, Kijas JMH, Amarger V, Carlborg O, Jeon J-T, Andersson L (2000) The origin of the domestic pig: independent domestication and subsequent introgression. Genetics 154:1785–1791

Goldman N, Yang Z (1994) A Condon-based model of nucleotide substitution for protein-coding DNA sequences. Mol Biol Evol 11:725–736

Gustafsson K, Germana S, Hirsch F, Pratt K, LeGuern C (1990) Structure of miniature swine class II DRB genes: conservation of hypervariable amino acid residues between distantly related mammalian species. Proc Natl Acad Sci U S A 87:9798–9802, doi:10.1073/pnas.87.24.9798

Gyllensten UB, Sundvall M, Erlich HA (1991) Allelic diversity is generated by intraexon sequence exchange at the DRB1 locus in primates. Proc Natl Acad Sci U S A 88:3686–3690, doi:10.1073/pnas.88.9.3686

Hedrick P (2004) Foxy MHC selection story. Heredity 93:237, doi:10.1038/sj.hdy.6800539

Ho C, Rochelle ES, Martens GW, Schook LB, Smith DM (2006) Characterization of swine leukocyte antigen polymorphism by sequence-based and PCR-SSP methods in Meishan pigs. Immunogenetics 58(11):873–882

Hosokawa, T., Tanioka Y., Tanigawa M., Matsumoto Y., Onodera, T., and Matsumoto Y (1998) Cloning and characterization of a new swine MHC (SLA) class II DQB allele. J Vet Med Sci 60(6):725–729

Hosokawa-Kanai T, Tanioka Y, Tanigawa M, Matsumoto Y, Ueda S, Onodera T, Matsumoto Y (2002) Differential alloreactivity at SLA-DR and -DQ matching in two-way mixed lymphocyte culture. Vet Immunol Immunopathol 85:77–84, doi:10.1016/S0165-2427(01)00416-0

Hughes A (1991) Testing for interlocus genetic exchange in the MHC: a reply to Andersson and co-workers. Immunogenetics 33:243–246

Hughes AL (2007) Looking for Darwin in all the wrong places: the misguided quest for positive selection at the nucleotide sequence level. Heredity 99(4):364–373, doi:10.1038/sj.hdy.6801031

Hughes A, Nei M (1992) Maintenance of MHC polymorphism. Nature 355:402, doi:10.1038/355402b0

Jonsson A, Andersson L, Rask L (1989) Selection for polymorphism in the antigen recognition site of major histocompatibility complex class II molecules. Scand J Immunol 30:409–417, doi:10.1111/j.1365-3083.1989.tb02444.x

Kijas JMH, Andersson L (2001) A phylogenetic study of the origin of the domestic pig estimated from the near-complete mtDNA genome. J Mol Evol 52:302

Kimura M, Crow JF (1964) The number of alleles that can be maintained in a finite population. Genetics 49:725–738

Klein J (1987) Origin of major histocompatibility complex polymorphism: the trans-species hypothesis. Hum Immunol 19:155–162, doi:10.1016/0198-8859(87)90066-8

Larson G (2005) Worldwide phylogeography of wild boar reveals multiple centers of pig domestication. Science 307:1618, doi:10.1126/science.1106927

Li W-H, Gojobori T, Nei M (1981) Pseudogenes as a paradigm of neutral evolution. Nature 292:237–239, doi:10.1038/292237a0

Maruyama T, Nei M (1981) Genetic variability maintained by mutation and overdominante selection in finite populations. Genetics 98:441

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16(3):1215, doi:10.1093/nar/16.3.1215

Nei M, Gojobori T (1986) Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 3:418–426

Okumura N, Kurosawa Y, Kobayashi E, Watanobe T, Ishiguro N, Yasue H, Mitsuhashi T (2001) Genetic relationship amongst the major non-coding regions of mitochondrial DNAs in wild boars and several breeds of domesticated pigs. Anim Genet 32:139–147, doi:10.1046/j.1365-2052.2001.00757.x

Randi E, Lucchini V, Hoong Diong C (1996) Evolutionary genetics of the suiformes as reconstructed using mtDNA sequencing. J Mamm Evol 3(2):163, doi:10.1007/BF01454360

Raymond M, Rousset F (1995) Testing heterozygote excess and deficiency. Genetics 140(4):1413–1419

Schwensow N, Eberle M, Sommer S (2008) Compatibility counts: MHC-associated mate choice in a wild promiscuous primate. Proc Biol Sci 275(1634):555–564, doi:10.1098/rspb.2007.1433

Shia YC, Bradshaw M, Rutherford MS, Lewin HA, Schook LB (1995) Polymerase chain reaction based genotyping for characterization of SLA-DQB and SLA-DRB alleles in domestic pigs. Anim Genet 26(2):91–100

Slatkin M (2000) Balancing selection at closely linked, overdominant loci in a finite population. Genetics 154:1367–1378

Smith DM, Lunney JK, Ho C-S, Martens GW, Ando A, Lee J-H, Schook LB, Renard C, Chardon P (2005) Nomenclature for factors of the swine leukocyte antigen class II system, 2005. Tissue Antigens 66:623, doi:10.1111/j.1399-0039.2005.00492.x

Takahata N, Nei M (1990) Allelic genealogy under overdominant and frequency-dependent selection and polymorphism of major histocompatibility complex loci. Genetics 124:967–978

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599, doi:10.1093/molbev/msm092

Vaiman M, Chardon P, Rothschild MF (1998) Porcine major histocompatibility complex. Rev Sci Tech 17(1):95–107

Widera G, Flavell RA (1984) The nucleotide sequence of the murine I-E immune response gene: evidence for gene conversion events in class II genes of the major histocompatibility complex. EMBO J 3(6):1221

Wimmers K, Schellander K, Ponsuksili S (2004) BF, HP, DQB and DRB are associated with haemolytic complement activity, acute phase protein reaction and antibody response in the pig. Vet Immunol Immunopathol 99:215–228

Yang Z, Bielawski J (2000) Statistical methods for detecting molecular adaptation. Trends Ecol Evol 15(12):496–503, doi:10.1016/S0169-5347(00)01994-7

Yeh FC, Boyle T, Rongcai Y, Ye Z, Xin JM (1999) POPGENE, version 1.31. A Microsoft Window Based Free Ware for population genetic analysis. University of Alberta, Edmonton

Yuhki N, Heidecker GF, O'Brien SJ (1989) Characterization of MHC cDNA clones in the domestic cat. Diversity and evolution of class I genes. J Immunol 142(10):3676–3682