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Polymorphisms in *ERAP1* and *ERAP2* are shared by *Caninae* and segregate within and between random- and pure-breeds of dogs

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ABSTRACT

Specific polymorphisms in the endoplasmic reticulum amino peptidase genes *ERAP1* and *ERAP2*, when present with certain MHC class receptor types, have been associated with increased risk for specific cancers, infectious diseases and autoimmune disorders in humans. This increased risk has been linked to distinct polymorphisms in both ERAPs and MHC class I receptors that affect the way cell-generated peptides are screened for antigenicity. The incidence of cancer, infectious disease and autoimmune disorders differ greatly among pure breeds of dogs as it does in humans and it is possible that this heightened susceptibility is also due to specific polymorphisms in *ERAP1* and *ERAP2*. In order to determine if such polymorphisms exist, the ERAP1 and ERAP2 genes of 10 dogs of nine diverse breeds were sequenced and SNPs causing synonymous or non-synonymous amino acid changes, deletions or insertions were identified. Eight ERAP1 and 10 ERAP2 SNPs were used to create a Sequenom MassARRAY iPLEX based test panel which defined 24 ERAP1, 36 ERAP2 and 128 ERAP1/2 haplotypes. The prevalence of these haplotypes was then measured among dog, wolf, coyote, jackal and red fox populations. Some haplotypes were species specific, while others were shared across species, especially between dog, wolf, coyote and jackal. The prevalence of these haplotypes was then compared among various canid populations, and in particular between various populations of random- and pure-bred dogs. Human-directed positive selection has led to loss of *ERAP* diversity and segregation of certain haplotypes among various dog breeds. A phylogenetic tree generated from 45 of the most common ERAP1/2 haplotypes demonstrated three distinct clades, all of which were rooted with haplotypes either shared among species or specific to contemporary dogs, coyote and wolf.

1. Introduction

Misfolded and aggregated proteins (Ruggiano et al., 2014), proteins generated by autophagy (Kobayashi, 2015), and foreign cellular proteins are all subjected to proteolytic digestion and the resulting peptides continuously screened for antigenicity by the major histocompatibility complex (MHC) class I receptors. The endoplasmic reticulum aminopeptidases 1 and 2 (*ERAP1, ERAP2*) are a unique class of proteases found on the luminal side of the endoplasmic reticulum that play a critical role not only in protein cleavage but in preparing the resulting peptides for proper receptor presentation (Hattori and Tsujimoto, 2013). Trimmed peptides resulting from the action of *ERAP1* and *ERAP2* are formed into a peptide-loading complex and transported to the MHC class I receptors by the action of the "transporter associated with antigen processing" genes *TAP1* and *TAP2* (Seyffer and Tampé, 2015). If the peptide is not recognized by any of the receptors, or binds with weak affinity, it will be further degraded and the amino acids recycled. However, if the peptide binds with sufficient affinity, the receptor will activate two processes that will end ultimately in death of the offending cell. The first process involves apoptosis, while the second process involves specific targeting of the cells for destruction by NK cells (innate immunity) or specific $CD8^+$ T cells (adaptive immunity). The affinity with which a particular peptide binds to the MHC class I receptor groove is ultimately dependent on the firmness of ligand/receptor binding. The final structure of both peptide ligand and MHC class I receptor is affected by a large number of nucleotide polymorphisms. Therefore, self/non-self (antigen) recognition is dependent on the makeup of both the peptide and the groove in which the peptide sits and different *ERAP1 and ERAP2*

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polymorphisms can alter that selection of the peptide repertoire within the same MHC class I allele (Reeves et al., 2014).

ERAP1 and *ERAP2* have been implicated in a number of disease processes involving MHC class I, including: 1) cancers, 2) infectious diseases, and 3) autoimmunity (Cifaldi et al., 2011). *ERAP1* and *ERAP2* and MHC class I have received increasing attention in cancer immunity. *ERAP1* and *ERAP2* are universally expressed in human cancers, and the level of MHC class I expression on the tumor cell surface correlates with the level of *ERAP1*, but not *ERAP2*, expression in the cells (Fruci et al., 2006). This suggests that *ERAP* has a role in the generation of the MHC class I repertoire. The expression of *ERAP1* and *ERAP2* varies according to the tumor type and is generally lower in neoplastic cells than normal tissues (Fruci et al., 2008). Down-regulation occurs in ovarian, mammary and lung carcinomas, whereas up-regulation has been observed in colon and thyroid carcinomas (Fruci et al., 2008). Interestingly, expression of ERAPs and MHC class I genes was enhanced by IFN-γ treatment, suggesting that they are both under the control of existing regulatory pathways. Variations in *ERAP1* (Mehta et al., 2009) and MHC class I (Mehta et al., 2008) have been associated with cervical tumor cell survival indicating a role of *ERAP1* and MHC class I for evasion of immune surveillance. This has been supported by experimental studies; treating ERAP1 in T-cell lymphoma lines with small interfering RNA interfered with *ERAP1* expression and enhanced tumor rejection in syngeneic animals by boosting NK- and T-cell-mediated cytolysis (Cifaldi et al., 2011).

Evidence for the role of ERAPs in immunity to infectious agents has come mainly from *ERAP1* deficient (*ERAP1*$^{-/-}$) transgenic mice. *ERAP1*$^{-/-}$ mice are not able to process the immunodominant HF10 decapeptide of *Toxoplasma gondii* and die following experimental infection (Blanchard et al., 2008). The hierarchy of T-cell responses to different immunodominant LCMV epitopes was also markedly changed in *ERAP1*$^{-/-}$ compared to wild-type mice (York et al., 2006). Transgenic mice demonstrate significantly different cytotoxic T cell (CTL) responses to the HLA-B27 associated immunodominant epitope of the influenza virus nucleoprotein depending on whether or not they have deleted or wild-type ERAP and HLA-B27 vs. HLA-B7 haplotypes (Akram et al., 2014). Nucleotide polymorphisms in *ERAP1* have also been associated with susceptibility to HIV-1. Draenert et al. (2004) showed that HIV isolated from individuals with the HLA-B57 MHC class I haplotype possessed a specific mutation in HIV-1*gag* that prevented terminal cleavage by *ERAP1* and diminished CTL responses. Some variants in *ERAP2* have been associated with resistance to HIV-1 infection possibly via the presentation of a distinctive peptide repertoire to CD8^{+} T cells (Cagliani et al., 2010).

Recent genome-wide association studies (GWAS) have linked ERAP1 and ERAP2 nucleotide polymorphisms, along with MHC class I, with autoimmune disease in humans (Fierabracci et al., 2012). A large multinational meta-analysis attributed 26% of the overall incidence for ankylosing spondylitis in a largely Northern European population to specific nucleotide polymorphisms in *ERAP1* (Burton et al., 2007). An interaction between *ERAP1* and *HLA-B27* affecting peptide handling was later shown to be involved in ankylosing spondylitis (Evans et al., 2011). A different ERAP1 variant was associated with the *HLA-B*2705* subtype in Hungarian patients with ankylosing spondylitis (Pazar et al., 2010). Two other recent GWAS identified *ERAP1* and implicated interactions between HLA-C and *ERAP1* in psoriasis (Strange et al., 2010; Sun et al., 2010). A recent meta-analysis of six Crohn's disease GWAS identified *ERAP2* as one of the most interesting candidate genes, bringing the total number of genetic risk factors for the disease to 71 (Franke et al., 2010). An epistatic association between *HLA-B*51* and *ERAP1* has been identified by GWAS for Behçet's syndrome, an arteritis that is often manifested by oral ulcers, retinitis and lesions in other organs (Kirino et al., 2013). Birdshot chorioretinopthy is strongly associated with HLA-A29 (OR = 157.5) and two nucleotide polymorphisms in *ERAP2* (Bakker et al., 2014; Kuiper et al., 2014).

Pure breed dogs have been recognized as excellent models for heritable disorders of humans, whether simple or complex in nature (Karlsson and Lindblad-Toh, 2008; Ostrander and Wayne, 2005). Two of the areas of particular interest to veterinary researchers and those modeling human disease are cancer and autoimmune disease. Increased susceptibility to certain infectious diseases does occur among dog breeds but has not been yet exploited to the degree of cancer and autoimmune disease.

It has been estimated that one-fourth of all dogs in the US will die of cancer with small dogs less susceptible than large dogs (Fleming et al., 2011). However, similar to human families and groups, there is a wide range in the cancer frequency depending on the breed, with the Bernese mountain dog and Golden Retriever being highest (54.6% and 49.9%), and the Pomeranian and Pekinese being the lowest (7.9%) (Fleming et al., 2011). There are also breed-associated differences in the types of cancers. Two of the most common cancers of Golden Retrievers, B-cell lymphoma and hemangiosarcoma, are of particular relevance to humans. Tonomura et al. (2015) conducted a genome wide association study (GWAS) in the breed and identified two loci that appeared to predispose to around 20% of the risk for these particular cancers. These germ-line mutations in B-cell lymphoma and hemangiosarcoma affected pathways involved in T-cell mediated immune response in the tumor, suggesting that an interaction between the immune system and malignant cells plays a common role in the tumorigenesis of these relatively different cancers.

Autoimmune disorders identical to those observed in humans also occur in dogs, and the incidence and breadth of disease tend to be much higher in purebred dogs, which have been associated with small founder size, artificial genetic bottlenecks, inbreeding and loss of heterozygosity (Pedersen et al., 2012a,b Pedersen et al., 2015a,b). The relationship of the canine MHC or dog leukocyte antigen (DLA) complex with autoimmune disease has received a great deal of study over the last two decades. The predisposition for autoimmune diseases of dogs appear mainly to be of an ancestral origin as for humans, and are increased through inadvertent human directed positive selection for certain phenotypic traits and associated inbreeding (Pedersen et al., 2012a,b Pedersen et al., 2015a,b). Many of the autoimmune disorders recognized in pure breeds of dogs have been associated with specific DLA class I and II haplotypes, but these associations have varied in strength and are not always apparent. However, DLA class I and II haplotype diversity has been greatly affected by pure breeding and certain haplotypes predominate in each breed studied (Pedersen et al., 2015a,b). It is possible, therefore, that the tendency of certain DLA class I and II polymorphisms to associate with autoimmune disease is also dependent on the ERAP1 and ERAP2 haplotypes that have been co-inherited by descent during breed development.

There are breed differences in susceptibility to infectious diseases, although these potentially heritable differences have not been as extensively investigated as cancer and autoimmune disease. A significant association was found between certain STR alleles in the DLA class II region and disseminated demodectic mange (It et al., 2010). Resistance to canine leishmaniasis has been linked to two loci on canine autosomes 1 and 2 associated with pathways involved in T helper cell function and macrophage signaling (Utsunomiya et al., 2015). There are other potentially genetic susceptibilities to infectious agents that remain to be determined. Rottweilers, Doberman Pinschers, Labrador Retrievers, American Staffordshire Terriers and German shepherd dogs develop more severe canine parvovirus associated enteritis with a higher mortality (Day, 1999; Glickman et al., 1985; Goddard and Leisewitz,

2010; Godsall et al., 2010). The German shepherd dog is also much more prone to developing systemic aspergillosis infection (Berry and Leisewitz, 1996; Kabay et al., 1985; Schultz et al., 2008), tropical canine pancytopenia (*Ehrlichia canis*) (Huxsoll et al., 1970) and Rocky Mountain spotted fever (*Rickettsia ricketsii*) (Greene and Breitschwerdt, 2006).

Dogs, like humans, have both *ERAP1* and *ERAP2* and their basic structure is conserved between species. Numerous genetic polymorphisms have been recognized in these genes between individuals and races of humans, and such polymorphisms presumably exist among dogs in general, pure breeds in particular, and between closely and distantly related canids. It is also possible that certain ERAP1/ERAP2 polymorphisms, when associated with certain DLA class I and II types, may also affect the incidence of cancer, infections, and autoimmune disorders. Therefore, the present study involved sequencing the exons of *ERAP1* and *ERAP2* of 10 dogs of 9 different breeds and identifying significant exonic SNPs leading to amino acid changes, insertions and deletions. These polymorphisms were found to be in strong linkage disequilibrium and therefore creating distinct haplotypes both within and between ERAPs. These SNPs were integrated by a Sequenom MassARRAY iPLEX based assay panel, which was used in turn for identifying the various ERAP1 and ERAP2 haplotypes in indigenous, mixed and pure breeds of dogs, several regional species of wolves, coyote, jackal, and red fox. The study has confirmed that polymorphisms exist in both *ERAP1* and *ERAP2* of dogs and that there is extensive haplotype sharing between dogs, wolves, coyotes, and jackals but not with red fox. The various ERAP1 and ERAP2 haplotypes segregated among various dog breeds, as has been previously shown for canine MHC (DLA) associated class I and II haplotypes (Pedersen et al., 2015a,b).

2. Materials and methods

2.1. DNA samples

DNA was isolated from either whole blood or cytology brushes (buccal swabs) by previously published procedures (Irion et al., 2003). All of the wolf, coyote, jackal, and red fox samples were from the collection of the Mammalian Ecology and Conservation Unit, School of Veterinary Medicine, UC Davis, Directed by Dr. Ben Sacks. This resource has been used in a number of past studies (Brown et al., 2011; Irion et al., 2005; Norén et al., 2015; Sacks et al., 2004, 2013; Safra et al., 2015; Statham et al., 2014). The 27 wolf samples included 19 wolves from the Yukon region of Canada, four Chinese wolves, two Iranian wolves, and two Eastern wolves from the USA. The 49 coyote samples were from California. Three Golden jackals were from Eurasia and one Black-backed jackal was from Africa. The 51 Red fox samples were from North America and Europe. The 16 dingo samples were from Australia and the 46 village dog samples came from across the Middle East, SE Asia, Taiwan and Island Pacific Nations. DNA of selected breeds was extracted from blood samples submitted for various clinical tests to the Hematology Laboratory of Veterinary Clinical Service, UC Davis.

2.2. Sequencing of ERAP1 and ERAP2 from dogs and SNP identification

The coding sequences of canine *ERAP1* and *ERAP2* were found at: http://uswest.ensembl.org/index.html, accessed 06/07/2016. Primers were from intronic regions flanking the desired exons and evaluated with NetPrimer www.premierbiosoft.com/netprimer/, accessed 06/07/2016. Forward and reverse primers used to amplify *ERAP1* and *ERAP2* are shown in Tables 1 and 2. Amplification included initial denaturation at 94 °C for 3 min followed by 30 cycles at 94 °C × 30 s, 60 °C × 30 s, 68 °C × 1 min followed by 10 min final extension at 68 °C. PCR products were digested with ExoSap (USB, Cleveland, OH) per manufacturer's recommendations and directly sequenced using the BigDye terminator Sequencing Kit v3.1 (Applied Biosystem/Life Technologies, Carlsbad, CA). The sequencing products were purified with Edge Biosystem Performa DT plates (Edge-Bio, Gaithersberg, MD) according to manufacturer's recommendations, and electrophoretically separated on ABI 3730 DNA analyzer (Applied Biosystems/Life Technologies, Carlsbad, CA). Sequences were verified and aligned using Vector NTI Advance Software (Invitrogen, USA).

2.3. Sequenom assay for SNPs identified from ERAP1 and ERAP2 sequencing of 10 dogs

Sequences from exons 3–20 of *ERAP1* and 1–20 of *ERAP2* were obtained from 10 dogs of the following breeds: Pug, Maltese, Beagle, Chihuahua, Golden Retriever, Yorkshire terrier, Miniature Poodle, Standard Poodle, and Italian greyhounds. It was not possible to amplify sequence from exon 1 and 2 of *ERAP1* using primers based on published sequence in Ensemble. Sequences of the 10 dogs were compared and SNPs coding for synonymous and non-synonymous amino acid changes and insertion/deletion mutations were identified (Figs. 1 and 2). Eight SNPs from *ERAP1* and 10 SNPs from *ERAP2* were then selected for optimal performance in Sequenom testing (Mass ARRAY® version 4.0.0.2 Compact 96 System with iPLEX Gold technology, Agena Bioscience Inc, San Diego, CA). The PCR and extension primer sequences of the SNPs used in this assay are given in Table 3. Haplotypes were identified with phase software (Stephens et al., 2001).

3. Results

3.1. Haplotypes based on phase analysis of ERAP1 and ERAP2 SNPs

The Sequenom assay was used to determine the SNPs in a number of indigenous and purebred dogs, wolves, coyotes, jackals and red fox (Tables 4 and 5). Phase analysis of SNPs defined 24 haplotypes for *ERAP1* and 36 in *ERAP2* among all of the species of *Caninae* tested.

3.2. Species specificity of canid ERAP1 and ERAP2 haplotypes

ERAP1-*24,* was found only in the wolf and ERAP1-*5* only in the coyote (Table 4). Only two haplotypes, ERAP1-*13* and *14* were detected in the red fox, but haplotype *13* was found in 99% of foxes tested. ERAP1-*7* and *8* were shared only by wolf and coyote, while ERAP1-*9, 11* and *17* were shared by wolf, coyote and Jackal, but were not found in dogs. Six haplotypes, ERAP1-*22, 21, 12, 20, 23* and 18, were found only in dogs. All other ERAP1 haplotypes were shared to varying degrees between dogs, wolf, coyote and jackal.

ERAP2-*9, 11, 25 26*, and *35* were found only in wolves and ERAP2-*22, 23*, and *31* only in coyote (Table 5). ERAP2-*16* was the sole haplotype observed in the jackal and red fox and shared at low frequency with wolf, coyote and village dogs. Twenty seven ERAP2 haplotypes were at various frequencies among the dog tested. Sixteen of the 27 haplotypes were found only in dogs and 11 shared at various frequencies with wolf, coyote, jackal and red fox.

3.3. Comparisons of ERAP1 and ERAP2 haplotype frequencies among various dog random- and pure-breed dogs

Randomly breeding village dogs possessed nine of the 16 ERAP1 haplotypes found among all dogs, while the dingo possessed five.

Table 1
Primer sequences for *ERAP1*.

Primer ID	Primer seqence		Location	
NP389(K9ERAP1-E1f)	CCGTCGGCCCCCTCAT	chr3	15761740	15761760
NP390(K9ERAP1-E2r)	CTGCTGCCTCTGCGGATTAG	chr3	15762567	15762586
NP391(K9ERAP1-E3f)	CGACCAAGAACCCTGACTGATAGA	chr3	15766706	15766729
K9ERAP1-E3r	CCTCCAGAACGGTGAGAGCATA	chr3	15767868	15767889
K9ERAP1-E4f	TAAGTTTTCATTATCTGTCAAGGCTATT	chr3	15769019	15769046
K9ERAP1-E4r	GCCCTCCTGTCAGCACCA	chr3	15769631	15769651
K9ERAP1-E5f	CGGTATTTAAGGTCAGGTGGACTAC	chr3	15771676	15771700
K9ERAP1-E5r	CGTTGTAAATACCAGCATCTGTGAC	chr3	15772639	15772663
K9ERAP1-E6f	GGTCCTACTGTTACTTCATTTCTGGC	chr3	15775074	15775099
K9ERAP1-E8r	GAAATAAGCCTTTGTGACACCTCTG	chr3	15776976	15777000
K9ERAP1-E9f	CACCTAAAATTGTTTGTTGGATGC	chr3	15778255	15778278
K9ERAP1-E9r	TGATAAGAGGCAGAGTTACTGTAGGC	chr3	15778813	15778838
K9ERAP1-E10f	GACTCTCCCAAGATAAAGTGTGATGA	chr3	15782084	15782109
K9ERAP1-E11r	ATGTGGAATAAGAGTGATATGATGAGG	chr3	15782593	15782619
K9ERAP1-E12f	GTGCCCAGTAGAACCACACAAAG	chr3	15783755	15783777
K9ERAP1-E12r	GCCGTGGCTTAGTTCAGAGTTC	chr3	15784452	15784473
K9ERAP1-E13f	AAGTCCAAGAAGAATGAGTCGTGC	chr3	15790052	15790075
K9ERAP1-E14r	GCTGCGGGATAAGTTGGTCTC	chr3	15791295	15791315
K9ERAP1-E15f	TTGTATCTGATTGAATGCTTGTTGG	chr3	15792209	15792233
K9ERAP1-E15r	TTCTCGTGCTGGGTCTTAGTGTC	chr3	15792651	15792673
K9ERAP1-E16f	CAGATACTTGAGGATGAGGCACG	chr3	15793569	15793591
K9ERAP1-E19r	GTTGATGTCCAAACACCACGG	chr3	15796724	15796744
K9ERAP1-E20f	CTGGGTGGACGCAGCAAC	chr3	15801075	15801096
K9ERAP1-E20r	CTTTAGAGGACAATGACTCAACAGC	chr3	15801894	15801918
K9ERAP1-E3s	TGTATCTCTATCTGTTAATCCTGGGTAG	chr3	15766870	15766897
K9ERAP1-E16s	CAGTGGTTGCGTATCTGCCTC	chr3	15793824	15793844
K9ERAP1-E17s	CATGTTGCTCCGATCACTGG	chr3	15795075	15795094
K9ERAP1-E18s	GAACAGGAGGGCTTCATGTAGG	chr3	15796198	15796219
K9ERAP1-E7s	GATTCCGCCCAAACCAGC	chr3	15776099	15776119

Table 2
Primer sequences for *ERAP2*.

Primer ID	Primer sequence		Location	
K9-ERAP2-E1f	GGACCTAGACGACTTGAATGGC	Chr3	12820025	12820046
K9-ERAP2-E1r	GTGTCTGGATAAGGAACCTAAGAGTAAG	Chr3	12819274	12819301
K9-ERAP2-E2f	TAACAGCAGGTTTTCTCCATTCAC	Chr3	12817109	12817132
K9-ERAP2-E2r	TGTCTCAACACAGTCACATACTCATCT	Chr3	12816090	12816116
K9-ERAP2-E3f	GGAGGTCATGTAAGTGGACTGTGC	Chr3	12813324	12813347
K9-ERAP2-E3r	AGTGCTGCTCTGTCTGGGTGG	Chr3	12812613	12812633
K9-ERAP2-E4f	GCATCAAGTGTCCTTCAAAGTGTC	Chr3	12809393	12809416
K9-ERAP2-E5r	GCTTCCTGGGCTAACTGAATCTC	Chr3	12807309	12807331
K9-ERAP2-E6f	GGTGCAGAAGCCATAATGATCA	Chr3	12805367	12805388
K9-ERAP2-E6r	ATGTATCTGCATAATCCATACCTTCC	Chr3	12804892	12804917
K9-ERAP2-E7f	TCTAATAACATTGAGTCTTTGCCTCCT	Chr3	12802214	12802240
K9-ERAP2-E9r	TCTGCCTTCTATGGACCCTGG	Chr3	12800637	12800657
K9-ERAP2-E10f	TGGACTTCCTATGGTCACTGCC	Chr3	12798182	12798203
K9-ERAP2-E11r	CGGAGAAAGAGCATCCAGCC	Chr3	12796465	12796484
K9-ERAP2-E12f	TAAGTCCTGGCAATGTCTATGTGTC	Chr3	12795109	12795133
K9-ERAP2-E14r	CCAACAATCAATGACCAACACTG	Chr3	12792777	12792799
K9-ERAP2-E13f	CTACCTGGGTCTGGCAATGTG	Chr3	12794245	12794265
K9-ERAP2-E15f	TGCCCCCATCATTGCTCTC	Chr3	12789240	12789259
K9-ERAP2-E18r	TAATTCCCAGCATGGCTTTCC	Chr3	12788209	12788229
K9-ERAP2-E19f	CAGGGGTGATTCTGTTATAGCAGTT	Chr3	12785771	12785795
K9-ERAP2-E20r	CAGGAGCAGGCTGGAGTGAG	Chr3	12784291	12784310
K9-ERAP2-E21f	ATAAATTTGATACACTTCATAGTTTTGGG	Chr3	12782982	12783010
K9-ERAP2-E23r	CAATGTAGATAATGTGCCGTGTGG	Chr3	12781557	12781580
K9-ERAP2-E7r	GCACCATCTTGTCCTACATCATTG	Chr3	12801923	12801946
K9-ERAP2-E20f	ATTAAAAATGTTAGCCAGGAAGGAA	Chr3	12785123	12785147

The number of ERAP1 haplotypes in specific pure breeds tested ranged from three (German shepherd) to eight (Standard Poodles, Jack Russell terrier) with average of 5.83 haplotypes/breed. ERAP1-*1, 3, 15* and *22* occurred at comparatively high frequency among all pure breeds of dogs, followed in frequency by ERAP1-*2, 6* and *21* (Table 4). ERAP1-*12* was common in dingo, village dogs and Standard Poodles, while ERAP1-*20* was prevalent among Alaskan and Siberian huskies. The lowest ERAP1 haplotype diversity, besides in the red fox, was in the dingo, German shepherd, Beagle and Alaskan husky, with a single haplotype dominating in over 50% of individuals.

The ERAP2 haplotypes identified in random- and pure-breeds of dogs also varied both in number and type. Among random-bred dog populations, village dogs possessed 15 of the 27 ERAP2 dog haplotypes, while the dingo possessed only four. The number of ERAP2 haplotypes in pure breeds of dogs ranged from three (Pug, Yorkshire terrier) to eight (Italian greyhound, Jack Russell terrier) with an average of 5.15 haplotypes/breed. ERAP2-*1, 27* and *34* were the predominant haplotypes among dogs in general, while ERAP2-*33* was common in Dingo. A single ERAP2 haplotype was found in 50% or more dingos (0.5), Alaskan huskys (0.67), German shepherds (0.75), Labrador retrievers (0.54) and Pugs (0.75).

Table 3
Sequenome primer sequences for ERAP1 and ERAP2 SNPs.

SNP_ID	1st-PCR primer	2nd-PCR primer	UEP_SEQ (Extension primer)	UEP_DIR	EXT1	EXT2
ERAP1-ex03a	ACGTTGGATGGGTATTCCAGGACTCTCAAG	ACGTTGGATGCTGCAAGTATCTAAGGCCAC	gGGGTTCTGCAGTCAG	R	G	A
ERAP1-ex03b	ACGTTGGATGCAAATCGCACTTGTGGCTTC	ACGTTGGATGCAATGACAACTGTGTACGGG	cCTTCCGAGCCTCTGGTG	F	G	T
ERAP1-ex07	ACGTTGGATGATGCTGAGAAGTCTTCTGCG	ACGTTGGATGATTTTGTGTACCTGGTGGGC	ggggCTGCGTCAGATAAGCTTG	F	A	G
ERAP1-ex08	ACGTTGGATGAACGACAGATCAACCTGTTA	ACGTTGGATGTGTGTCTGTCAGTGTGACTC	AATAAGTAAATACTTACAACTTTCA	R	C	A
ERAP1-ex11b	ACGTTGGATGTGGATGGCTTTTGCTCTAGG	ACGTTGGATGGTAAAAACCTGGGCTTAGGG	cccCTCTAGGGGCTCACGTTCATC	F	DEL	TTC
ERAP1-ex13	ACGTTGGATGGGCATGTTCCATTGACCTTC	ACGTTGGATGCCAGTCACTTCTGCAATACG	ccCCTTCATTACCAGCAAATC	F	A	G
ERAP1-ex14	ACGTTGGATGTTGTTAATGAGACTGGCCCG	ACGTTGGATGGAGGATGATGGATGGGATTC	CGCCCCTTTTAAAAGAG	R	G	A
ERAP1-ex18	ACGTTGGATGTTCCTGCCAACAGTTCTAAG	ACGTTGGATGTCTAGGCTACTAGATGAAAG	ggGGAAACTCTTGAAGTTTTATTAC	R	T	C
ERAP2-ex01	ACGTTGGATGGGGCTTGAAGCAAGACAAAG	ACGTTGGATGCACTGGCTGGTTCATTATGG	AGCAAGACAAAGTCTTGTG	F	C	G
ERAP2-ex02a	ACGTTGGATGACAGGAAACCAGGGAAAAGG	ACGTTGGATGTTCTCTGGAACCAACAGTGC	GGAAAAGGCTGAACATTT	F	C	T
ERAP2-ex02c	ACGTTGGATGCTCTGTGGCAATTGATTTCC	ACGTTGGATGCACCATGAGTTCTGTATGTG	ctccATTGATTTCCAGGCCAA	F	C	T
ERAP2-ex04	ACGTTGGATGTACGTAGGCTACAAGGTATG	ACGTTGGATGCAATTGAACTTGAGGGAGGC	ACAAGGTATGTACTCATTTTTAC	R	T	A
ERAP2-ex11a	ACGTTGGATGGAAGTCCTTTCTGCAGAGTC	ACGTTGGATGGTCATTTCAGCTGGCCTTTC	CCAGGTCCTCATCATCT	R	G	C
ERAP2-ex11b	ACGTTGGATGTCCTTGAAGACTCCGCTCAG	ACGTTGGATGCCTGGTGATTGAACGAGAAG	gaatCGCTCCTGCCTCAGTTT	R	T	C
ERAP2-ex13	ACGTTGGATGGAACGTGTCTATGAACCACA	ACGTTGGATGTCTGACATAGGTTCCTCTGG	agGAACCACATTAGAGGAACT	R	G	A
ERAP2-ex14	ACGTTGGATGTGTAGTAGCCGTTTGAGTCC	ACGTTGGATGTCCTCCTTCTAGACACTCTG	CCACATTGAATTTCACCCA	R	T	A
ERAP2-ex15	ACGTTGGATGTTCAGTGCAGGAAGGTTGAC	ACGTTGGATGAGTGCAGGGATGCTTGTTTC	ACCCTAGACAAAGCCCTTGA	F	C	T
ERAP2-ex18	ACGTTGGATGTCACTCCAGCTTTGCATGTC	ACGTTGGATGTGTGCTTTCTGCAGCGTTAC	ATGTCAATCACCGGC	R	G	A

Table 4
ERAP1 haplotypes and their frequency in various canid populations. Other breeds consists of 53 dogs of a number of breeds in addition to the 15 pure breeds tested.

ERAP1	ERAP1-hap	Wolf	Coyote	Jackal	Fox	Dingo	Village-dog	Ita. Greyhound	ST. Poodle	Alaskan Husky	Siberian Husky	Aus. shpeherd	Germ. shpherd	Beagle	Boxer	Eng. Bulldog	Chihuahua	Gold. Retriever	Jack Russell	Labrador	Pug	Yorkshire	Other breeds
24	ATACTGAT	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
5	GGACTGAC	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
19	GTGCTGGT	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0
7	GGACDAGC	0.04	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8	GGACDAAC	0.13	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	GGACDGGT	0.2	0.01	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	GGACDGAT	0.04	0.06	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
17	GGGCTGAT	0.06	0.01	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10	GGACDGAC	0.02	0.05	0	0	0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
14	GGGCTAGT	0	0	0.13	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16	GGGCTGGT	0	0.35	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0.03
13	GGGCTAGC	0	0.05	0	0.99	0	0.01	0	0	0	0.07	0	0	0	0	0.05	0	0	0	0	0	0	0.01
4	GGACTGGT	0.06	0.05	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0.01
6	GGACTGAT	0.19	0	0.13	0	0	0	0	0.01	0	0	0.1	0	0	0	0	0.1	0	0.15	0.29	0	0.05	0.08
2	GGACTAGT	0.02	0	0	0	0	0.06	0.14	0.07	0	0	0.25	0	0	0	0	0.1	0.05	0.05	0	0	0	0.03
1	GGACTAGC	0.19	0.02	0.13	0	0.03	0.15	0.46	0.1	0	0.14	0.1	0.3	0.55	0.1	0.25	0.1	0.15	0.35	0.21	0.15	0.45	0.26
3	GGACTGGC	0.04	0.1	0	0	0.25	0.24	0.06	0.19	0.04	0.07	0.05	0	0.25	0.3	0.3	0.15	0	0.15	0.08	0.35	0.1	0.24
15	GGGCTGGC	0	0.26	0.13	0	0.06	0.13	0.14	0.16	0	0.07	0.2	0.1	0.1	0.5	0.35	0.15	0.3	0.1	0.08	0.05	0.1	0.13
22	ATACTGGC	0	0	0	0	0	0.07	0.01	0.09	0.29	0.29	0.1	0.6	0.1	0.05	0.05	0.3	0.5	0.05	0.29	0.45	0.3	0.14
21	ATACTAGT	0	0	0	0	0	0.07	0.18	0.01	0.08	0.07	0.2	0	0	0	0	0.05	0	0	0.04	0	0	0.02
12	GGAADGAC	0	0	0	0	0.63	0.23	0	0.37	0	0	0	0	0	0	0	0	0	0	0	0	0	0.04
20	ATACTAGC	0	0	0	0	0	0	0	0	0.58	0.29	0	0	0	0	0	0	0	0	0	0	0	0
23	ATACTGGT	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0	0
18	GTGCTAGC	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
	# Individual	**27**	**49**	**4**	**51**	**16**	**41**	**198**	**260**	**12**	**7**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**10**	**12**	**10**	**10**	**52**

Table 5
Forty five of the 128 pruned ERAP2 haplotypes and their frequency in various canid populations. Other breeds consists of 53 dogs of a number of breeds in addition to the 15 pure breeds tested.

ERAP2	ERAP2-hap	Wolf	Coyote	Jackal	Fox	Dingo	Village-dog	Ita. Greyhound	ST. Poodle	Alaskan Husky	Siberian Husky	Aus. shpeherd	Germ. shpherd	Beagle	Boxer	Eng. Bulldog	Chihuahua	Gold. Retriever	Jack Russell	Labrador	Pug	Yorkshire	Other breeds
9	GTTAGCGTCG	0.11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11	GTTACCGTCA	0.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26	CTTAGCGTTA	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
25	CTTAGCGTCG	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
35	CTTACCAACA	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
32	CTTACCATCA	0.06	0.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
31	CTTACCGTCA	0	0.09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
22	CTTAGTGTCG	0	0.03	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
23	CTTAGTGTTA	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
29	CTTAGCATCA	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
19	CTTTCCATCA	0	0.08	0	0	0	0	0	0	0	0.07	0	0	0	0	0	0	0	0	0	0	0	0
14	CTTTGTGTCA	0	0.08	0	0	0	0.01	0	0	0	0.14	0	0	0	0	0	0	0	0	0	0	0	0.05
7	GTTTGTGTCA	0	0.06	0	0	0	0.02	0	0	0	0	0.25	0	0	0	0	0.05	0.1	0	0	0	0	0
16	CTTTGCGTCA	0.04	0.03	1	1	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	CTTAGTGTCA	0.07	0.43	0	0	0	0.02	0.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
28	CTTAGCGACG	0.24	0	0	0	0	0.2	0	0.37	0	0	0	0.05	0	0	0	0	0	0.2	0	0	0	0.04
24	CTTAGCGTCA	0.04	0.02	0	0	0	0.01	0	0	0	0	0	0	0	0.05	0.05	0.05	0	0.05	0	0	0	0.01
27	CTTAGCGACA	0.28	0	0	0	0	0.13	0.18	0.12	0.67	0.29	0.1	0.1	0.2	0	0	0.1	0	0	0.04	0	0	0.13
34	CTTACCATTA	0.02	0.01	0	0	0	0.06	0.02	0.06	0.08	0	0.5	0.75	0.1	0.3	0.3	0.2	0.5	0.15	0.54	0.75	0.45	0.23
33	CTTACCATCG	0.02	0	0	0	0.5	0.06	0	0.06	0	0	0	0.05	0	0	0	0.1	0	0.05	0	0.15	0	0.01
1	GTCTGTGTCA	0	0	0	0	0.06	0.32	0.31	0.33	0.13	0	0.1	0	0.25	0.35	0.3	0.3	0.35	0.35	0.21	0.1	0.5	0.28
30	CTTAGCATCG	0	0	0	0	0.19	0.05	0.01	0	0	0	0	0.05	0	0	0.3	0.1	0	0	0.17	0	0.05	0.07
17	CTTTGCGACG	0	0	0	0	0	0.02	0.18	0.03	0	0	0	0	0.3	0	0	0	0	0	0	0	0	0
12	CTCTGTGTCA	0	0	0	0	0	0.01	0	0.02	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0.02
15	CTTTGTGACA	0	0	0	0	0	0.01	0	0	0.08	0.43	0	0	0	0	0	0	0	0	0	0	0	0.01
36	CCTTGTGTCA	0	0	0	0	0	0.01	0	0	0	0.07	0	0	0	0.3	0	0	0	0	0	0	0	0.03
20	CTTTCCATCG	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0	0
5	GTCAGCATCG	0	0	0	0	0	0	0.15	0	0	0	0	0	0.05	0	0	0	0	0	0.04	0	0	0.05
13	CTCTGCGACA	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0.05	0.05	0	0	0	0.01
8	GTTTGCGACG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0.05	0	0	0	0
4	GTCAGCGACA	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	GTCACCATTA	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0	0	0	0	0
2	GTCTGTGTCG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0.03
3	GTCTGTGTTA	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02
18	CTTTGCATCG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
37	CCTTGCGACG	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01

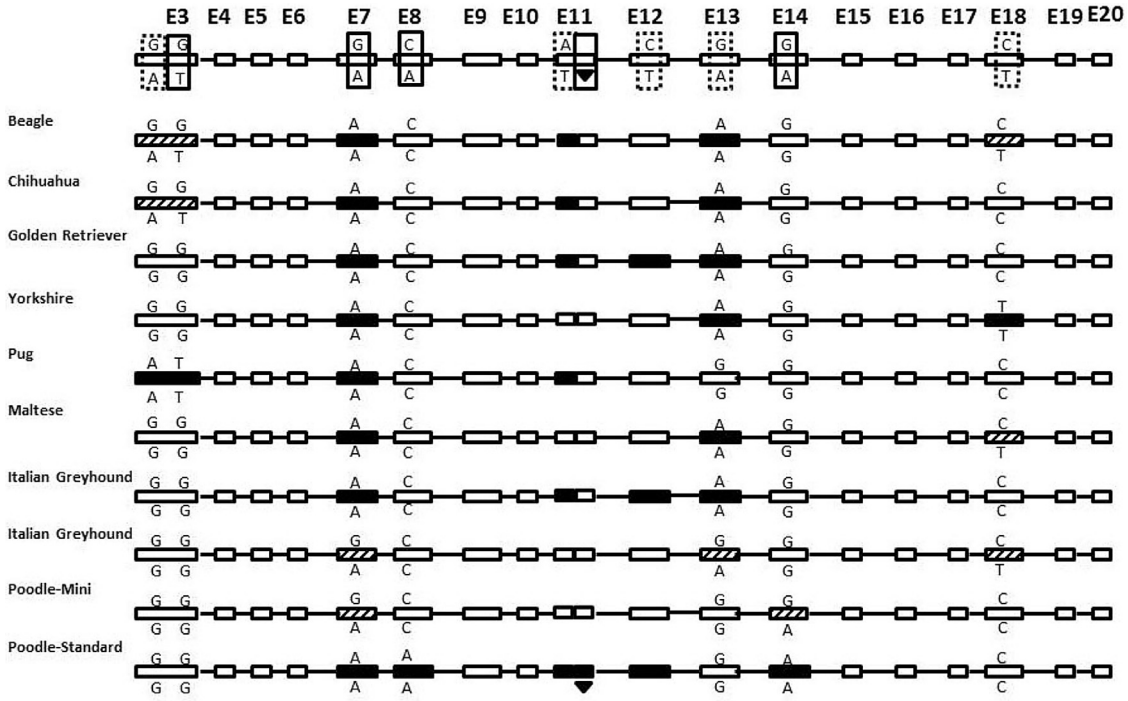

Fig. 1. Depiction and characterization of polymorphisms identified in exons 3–20 of canine *ERAP1*.

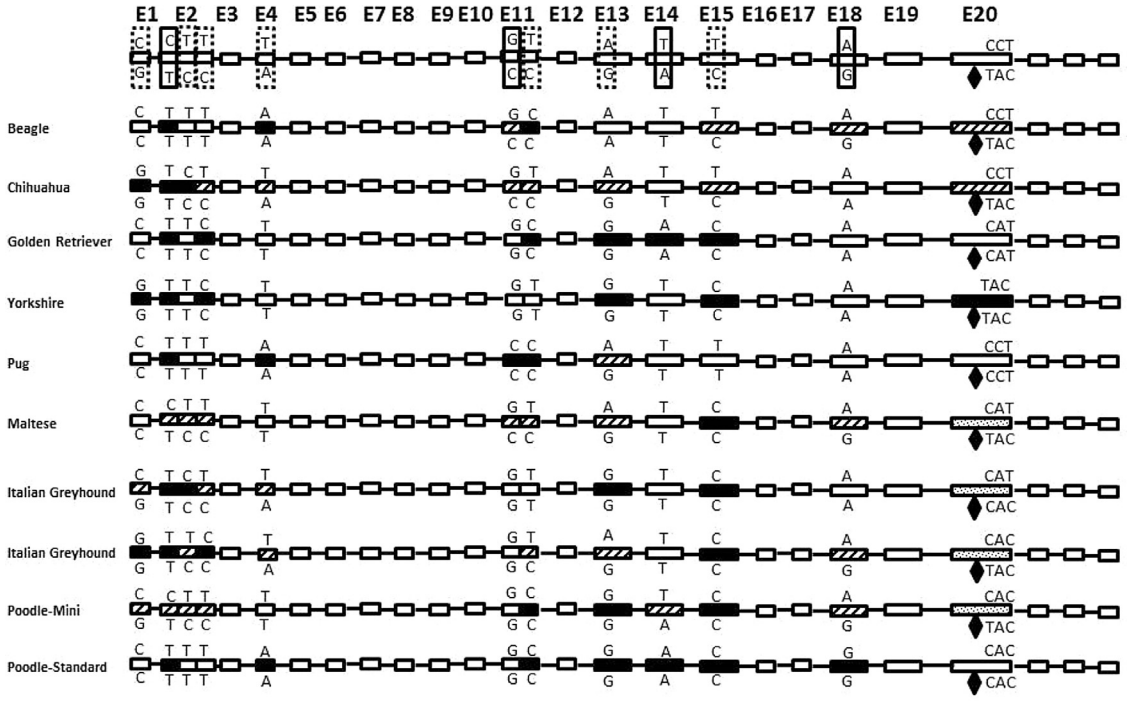

Fig. 2. Depiction and characterization of polymorphisms identified in exons 1–20 of canine *ERAP2*.

3.4. Recombination between ERAP1 and ERAP2 haplotypes

There was significant linkage disequilibrium between *ERAP1* and *ERAP2*. Although linkage might be expected given their 98 kb proximity on CFA3, recombination between ERAP1 and ERAP2 haplotypes was actually common as identified by Phase analysis. Recombination resulted in 128 extended ERAP1/2 haplotypes, of which 45 were identified at least four or more times among the 1658 total haplotypes that were interrogated (Table 6). This pruning was done to eliminate possible errors in phasing that might occur between rare haplotypes.

3.5. Phylogenetic relationship of ERAP1 and ERAP2 haplotypes among Caninae

The 45 extended ERAP1/ERAP2 haplotypes were used to construct a phylogenetic tree of haplotype evolution between the various canids (Fig. 3). Three major clades (1–3) and six subclades

Table 6
Forty five common ERAP1/2 haplotypes and their frequency in various canid populations. Other breeds consists of 53 dogs belonging to breeds other than the 15 pure breeds listed.

ERAP1/2	Wolf	Coyote	Jackal	Red Fox	Dingle	Village dog	Ita.Greyh.	ST. Poodle	A.Husky	S.Husky	Aus.Sheph	Germ.Sheph	Beagle	Boxer	Eng.Bulldog	Chihuahua	Gold	Jack Retriv.	Lab	Pug	York	Other breeds
9/28	0.13	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6/9	0.09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
8/27	0.07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15/32	0	0.09	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15/21	0	0.07	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15/19	0	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
10/21	0	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
16/32	0	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13/16	0	0	0	0.99	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1/27	0.15	0	0	0	0	0.02	0	0	0	0.07	0	0.1	0.1	0	0	0	0	0	0	0	0	0.03
8/28	0.06	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2/21	0.04	0	0	0	0	0	0.14	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3/16	0.04	0.01	0.13	0	0	0.02	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6/34	0.02	0	0	0	0	0	0	0	0	0	0.1	0	0	0	0	0.05	0	0	0.29	0	0.05	0.03
16/21	0	0.16	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
16/31	0	0.08	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0
3/21	0	0.06	0	0	0	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
4/14	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
15/7	0	0.02	0	0	0	0.02	0	0	0	0	0	0	0	0	0	0.05	0.05	0	0	0	0	0
15/34	0	0.01	0	0	0	0.01	0.02	0	0	0	0.15	0.05	0	0.25	0.3	0	0	0.05	0.04	0.05	0.1	0.04
1/1	0	0	0	0	0	0.09	0.12	0.04	0	0	0.05	0	0.15	0.1	0.25	0.1	0.1	0.25	0.13	0.1	0.4	0.13
1/5	0	0	0	0	0	0	0.15	0	0	0	0	0	0.05	0	0	0	0	0	0.04	0	0	0.04
1/17	0	0	0	0	0	0.01	0.18	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0
1/33	0	0	0	0	0	0.01	0	0.05	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0
1/34	0	0	0	0	0	0.01	0	0	0	0	0.05	0.2	0	0	0	0	0	0.05	0	0	0	0.04
2/1	0	0	0	0	0	0.06	0.01	0.07	0	0	0	0	0	0	0	0	0	0	0	0	0	0.02
2/7	0	0	0	0	0	0	0	0	0	0	0.25	0	0	0	0	0	0	0	0	0	0	0
3/1	0	0	0	0	0	0.1	0.05	0.16	0	0	0	0	0	0	0	0.1	0	0.1	0.04	0	0.1	0.05
3/30	0	0	0	0	0.09	0.04	0.01	0	0	0	0	0	0	0	0.3	0.05	0	0	0	0	0	0.06
3/33	0	0	0	0	0.13	0.01	0	0	0	0	0	0	0	0	0	0	0	0	0	0.1	0	0
3/34	0	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0	0	0.04	0.25	0	0.02
3/36	0	0	0	0	0	0.01	0	0	0	0.07	0	0	0	0.3	0	0	0	0	0	0	0	0.02
6/1	0	0	0	0	0	0	0	0.01	0	0	0	0	0	0	0	0.05	0	0.05	0	0	0	0.02
12/20	0	0	0	0	0.19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
12/28	0	0	0	0	0	0.18	0	0.36	0	0	0	0	0	0	0	0	0	0	0	0	0	0.01
12/33	0	0	0	0	0.41	0.04	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15/1	0	0	0	0	0.06	0.05	0.13	0.04	0	0	0.05	0	0.1	0.25	0.05	0	0.25	0	0.04	0	0	0.06
15/27	0	0	0	0	0	0.02	0	0.11	0	0	0	0	0	0	0	0	0	0	0	0	0	0.05
20/15	0	0	0	0	0	0	0	0	0.08	0.21	0	0	0	0	0	0	0	0	0	0	0	0
20/27	0	0	0	0	0	0	0	0	0.5	0.21	0	0	0	0	0	0	0	0	0	0	0	0
21/27	0	0	0	0	0	0.05	0.18	0	0.08	0	0.1	0	0	0	0	0.05	0	0	0.04	0	0	0.01
22/1	0	0	0	0	0	0.01	0	0.01	0.13	0	0	0	0	0	0	0.05	0	0	0	0	0	0
22/15	0	0	0	0	0	0.01	0	0	0	0.21	0	0	0	0	0	0	0	0	0	0	0	0.01
22/30	0	0	0	0	0	0	0	0	0	0	0	0.05	0	0	0	0.05	0	0.05	0.13	0	0	0.01
22/34	0	0	0	0	0	0.04	0	0.05	0.08	0	0.15	0.5	0.05	0.05	0	0.15	0.5	0	0.17	0.45	0.3	0.1

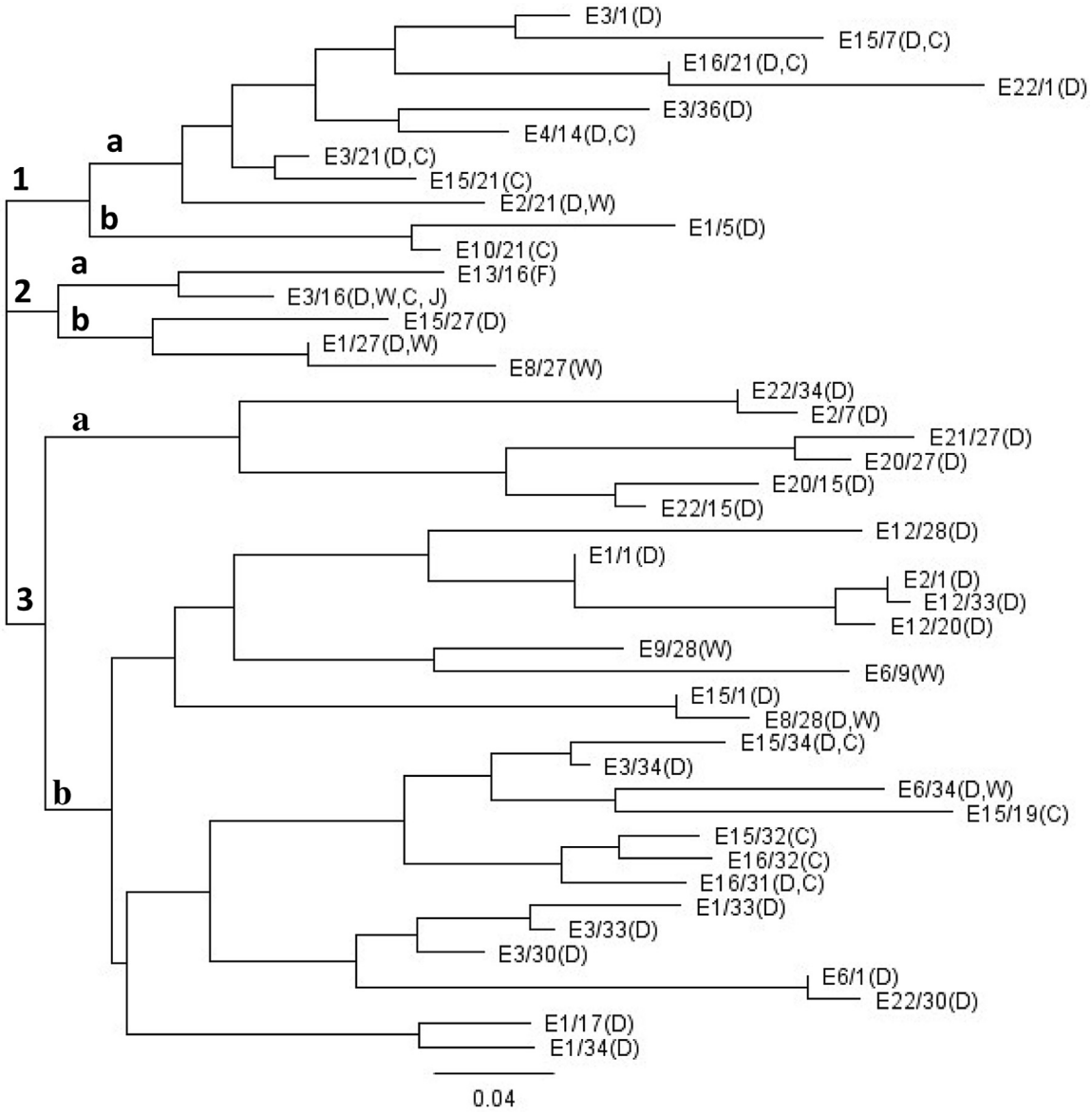

Fig. 3. Polygenic tree based on ERAP1/2 haplotypes. Haplotypes detected less than 4 times in 829 individuals were excluded. The tree was generated using Tree Builder of Geneious 8.0.5 based on genetic distance model of Jukes-Cantor with Neighbor-joining option.

(1a, 1b, 2a, 2b, 3a, 3b) were identified. The 3b subclade was the most diverse of these six branches. Dog, wolf, and coyote specific haplotypes were rooted in all three clades. Each of the four Jackals in the study were heterozygous and possessed eight ERAP1/2 haplotypes, with only one (E3/16) shared by dog, wolf and coyote. All of the red fox possessed the E13/16 haplotype, which was not shared by any of the *Caninae*. The E13/16 was found in the 2a subclade and the lack of sharing with dog, wolf, and coyote suggested that their genetic contribution to the dog was minor. Twenty-five dog specific haplotypes were present in the tree, compared to five coyote and three wolf specific haplotypes.

4. Discussion

The objective of this study was to identify polymorphisms within canine *ERAP1 and ERAP2* that might be useful in future research on genetic factors related to immune recognition and their effects in the incidence of cancer, infectious diseases and autoimmune disorders in certain pure breeds of dogs. The simplicity and economy of identifying ERAP1 and ERAP2 haplotypes using high throughput SNP identification technology like Sequenom MassARRAY iPLEX should help facilitate such research. This test can then be merged with a simple and highly efficient procedure recently described for identifying DLA class I and II haplotypes using linked STR markers (Pedersen et al., 2015a,b), making it possible to interrogate the relationships between certain ERAP1 and ERAP2 and DLA class I or II polymorphisms with various canine diseases that might involve how cellular peptides are screened for antigenicity.

The structure of canine *ERAP1* and *ERAP2* was typical of humans, with numerous single nucleotide polymorphisms forming definite haplotypes (Reeves et al., 2013). Dog ERAP1 and ERAP2 haplotypes were shared by *Caninae* with karyotypes consisting of 76 acrocentric autosomes and a pair of metacentric sex chromosomes (Reimann et al., 1999). These species include the dog, gray wolf, coyote and jackal. Only two ERAP1 and one ERAP2 haplotypes found in these canids were identified in the red fox, which has a more typical mammalian karyotype consisting of 32–34 large metacentric- and 1–2 pairs of micro-chromosomes (Vogt and Arakaki, 1971).

Single nucleotide polymorphisms were common in both *ERAP1* and *ERAP2* which can form a number of specific haplotypes. Only

select polymorphisms leading to amino acid changes or deletions were selected for Sequenom SNP testing, but even with a limited number of SNPs, 24 ERAP1 and 36 ERAP2 haplotypes were identified by Phase analysis. Although canine *ERAP1* and *ERAP2* are only 98kb apart on CFA3, 128 potential ERAP1/ERAP2 haplotypes resulted from recombination. Forty-five of the 128 haplotypes occurred in four or more individuals and were unlikely to be results of errors in phasing. The genetic plasticity of canine *ERAP1* and *ERAP2*, the tendency for certain SNPs to form specific haplotypes, and recombination between ERAP1 and ERAP2 haplotypes supports the importance of recombination hot spots in balancing selection in ERAP as in the MHC.

There has been a question on how much of the original dog diversity still exists in contemporary indigenous and randomly breeding dog populations compared to the collective diversity of all known dog breeds and even among all *Caninae*. Pilot et al. (2015) believe that current random breeding dogs in Eurasia retain much of diversity of the original dogs. Wade (2011) estimated from DNA analysis that each modern dog breed retains on average 87% of the diversity present in their founding populations. This estimate may be correct for diversity lost after breed creation, but it is not correct when comparing diversity in a given contemporary breed to the diversity that remains among all dogs. Pure breeds possess only 3–9 of 15 dog ERAP1 haplotypes and 3–8 of 27 ERAP2 haplotypes. No single existing dog population in the present study contained all of the ERAP1 and ERAP2 polymorphisms that still exist among all dogs, regardless of population size, geographic location, or randomness of breeding.

This loss and imbalance of ERAP1/2 haplotype diversity among dog breeds mirrored the situation observed for DLA class I and II haplotypes (Pedersen et al., 2015a,b). A diminution in the repertoire of ERAP1 and ERAP2 haplotypes, especially when coupled with similar diminutions in DLA class I polymorphisms, would be counter to normal evolution. Maintaining the total ancestral diversity of ERAPs and MHC class I receptors is accomplished by a process known as balancing selection (Garrigan and Hedrick, 2003). Andrés et al. (2009) concluded "that although balancing selection may not have an obvious impact on a large proportion of human genes; it is a key force in affecting the evolution of a number of genes in humans." Under normal circumstances, a new allele that favors survival would be expected to dominate over the less fit ancestral allele. Balancing selection prevents this by what is known as heterozygote advantage (Sellis et al., 2011). Natural selection in the MHC is largely pathogen driven; the objective being temporary expansion of certain receptor haplotypes rather than permanent selection of one haplotype over another. Although balancing selection has not been the most pervasive force in shaping the human genome, it has played an essential role in maintaining polymorphisms that are critical for species survival in whatever form evolution will dictate. There was evidence for balancing selection among ERAP1 and ERAP2 haplotypes when looking at all dogs as a whole. However, the positive selection forces created by breed development, which has been most intense in the last 150 years or so, has negated balancing selection forces and limited the number and specific form of ERAP1 and ERAP2 haplotypes inherited by descent in each breed of dogs. This non-balancing selection within the ERAPs and MHC class I receptors might help to explain differences in susceptibility to cancers, infectious disease, and autoimmune disorders between different pure breeds of dogs. Whether this is indeed the case remains to be determined by further studies, but the necessary tests are now available.

The original goals for the study did not include canid evolution. However, the dog ERAP1 and ERAP2 haplotypes provided a large and highly polymorphic region of the genome, which has been under strong linkage disequilibrium and subjected to balanced selection until recently. Three major ERAP1/2 clades were identified among all of the dogs tested, which mirrored the three major population clades derived from mitochondrial sequences (Vilà et al., 1997 Savolainen et al., 2002). Other genetic evidence indicates that dogs descended from at least 51 female Gray wolf founders (Webb and Allard, 2010), and that all dog breeds descend from 67 paternal lineages (Bannasch et al., 2005). These figures are in range to the 45 most common ERAP1/2 haplotypes that occur in the phylogenetic tree of the dogs that were tested.

Although the contemporary Gray wolf is presumed to share ancestry with the modern dog, the paucity of wolf specific or wolf/dog shared haplotypes in the genetic tree of present day dogs requires comment. Dog specific haplotypes may have predominated because the SNP polymorphisms used for testing were of dog origin. However, many dog ERAP1 and ERAP2 SNPs and SNP haplotypes were both shared and unique among various *Caninae*. The paucity of wolf-specific ERAP1/2 haplotypes may also be a reflection of the estimated 16-fold reduction in effective population size of wolves beginning soon after their genetic divergence from dogs (Freedman et al., 2014). The larger presence of coyote (five) or coyote/dog (six) haplotypes compared to wolf (three) or wolf/dog (four) haplotypes in the dog genetic tree also suggests a genetic contribution of coyote-like canids to dog ancestry, evoking an ancestral species less like modern Gray wolves than assumed by sharing of mitochondrial DNA (Vilà et al., 1997). At least one recent study suggests that present-day wolves are genetically monophyletic from indigenous dogs in the same regions and that the true nearest wild ancestor of dogs may be extinct (Freedman et al., 2014).

Although the origin and timing of the emergence of dogs has been debated, there is little doubt that the greatest changes in the genetic and phenotypic make-up of dogs has been in the Victorian and Post-Victorian eras with the creation of closed registries, pure breeding and dog showing (Pedersen et al., 2013). This is also supported by the present study, with random bred village dogs having more ERAP diversity than most pure breeds, and more randomly bred breeds such as Chihuahua and Jack Russell terriers having more diversity than highly inbred breeds such as Pug, English bulldog, and Yorkshire terrier. There is also evidence that heritable traits responsible for cancer, infectious disease susceptibility, and autoimmune disease are also ancestral in origin and inherited by descent in both modern random- and pure-breeds of dogs (Bellumori et al., 2013 Pedersen et al., 2015a,b). Inbreeding, which concentrates undesirable traits by interfering with balancing selection and heterozygote advantage, has been implicated in the increasing incidence of autoimmune disorders in breeds such as the Italian greyhound and Standard poodle (Pedersen et al., 2015a,b). The exact role of evolved ERAP1, ERAP2 and DLA class I polymorphisms in the increased incidence of certain diseases among purebred *vs.* random bred dogs remains to be determined.

Competing interests

The authors declare that they have no competing interests.

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