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EDITED BY

Sabyasachi Das,
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REVIEWED BY

Masayuki Hirano,
Emory University, United States
Karel Novák,
Institute of Animal Science, Czechia
Sayaka Miura,
University of Mississippi, United States

*CORRESPONDENCE

Petr Horin
✉ horin@ics.muni.cz

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Comparative genomics of the Natural Killer Complex in carnivores

Jan Futas¹, April L. Jelinek², Pamela A. Burger³
and Petr Horin^{1,2*}

¹Research Group Animal Immunogenomics, Central European Institute of Technology (CEITEC) VETUNI, Brno, Czechia, ²Department of Animal Genetics, Faculty of Veterinary Medicine, University of Veterinary Sciences Brno (VETUNI), Brno, Czechia, ³Research Institute of Wildlife Ecology, University of Veterinary Medicine Vienna (VETMEDUNI), Vienna, Austria

Background: The mammalian Natural Killer Complex (NKC) harbors genes and gene families encoding a variety of C-type lectin-like proteins expressed on various immune cells. The NKC is a complex genomic region well-characterized in mice, humans and domestic animals. The major limitations of automatic annotation of the NKC in non-model animals include short-read based sequencing, methods of assembling highly homologous and repetitive sequences, orthologues missing from reference databases and weak expression. In this situation, manual annotations of complex genomic regions are necessary.

Methods: This study presents a manual annotation of the genomic structure of the NKC region in a high-quality reference genome of the domestic cat and compares it with other felid species and with representatives of other carnivore families. Reference genomes of Carnivora, irrespective of sequencing and assembly methods, were screened by BLAST to retrieve information on their killer cell lectin-like receptor (KLR) gene content. Phylogenetic analysis of *in silico* translated proteins of expanded subfamilies was carried out.

Results: The overall genomic structure of the NKC in Carnivora is rather conservative in terms of its C-type lectin receptor gene content. A novel KLRH-like gene subfamily (KLRL) was identified in all Carnivora and a novel KLRJ-like gene was annotated in the Mustelidae. In all six families studied, one subfamily (KLRC) expanded and experienced pseudogenization. The KLRH gene subfamily expanded in all carnivore families except the Canidae. The KLRL gene subfamily expanded in carnivore families except the Felidae and Canidae, and in the Canidae it eroded to fragments.

Conclusions: Knowledge of the genomic structure and gene content of the NKC region is a prerequisite for accurate annotations of newly sequenced genomes, especially of endangered wildlife species. Identification of expressed genes, pseudogenes and gene fragments in the context of expanded gene families would allow the assessment of functionally important variability in particular species.

KEYWORDS

CLEC, KLR, Natural Killer Complex, carnivore, Felids, genomes

1 Introduction

Natural killer (NK) cells constitute a heterogeneous lymphocyte population (1). Conventional NK (cNK) cells found in the peripheral blood and spleen are endowed with potent cytotoxic capacity. They are primarily involved in innate immune responses against intracellular pathogens and tumor cells and contribute to the recognition of allogeneic cells. They also influence adaptive immune responses via the production of inflammatory cytokines (2) and crosstalk with dendritic cells (3). Tissue-resident NK (trNK) cells present in the liver, skin, kidney and virgin uterus (4) represent different NK lineages. Both uterine trNK and cNK cells orchestrate the implantation of semi-allogeneic embryo(s) and successful pregnancy in mice (5) and humans (6).

The diversity of the NK cell receptor repertoire represented by inherited haplotypes of NK cell receptor genes is essential for multiple NK cell functions. Stochastic expression of NK cell receptors on individual NK cells results in different sets of inhibitory and activation receptors on their surface. NK cell inhibitory receptors signal via cytoplasmic immunoreceptor tyrosine-based inhibitory motifs (ITIM) that recruit tyrosine phosphatase SHP1. Contrarily, activation receptors mostly couple through a cationic amino acid in their transmembrane domain to molecules containing immunoreceptor tyrosine-based activation motifs (ITAM), e.g. adaptor proteins CD3 ζ , Fc ϵ RI γ , DAP10 or DAP12. Integration of activating and inhibitory signals originating from various surface receptors determines the activation status of an individual NK cell, providing the capacity to discriminate between self and non-self or altered self (7).

The mechanism of missing-self recognition is based on the capacity of inhibitory NK receptors to bind MHC class I molecules on target cells, preventing NK cell degranulation and overriding activation signals mediated by different ligands. The molecules recognizing MHC class I ligands are killer cell lectin-like receptors (KLR) Ly49 in mice and killer cell immunoglobulin-like receptors (KIR) in humans. Both of these show high allelic and haplotypic variability in individuals. Activating members of these gene families binding MHC class I molecules are crucial for successful pregnancy, as exemplified by the differential outcomes of various combinations of maternal KIR haplotypes and fetal HLA-C2 in humans (8) and by a Ly49 knock down model in mice (9). The mechanism of altered-self recognition relies on different activating NK receptors which, upon binding various stress-induced ligands expressed on target cells, can override inhibitory signals and activate NK cells.

The natural killer gene complex (NKC) is located on mouse chromosome 6 and human chromosome 12 and contains a number of C-type lectin-like receptor (CTLR) genes. The mouse and human NKC genomic regions have been well characterized (10). NKC genes have been classified as 'killer cell lectin-like receptor' (*KLR*) genes expressed by NK cells and 'C-type lectin receptor' (*CLEC*) genes expressed on myeloid cells. Eleven sub-families/loci of *KLR* genes are currently recognized in mammals (*KLRA-KLRK*). Within the mouse NKC, genes encoding *CLECA* receptors which bind carbohydrates in the presence of Ca²⁺ ions constitute a gene family located between the *KLRG* and *KLRB* loci. The *KLRG1*

gene has been relocated in the human NKC. It encodes an inhibitory homodimeric receptor for cadherin molecules in the mouse and humans (11). Genes of the *KLRB* and/or *KLRF* family are intercalated with genes of the *CLEC2* family, thus forming pairs of genetically linked receptors and ligands, respectively (12). These are followed by the genes of the Dectin-1 cluster. The functions and known ligands of myeloid receptors were reviewed by Scur et al. (13). The *KLRE* gene is adjacent to this cluster in the mouse, but not in the human NKC. Genes of the *KLRE* and *KLRI* loci, which code for heterodimeric receptors in the mouse and rat (14), have also been identified in even-toed ungulates (15, 16). The *KLRD* gene codes for the CD94 protein, one chain of the heterodimeric receptors for non-classical MHC class I molecules Qa-1(b) in mice (17) and HLA-E in humans (18). The second chain of these receptors is encoded by genes of the *KLRC* family. *KLRC1* codes for an inhibitory receptor with two ITIM motifs (NKG2A) (19); *KLRC2* encodes an activating receptor (NKG2C) with arginine in the transmembrane domain (20). The *KLRK* gene encodes an homodimeric activating receptor NKG2D for diverse stress ligands in humans (MICA, MICB, ULBL1-6) and mice (H60, Rae1, Mult1) as reviewed by Lanier (21). In rats, *KLRH1* codes for a homodimeric inhibitory receptor recognizing MHC class I ligands (22); however, the mouse reference genome contains a *KLRH1* gene with a mutated ITIM. The *KLRH* gene family was identified in ungulates with a varying extent of pseudogenization (15) as well as in rabbits (23). In rodents, the *KLRA* (Ly49) genes encode homodimeric receptors for MHC class I molecules. They have expanded into a huge family containing both inhibitory and activating receptors (24). Similarly, the horse (25) and equids (26) possess at least five functional *KLRA* genes. The human *KLRA* counterpart is a pseudogene. It marks the boundary of the NKC.

The organization of the NKC in the draft genomes available to date of species other than rodent or human was compared by Hao et al. (27). Both expansion and reduction of gene numbers was observed for some gene families in cattle and dogs. Although mRNA for bovine *KLRJ* was described (28), its product has not yet been characterized as a receptor or co-receptor. A more comprehensive comparison between KLR gene clusters located within the NKCs of ruminants (cow, goat, sheep), ungulates (pig, horse), lemurs, rats and humans was made by Schwartz et al. (15). Long-read assemblies of cow and goat genomes made a detailed characterization of their expanded *KLRC* and *KLRH* genes possible. An outline of the NKC evolution in mammals documenting the plasticity of the KLR genes was presented by Hilton et al. (23).

The accuracy of the annotation of complex genomic regions such as the Leukocyte Receptor Complex (LRC), NKC and MHC is limited by the methods used for whole genome sequencing. Automatic annotation of genomes has proved to be insufficient for immune genes in non-model species (59% inaccurately annotated and 21% of genes not annotated) due to poor support of gene models by RNA-sequencing data and to genes missing in model databases (29). Manual annotations based on long contigs scaffolded with Hi-C may lead to a near-complete reconstruction of immune gene clusters. As such, they represent the approach of choice for complex regions of non-model species (29). Recently, we compared the LRC regions of carnivores (30). Here, we made a

similar comparison and evolutionary analysis for the carnivore NKC with special focus on the KLR genes that represent the most variable part of the mammalian NKC.

2 Methods

2.1 Genomic resources

Eighty-nine NCBI reference or other genomes of 74 carnivore species were analyzed (<https://www.ncbi.nlm.nih.gov/genome>). A full list of these along with corresponding links and abbreviations used throughout the text is provided in **Supplementary Table 1**. Families of carnivores were represented as follows: Felidae (22 species), Viverridae (1 species), Canidae (14 species), Mustelidae (19 species), Otariidae (4 species), Phocidae (8 species) and Ursidae (6 species). Short-read based genomes of three Feliformia families (Eupleridae, Herpestidae, and Hyaenidae) and four Caniformia families (Ailuridae, Mephitidae, Odobenidae, and Procyonidae) were excluded because their fragmented genes hampered the analysis.

2.2 Annotations

We followed the nomenclature rules of the HUGO Gene Nomenclature Committee for orthologues in humans (<https://www.genenames.org/>) (Table 1) and VGNC for the cat and dog (<https://vertebrates.genenames.org/>). According to rules suggested for KLR genes in non-model animals (15), numbers should be assigned to genes and pseudogenes consecutively from centromere to telomere. Our numbering of genes in expanded gene families is provisional. Two categories of pseudogenes were distinguished: the full-length genes with a 1-2 bp insertion/deletion or premature stop codon and obvious pseudogenes with frameshift mutations or missing parts. For the sake of clarity, numbers were given only to full length genes identified between chosen framing genes, ordered from the mannose-6-phosphate receptor gene (*M6PR*) to the protein mago nashi homolog 2 (*MAGOHB*), irrespective of the centromere to telomere orientation of the NKC. As usual in mammals, *KLRC1* denotes inhibitory receptors, while *KLRC2* designates activating receptors. A new category (*KLRC3*) was introduced for dual purpose receptors containing both inhibitory and activating signaling motifs. Likewise, a new name was introduced for *KLRH-like* genes: *KLRL*. The acronym *KLRL1* was already used in two papers (31, 32). However, according to the current nomenclature, the name *CLEC12A* is used for the corresponding gene in humans and mice. Therefore, we prefer *KLRL* to the next acronym *KLRM* for this newly identified gene family in carnivores.

The presence of NKC genes was assessed in the NCBI genome annotations of the highly contiguous genomes of five Carnivora representatives: domestic cat (*Felis catus*, GCF_018350175.1), ermine (*Mustela erminea*, GCF_009829155.1), California sea lion (*Zalophus californianus*, GCF_009762305.2), northern elephant seal (*Mirounga angustirostris*, GCF_021288785.2), and brown bear (*Ursus*

arctos, GCF_023065955.2). Model mRNA sequences of full length proteins were retrieved for each species and compared across the entire group using the NCBI Splign tool (33) (stopped in March 2024, https://www.ncbi.nlm.nih.gov/sutils/splign/splign_note.html) against the NKC regions of other species. For genomes not annotated by NCBI, the annotation was made by comparison with closely related species. The grey wolf NKC (*Canis lupus*, GCA_905319855.2) was annotated based on the dog NCBI RefSeq annotation (GCF_014441545.1). Similarly, the masked palm civet NKC (*Paguma larvata*, GCA_030068075.1) was annotated based on the domestic cat reference genome. Corrected annotations of seven genomic regions between the *M6PR* and *MAGOHB* genes are provided in **Supplementary Table 2**. Coding sequences (CDS) of CTLR genes are listed in **Supplementary Data Sheet 1**. Typically, CTLRs are type II transmembrane glycoproteins with an N-terminal cytoplasmic tail, a transmembrane domain, a stalk and a single extracellular C-type lectin-like domain (CTLD). Transmembrane domains were checked by DeepTMHMM prediction (<https://dtu.biolib.com/DeepTMHMM>) and CTLD domains were checked by NCBI Conserved Domain Search on presumed protein sequences (<https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>).

TABLE 1 Comparison of CTLRs of mouse and human NKC.

Locus	Mouse proteins	Human proteins	Signaling motif
<i>KLRG</i>	Klrg1	MAFA	ITIM
<i>CLEC4</i>	Dcar, Dcar1 – Dcar4, Dectin-2, Mcl, Mincle	BDCA2, DCIR, DECTIN-2, MCL, MINCLE	ITAM/ITIM
<i>KLRB</i>	Nkrp1-a – Nkrp1-g	NKR-P1A	ITIM/arginine
<i>CLEC2</i>	Clr-a – Clr-h, Cd69	AICL, KACL, LLT1, CD69	
<i>KLRF</i>	Not applicable	NKp80, NKp65	hemITAM
<i>CLEC12</i>	Micl, Clec12b	MICL (KLRL1), CLEC12-B	ITIM
<i>CLEC1</i>	Clec-2, Clec-1	CLEC-2	hemITAM
<i>CLEC9</i>	Dngr-1	DNGR1	hemITAM
<i>CLEC7</i>	Dectin-1	DECTIN-1	hemITAM
<i>OLR1</i>	Lox-1	LOX-1	
<i>KLRE</i>	Nkg2i	Not applicable	
<i>KLRD</i>	Cd94	CD94	
<i>KLRK</i>	Nkg2d	NKG2D	arginine
<i>KLRC</i>	Nkg2a/b, Nkg2c, Nkg2e	NKG2A, B, C, E	ITIM/arginine
<i>KLRI</i>	Klri1, Klri2	Not applicable	ITIM/lysine
<i>KLRH</i>	Klrh1	Not applicable	(ITIM)
<i>KLRA</i>	Ly49a-Ly49q	(pseudogene)	ITIM/arginine

To mine data from Carnivora genomes regarding *KLR* gene content, high quality mRNA models of *KLR* sequences were used to BLAST® search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) other genomes within the relevant family to retrieve fragments with similar sequences. These BLAST hits were identified by Spleen tool alignment. The coding sequences of full length genes were extracted to BioEdit version 7.2.6 (34) and are available in FASTA format in [Supplementary Data Sheet 2](#). VISTA comparisons (<https://genome.lbl.gov/vista/index.shtml>) were used for uncertain sequences/regions identified during manual annotation.

2.3 Phylogenetic analysis

The phylogeny of Carnivora CTLR genes was reconstructed using MEGA X version 11.0.13 (35). Sequences were aligned using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) algorithm as implemented in the software. The Neighbor-Joining method was used both for nucleotide (CDS, p-distance) and protein sequences (p-distance) with 1000 bootstrap replicates to construct phylogenetic trees. A set of murine reference genes was used as an outgroup; their CDS are available in [Supplementary Data Sheet 1](#).

3 Results

3.1 Structure of the NKC genomic region in carnivores

A comparison of the NKCs of seven representatives of carnivores can be found in [Figure 1](#). Details concerning the names of genes and mRNA models used are summarized in [Supplementary Table 2](#). Based on these data, the NKC of carnivores can be sub-divided into two parts.

The first part, delimited by *M6PR* and *TMEM52B*, comprises mostly CLEC genes ([Figure 1](#)). It is rather conservative in terms of the number of genes. The few exceptions are mainly *KLR* genes (*KLRB* and *KLRF*) and *CLEC2* genes. Eleven CLEC genes were found in all species and their orthologues/homologues are present in mice ([Figure 2](#)). Three *CLEC2* genes (*CLEC2A*, *CLEC2D-like* and *CLEC2F*) were found only in some of the species analyzed. *KLRG* is a single gene in all species except the masked palm civet, in which a processed pseudogene was identified on the chromosome containing the NKC. The *KLRB* gene is present as a single gene in all species except the masked palm civet and ermine, in which subfamilies consisting of two genes were identified. The *KLRF* subfamily of three genes (*KLRF1*, *KLRF2* and *KLRF3*) was identified in all species except marine carnivores. The *KLRF2* gene was not found in the sea lion and elephant seal.

The second part of the NKC of carnivores is delimited by *GABARAPL1* and *MAGOHB* ([Figure 1](#)). It comprises four *KLR* subfamilies, *KLRA*, *KLRC*, *KLRH* and *KLRL* (a novel *KLRH-like*

subfamily), with variable numbers of genes. The *KLRD*, *KLRK* and *KLRJ* genes are present as single genes in all species. A *KLRJ-like* gene was identified in the ermine ([Figure 3](#)).

The phylogenetic reconstruction of the coding sequences of CLEC genes ([Figure 2](#)) shows rather conservative evolution and no gene was identified as arising from a recent duplication event. The phylogenetic tree for *KLR* genes ([Figure 3](#)) presents different evolutionary paths for individual lineages of *KLR* genes. While there are some conserved single genes in all seven species (*KLRD*, *KLRG*, *KLRJ* and *KLRK*) or with duplication in one species (*KLRA* and *KLRB*), the duplications of *KLRF* gene must have occurred in a common ancestor of Feliformia and Caniformia. The lineages of the *KLRC*, *KLRH* and *KLRL* families document relatively recent duplication events for some of their genes with species-specific amplification.

3.2 The *KLR* gene contents of the NKC in carnivores

The structure of a *KLR* gene typically involves the 5'-untranslated region, an exon coding for the cytoplasmic tail, an exon for the transmembrane domain, an exon for the stalk region and three exons for the CTLD, accompanied by the 3'-untranslated region. The feline *KLRL* ([Figure 4A](#)) provides an example of the corrected CDS for a gene based on cross-species comparisons. The organization of its polypeptide was compared to feline *KLRH1* and *KLRA* ([Figure 4B](#)).

A full set of model mRNA sequences of *KLR* genes was searched against the genomes of six carnivore families. The numbers of genes (full length and pseudogenes) found are summarized in [Table 2](#). Individual species are described in [Supplementary Table 3](#). The organization of the *KLR* sub-regions of the NKC in carnivores is compared in [Supplementary Figures 1-6](#).

3.2.1 Felidae

The structure of the NKC of the Felidae is the most variable among carnivores. Their NKC is characterized by single *KLRA*, *KLRB*, *KLRD*, *KLRG*, *KLRJ*, *KLRK* and *KLRL* genes, three *KLRF* and various numbers of functional *KLRC* and *KLRH* genes ([Supplementary Figure 1](#)). One dual-purpose receptor, *KLRC3*, is found in all Felid genomes along with five to eleven predominantly inhibitory *KLRC* genes. Likewise, one activating *KLRH* in all Felids is accompanied by one to four *KLRH* genes for activating and/or inhibitory receptors.

3.2.2 Canidae

Low numbers and low variability of functional *KLR* genes were observed in the NKC genomic region of dogs and wolves. In addition to single *KLRA*, *KLRB*, *KLRD*, *KLRF1*, *KLRG*, *KLRH*, *KLRJ* and *KLRK* genes, the region contains three to four *KLRC* genes. In some assemblies, one inhibitory and two dual-purpose receptors are accompanied by another activating receptor. Foxes possess a very similar NKC with two to three *KLRC* genes ([Supplementary Figure 2](#)). The red fox and Corsac fox have two functional *KLRF* genes (*KLRF1* and *KLRF3*).

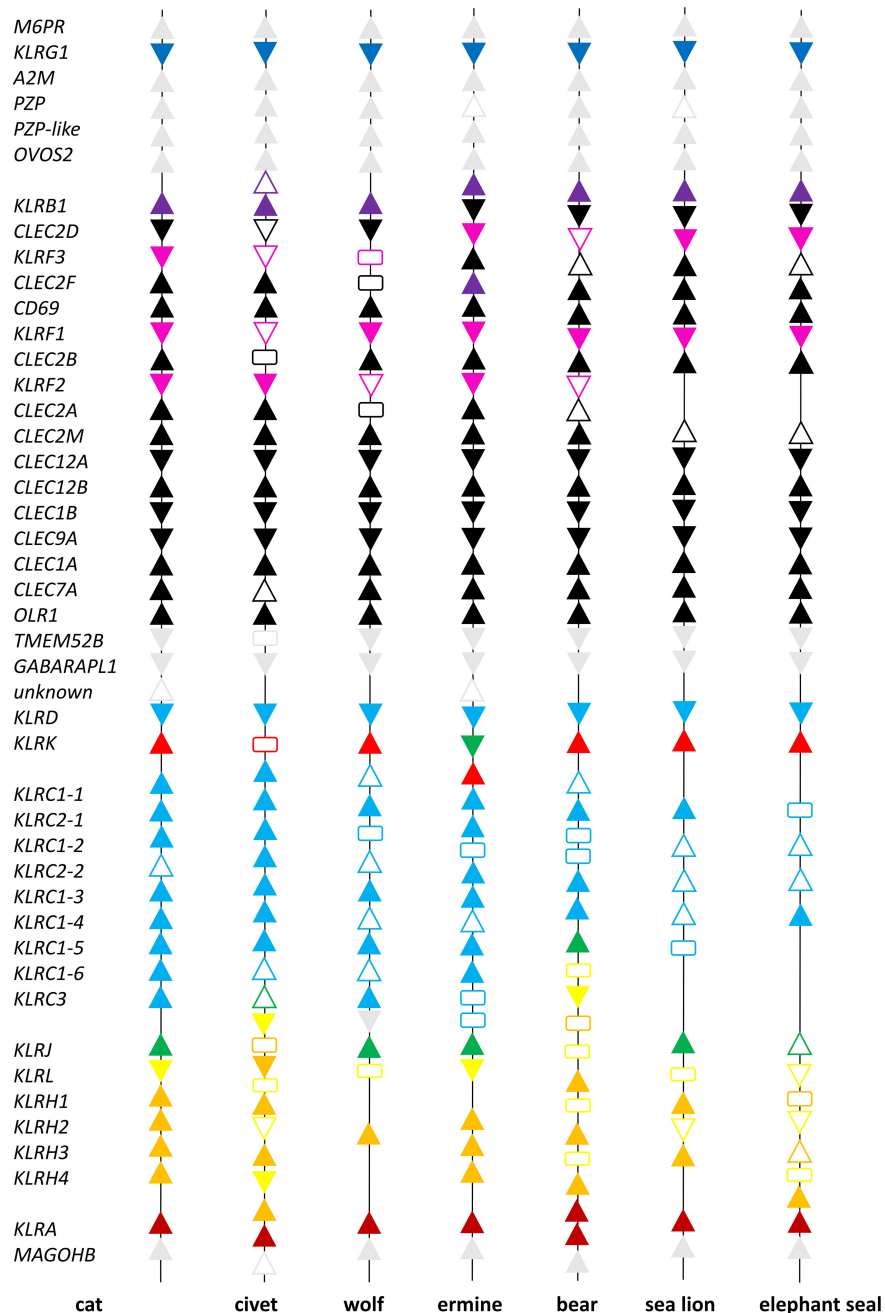


FIGURE 1
 Comparison of the Natural Killer Complex structure in carnivores. Seven representatives of Carnivora are depicted: the domestic cat (*Felis catus*), masked palm civet (*Paguma larvata*), gray wolf (*Canis lupus*), ermine (*Mustela erminea*), brown bear (*Ursus arctos*), California sea lion (*Zalophus californianus*) and northern elephant seal (*Mirunga angustirostris*). Individual NCBI reference genome sequences of the NKC were manually annotated based on cross-species comparisons. The scheme is not to scale, functional genes are represented by *solid color triangles* pointing in the direction of transcription, full length pseudogenes by *open triangles* and fragments of genes by *rectangles*. Color scheme: grey - framing genes; black - CLEC genes; blue - KLRG; violet - KLRB; magenta - KLRF; cyan - KLRD and KLRC; red - KLRK; green - KLRJ; yellow - KLRH; orange - KLRH; and brown - KLRA genes. For details on gene names see [Supplementary Table 2](#).

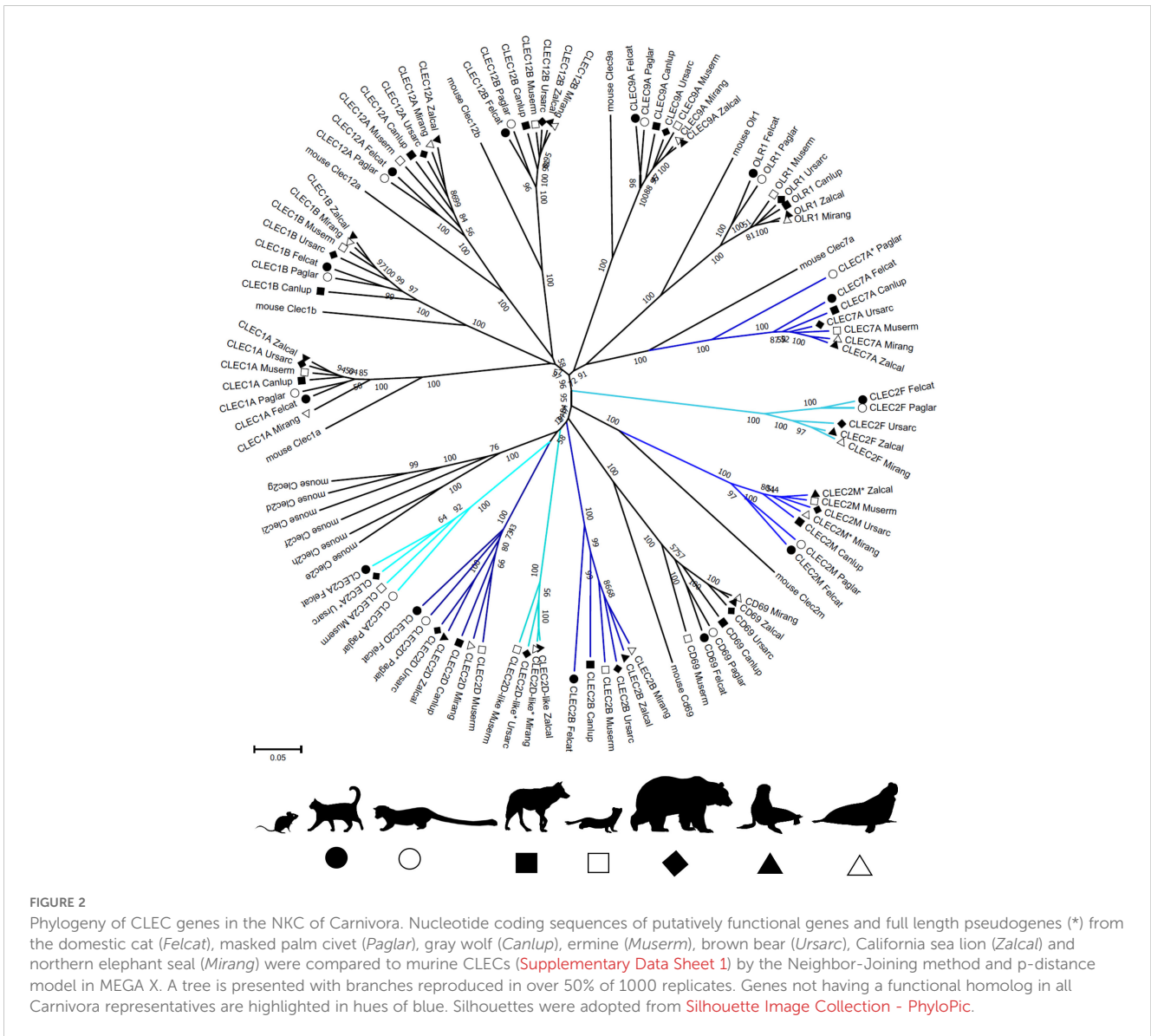
3.2.3 Mustelidae

Mustelids have the most complicated and diverse structure of the NKC among Caniformia ([Supplementary Figure 3](#)). Single genes of *KLRA*, *KLRD*, *KLRG*, *KLRK* and *KLRL* are accompanied by two *KLRB*, three *KLRF* and two *KLRJ* genes (*KLRJ* and *KLRJ-like*). Four to six *KLRC* genes encoding various types of receptors (*KLRC1*,

KLRC2, *KLRC3*) are complemented by one to four *KLRH* with activating, inhibitory or both signaling motifs.

3.2.4 Otariidae

The genomes of the Guadalupe fur seal, the northern fur seal, the Steller sea lion, and the California sea lion contain the smallest



numbers of functional KLR genes among carnivores. Except for the Guadalupe fur seal, all possess *KLRA*, *KLRB*, *KLRD* with inhibitory *KLRC1* (*CD94/NKG2*), *KLRG*, *KLRJ*, and *KLRK* accompanied by two inhibitory *KLRH* genes ([Supplementary Figure 4](#)). *KLRK* and *KLRH2* are missing in the Guadalupe fur seal. Two functional *KLRF* genes (*KLRF1* and *KLRF3*) were found in all but the northern fur seal (*KLRF1* only).

3.2.5 Phocidae

The gray seal, the Weddell seal, the northern elephant seal, the southern elephant seal, the Hawaiian monk seal, the harbor seal, the Saimaa ringed seal, and the Baikal seal possess a set of functional KLR genes comparable to Otariidae along with a putative *KLRL* gene without a signaling motif ([Supplementary Figure 5](#)). Their functional inhibitory *KLRC1* is a different gene from the gene found in the Otariidae. The genomes of the Hawaiian monk seal, the harbor seal, and the Saimaa ringed seal have an additional

inhibitory *KLRH* gene (three *KLRH* total). The Weddell seal and Hawaiian monk seal have only one functional *KLRF* gene (*KLRF1*).

3.2.6 Ursidae

The giant panda, the Malayan sun bear, the spectacled bear, the American black bear, the brown bear, and the polar bear are a more diverse group in terms of their NKC compared to marine carnivores, but less variable in comparison to mustelids. The *Ursus* species all possess the same set of functional KLR genes (two inhibitory *KLRA*, single *KLRB*, *KLRD* with three inhibitory *KLRC1* (*CD94/NKG2*), *KLRF1*, *KLRG*, two inhibitory *KLRH*, *KLRJ*, *KLRK*, and *KLRL*) with the exception of one activating *KLRH* which is functional in the brown bear and the polar bear but pseudogenized in the American black bear ([Supplementary Figure 6](#)). Although probably improperly assembled, the NKC of the Malayan sun bear seems to be similar to the NKC of the spectacled bear with one *KLRB*, *KLRD* with four inhibitory *KLRC1*,

KLRF1, *KLRG*, one inhibitory *KLRH*, *KLRJ*, *KLRK*, and *KLRL*. The giant panda reference genome also shows similarities to the spectacled bear *NKC*, although its *KLRH* is activating and functional *KLRA* has both inhibitory and activating signaling motifs (an ITIM and a cationic amino acid in the transmembrane domain). However, another giant panda genome differs from the reference genome by three *KLRA* genes (one activating, two inhibitory) and a stop codon in *KLRL2*.

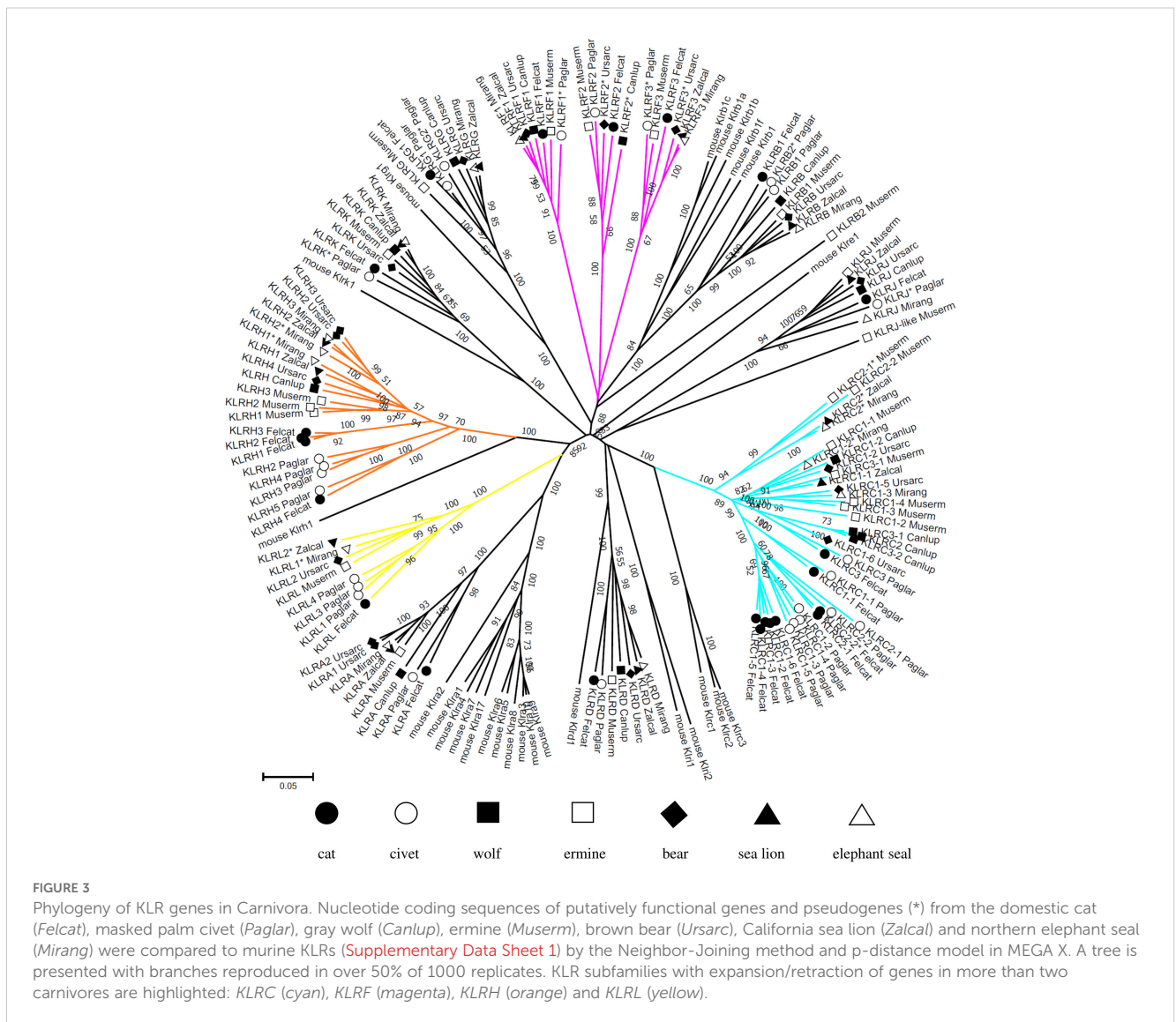
3.3 Phylogeny of KLR receptors

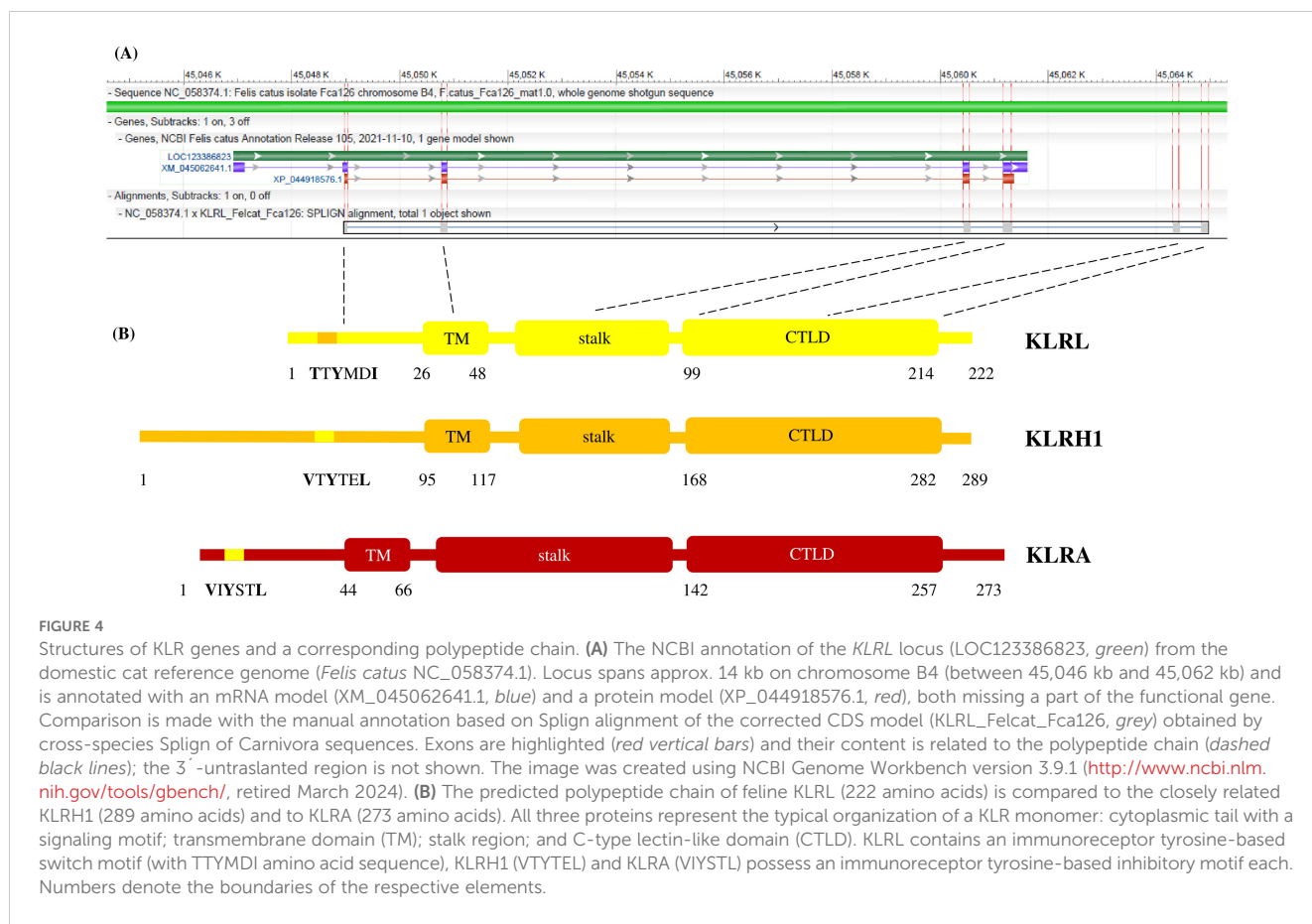
A comparison of the phylogenetic tree for *KLRA*, *KLRH* and *KLRL* to the tree for *KLRC* and *KLRD* protein sequences identified in Carnivora families is depicted in **Figure 3**.

A phylogenetic reconstruction of the expanded *KLRC* was compared to the *KLRD* co-receptor proteins (for details see

Supplementary Figure 7). While it suggests conservative evolution of *KLRD* from a common ancestor in all six carnivore families, a family-specific expansion of *KLRC* genes (except *KLRC3*) occurred in the Felidae. It seems that feline *KLRC3* probably has a common ancestor with both *KLRC3* and *KLRC2* of Canidae and *KLRC1-6* of Ursidae. There appear to be at least three other *KLRC* lineages in the common ancestor of the Caniformia. Canidae, Otariidae and Phocidae have retained only one lineage each, Ursidae and Mustelidae have retained all three lineages, and in Mustelidae duplications have occurred in two of the lineages.

Likewise, the expanded *KLRH* proteins were compared to the *KLRA* and *KLRL* proteins (for details see **Supplementary Figure 8**). Comparable to *KLRA* in all six families, the *KLRL* protein in the Felidae, Mustelidae and Ursidae can be traced back to a common ancestor of carnivores. The *KLRH* amplifications in the Felidae and Mustelidae are family-specific, while there were probably two lineages of *KLRH* in a common ancestor of other Caniformia.





The Canidae have retained only one lineage, but both lineages remain in the Otariidae. The Ursidae and Phocidae experienced duplication in one of the lineages.

3.4 Signaling motifs of carnivore KLR receptors

A comparison of the signaling motifs of carnivore KLR receptors to their murine counterparts shows their common as well as contrasting features (Figure 6). There are three KLR lineages concordant with murine receptors. The *KLRA* receptors of carnivores possess canonical ITIMs like some of murine *Klra* (Ly49), but one receptor with a mutated ITIM and a transmembrane arginine was identified in the

giant panda. The *KLRB* molecule of carnivores contains an ITIM in its cytoplasmic tail as seen in two of murine *Klrb* receptors. As in mice, *KLRD* contains no signaling motif in any of the carnivores.

Two KLR lineages are comparable to murine homologs. The two opposing murine *Klrc* receptors are mirrored in carnivores by an inhibitory *KLRC1* containing two ITIM motifs and by activating *KLRC2* with a cationic amino acid in the transmembrane domain. In those Carnivora with functional genes, the *KLRC2* transmembrane domain contains an arginine, while all Felidae have a lysine in this domain. The existence of *KLRC3* dual-purpose receptors with both ITIM(s) and a transmembrane arginine is specific to Carnivora. Comparably to rat *Klrh* with inhibitory function, the *KLRH* of Carnivora contain ITIM(s). But again, some dual purpose or activating receptors with an ITIM (or a

TABLE 2 Complement of KLR genes/pseudogenes in carnivores.

Family (number of species)	<i>KLRA</i>	<i>KLRB</i>	<i>KLRC</i>	<i>KLRD</i>	<i>KLRF</i>	<i>KLRG</i>	<i>KLRH</i>	<i>KLRJ</i>	<i>KLRK</i>	<i>KLRL</i>
Felidae (22)	1	1	7-19	1	3	1	3-26	1	1	1
Canidae (14)	1	1	4-11	1	3	1	1-2	1	1	1
Mustelidae (19)	1	2	8-13	1	2-3	1	1-8	2	1	1-2
Otariidae (4)	1	1	4-5	1	2	1	2	1	1	1-2
Phocidae (8)	1	1	4-5	1	2	1	3-5	1	1	3-4
Ursidae (6)	0-3	1	6	0-1	3	1	1-4	1	1	2-5

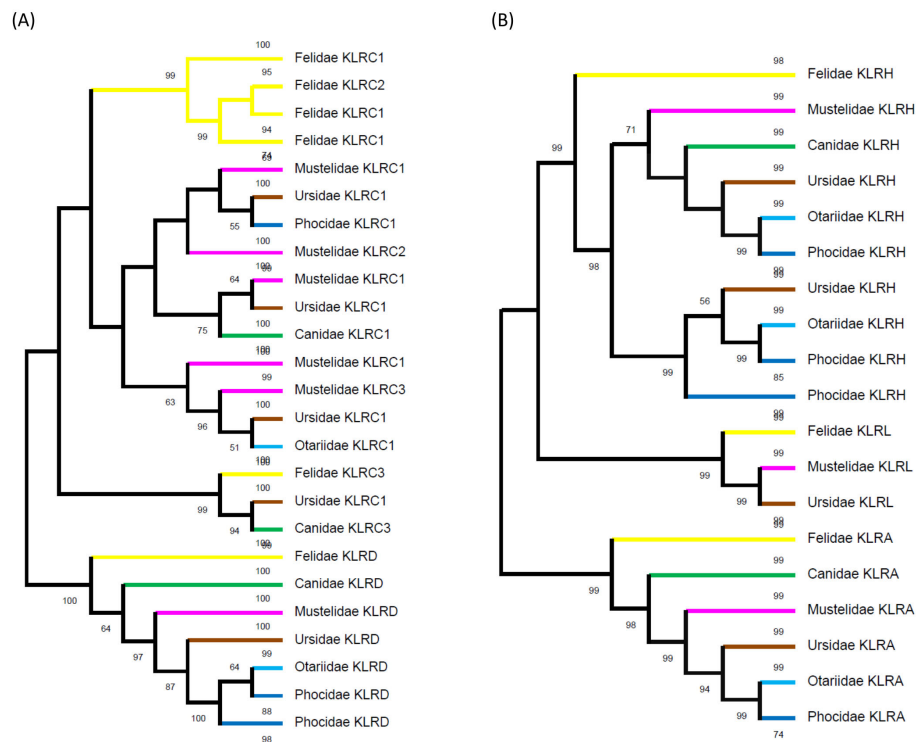


FIGURE 5

Phylogeny of expanded lineages of KLR receptors. (A) Overview of the phylogenetic tree for KLRC proteins identified in families of Carnivora related to co-receptor KLRD amino acid sequences. While the KLRD lineage is conserved, the KLRC lineages split in ancestors of Caniformia and Feliformia independently. The KLRC3 lineage was present in a common ancestor of Carnivora. For details see [Supplementary Figure 7](#). (B) Simplified view of the phylogenetic tree for KLRH and KLRL proteins identified in families of Carnivora related to KLRA amino acid sequences. KLRA and KLRL lineages were present in a common ancestor of Carnivora. The KLRH lineage split in ancestors of Caniformia and the expansion of KLRH receptors seen in Felidae is family-specific. For details see [Supplementary Figure 8](#).

mutated ITIM) and/or a transmembrane arginine were also found across carnivores.

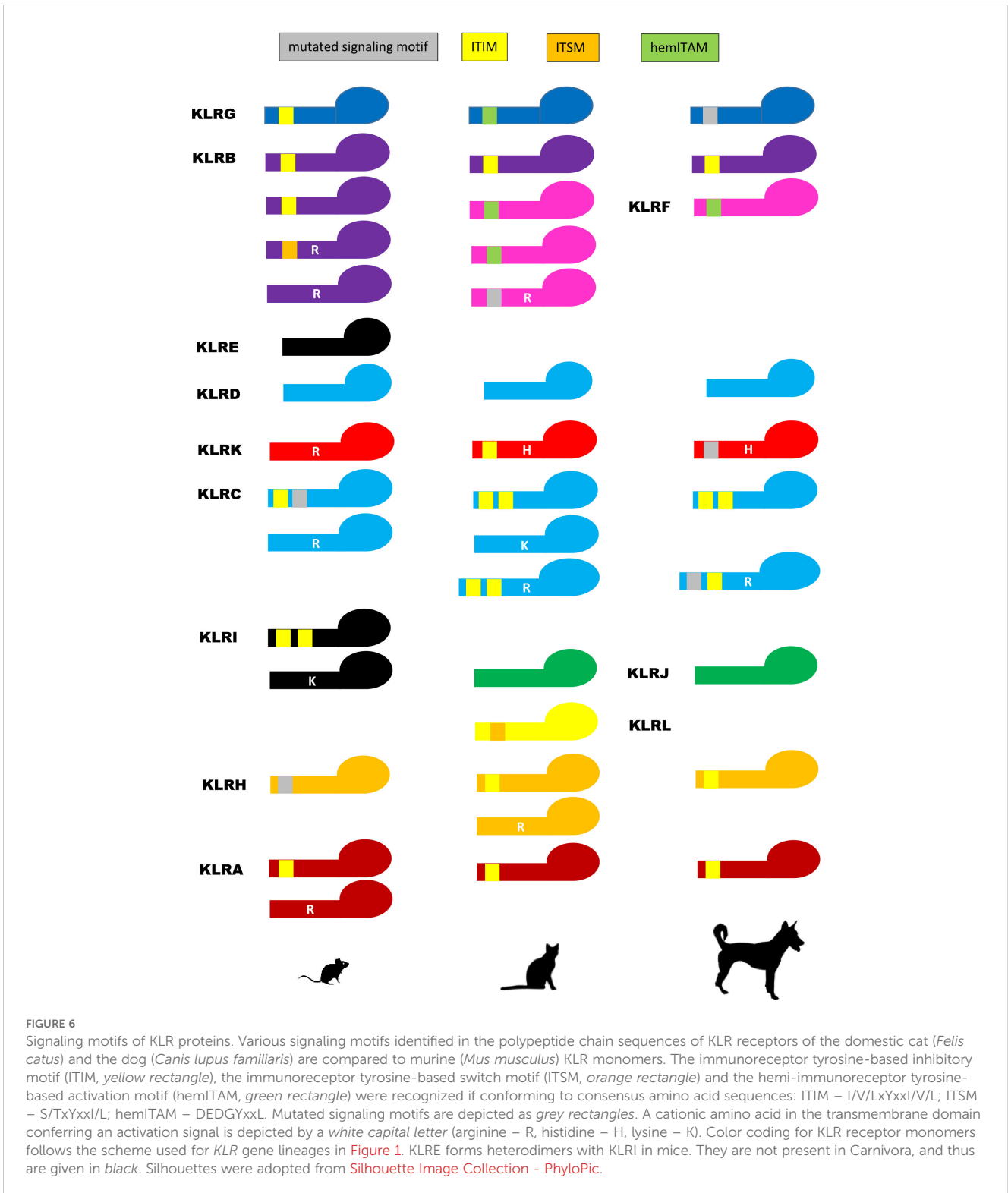
Two lineages differ between carnivores and mice. In contrast to the murine (and human) ITIM-containing gene *Klrg1*, the KLRG molecule of the domestic cat possesses a signal resembling the hemi-immunoreceptor tyrosine-based activation motif (hemITAM) (36). This feature is common to all Felidae, Otariidae (except the northern fur seal KLRG which has an ITIM domain) and Phocidae. In the Ursidae, this motif differs (DDGIYSEL/DGGIYSEL), while in the Canidae and Mustelidae, only mutated motifs were identified. Likewise, the KLRKs of all Caniformia possess a histidine in the transmembrane region while the murine activating *Klrk* contains arginine. In all Felidae, this signaling motif is accompanied by an ITIM-like motif in the cytoplasmic tail of the molecule.

There are few lineages without homologs between mice and carnivores. KLRE and KLRI are not present in Carnivora. KLRF, KLRJ, KLRJ-like and KLRL can be found in carnivores but not in mice. The KLRF proteins are characterized by hemITAM motifs in all carnivores except KLRF3 which bears an arginine in its transmembrane region. KLRJ contains no signaling motif, contrary to the KLRJ-like of mustelids which bears an ITIM. The newly identified KLRL molecules have a predicted immunoreceptor tyrosine-based switch motif (ITSM) in all Felidae, but no signaling motif was identified in the Mustelidae or the Ursidae.

4 Discussion

The remarkable evolutionary plasticity of the NKC and LRC gene clusters has been established by previous studies. Even closely related species may differ markedly as a result of rapid, lineage-specific expansions or contractions of sets of loci (10). Therefore, interspecific extrapolations are of limited value and a detailed annotation of these complex regions for each individual non-model species is necessary, as demonstrated by this study of Carnivora.

The advantage of exploring genomes obtained by a 'kitchen sink approach' to genome sequencing and assembly is that it allows the integration of information from a variety of different methods leading to a reconstruction of complex gene families such as the MHC, LRC, and NKC regions (29). This approach combines multiple methods (long-reads, short-reads, HiC scaffolding and optical mapping) and benefits from the complementary pros and cons of individual methods. Highly continuous genomes are required to evaluate the genomic architecture of the NKC; these were available for seven out of 89 species present in NCBI databases, representing seven out of 14 carnivore families. Due to the high evolutionary conservation of CLEC genes (Figure 2) and their good annotation in reference genomes, we focused on the characterization of the KLR sub-region of the NKC in carnivores.



Not all lineages of KLR genes identified in mammals (27) were found within the natural killer complex of Carnivora; namely, *KLRE* and *KLRI* are missing. On the other hand, the *KLRL* lineage was identified as a novel KLR gene subfamily specific to carnivores. While *KLRL* is a single gene in all Felidae, this locus is amplified in Viverridae. In Caniformia, there is no functional *KLRL* gene in the

Canidae or Otariidae, and the Phocidae probably do not possess a functional gene despite the amplification of this locus. Amplification of *KLRL* has led to one functional gene accompanied by a various number of pseudogenes in the Mustelidae and Ursidae.

There are four lineages of KLR genes represented by a single gene. *KLRD* is conserved and present as a functional gene in all

Carnivora families studied. *KLRG* was also found as a single gene in Carnivora. Although there are *KLRG2* genes annotated in the genomes of carnivores (cat, dog, ermine, bear, Californian sea lion and northern elephant seal) based on the mouse and human *KLRG2* genes. However, as *KLRG2* in humans (and mice) differs from the structure of *KLRG1* and their C-type lectin-like domains have only 33% homology, we suppose this is an erroneous designation for a different gene. *KLRJ* is highly conserved. It is present in all carnivores but has no evident signaling motifs, and its function remains unknown. Strikingly, a related *KLRJ-like* gene with an inhibitory motif was identified in the Mustelidae. The *KLRK* gene is also highly conserved and present in all carnivores. It is presumably an activating receptor in most carnivore families, while it shows features of a dual-purpose receptor in the Felidae.

Two lineages underwent duplication of the gene in only some carnivores. *KLRA* is present as a single gene in most carnivores studied except the Ursidae, in which this locus amplified. This is in agreement with the previous findings of an analysis of three seal and one sea lion species focused on specific loci (*KLRA* and *KIR3DL*) (37). The *KLRB* gene encodes an inhibitory receptor in all Carnivora; only Mustelidae have an amplified *KLRB* locus comparably to mice.

Three other lineages are characterized by the amplification of *KLRC* genes. *KLRC1* appears to have experienced the most diversified expansion and pseudogenization, and is amplified in all studied families of carnivores. *KLRC2* is pseudogene in the Otariidae and Phocidae, while it is missing in the Ursidae. In other families, it underwent amplification and pseudogenization. A *KLRC3* gene encoding a dual-purpose receptor is present in the Felidae, Viverridae and Mustelidae, while it is missing in the Otariidae and Phocidae. On the other hand, it underwent amplification in the Canidae. Phylogenetic comparisons (Figures 3, 5) showed that in the Ursidae this gene lost its arginine from the transmembrane domain, while in the Mustelidae another *KLRC* gene acquired one. *KLRF* amplified in a common ancestor of Carnivora with the genes for their presumed ligands (*CLEC2*) in their vicinity. Three *KLRF* loci followed different evolutionary paths. All three remain functional in the Felidae and Mustelidae, while two loci function in the Otariidae, Phocidae (with exceptions) and in some foxes. Only one locus works in the Viverridae, Canidae and Ursidae. As for *KLRH*, each Carnivora family contains species with at least duplicated or even expanded genes.

Expansions of *KLR* subfamilies (*KLRC* and *KLRH*) in carnivores seem to be family-specific, although some of their genes may have been duplicated in common ancestors. Receptors with presumed inhibitory signaling predominate in both the *KLRC* and *KLRH* subfamilies, partly due to preferential pseudogenization of activating receptors. A striking feature of the *KLR* genes of carnivores is the existence of some putative dual-purpose receptors containing both activating and inhibitory signaling motifs in the *KLRC* and *KLRH* subfamilies. Nothing is known about the signaling mechanisms of such receptors or their ligands, and functional studies are needed to address this issue. Some of these genes may represent an intermediate state in the evolution as they appear independently in only few species, and their expression could be silenced or cells bearing such a receptor could be

eliminated during NK cell development. A distinct functional role may be supposed for *KLRC3* as these receptors are likely to have been present in ancestors of Feliformia and Caniformia and the recent families of Felidae, Viverridae and Canidae have retained them. On the other hand, the role might be only a subtler regulation of cell activation as Ursidae have lost *KLRC3* receptor and Mustelidae have acquired another one.

In general, the mammalian NKC is very variable in terms of its *KLR* gene content. At one extreme, the naked mole-rat (missing NK cells) has a very simplified complement of 6 genes (*KLRA*, *KLRC*, *KLRD*, *KLRE*, *KLRG* and *KLRK*) and 4 pseudogenes (two *KLRA*, *KLRC*, and *KLRI*). In contrast, the platypus possesses one *KLRB*, *KLRH*, and *KLRJ* gene, two *KLRA* and *KLRI* genes, expanded *KLRC*, *KLRF* and *KLRK* families (dozens of genes), and an extreme expansion of the *KLRD* family (54 genes) (23).

Carnivora represent an intermediate point in this diversity. Many aspects of Carnivora NKCs are typical for mammals. For example, although an expanded *KLRA* family has been identified in Rodentia, Lagomorpha and odd-toed ungulates, Carnivora are similar to the majority of mammals with only one *KLRA* gene, with the exception of Ursidae possessing two to three *KLRA* genes. The *KLRB*, *KLRD*, *KLRG* and *KLRK* genes are quite stable in their numbers. Again, in this aspect, Carnivora do not differ from the majority of other mammals. Moreover, the *KLRE* and *KLRI* lineages have been identified only in Rodentia and ungulates. This is in alignment with our finding that Carnivora do not possess *KLRE* and *KLRI* sequences. Amplifications of *KLRC* genes, such as those seen in carnivores, are present in the majority of mammalian species.

On the other hand, some carnivore *KLR* families are less typical. *KLRF* is duplicated in primates and further amplified in Carnivora, ungulates and Chiroptera. *KLRJ*, identified in all Carnivora families included in the present study, is not commonly found in mammals other than ungulates or Chiroptera. Further, *KLRH* appears to be specific to Carnivora, ungulates, Rodentia and Lagomorpha.

In comparison with other previously studied mammalian NKCs, ruminants are the most comparable to Carnivora. NKCs with expanded *KLRC* and *KLRH* genes and with duplicated *KLRD* have been identified in ruminants. Bovine and caprine *KLRC* genes located between the *KLRI* and *KLRA* loci are interspersed with specific *KLRH-like* genes (15). These are combinations of *KLRH* CTLD with *KLRC2* cytoplasmic, transmembrane and stalk regions, unlike Carnivora *KLRH* which is fully homologous to mouse *Klrh1*.

To mine as much information on *KLR* genes as possible, we have not limited our analyses solely to high-quality genomes. In so doing, some pitfalls must be considered. Short-read sequencing technologies limit the reassembly of repetitive and complex immune sequences. This leaves a number of genes fragmented on short scaffolds and can potentially produce false pseudogenization. On the other hand, long-read technologies help to span complex and repetitive sequences, resulting in long contigs and scaffolds that contain multiple immune genes with complete coding sequences (29), but their raw reads are error-prone. Comparisons of the NKC in draft-quality genomes of closely related species could be beneficial, but caution should be taken when assessing variability within species.

This point is well illustrated by the example of the giant panda. The NCBI reference genome 'Jingjing' (Illumina, 10X Genomics, Flow-sorted chromosome sequencing) and another giant panda genome 'CPB_GP_2021' (PacBio) contain seemingly complete NKC and suggest intraspecific variability in the number of *KLRA* genes (Supplementary Figure 6). An unusual single *KLRA* gene with both inhibitory and activating signaling motifs in the 'Jingjing' genome is different from the three *KLRA* genes in the 'CPB_GP_2021' genome, of which one gene has activating and mutated inhibitory motifs and two are inhibitory genes. A VISTA alignment of these genomic regions (Supplementary Figure 9) revealed the combined nature of the *KLRA* gene (parts of *KLRA1* and *KLRA3* of 'CPB_GP_2021') in 'Jingjing'. A BLAST search of the 'Jingjing' genome identified another eight *KLRA* fragments scattered on various unplaced contigs. Their VISTA alignment against 'CPB_GP_2021' (Supplementary Figure 10) suggests that the reference genome could in fact also have three *KLRA* genes. Further, the *KLRL2* gene contains a stop codon in 'CPB_GP_2021', while an intact gene was found in the 'Jingjing' genome. It is plausible that at least some of this intraspecific variability is in fact an artifact of the different sequencing and assembly technologies employed and errors in the resultant genomes.

A comparison of different assemblies of the domestic cat genome is another example which seemingly suggests the existence of within-species variability. In this case, it is the number of *KLRC* and *KLRH* genes which appear variable (Supplementary Figure 1). As these assemblies were obtained by different methods, this presumed variability needs to be confirmed.

For comparison, two dingo genomes constructed using a combination of long- and short-read sequencing as hybrid assemblies also differ in the number of *KLRC* genes. However, in this case, one haplotype is in common with domestic dog genomes while the second one is found in the grey wolf, which supports the idea of such variability in the dingo (Supplementary Figure 2). In this context, we assume that even *KLR* sequences assembled based on short reads could be helpful for identifying their presence/absence in their respective species and for phylogenetic analyses based on interspecific differences. Nevertheless, we cannot exclude the possibility of technical artifacts (such as the number of functional genes vs. pseudogenes) produced by our analyses of genomes obtained by different methods, and we thus have to consider the comparative view of the carnivore NKC as a still-emerging picture of such a complex genomic region.

A good knowledge of the structure of the complex immune response related genomic regions may contribute to a better understanding of NK cell development and function with implications for veterinary medicine (38). Single-cell RNA sequencing of peripheral blood mononuclear cells has been used to identify NK cell populations as documented for the domestic cat, tiger and dog (39). Recently, a targeted analysis of *KLRA* showed its almost exclusive expression in canine NK cells (40). A high-quality annotation of the reference transcriptome is a prerequisite for single-cell RNA sequencing. The data obtained in this study cover an important part of the carnivore families and may help to characterize the NKC genomic region of other wild felid species.

This knowledge may then be used for conservation efforts in endangered species (41).

Data availability statement

The original data are freely available in the public database NCBI Genome (RRID:SCR_002474) and are referenced in Supplementary Table 1. The contributions presented in the study are included in the article and Supplementary Material. Further inquiries can be directed to the corresponding author.

Author contributions

JF: Investigation, Formal analysis, Methodology, Validation, Writing – original draft, Writing – review & editing. ALJ: Conceptualization, Methodology, Writing – review & editing. PAB: Conceptualization, Funding acquisition, Project administration, Writing – review & editing. PH: Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2024.1459122/full#supplementary-material>

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