

Article

The genomic organisation of the TRA/TRD locus validates the peculiar characteristics of dromedary δ -chain expression

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Abstract: The role of $\gamma\delta$ T cells in vertebrate immunity is still an unsolved puzzle. Species such as humans and mice display a low percentage of these T lymphocytes (i.e., " $\gamma\delta$ low species") with a restricted diversity of $\gamma\delta$ T cell receptors (TR). Conversely, artiodactyl species (i.e., " $\gamma\delta$ high species") account for a high proportion of $\gamma\delta$ T cells with large γ and δ chain repertoires. The genomic organisation of the TR γ (TRG) and δ (TRD) loci has been determined in sheep and cattle, noting that a wide number of germline genes that encode for γ and δ chains characterise their genomes. Taking advantage from the current improved version of the genome assembly, we have investigated the genomic structure and gene content of the dromedary TRD locus, which, as in the other mammalian species, is nested within the TR α (TRA) genes. The most remarkable finding was the identification of a very limited number of variable germline genes (TRDV) compared to sheep and cattle, which supports our previous expression analyses for which the somatic hypermutation mechanism is able to enlarge and diversify the primary repertoire of dromedary δ chains. Furthermore, the comparison between genomic and expressed sequences reveals that *D* genes, up to four incorporated in a transcript, greatly contribute to the increased diversity of the dromedary δ chain antigen binding-site.

Keywords: $\gamma\delta$ T cell; T cell receptor; TRA/TRD locus; Variable, Diversity, Joining and Constant genes; complementarity determining region-3; δ chain; somatic hypermutation; Camelidae.

1. Introduction

The Camelidae family, belonging to the Artiodactyl order, comprises two groups of living camels. One, which resides in Northern Africa and Central Asia, consists of one-humped camels (*Camelus dromedarius*) and two-humped camels (*Camelus bactrianus* and *Camelus ferus*). The other group, the South American camelids, includes llama (*Lama glama*), guanaco (*Lama guanicoe*), alpaca (*Vicugna pacos*) and vicugna (*Vicugna vicugna*).

This small number of animal species has unique behavioural, anatomical and physiological characteristics compared to other species of artiodactyls, which have aroused great interest in the scientific community.

Camels are large herbivores and have a digestive system adapted to consume large amounts of different forages containing fibrous and thorny plants that are frequently found in the desert. This allows them to live under severe environmental conditions becoming essential for the economy and food security of local populations. Although they ruminate, distinct morphological features separate them from true ruminants such as the presence of a three-chambered foregut, different from the four-chambered foregut of sheep, cows and goats. Furthermore, camels have the ability to retain ingested material in the rumen for longer than other ruminants, and the pH in camel rumen is closer to neu-

tral, which supports the lignocellulose degradation [1]. For this reason, camels are commonly called pseudo ruminants [2] and classified separately from true ruminants, in a family within Tylopoda, an Artiodactyla suborder.

Beside their adaptation to harsh environments, camels are multipurpose animals used for milk and meat production, racing, transportation and tourism.

Camels possess unusual features in the immune system [3–4]. In the context of the cellular-mediated response of the adaptive immunity, camels are similar to other artiodactyls, such as sheep, cows and pigs, with higher frequencies of blood $\gamma\delta$ T cells (up to 35%) in younger animals. Therefore, camels belong to the “ $\gamma\delta$ high species”, in contrast to “ $\gamma\delta$ low species”, like humans and mice, where $\gamma\delta$ T cells represent only a minor subpopulation (< 5%) of circulating lymphocytes. Furthermore, it has been shown that in *Camelus dromedarius*, the rearranged variable domains of γ and δ chains of the T cell receptor (TR) display somatic mutations as a result of a mechanism that enhances the $\gamma\delta$ T cell repertoire diversity [5–7].

The TR genes are multigene families organised into four TR loci distinct for each TR chain: TRA for α , TRB for β , TRG for γ and TRD locus for δ chain. The chains combine to form the heterodimeric receptor of the $\alpha\beta$ or $\gamma\delta$ T cells. Each TR locus consists of arrays of different gene types, including *variable* (V), *diversity* (D), *joining* (J) and *constant* (C) genes. Productive TRs are produced during the development of T lymphocytes in the thymus by somatic rearrangements of V and J genes in the TRA and TRG loci, and between V, D, and J genes in the TRB and TRD loci. After transcription, the resulting rearranged V–(D)–J region, encoding the variable domain of the TR chain, is spliced to the C gene, which encodes the constant domain of the receptor. The resulting TR chains are proteins with a variable domain at the N-terminal end, and a constant domain in the C-terminal end. Each variable domain comprises nine beta sheets forming four framework regions or FR, which support three hypervariable loops (complementarity determining regions or CDR) [8–9]. Both CDR1 and CDR2 are encoded by the germline V gene; the third, CDR3, results from the V–(D)–J rearrangement.

The current development of improved versions of genome assemblies provides invaluable help for the study of complex genomic regions such as TR loci. In this context, the genomic organisation of the dromedary TRG locus has been recently completed by combining previous cloning data [6] with the analysis of genomic assemblies [10]. The locus is single with respect to ruminants' TRG1 and TRG2 loci [11–12], and as in cows, sheep and goats it is organised in V–J–J–C cassettes (only three) with only six functional TRGV germline genes. In contrast, the ruminant TRG loci, located in two distinct positions of the same chromosome, display a higher number of functional TRGV germline genes (eleven in sheep and goats, 16 in cattle) distributed, as in dromedary, in reiterated V–J–J–C cassettes (six in sheep and seven in cattle and goats). However, the reduced potential diversity of dromedary TRG repertoire due to the lower number of germline genes is overcome thanks to the somatic hypermutation (SHM) mechanism that allows the expansion of the primary repertoire [6–7].

Similar to the γ chain, the ruminant as well as pig T cell receptor δ chain repertoire is determined by a high number of germline genes, with a marked expansion and preferential usage of the TRDV1 multigene subgroup [13–16]. The recent annotation of the TRD genes [17], which notoriously are organised together with the TRA genes in a more complex TRA/TRD locus, revealed the existence up to 50 and 65 germline TRDV1 genes in cattle and in sheep respectively, compared with the single TRDV1 gene present in the human locus (IMGT®, <http://www.imgt.org>). Four additional germline TRDV subgroups, nine TRDD and four TRDJ genes located in the TRD locus contribute to the δ chain repertoire in these ruminant species.

In camel species, genomic data about the TRD locus are still poor and fragmented. It has been proposed that also the diversification of the δ chain repertoire in *Camelus dromedarius* is the result of SHM, particularly of the TRDV1 genes, which are poorly represented in the dromedary genome compared to the other artiodactyls [5]. The lack of solid information on the genomic organisation of the dromedary TRD locus has not allowed to

substantiate this finding and, therefore some aspects of the δ repertoire could not be completely evaluated.

The latest release of a new high-quality version of dromedary reference genome assembly [18], which is chromosomally assigned and improved in terms of contiguity and increased size, allowed us to perform in-depth studies for understanding the genome architecture of the TRD locus that is critical for the discovery of the genetic basis of TR δ chain repertoire.

2. Materials and Methods

2.1. Genome analysis

To determine the TRA/TRD locus location, the upgraded dromedary CamDro3 genome assembly [18] was searched using the BLAST algorithm. A sequence of 877081 bp was retrieved directly from the reference sequence NC_044516.1 (*Camelus dromedarius* chromosome 6 genomic sequence) available at NCBI from 31696654 to 32573734 positions. Particularly, the analysed region extended from the *TRAV1* to the *TRAC*, respectively, the first and the last gene of the TRA/TRD locus. The *TRAV1* gene was located 6154 bp downstream of the *OR10G3* gene that borders all mammalian TRA/TRD loci studied so far. Likewise, the *TRAC* gene ends all the TRA/TRD loci at the 3'.

The available dromedary TRD germline gene sequences [5] as well as the human and sheep TRA/TRD genomic sequences (IMGT®, <http://www.imgt.org>) [17] were used against the *Camelus dromedarius* genome sequence to identify, based on homology by the BLAST program, the corresponding genomic *V*, *D*, *J* and *C* genes. Moreover, the homology-based method was used, aligning the dromedary retrieved sequence against itself with the PipMaker program [19]. The beginning and end of each coding exon were identified with accuracy by the presence of splice sites or flanking recombination signal (RS) sequences of the *V*, *D* and *J* genes.

Locations of the *TRA* and *TRD* genes are provided in Supplementary Table S1. The locations of the *olfactory receptor* (*OR*) genes intermingled with the *TRV* genes at the 5' of the locus are also provided (Supplementary Table S1).

Moreover, computational analysis of the dromedary TRA/TRD locus was conducted using the RepeatMasker for the identification of genome-wide repeats and low complexity regions (Smit, A.F.A., Hubley, R., Green, P. RepeatMasker open-4.0. at <http://www.repeatmasker.org>) and Pipmaker [19] for the alignment of the determined dromedary sequence with itself. The inspection of the obtained dot-plot matrix allowed us to identify portions of the sequence that align with more regions within the sequence itself.

The CamBac2 and CamFer2 genome assemblies, which are the improved versions of the two Old World camelid genome sequences, and BCGSAC_Cfer_1.0 [18,20] were screened for the presence of the TRA/TRD region. A sequence from *TRAV1* to *TRAC* orthologous genes was retrieved from each assembly. Hence, the obtained sequences were compared with Mauve, a genome multiple alignment software available at <http://darlinglab.org/mauve/mauve.html>. For the genomic comparison among *Camelus dromedarius* (CamDro3), *Camelus bactrianus* (CamBac2) and *Camelus ferus* (CamFer2 and BCGSAC_Cfer_1.0) TRA/TRD regions, the dromedary sequences of the first and the last genes of the locus (*TRAV1* and *TRAC*, respectively pos. 31696654-31697280 and pos. 32569624-32573734 in NC_044516.1) were used to identify the corresponding sequences within CamBac2, CamFer2 and BCGSAC_Cfer_1.0 assemblies. CamBac2 and CamFer2 sequences are available in fasta format from Dryad repository at <https://doi.org/10.5061/dryad.qv9s4mwb3>. The wild Bactrian camel BCGSAC_Cfer_1.0 assembly is available from GenBank (GCA_009834535.1) and a sequence of approximately 605 kb was retrieved by similarity comparison in a chromosome 6 contig (NC_045701). CamBac2, CamFer2 and BCGSAC_Cfer_1.0 TRA/TRD sequences were multi-aligned with CamDro3 by MAUVE program (Multiple Genome Alignment,

<http://darlinglab.org/mauve/mauve.html>). The position of the annotated *TRA/TRD* genes was inserted in the dromedary sequence by using SnapGene utility (<https://www.snapgene.com>).

2.2 Classification of the dromedary TR genes

The functionality of the *V*, *D*, *J* and *C* genes was predicted through the manual alignment of sequences adopting the following parameters: (a) identification of the leader sequence at the 5' of the *V* genes; (b) determination of proper RS sequences located at 3' of the *V* (*V*-RS), 5' and 3' ends of the *D* (5'D-RS and 3'D-RS) and 5' of the *J* (*J*-RS), respectively; (c) determination of conserved acceptor and donor splicing sites; (d) estimation of the expected length of the coding regions; (e) absence of frameshifts and stop codons in the coding regions of the genes. Conversely, a germline gene is qualified as ORF (open reading frame) if the coding region has an open reading frame, but alterations have described in the splicing sites and/or RS sequences, and/or in changes of conserved amino acids. Finally, a germline gene is qualified as pseudogene (P) if its coding region has stop codon(s) and/or frameshift mutation(s).

The *TRAV* and *TRDV* genes were grouped in different subgroups based on the percentage of nucleotide identity by using Clustal Omega alignment tool, which is available at the EMBL-EBI website (<http://www.ebi.ac.uk/>), adopting the criterion that sequences with a nucleotide identity of more than 75% in the V-REGION (i.e., coding region of a TR *V* gene) belong to the same subgroup [21]. For some *V* pseudogenes exhibiting a nucleotide identity less than 75% in the V-REGION respect to some but not all member genes of its own subgroup, the entire nucleotide sequence, L-PART1 (exon encoding the first part of the leader peptide of a *V* gene), intron and V-EXON (germline sequence that comprises L-PART2 and V-REGION), was compared and the common structure with all subgroup genes was checked. The same structural analysis was applied to classify and/or assign to a subgroup, *V* pseudogenes with marked defects along the sequence.

Each subgroup was then classified by comparison with the sheep and/or human gene subgroups, taking into account the percentage of nucleotide identity, by means of BLAST program (<https://blast.ncbi.nlm.nih.gov>) and IMGT/V-QUEST tool [22]. Hence, based on the genomic position within the dromedary locus, each *TRAV* and *TRDV* gene was named following the IMGT nomenclature (<http://www.imgt.org>).

The *TRDD*, *TRDJ* and *TRAJ*, and *TRDC* and *TRAC* genes were annotated and classified in accordance with the international nomenclature (IMGT®, <http://www.imgt.org>), [23–24].

To confirm the presence of an additional *TRDD* gene, the *TRDD* region (from *TRDV2* to *TRDJ1*) was retrieved from *Camelus bactrianus* (CamBac2) and *Camelus ferus* (BCGSAC_Cfer_1.0) genomic assemblies and analysed comparing with the corresponding region of the dromedary assembly (CamDro3). The comparison showed that the dromedary sequence was approximately 7.8 Kb shorter than the Bactrian one, with a gap between the *TRDD4* gene and the *TRDD5* gene where an additional *TRDD* gene in the Bactrian region is located. This gene had a sequence similar to the *TRDD2* gene. The orthologous gene has been found on the chromosome 6 contig (NC_045701, pos. 31422287-31422298) of the wild Bactrian assembly BCGSAC_Cfer_1.0; whereas the same *TRDD* gene was not present within CamFer2 because of a gap.

2.3 Phylogenetic analyses

The sheep and human *TRAV*, *TRDV* and *TRDJ* gene sequences used for the phylogenetic analyses were retrieved from the IMGT database (IMGT Repertoire, <http://www.imgt.org>) [25, 17].

The dromedary *TRAV*, *TRDV* and *TRDJ* genes were retrieved from the reference sequence NC_044516.1 (this work).

Only one gene per each sheep and the human TRAV subgroup was included, first preferring potential functional genes when present. The human *TRAV31* pseudogene, as a single member of its own subgroup, was excluded from the analysis because of several defects along the sequence. For the same reason, the dromedary *TRAV8-3*, *TRAV16-1*, *TRAV16-2*, *TRAV19-4*, *TRAV22-5* and *TRAV31* pseudogenes were not included in the tree.

Conversely, all dromedary and human genes were included in the TRDV tree, while only potential functional sheep *TRDV* genes and in-frame pseudogenes (except for the multimer TRDV1 subgroup genes indicated by “D”, duplicated genes, and “S”, provisional genes) were included.

We combined the nucleotide sequences of the V-REGION of the dromedary *TRAV* or *TRDV* genes with the corresponding gene sequences of sheep and human.

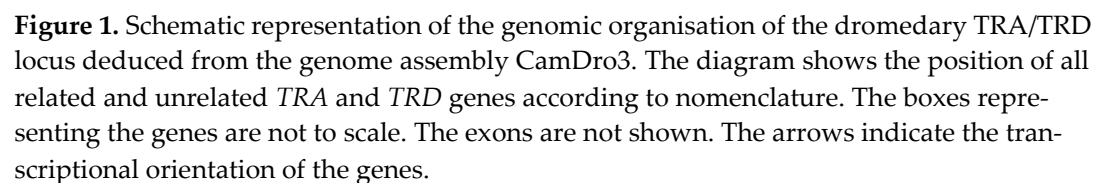
The phylogenetic relationship of the *TRDJ* genes were investigated by aligning the nucleotide sequences of all dromedary *TRDJ* genes (coding region plus RS) with those of sheep and humans.

Multiple alignments of the gene sequences under analysis were carried out with the MUSCLE program [26]. The evolutionary analyses were conducted in MEGAX [27–28]. We used the neighbour-joining (NJ) method to reconstruct the phylogenetic tree [29]. The evolutionary distances were computed using the p-distance method [30] and are in the units of the number of base differences per site.

3. Results

3.1. Genomic organisation of the dromedary TRA/TRD locus deduced from the latest version of the *Camelus dromedarius* genome assembly

The availability in the public database of a more accurate dromedary genome assembly (CamDro3) has prompted us to study in this camel species the genomic organisation of a complex locus such as the TRA/TRD. Thus, we retrieved a sequence of 877 kb in length from the *Camelus dromedarius* whole chromosome 6 genomic contig. The genomic sequence extended from the *TRAV1* gene, the first gene at the 5' end of the locus embedded among three *OR* genes in conserved synteny with other mammalian species (IMGT®, <http://www.imgt.org>) [31], to the *TRAC* gene, which is located at the most 3' end. The sheep and human corresponding genomic sequences (IMGT®, <http://www.imgt.org>) [17] were used as a reference to identify and annotate all dromedary *TRA* and *TRD* genes. Basically, the deduced genomic structure of the dromedary TRA/TRD locus was found to be conserved with respect to the sheep and human as well as to the other species of mammals, with the TRD locus nested within the TRA locus. It includes, from 5' to 3', 81 *TRAV* and two *TRAV/TRDV* genes, intermingled with eleven *TRDV* genes, followed by six *TRDD*, four *TRDJ*, one *TRDC* and a single *TRDV* gene with an inverted transcriptional orientation (Figure 1). Beside the *TRDV3* gene, 26 *V* genes lie in opposite orientation with respect to the transcriptional direction of the entire locus. At the 3' end, the locus is completed with 60 *TRAJ* genes and one *TRAC* gene.



The TRAV genes were assigned to 33 different subgroups by sequence analyses (see Section 2). Fifteen TRAV subgroups consist of more than one member genes with maximum expansion of the TRAV22 and TRAV45 (eight genes each), and the TRAV23 and TRAV24 (seven genes each). The TRAV9 subgroup consists of five genes, while the TRAV14, TRAV16, TRAV19 and TRAV25 subgroups contain four genes. Moreover, the TRAV8 and TRAV13 comprise three genes; and, finally, the TRAV12, TRAV33, TRAV43 and TRAV44 consist of two genes. It should be noted that the TRAV33 genes have been annotated as TRAV33/DV6 because they were found to rearrange with TRD(D)J genes within δ -chains productive cDNAs (see Section 3.6). Out of the 83 TRAV genes, 37 (approximately 45%) are predicted to be functional genes as defined by the IMGT rules (IMGT®, <http://www.imgt.org>), and 46 (55%) are not functional (pseudogenes and ORF) (Table 1 and Supplementary Table S1). Most of the expanded TRAV subgroups consist predominantly of non-functional genes, with nine TRAV subgroups consists of only pseudogenes. Therefore, the diversity of the functional germline repertoire is considerably reduced.

The deduced amino acid sequences of the potential functional germline *TRAV* genes, ORF and in-frame pseudogenes are shown in Supplementary Figure S1, where they are aligned according to IMGT unique numbering for the V-REGION [32] to maximise the percentage of identity. The *TRAV* gene subgroups showed a high structural diversity both in amino acid composition and in length. In particular, the CDR1-IMGT (definition according to the IMGT unique numbering for V-REGION) was five, six or seven amino acid (AA) long. The CDR2-IMGT ranged in length from four to eight AA and the germline CDR3-IMGT was two to five AA long. Conversely, FR-IMGT varied above all in amino acid composition.

Table 1. Description of the *TRAV* subgroups

Subgroups	Gene number	Functional	ORF/P
TRAV1	1	1	-
TRAV2	1	-	1
TRAV3	1	1	-
TRAV4	1	1	-
TRAV8	3	-	3
TRAV9	5	4	1
TRAV12	2	1	1
TRAV13	3	2	1
TRAV14	4	1	3
TRAV16	4	-	4
TRAV17	1	-	1
TRAV18	1	1	-
TRAV19	4	1	3
TRAV22	8	4	4
TRAV23	7	2	5
TRAV24	7	2	5
TRAV25	4	4	-
TRAV26	1	-	1
TRAV27	1	-	1
TRAV28	1	1	-
TRAV29	1	-	1
TRAV31	1	-	1
TRAV33/DV6	2	2	-
TRAV35	1	-	1
TRAV36	1	1	-
TRAV37	1	-	1
TRAV38	1	1	-
TRAV39	1	1	-
TRAV40	1	1	-
TRAV41	1	1	-
TRAV43	2	1	1
TRAV44	2	2	-

TRAV45	8	1	7
Total	83	37	46

The classification and the membership of the dromedary *TRAV* genes to the subgroups have been validated by a phylogenetic analysis with the corresponding sheep and human genes. Sheep was chosen as representative of the order of artiodactyls in which the TRA/TRD locus has been well characterised and recently annotated [17]; while the human germline genes represent the annotated reference repertoire. Adopting as the sole selection criterion the inclusion of only one sheep and human gene per subgroup, the V-REGION nucleotide sequences of all selected *TRAV* genes were combined in the same alignment and an unrooted phylogenetic tree was constructed using the NJ method [29] (Figure 2).

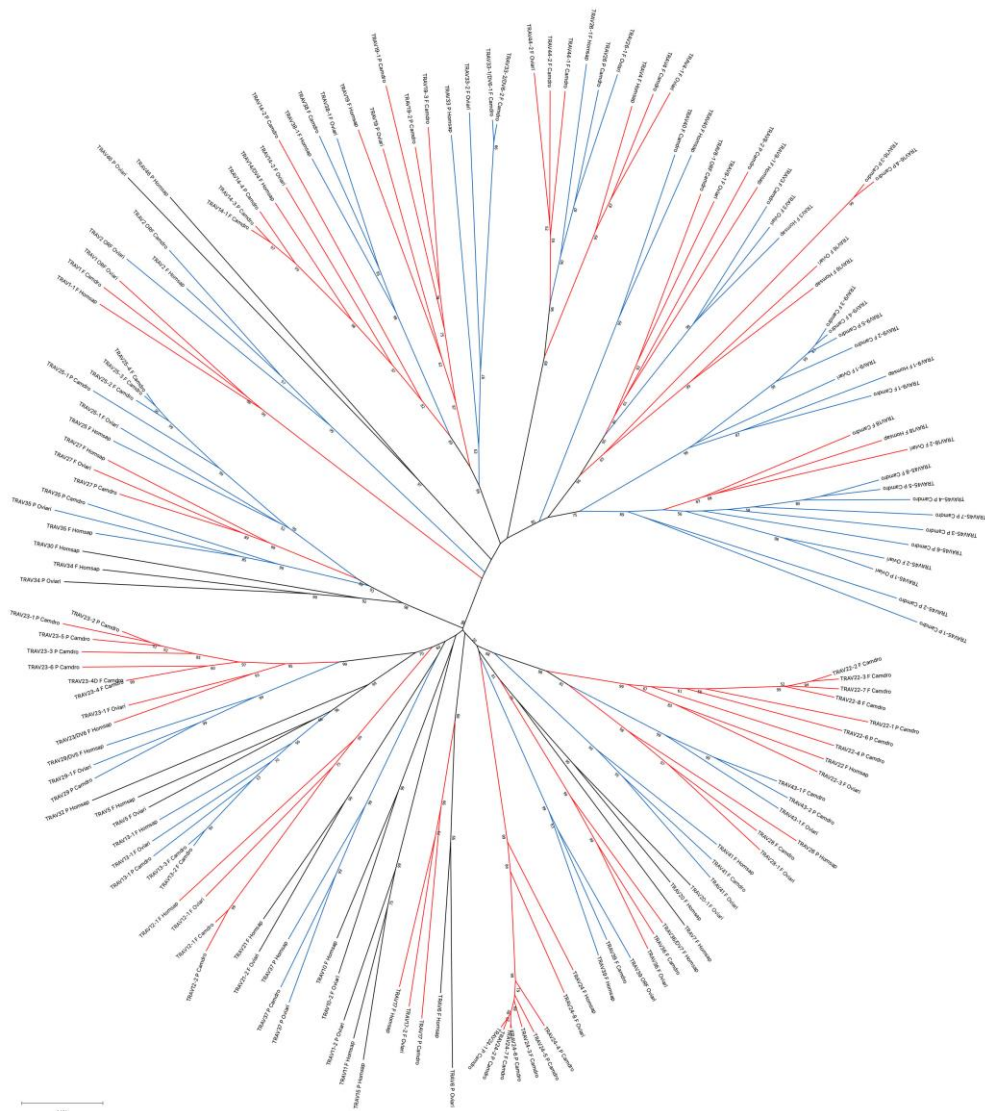


Figure 2. The neighbour-joining (NJ) tree inferred from the dromedary, sheep and human TRAV gene sequences. The evolutionary analyses were conducted in MEGA X [27-28]. The optimal tree with the sum of branch length = 19.33009914 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) is shown next to the branches [33]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [30] and are in the units of the number of base differences per site. This analysis involved 159 nucleotide sequences. Codon positions included were 1st +

2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 405 positions in the final dataset. The orthologous *TRAV* genes formed monophyletic grouping that are alternatively highlighted in red and blue. The gene functionality according to IMGT rules (F: functional, ORF: open reading frame, P: pseudogene) is indicated. The IMGT 6-letter for species (Camdro, Oviari and Homsap) standardized abbreviation for taxon is used.

The tree shows that each dromedary *TRAV* subgroup form a monophyletic group with corresponding sheep and/or human genes, consistent with the birth of distinct subgroups prior to the divergent of the different species, with the exception of the *TRAV45-1* and *TRAV45-2* pseudogenes. Twelve *TRAV* subgroups are missing in the dromedary with respect to the human locus, and of these, four are missing also in the sheep locus (*TRAV7*, *TRAV15*, *TRAV30* and *TRAV32* subgroups). Moreover, three *TRAV* subgroups (*TRAV43*, *TRAV44* and *TRAV45*) are shared between dromedary and sheep only; while the *TRAV40* subgroup is missing in the sheep locus. Hence, the phylogenetic clustering confirmed the previous classification by sequence analysis of each dromedary *TRAV* genes as orthologous to the corresponding sheep and human genes.

3.3. Classification, structure and phylogenetic analysis of the *TRDV* genes

Twelve *TRDV* genes were assigned to five different subgroups by sequence analyses (see Section 2). Only the *TRDV1* is a multimember gene subgroup with eight genes that are intermingled with the *TRAV* genes. The other subgroups (*TRDV2*, *TRDV3*, *TRDV4* and *TRDV5*) consist of one member gene each, which are located after the *TRAV* gene pool, within the *TRD* locus (Figure 1). Three out of twelve *TRDV* genes (25%) have been predicted to be pseudogenes (Supplementary Table S1).

The classification of the dromedary *TRDV* subgroups was established by comparing all dromedary genes with available corresponding genes in sheep and human (Figure 3).

The phylogenetic clustering of the genes indicates that the definition and the adopted nomenclature of the five dromedary *TRDV* subgroups are consistent with the corresponding sheep and human gene subgroups. The tree is a revised and updated version of a previous evolutionary tree [5] in order to define the nomenclature as well as to better investigate the evolutionary relationship of the dromedary germline *TRDV* with the corresponding sheep and human genes. It shows that the dromedary *TRDV* genes are clustered in five groups, A–E.

Group A contains the artiodactyl *TRDV1* multigene subgroups with the related human gene. As already showed [5], the dromedary *TRDV1* genes do not intermingle with the numerous sheep corresponding genes, indicating that the gene duplications aimed at expanding the *TRDV1* subgroup occurred independently within the two species.

Group B and D are representative of genes belonging to artiodactyls as they include dromedary and sheep *TRDV5* and *TRDV4* genes, respectively.

In C, the dromedary, sheep and human *TRDV3* genes are grouped, that stand in an inverted transcriptional orientation downstream the *TRDC* gene (Figure 1) (IMGT®, <http://www.imgt.org>) [17]. Finally, group E contains the *TRDV2* corresponding genes of all the three species.

Supplementary Figure S2 shows the protein display of the dromedary *TRDV* genes. The deduced amino acid sequences of the functional genes were manually aligned according to IMGT unique numbering for the V-REGION [32]. The *TRDV* gene subgroups show a structural diversity essentially in amino acid composition. The amino acid lengths of the CDR-IMGT are identical for all genes (7.3.4) except for the CDR2-IMGT of the *TRDV3* gene (three AA longer) and for the CDR3-IMGT of the *TRDV2* (one AA shorter).

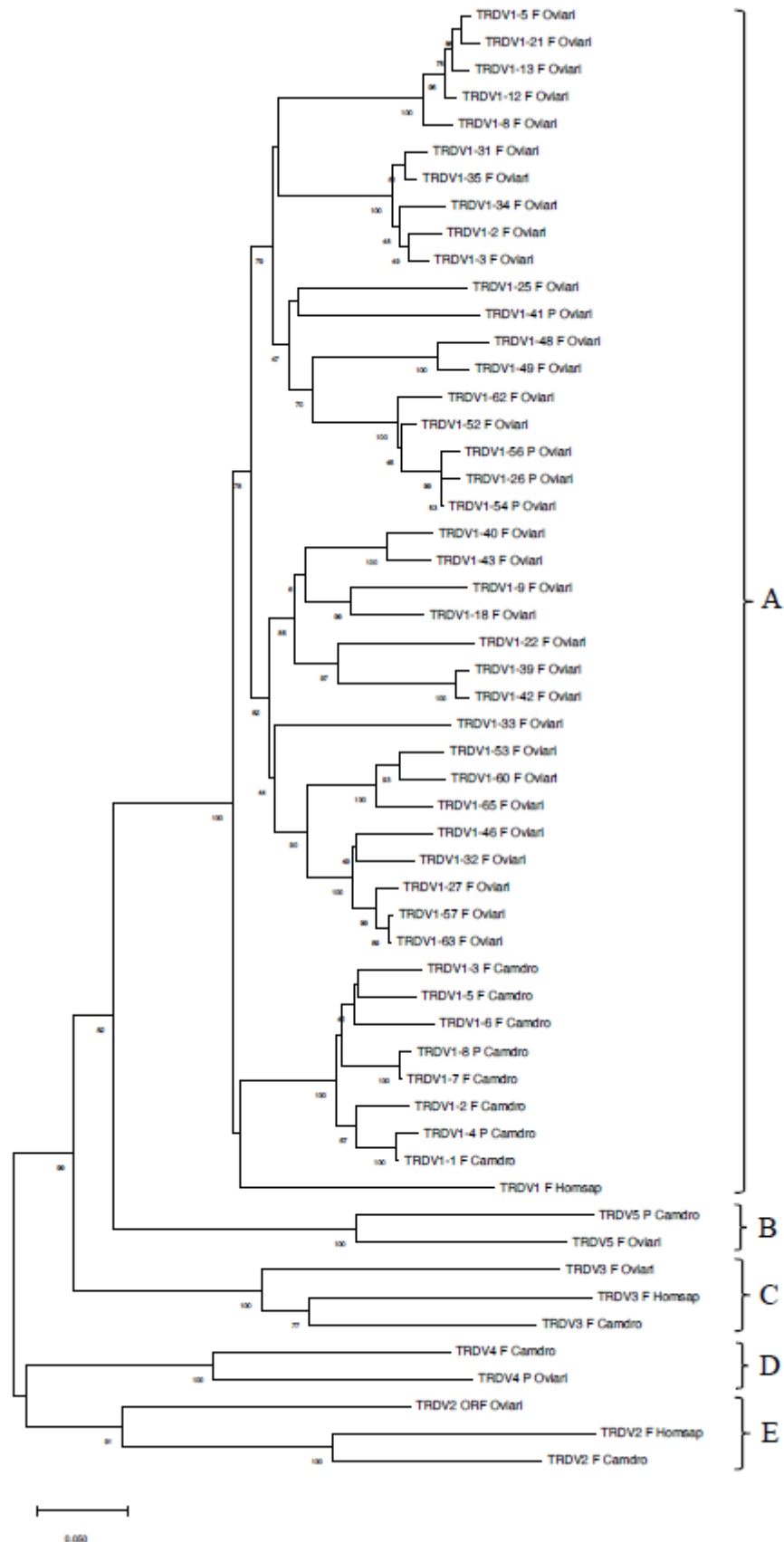


Figure 3. The NJ tree inferred from the dromedary, sheep and human TRDV gene sequences. The five groups (A-E) of the *TRDV* genes drawn by the tree are indicated by brackets. The evolutionary analyses were conducted in MEGA X [27-28]. The optimal tree with the sum of branch length = 4.03998833 is shown. The percentage of replicate trees in which the associated taxa

clustered together in the bootstrap test (100 replicates) are shown below the branches [33]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [30] and are in the units of the number of base differences per site. This analysis involved 54 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 411 positions in the final dataset. The gene functionality according to IMGT rules (F: functional, ORF: open reading frame, P: pseudogene) is indicated. The IMGT 6-letter for species (Camdro, Oviari and Homsap) standardized abbreviation for taxon is used.

3.4. Architecture of the dromedary V-CLUSTER containing region

The genomic structure of the V-CLUSTER of the dromedary TRA/TRD locus was investigated aligning the masked corresponding sequence (from *TRAV1* to *TRDV2* gene) against itself with Pipmaker program (Figure 4).

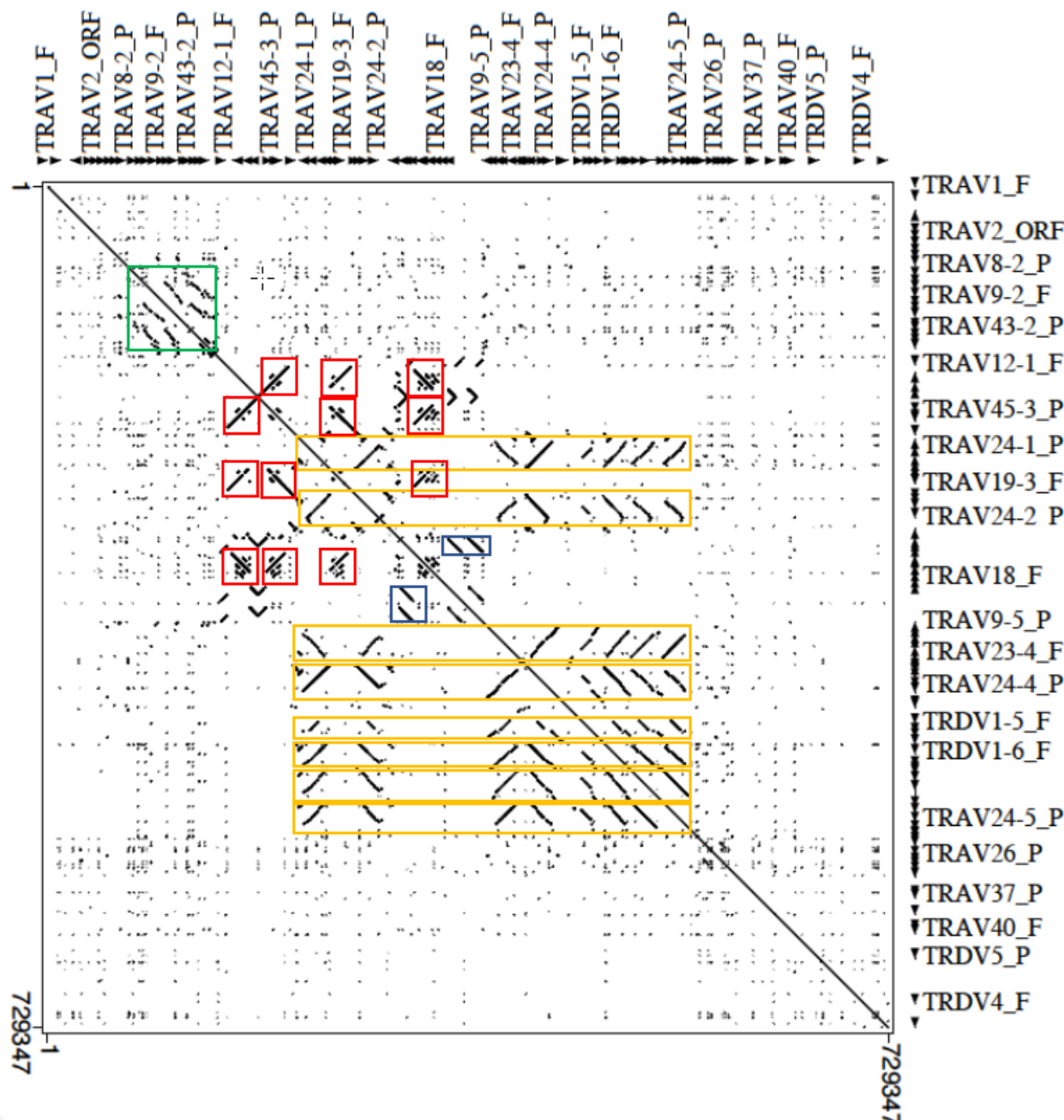


Figure 4. Dot-plot matrix of the dromedary V-cluster sequence against itself. With the exception of the main diagonal line for the match of each base with itself, dots and lines indicate internal homology units in the sequence. The coloured boxes show the V region underwent to duplication events (see text). Orthogonal lines indicate units of homology between regions with an opposite orientation.

The dot-plot matrix highlights the high level of nucleotide identity between V genes as indicated by dots and lines. Furthermore, parallel and orthogonal lines to the perfect main diagonal line, which indicates the match of each base of the sequence with itself, detect units of internal homology due to the duplicative events that have arisen within the multimember TRV genes subgroups. Notable features are the multiple homology regions (parallel or perpendicular lines depending on the orientation of regions within the genomic sequence) due to the expansion of the genes (eight members) of the TRDV1 subgroup (eight orange rectangles in Figure 4). Each homology lines also includes members of expanded TRAV subgroups intercalated with the TRDV1 genes: TRAV22 (eight copies), TRAV23 and TRAV24 (seven copies) and TRAV25 (four copies) subgroups. In fact, the longest homology unit, of about 24 kb,

(TRAV22-6/TRAV23-5/TRDV1-4/TRAV24-4/TRAV25-2) contains member genes of all these subgroups. Therefore, the *TRDV1* amplification mechanism may have facilitated the generation of multigene subgroups even between *TRAV* genes or vice versa. Such concomitant expansion of the *TRDV1* and *TRAV* genes has been also shown in the sheep locus where the *TRDV1* genes are always co-localised with genes of expanded *TRAV* subgroups, mostly belonging to corresponding dromedary *TRAV* subgroups [17,31].

Similarly, parallel or perpendicular lines, in the dot, identify duplication areas in which the *TRAV19* subgroup genes have arisen through duplication events which have involved also the *TRAV45* subgroup (red boxes in Figure 4), generating *TRAV19*–*TRAV45*–*TRAV45* blocks.

Finally, parallel lines that identify multiple tandem duplications of an internal homology unit consisting of the *TRAV9*, *TRAV13*, *TRAV14* and *TRAV43* (green box in Figure 4) subgroup genes and parallel lines due to the tandem duplication of the *TRAV45* genes (blue boxes in Figure 4) were observed.

3.5. Description of the D, J and C genes at the 3' region of the TRA/TRD locus

Following the V-CLUSTER, the TRA/TRD locus continues with the *TRD* genes (Figure 1 and Supplementary Figure S3). Six *TRDD* genes were localised in a region spanning about 49 kb between the *TRDV2* and the first *TRDJ* gene. To date, nine *TRDD* genes in a region of about 112 kb have been demonstrated in sheep TRD locus [17], whereas only three *TRDD* genes lie in the human region of about 27 kb (IMGT®, <http://www.imgt.org>). The nucleotide and deduced amino acid sequences of the dromedary *TRDD* genes identified in the region are shown in Supplementary Figure S4A. They were annotated and classified, according to the international nomenclature (IMGT®, <http://www.imgt.org>), [23–24] with a number corresponding to their position from 5' to 3' within the locus. The six *TRDD* genes consist of nine (*TRDD3*), 11 (*TRDD2* and *TRDD6*) and 13 pb (*TRDD1*, *TRDD4* and *TRDD5*) sequence that can be productively read through their three coding phases. The 5'D–RS and 3'D–RS sequences that flank the D-REGION (i.e., coding region of a *TRD* gene) are well conserved with respect to the consensus sequence except for the non-canonical RS of *TRDD3* 3' heptamer, where the C nucleotide located in the third nucleotide position is mutated to T.

Consistent with sheep and human (IMGT®, <http://www.imgt.org>) [17], four *TRDJ* genes are located upstream of the *TRDC* gene. The nucleotide and deduced amino acid sequences of the *TRDJ* genes are shown in Supplementary Figure S4B. They are typically 49–60 bp long. Each *TRDJ* gene is flanked by the J–RS at the 5' end, and a donor splice site at the 3' end. The coding regions were all predicted to be functional and conserve the canonical F–G–X–G amino acid motif, whose presence characterises the functional *J* genes. The dromedary *TRDJ* genes were classified in accordance with their high nucleotide identity with the sheep and human orthologues. A phylogenetic tree confirms the evolutionary relationship between the clustering distribution of the corresponding *TRDJ* genes in the three species and their order within each TRD locus (Supplementary Figure S5).

The exon–intron organisation of the *TRDC* gene was also determined (Supplementary Figure S4C). The dromedary *TRDC* gene encodes a protein of 152 AA. The human *TRDC* gene encodes a protein with a same length, while the sheep protein is three AA longer (IMGT®, <http://www.imgt.org>), [17]. As in the other mammalian species, it is composed of three translated exons plus a fourth untranslated one. The C-DOMAIN is encoded by EX1 and is 93 AA long as in sheep and human. The C region also comprises the connecting region (CO) of 34 AA (encoded for 22 AA by EX2 as in human, while in sheep EX2 encodes for 25 AA, and for 12 AA by EX3) with a cysteine involved in the interchain disulphide bridge, the transmembrane (TM) of 25 AA (encoded by the 3' part of EX3) and the cytoplasmic region (CY) of five AA encoded by the last part of EX3).

The transcriptionally inverted *TRDV3* gene ends the TRD locus. Sixty *TRAJ* genes lie in the genomic region between *TRDV3* and *TRAC* genes (Supplementary Figure S3 and

Supplementary Figure S6A). Fifty-four *TRAJ* genes were assessed as functional genes and they conserve the canonical F/W–G–X–G amino acid motif (where F is phenylalanine, W tryptophan, G glycine and X any AA) (IMGT®, <http://www.imgt.org>) [34]. Each *TRAJ* gene is flanked by the J–RS at the 5′ end, and a donor splice at the 3′ end. The dromedary *TRAJ* genes were named on the basis of their homology with sheep and bovine genes [17].

The *TRAC* gene surrounds the TRA/TRD locus (Supplementary Figure S3 and Supplementary Figure S6B). The dromedary *TRAC* gene consists of three translated exons and a fourth untranslated exon. The first, second and third exons are 261, 45 and 108 bp in length, respectively. The fourth exon is 573 bp. These four exons are separated by introns that are 1456, 1008 and 660 bp in length, respectively. The exons encode a protein of 121 AA. The C-DOMAIN is encoded by EX1 and is 87 AA long. The C region also comprises the connecting region (CO) of 27 AA (encoded for 15 AA by EX2 and for 12 AA by EX3) with a cysteine involved in the interchain disulphide binding, the transmembrane (TM) of 20 AA (encoded by the 3′ part of EX3) and the cytoplasmic region (CY) of three AA (encoded by the last part of EX3).

3.6. Relationship of the dromedary TRDV germline genes with public available gene sequences and analysis of the δ -chain repertoire

A suggestive implication of the dromedary *TRA/TRD* gene annotation is represented by the possibility of a detailed assessment of the functional δ chain repertoire earlier determined in *Camelus dromedarius* [5]. By a combination of rapid amplification of cDNA ends (RACE) and reverse transcription-polymerase chain reaction (RT-PCR), Antonacci and colleagues evaluated the expressed δ chain repertoire and identified *TRDV* genes classified into three distinct subgroups: TRDV1, TRDV2 and TRDV4. Hence, δ chain transcripts were used to identify germline *TRDV* genes in the dromedary genome by genomic PCRs. Six distinct functional *TRDV1*, two *TRDV2* genes and one *TRDV4* gene were isolated. The amount of *TRDV* germline genes, especially the low number of *TRDV1*, in the dromedary genome was then demonstrated by real-time PCR (qPCR) data, too. In the present work, the characterisation of the *TRD* genes in the dromedary genome assembly has showed the presence of eight *TRDV1* genes (six functional genes and two pseudogenes), which is perfectly in line with the data already described [5]. In order to find any correspondences, we have aligned the nucleotide sequence of the two TRDV1 gene groups: six functional germline genes retrieved from the genome assembly with six distinct *TRDV1* genes isolated before. The comparison between the two groups of *TRDV1* genes allowed us to establish that five genes match perfectly each other (Table 2), based on an operational criterion according to which sequences sharing >97% of nucleotide identity represent the same genes.

Table 2. Correspondence between *TRDV* genes and nomenclatures

This work	Antonacci et al. [5]	% NN identity
TRDV1-1_F	-	from 93.3 to 94.7*
TRDV1-2_F	TRDV1-6	99.71
TRDV1-3_F	TRDV1-3	99.71
TRDV1-5_F	TRDV1-1	99.71
TRDV1-6_F	TRDV1-2	99.37
TRDV1-7_F	TRDV1-4	99.37
-	TRDV1-5	from 92.1 to 96.9*
TRAV33-1/DV6-1	TRDV2.2	99.1
TRAV33-2/DV6-2	TRDV2.1	100

TRDV3	TRDV4	100
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* indicates the identity range with all compared *TRDV1*

Otherwise, two genes, the *TRDV1-1* gene annotated within the locus and the *TRDV1-5* gene [5], showed in the comparison a range of percentage of nucleotide identity <97%, indicating that the *TRDV1-1* could represent a distinct gene not found in the previous analysis, while, the absence of the corresponding *TRDV1-5* gene in the TRA/TRD locus would suggest the presence of a gap in the current assembly. However, polymorphisms or sequencing errors cannot be ruled out.

Similarly, we aligned the nucleotide sequences of the two *TRDV2* genes and the single *TRDV4* gene previously isolated [5] with the gene sequences annotated in this study in order to find any correspondences between them. The comparison showed that the two genes, previously named *TRDV2* (*TRDV2-1* and *TRDV2-2*) (Table 2) because of their homology with the corresponding sheep sequence [13], actually correspond to the *TRAV33* genes (*TRAV33-1* and *TRAV33-2*). As these genes, structurally belonging to the group of *TRAV* genes, have been found rearranged with *TRDD* and/or *TRDJ* genes and therefore used in the synthesis of δ chains in sheep [13] as well as in dromedary [5], they have been classified as *TRAV33/DV6* (Table 2 and Figure 1).

Finally, the *TRDV4* gene, classified according to the initial sheep nomenclature [16], perfectly matches the *TRDV3* gene that stands in an inverted transcriptional orientation downstream the *TRDC* gene within the TRA/TRD locus (Table 2 and Figure 1).

Definitively, our genomic analysis confirms the substantial difference in regard to the number of the *TRDV1* germline genes observed when dromedary is compared with the other artiodactyl species [16–17, 35–36].

Despite of somatic mutation, we tentatively evaluated the contribution of each annotated germline genes in the formation of the δ chain repertoire with particular reference to the CDR3-IMGT region. A total of 61 cDNA clones isolated from peripheral lymphoid tissues of an adult healthy dromedary (22 from spleen, 15 from tonsils and 24 from blood) and containing unique rearranged productive V–(D)–J–C transcripts, were selected from the cDNA library [5] and analysed.

Although nucleotide changes with respect to the germline sequences, 39 cDNA clones (nine in spleen, eight in tonsils and 22 in blood) consist of a member of the *TRDV1* subgroup, 11 clones (seven in spleen and four in tonsils) contain the *TRAV33/DV6* genes and 11 clones (six in spleen, three in tonsils and two in blood) retain the *TRDV3* gene.

The comparison between germline and cDNA sequences has also allowed us to establish that all annotated *TRDJ* genes are represented, with the germline *TRDJ1* gene corresponding to the *TRDJ5* classified within the cDNAs [5].

For a close inspection of the enlarge CDR3-IMGT region, the nucleotide (nt) sequences from codon 105 (codon following the 2nd-CYS codon 104 of the V-REGION) to codon 117 (codon preceding the J-PHE 118 or J-TRP 118) that belongs to the F/W–G–X–G motif characteristic of the J-REGION (i.e., coding region of a *J* gene) (IMGT®, <http://www.imgt.org>), was excised from each clone and reported in Supplementary Figure S7. By comparison with the *TRDD* genomic sequences, the nt located in each sequence were considered to belong to a *TRDD* gene if they constituted a stretch of at least five consecutive nt corresponding to one of the six *TRDD* germline sequences. All *TRDD* but one (*TRDD3*) gene, with no preference in the use, were recovered within the cDNA clones. Moreover, in 12 clones the presence of an additional *TRDD* gene (*TRDD** in Supplementary Figure S7) with a nucleotide sequence similar to the *TRDD2* and missing in the current genome assembly has been hypothesised. We proved the presence of this additional *TRDD* gene with an analysis aimed at the *TRDD* region in *Camelus Bactrianus* (CamBac2) and *Camelus ferus* (BCGSAC_Cfer_1.0) genome sequences, which revealed the presence of seven *TRDD* genes, indicating a probably gap between the dromedary *TRDD4* and *TRDD5* where one more *TRDD* similar to the *TRDD2* gene would lie (see Section 2).

Hence, we observed four clones with no recognizable *TRDD* gene that could be interpreted as direct V–J junction. However, it is also possible that nucleotide trimming as well as somatic mutation masked the participation of *TRDD* gene during the rearrangement. Nevertheless, the presence of an unidentified *TRDD* gene in the genome assembly cannot be excluded. In 14 clones, there is the presence of a single *TRDD* gene, while 20 and 17 clones have two or three *TRDD* genes, respectively. Six clones even contain four *TRDD* genes.

In some cases, two or three or four *TRDD* genes are continuous, in others they are separated by P/N nucleotides. As expected, the order of the *TRDD*, within the CDR3 junction with more than one gene, corresponds to that found in the genome.

The mean length of CDR3-IMGT is 20.4 AA (range 9–37 AA) without significant difference in the three tissues but with an appreciable increase in length in clones of the TRDV1 subgroup compared to the TRAV33 and especially the TRDV3 (Table 3).

Table 3 . CDR3 length of the cDNAs

		TRDV1	TRAV33/DV6	TRDV3	Total
Spleen	N° clones	9	7	6	22
	<i>CDR3 length</i>				
	mean value	22.8	21.1	20.3	21.6
	range	13-37	18-28	19-27	13-37
Tonsils	N° clones	8	4	3	15
	<i>CDR3 length</i>				
	mean value	21.2	19.2	13.3	19.1
	range	15-32	17-22	9-18	9-32
Blood	N° clones	22	-	2	24
	<i>CDR3 length</i>				
	mean value	20.4	-	18.5	20.3
	range	12-32	-	18-19	12-32
Total	N° clones	39	11	11	61
	<i>CDR3 length</i>				
	mean value	21.7	20.4	18.0	20.4
	range	12-37	17-28	9-27	9-37

The CDR3-IMGT length seems to be correlated to the number of the *TRDD* genes incorporated in the loop (Table 4).

In fact, when we analysed the CDR3-IMGT region of all except four clones in which it was not possible to recognize any *TRDD* gene, we found a progression in the CDR3-IMGT length from clones with one *TRDD* (mean 18.6 AA, range 9–27 AA), two (mean 19.2 AA, range 12–28 AA) and three *TRDD* genes (mean 21.1 AA, range 15–28 AA), reaching a maximum of the CDR3-IMGT length (mean 28.3 AA, range 18–37) in the clones with four *TRDD* genes. It should be noted that the CDR3-IMGT, consisting of four *TRDD* genes, was found only in clones of the TRDV1 subgroup.

Table 4. Correlation between the CDR3 length and the number of *TRDD* genes

Subgroups		1 TRDD	2 TRDD	3 TRDD	4 TRDD
TRAV1	N° clones	5	11	13	6
	CDR3 length				
	mean value	19.6	18.3	21.6	28.3
	range	17-22	12-25	15-28	18-37
TRAV1 TRAV33/DV6	N° clones	2	6	3	-
	CDR3 length				
	mean value	19.0	21.3	19.6	-
	range	19	17-28	17-22	-
TRDV3	N° clones	7	3	1	-
	CDR3 length				
	mean value	17.8	18.3	19.0	-
	range	9-27	18-19	19	-
Total	N° clones	14	20	17	6
	CDR3 length				
	mean value	18.6	19.2	21.1	28.3
	range	9-27	12-28	15-28	18-37

3.7. Comparative analysis of the TRA/TRD locus in the *Camelus* genus

The dromedary TRA/TRD sequence was used as a reference for an overview, by computational method, of the orthologous regions in three genome assemblies (CamBac2, CamFer2 and BCGSAC_Cfer_1.0) of the most closely related species available in databases, i.e., *Camelus bactrianus* and *Camelus ferus*.

The CamDro3 chr6:31696654-32573734 region (877081 bp) matched with CamBac2 chr6:29891560-30751197 region (859638 bp), CamFer2 chr6:30383898-31277736 region (893839 bp), and a very short region in a contig of BCGSAC_Cfer_1.0 (NC_045701:30928671-31534193).

A multiple-alignment analysis identified conserved blocks of synteny within each assembly as presented in Figure 5.

The distribution of the corresponding blocks, indicated by identical colours, reveals wide co-linearity between the different TRA/TRD sequences. This is in accordance with previous comparative genomic analysis that reveals the existence of a co-linearity between the three Old World camel TRB genomic sequences [37]. Indeed, large blocks of synteny are interrupted by shortest blocks, due to the presence of sequencing gaps (grey lines in Figure 5) or micro rearrangements (e.g., local inversion, blocks below the central line of each graphic in Figure 5) in some of the three genomic sequences. Taking into account that the present gaps are rightly estimated in size, the length of the entire TRA/TRD locus appears to be comparable in the recent draft of the genome sequences belonging to the three camel species [18]. Instead, sequence gap annotation lacks in the assembly BCGSAC_Cfer_1.0 of the wild Bactrian camel [20].

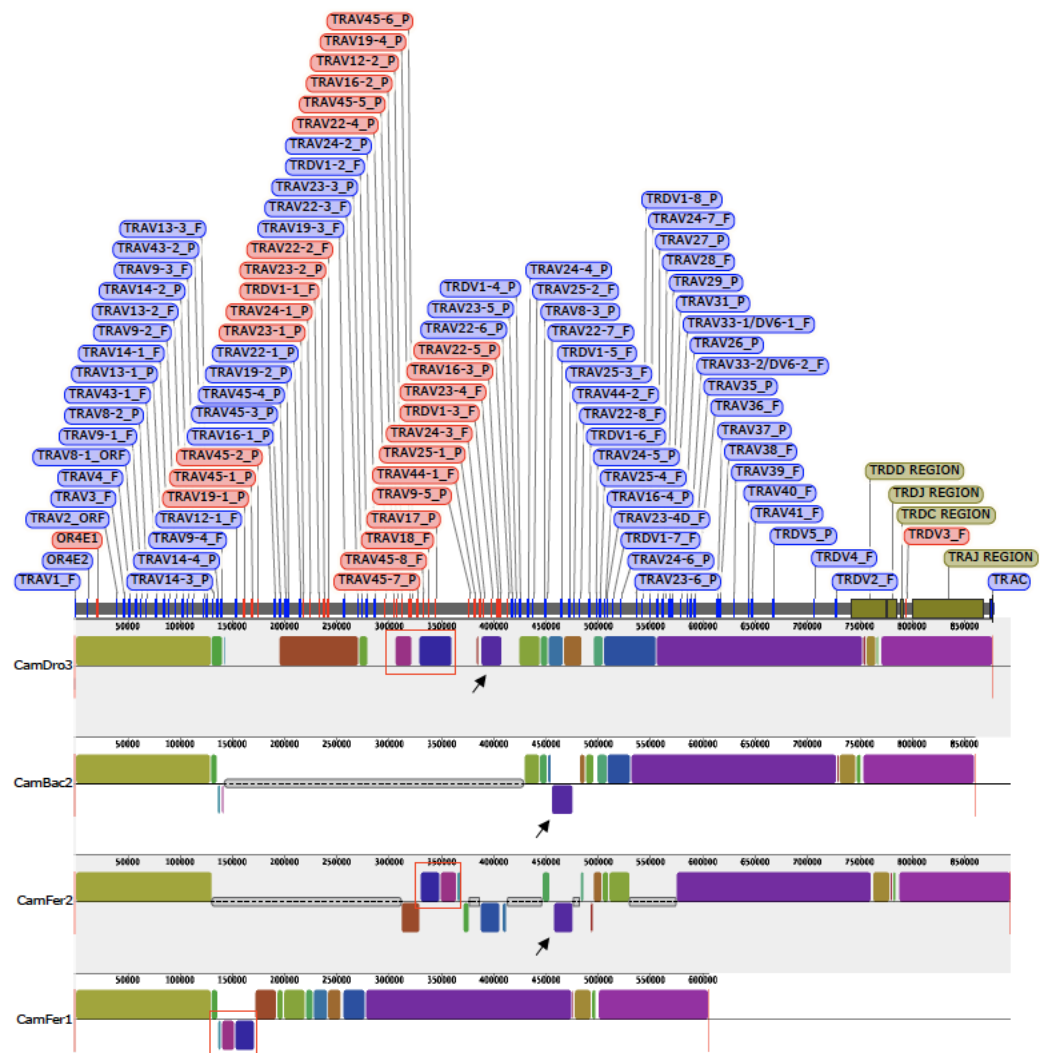


Figure 5. Schematic representation of the comparison of the dromedary (CamDro3) TRA/TRD genomic region with Bactrian (CamBac2) and wild Bactrian (CamFer2 and CamFer1) sequences. Coloured blocks highlight syntenic regions between the different assemblies. Blocks below the line indicate sequences of inverted orientation with respect to the reference CamDro3. The grey thin boxes represent sequencing gaps. The position of all dromedary TRA and TRD genes is reported. The genes with an opposite orientation within the dromedary TRA/TRD locus are marked in red. The black arrows and the red boxes are described in the text.

Mostly, the sequencing gaps affect the regions where the TR V genes have been found to be in opposite orientation respect to the 5'–3' direction of the locus. It is possible that the presence of regions with duplicated genes organised in an inverted transcriptional orientation makes difficult to assemble them in a continuous genomic sequence and, therefore, inconsistencies and sequencing gaps are more frequent. For instance, black arrows in Figure 5 indicate that the corresponding purple blocks, consisting of dromedary TRAV24-3, TRDV1-3, TRAV23-4 and TRAV16-1 genes, are not located in a syntenic region in CamBac2 as well as in CamFer2 sequences. Moreover, both in CamBac2 and CamFer2, the purple block has been assembled in opposite orientation with respect to the CamDrom3 one. Finally, the corresponding purple block is completely missing within the other *Camelus ferus* sequence (BCGSAC_Cfer_1.0), showing that CamFer2 assembly represent an improvement version also for the TRA/TRD region.

Similarly, the blocks containing the *Camelus dromedarius* inverted genes, from TRAV22-4 to TRAV17 (red boxes in Figure 5), lack in CamBac2 but are present in Cam-

Fer2 as well as in BCGSAC_Cfer_1.0, even if the orientation in the last assembly is different.

4. Discussion

In this work, the latest improved version of the genomic assembly [18] allowed us to fill the gap in our knowledge [5] regarding the genomic organisation of the dromedary TRD locus, which, represents the most complex among the TR loci.

As in all mammalian species studies so far, the dromedary TRA/TRD locus has the common feature of the presence of *TRD* genes intermingled with the *TRA* genes (Figure 1 and Supplementary Figure S3). The dromedary TRA/TRD locus spans about 870 Kb long, making it smaller in size with respect to the homologous region of human (1000 Kb) and sheep (2882 Kb) (IMGT® Repertoire, <http://www.imgt.org>) [17]. These two species were considered for the comparison, taken the first as a reference sequence, and the second as representative of the artiodactyl species.

Although the general structure is shared between the three species, interesting differences in the gene content can be observed. In dromedary, the total number of *TRAV* genes (83 *TRAV* genes)(Table 1) is higher than in human (56 *TRAV* genes) (IMGT® Repertoire, <http://www.imgt.org>) but much lower than in the sheep (277 *TRAV* genes) [17]; whereas the number of the dromedary *TRAV* subgroups (33 subgroups) is lower with respect to both human (42 subgroups) and sheep (39 subgroups). Therefore, the dromedary germline *TRAV* repertoire is mainly due to the complexity of the duplication events that have caused the expansion of genes within some subgroups rather than the birth and/or the maintenance of other subgroups. As a matter of fact, the clustering of the genes in the phylogenetic tree (Figure 2) shows that gene duplication, involving ancestral *TRAV* gene subgroups followed by diversification, is the major mode of evolution of the dromedary *TRAV* genes. However, the dromedary *TRAV*42, *TRAV*43 and *TRAV*45 subgroups, shared with sheep but not with human, are distinctive of the artiodactyl lineage, only.

It is possible that the expansion in the dromedary genome of some multigene *TRAV* subgroups could be in part the direct consequence of the gene expansion of the *TRDV1* subgroup. How the mechanism of *TRDV1* amplification could have also facilitated the generation of multigene *TRAV* subgroups is evident in the dot-plot matrix (Figure 4). A noticeable feature is the presence in the central part of the matrix of multiple homology units, due to the expansion of the eight dromedary *TRDV1* subgroup members together with genes of the *TRAV*22, *TRAV*23, *TRAV*24 and *TRAV*25 subgroups. The corresponding *TRAV* subgroups, which consist of only one member gene, is flanked by the single *TRDV1* gene in the human TRA/TRD locus (IMGT® Repertoire, <http://www.imgt.org>), indicating that the *TRAV*22-*TRAV*23/*DV*-*TRDV1*-*TRAV*24-*TRAV*25 region represents an ancestral block. The concomitant expansion of *TRDV* and *TRAV* gene repertoires appears to be occurred also in the sheep locus [17,31] where the expansion of 65 members of the *TRDV1* subgroup involved more *TRAV* subgroups including the corresponding dromedary, too. As a direct consequence of the wide *TRAV* expansion it is the presence, in both species, of a high level (55%) of not functional *TRAV* genes, mostly belonging to the expanded *TRAV* subgroups. Differently, in human only 32% of *TRAV* genes are not functional.

The dromedary matrix also highlights the presence within the V cluster of extensive inversion regions also exhibited in the sheep as well as in other ruminant TRA/TRD locus [17].

The dromedary TRA locus is completed by 60 *TRAJ* genes and one *TRAC* gene (Supplementary Figure S3), similarly to human but differently to sheep, where 61 and 79 *TRAJ* genes were found, respectively.

In the dromedary TRD locus (Figure 1 and Supplementary Figure S3), twelve germline *TRDV* genes, distributed in five subgroups, were identified. Only eight mem-

bers with respect to the high number in sheep as well as in other artiodactyls [16–17, 35–36], belong to the TRDV1 subgroups. Six out of eight *TRDV1* are functional genes (Supplementary Table 1) and present a high level of nucleotide identity (from 93% to 97%), with a low level of structural diversity, similar length and amino acid composition in the CDR (Supplementary Figure S2). Differently, in sheep 65 TRDV1 subgroup genes are largely diversified by a structural variability that includes increased area of CDR and a high degree of amino acid variations, with a range of nucleotide identity from 78 to 97% [16]. The diversification at the CDR has probability guaranteed the maintenance of functional multiple copies of the genes of the TRDV1 subgroup in this species.

Hence, our data confirm that, given the low number of *TRDV1* germline genes in the dromedary locus, the SHM process plays a key role in the generation of the δ chain repertoire in this species, allowing it to achieve diversity comparable to that of sheep [5].

The remaining dromedary TRDV subgroups (TRDV2, TRDV3, TRDV4 and TRDV5) consist of single gene each (Supplementary Figure S2), with the *TRDV5* gene classified as a pseudogene (Supplementary Table S1). The phylogenetic approach demonstrated that *TRDV4* and *TRDV5* genes are typical of the artiodactyl species (Figure 3). Moreover, the phylogenetic tree also attests that the gene duplications that affected the birth of the expanded TRDV1 subgroup occurred independently in sheep and dromedary genome, although the model of duplication can be the same as indicated by the dot plot matrix information.

The *TRDD* genes greatly contribute to the dromedary δ chain repertoire since six or more probably seven *TRDD* genes is capable of generating many potential V–(D)–J recombinations similar to sheep (nine *TRDD* genes) rather than to human (three *TRDD* genes). Differently, dromedary, sheep and human share the same number of *TRDJ* genes. Therefore, *TRDD* genes strongly participate to the diversity of dromedary δ CDR3. Recent evidence [38] suggests that $\gamma\delta$ T cell antigen specificity lies predominantly in the CDR3 loop of the TR δ chain, as it does in immunoglobulins, in which the major contribution to the interaction with the antigen is provided by the CDR3 of the heavy chain [39]. Our cDNA analysis revealed that the length of dromedary δ CDR3 (mean length 20.4 AA; range 9–37 AA) mainly depends on the incorporation of more sequential *TRDD* genes during the recombination process rather than the reduced level of trimming at the ends of *TRDV* and *TRDJ* genes. The maximum number of incorporated *TRDD* genes is 4 in six out of 61 clones, with CDR3 length of 28.3AA (range 18–37) (Table 3, Table 4 and Supplementary Figure S7).

By comparison, in sheep the mean length of δ CDR3 is 17.15 AA (range 8–26 AA) with only one clone out of 56 containing four *TRDD* genes [31]. Similarly, pig δ chains can involve up to four *TRDD* genes [36], and the cattle δ CDR3 shows combinations from one up to five *TRDD* genes with a range in length from nine to 37 AA [35].

Differently, looking at the β CDR3 also formed by V–D–J junctions, the mean length is 12.8 AA (range 9–19 AA) in dromedary spleen, with one clone out of 35 containing two *TRBD* genes, although the different genomic organisation of the TRB locus [40]. A similar length was reported in sheep spleen β CDR3 that is 13.18 AA long (range 9–20 AA) [41]. Rarely, two *TRBD* genes are also present in the pig β CDR3 region [42], where the mean length of β CDR3 loop was 12.7 AA (range 9–18 AA) for thymus and 12.2 AA (range 5–18 AA) for peripheral blood, indicating that a reduced CDR3 length in the β chain is essential for $\alpha\beta$ T cell function.

Altogether these results suggest that a particularly long δ CDR3 may be a prerequisite to perform appropriate T cell functions in artiodactyl species and this is achieved by increasing the number of *TRDD* genes used in the rearrangement. This situation is quite different from humans and mice, confirming that differences between “ $\gamma\delta$ high” and “ $\gamma\delta$ low” species in distribution, diversity and function of $\gamma\delta$ T cells may be substantial.

Furthermore, it should be noted that also in dromedary as in humans there are *TRAV* genes (*TRAV33/DV6* genes), which are used for the production of δ chains. The two *TRAV33/DV6* genes and the unique *TRDV3* gene that our genomic analysis confirms to be

positioned after the *TRDC* gene in inverted orientation, contribute strongly to the adult dromedary δ repertoire through hypermutation [5].

Finally, the genomic characterisation of the entire TRA/TRD region in *Camelus dromedarius* allowed us to roughly investigate the corresponding region in *Camelus bactrianus* and *Camelus ferus* highlighting how omics data can be transferred to more closely related evolutionarily species (Figure 5).

The genomic comparison stressed that the development of improved versions of genomic assemblies are needed to decipher complex genomic regions such as TRA/TRD genes. Moreover, high-quality genome assemblies serve not only as a reference to further genome assembly improvements, but also, they allow detailed studies of the diversity between genomes.

However, if high-quality genome assemblies are essential for the characterisation of complex genomic regions, detailed annotations of these regions are useful for validating the reliability of the assembly.

5. Conclusions

$\gamma\delta$ T cells are an enigmatic cell population with unique features compared with $\alpha\beta$ T cells. Their functional plasticity allows these T cells to be involved in adaptive and innate immune responses [11]. However, $\gamma\delta$ T cells can be highly divergent between species and play specialised roles within a species [43]. In humans and mice (“ $\gamma\delta$ low species”), $\gamma\delta$ T cells make up a minor fraction of T cells in the spleen, lymph nodes and peripheral blood after birth, but are greatly enriched in epithelial tissues, such as the epidermis and mucosa of the digestive and reproductive tracts, suggesting a critical role in early immune responses. In addition to constituting a minority of the T cell pool in these species, the $\gamma\delta$ TR phenotypes generating by somatic recombination are very limited and also the germline gene repertoire is very restricted.

In artiodactyls (sheep, cows, goats and pigs), $\gamma\delta$ T cells represent an intriguing set of T population whose functions and characteristics have to be defined. However, in these species, $\gamma\delta$ T cells exhibit a higher frequency and a wider physiological distribution with respect to “ $\gamma\delta$ low species” [13–16]. They comprise up to 60% of circulating lymphocytes in young sheep, cattle and swine and, although the percentage decreases with age, they still represent up to 30% in adults. For this reason, ruminants and pigs are considered “ $\gamma\delta$ T cell high species”. Moreover, looking at the expressed $\gamma\delta$ TR repertoire, the diversity is significantly higher in artiodactyl species than in humans and mice. This feature is favoured by the large number of germline genes that encode for γ and δ chains, as proved by the genomic analysis of the TRG and TRD loci in the major livestock species (sheep, goat and cattle)[11–12, 17].

Among artiodactyls, also camels have been defined “ $\gamma\delta$ high species” [4]. In fact, $\gamma\delta$ T cells account for up to 35% of blood lymphocytes in newborn and young animals and, despite the lower percentage compared to the other species, they are still a major population in blood.

Previous expression studies on γ and δ chains have showed that also in dromedary, as in the other “ $\gamma\delta$ high species”, the primary $\gamma\delta$ repertoire is wide and diversified, even if its diversity seems to be largely due to SHM rather than the number of the germline genes [5–7]. This assumption has been proved by analysing the genomic organisation of the dromedary TRG locus that revealed how the total number of TRG germline genes is certainly lower compared to that of sheep and cattle, and the γ chain diversity due to the potential gene rearrangements is therefore more limited.

The results of our study on the genomic organisation of the dromedary TRD locus definitively confirmed this finding, highlighting a lower number of germline genes available to generate δ chains compared with the other artiodactyls. Therefore, the observed diversity of dromedary $\gamma\delta$ TR is higher than that expected due to the SHM effect.

Furthermore, the incorporation up to four *D* genes greatly contribute to the diversity of the antigen binding-site of the receptor.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, **Figure S1:** Structure of the *TRAV* genes. The IMGT Protein display of the dromedary *TRAV* genes. The deduced amino acid sequences of the *TRAV* genes were manually aligned according to IMGT unique numbering for the V-REGION [32] to maximise homology. Only functional genes, ORF and in-frame pseudogenes are shown. All sequences exhibit the typical framework regions (FR) and complementarity determining regions (CDR) and the four amino acids (depicted and indicated in bold): cysteine 23 (1st-CYS) in FR1-IMGT, tryptophan 41 (CONSERVED-TRP) in FR2-IMGT, hydrophobic (here L, V and I) 89, and cysteine 104 (2nd-CYS) in FR3-IMGT, with the exception of *TRAV23-2* and *TRAV24-5* pseudogenes that lack the CONSERVED-TRP. Conversely, CDR-IMGT vary in amino acid composition and length. The description of the strands and loops and of the FR-IMGT and CDR-IMGT is according to the IMGT unique numbering for V-REGION [32]. The amino acid length of the CDR-IMGT is also indicated in square brackets., **Figure S2:** The IMGT Protein display of the dromedary *TRDV* genes. The deduced amino acid sequences of the *TRDV* genes were manually aligned according to IMGT unique numbering for the V-REGION [32] to maximise homology. Only functional genes are shown. All sequences exhibit the typical framework regions (FR) and complementarity determining regions (CDR) and the four amino acids (depicted and indicated in bold): cysteine 23 (1st-CYS) in FR1-IMGT, tryptophan 41 (CONSERVED-TRP) in FR2-IMGT, hydrophobic (here L) 89, and cysteine 104 (2nd-CYS) in FR3-IMGT. Conversely, CDR-IMGT vary in amino acid composition and length. The description of the strands and loops and of the FR-IMGT and CDR-IMGT is according to the IMGT unique numbering for V-REGION [32]. The amino acid length of the CDR-IMGT is also indicated in square brackets., **Figure S3:** Zoom of the schematic representation of Figure 1 for the genomic organisation of the dromedary TRD D-J-C and TRA J-C clusters as deduced from the genome assembly CamDro3. The diagram shows the position of all *TRA* and *TRD* genes according to nomenclature. The boxes representing the genes are not to scale. The exons are not shown. The arrows indicate the transcriptional orientation of the genes., **Figure S4:** Nucleotide and deduced amino acid sequences of the dromedary *TRDD* (A), *TRDJ* (B) and *TRDC* (C) genes. The consensus sequence of the heptamer and nonamer is provided at the top of the figure and is underlined. The numbering adopted for the gene classification is reported on the left of each gene. In (A), the inferred amino acid sequence of the *TRDD* genes in the three coding frames are reported. In (B), the donor splice site for each *TRDJ* is shown. The canonical F-G-X-G amino acid motifs are underlined. In (C), IMGT Protein display of the dromedary *TRDC* gene. The descriptions of the strands and loops were collected according to the IMGT unique numbering for the C-DOMAIN [44]. The abbreviation for the connecting (CO), transmembrane (TM) and cytoplasmic (CY) regions is used., **Figure S5:** The NJ tree inferred from the dromedary, sheep and human *TRDJ* gene sequences. The orthologous genes of the three species form monophyletic groups. The evolutionary analyses were conducted in MEGA X [27]. The optimal tree with the sum of branch length = 1.64240724 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) are shown next to the branches [33]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method [30] and are in the units of the number of base differences per site. This analysis involved 12 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 94 positions in the final dataset. The IMGT 6-letter for species (Camdro, Oviari and Homsap) standardized abbreviation for taxon is used., **Figure S6:** Nucleotide and deduced amino acid sequences of the dromedary *TRAJ* (A) and *TRAC* (B) genes. In (A), nucleotide and deduced AA sequences of the dromedary *TRAJ* genes. The numbering adopted for the gene classification is reported on the left of each gene. The functionality is also reported. The consensus sequences of the J-heptamer and J-nonamer are provided at the top of the figure and underlined. The donor splice site for each *TRAJ* is also shown. The canonical F/W-G-X-G amino acid motifs are underlined. In (B), IMGT Protein display of the *TRAC* gene. Description of the strands and loops is according to the IMGT unique numbering for C-DOMAIN [44]. The abbreviation for the connecting (CO), transmembrane (TM) and cytoplasmic (CY) regions is used., **Figure S7:** CDR3 nucleotide and predicted amino acid sequences retrieved from the TRD cDNA clones isolated from spleen, tonsils and peripheral blood. CDR3-IMGT sequences are shown from codon 105 (the codon after the 2nd-CYS 104 of the V-REGION) to codon 117 (the codon before J-PHE 118

of the J-REGION) according to the unique numbering [32]. The CDR3 nucleotide/amino acid sequence, and the classification of the *TRDV* and *TRDJ* genes of each clone are also listed. The *TRDV* genes were assigned to the corresponding germline subgroup only. Nucleotides of the 3'-V-REGION and of the 5'-J-REGION are indicated in uppercase letters. The sequences considered to present recognizable *TRDD* genes are indicated in coloured lowercase letters (pink for *TRDD1*, blue for *TRDD2*, red for *TRDD3*, orange for *TRDD5*, green for *TRDD6* and dark purple for the *TRDD* missing in the current assembly), and the nucleotide substitutions are underlined. The amino acids belonging to a *TRDD* gene are in bold. Nucleotides that cannot be attributed to any V, D or J region (P/N-nucleotides), are indicated in lower cases on the left and on the right sides of the *TRDD* regions. The CDR3 length and the name of each clone are also reported., **Table S1:** Description of the *TRA/TRD* genes in the *Camelus dromedarius* chromosome 6 genome assembly (GeneBank ID: NC_044516.1). The position of all genes and their classification and functionality are reported.

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