RESEARCH ARTICLE

A Comprehensive Analysis of the Phylogeny, Genomic Organization and Expression of Immunoglobulin Light Chain Genes in *Alligator sinensis*, an Endangered Reptile Species

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Abstract

Crocodilians are evolutionarily distinct reptiles that are distantly related to lizards and are thought to be the closest relatives of birds. Compared with birds and mammals, few studies have investigated the Ig light chain of crocodilians. Here, employing an *Alligator sinensis* genomic bacterial artificial chromosome (BAC) library and available genome data, we characterized the genomic organization of the *Alligator sinensis* IgL gene loci. The *Alligator sinensis* has two IgL isotypes, λ and κ, the same as *Anolis carolinensis*. The Igλ locus contains 6 Cλ genes, each preceded by a Jλ gene, and 86 potentially functional Vλ genes upstream of (Jλ-Cλ)n. The Igκ locus contains a single Cκ gene, 6 Jκs and 62 functional Vκs. All VL genes are classified into a total of 31 families: 19 Vλ families and 12 Vκ families. Based on an analysis of the chromosomal location of the light chain genes among mammals, birds, lizards and frogs, the data further confirm that there are two IgL isotypes in the *Alligator sinensis*: Igλ and Igκ. By analyzing the cloned Igλ/κ cDNA, we identified a biased usage pattern of V families in the expressed Vλ and Vκ. An analysis of the junctions of the recombined VJ revealed the presence of N and P nucleotides in both expressed λ and κ sequences. Phylogenetic analysis of the V genes revealed V families shared by mammals, birds, reptiles and *Xenopus*, suggesting that these conserved V families are orthologous and have been retained during the evolution of IgL. Our data suggest that the *Alligator sinensis* IgL gene repertoire is highly diverse and complex and provide insight into immunoglobulin gene evolution in vertebrates.
Introduction

Immunoglobulin (Ig) is one of the most important primary effector molecules in the adaptive immune system of jawed vertebrates [1]. Each immunoglobulin is composed of a heavy (H) chain and one of two light (L) chain types: λ or κ in mammals. Each of these L chains typically covalently links to H by disulfide bonds formed by positionally conserved cysteine residues [2]. As exceptions, shark IgNAR and camelid IgGs are only composed of heavy chains [3, 4]. The Ig light chain is encoded by λ and κ loci, which differ significantly in their genomic organization. At the λ locus, multiple V_λ segments are followed by J_λ-C_λ repeats. In contrast, the cluster of V_κ gene segments is followed by a cluster of J_κ gene segments and then by a single C_κ gene [5–7]. Lymphocytes can generate specific immunoglobulins against diverse antigens by a somatic recombination process, known as V (D) J recombination [8–10]. A pair of recombination signal sequences (RSSs) are composed of conserved heptamer and nonamer sequences and are separated by a relatively non-conserved spacer of either 12 or 23 bp, which is recognized by RAG1 and RAG2. Then, RAG introduces a double-strand break (DSB) between the RSS and the coding segments [11, 12]. Each of the L chains is the result of the imprecise and random combinatorial assembly of several gene fragments by a non-homologous end joining (NHEJ) pathway with the removal or addition of a random number of nucleotides [10, 13]. This imprecision in the coding joint arises from short additions of self-complementary (P) or random (N) nucleotides [9], small deletions, or a combination of these and contributes to the antigen receptor diversity generated by V (D) J joining [14].

IgL genes in cartilaginous fishes belong to four major groups: κ, λ, σ and σ-cart [13]. Among cartilaginous fish, the Ginglymostoma cirratum L chain genes have been studied most comprehensively. In a previous study, four L chain isotypes were identified in Ginglymostoma cirratum: type I (NS5), type II (NS3), type III (NS4) and type IV. The type III L chain is clearly κ, the type II light chain is somewhat more λ-like, the type I gene is closely related to but distinct from the σ gene [15–17] and is referred to as σ-cart, and type IV is homologous with the L chain isotype σ, found first in Xenopus and later in bony fish [13, 17]. The IgL isotypes currently found in teleost belong to κ (L1/G and L3/F), λ and σ(L2). These have been found in a cluster assemblage and, depending on the species, the number of IgL isotypes is different [17–26].

Three types of light chains have been identified in amphibians as well, based on studies of Xenopus laevis: ρ, σ and type III [17, 27–30]. Qin and colleagues completely characterized all three gene loci in Xenopus tropicalis [31] and supported the classification of amphibians in which the ρ gene belongs to the κ gene family and type III appears λ-like [17, 29]. Evolutionarily, mammals express two types of Ig light chain, λ and κ, which are expressed in varying ratios in different species [5, 32–36]. In Mus musculus serum, 95% of the light chains are κ and 5% are λ [5], whereas Bos taurus exhibit a biased usage pattern of λ chain [32]. Like Homo sapiens, Sus scrofa do not show any preference for the usage of the light chain [36]. Surprisingly, unlike reptiles and mammals, birds possess only one light chain, which is orthologous to the Homo sapiens/Mus musculus λ chain [37–41]. The genomic organization of the λ chain is similar to the heavy chain in birds: only one functional V_λ and I_λ are 1.8 kb apart and are located upstream from the C_λ gene in the Gallus gallus [42]. The light chain has evolved an exceptional mechanism of generating diversity due to multiple V_λ pseudogenes that modify the functional V_λ gene and can act as donors to form intrachromosomal gene conversion [43]. These results suggested that the typical birds IgL was likely already present in the common ancestor and remained unchanged over a long period of evolution [40].

Reptilia can be divided into two main evolutionary lineages: one gave rise to Squamata, while the other gave rise to Testudines, Crocodylia, and birds [44]. Some studies have been
conducted to investigate Ig gene isotypes and their genomic organization in reptilia. Until now, IgM, IgD and IgY encoding genes have been identified in all Squamata species studied to date [45–47]. While it was shown that the Anolis carolinensis express two types of light chains: λ and κ [7, 39, 48], snakes lack the Igκ light chain isotype [45]. In the Testudines, IgM, IgD, IgY and IgD2 encoding genes were described, and two immunoglobulin domains of IgD2 are shown to be homologous to bird IgA domains, suggesting that they may originate from a common ancestral gene [49–51]. Crocodilians appeared during the Middle Triassic, approximately 240 million years ago (MYA). Although similar in appearance, crocodilians, as reptiles, are only distantly related to lizards and are thought to be the closest relatives of birds and have thus occupied an important position in evolution [52, 53]. According to phylogenetic studies, crocodilians provide a phylogenetic link to other reptiles and birds, and analysis of their Ig genes may provide important clues to understanding Ig evolution. In addition, despite living in poor conditions, crocodilians are rarely subject to infections caused by bacteria and viruses because of their strong immune systems [54, 55]. However, there have been few studies on the crocodilian immune system. Recently, IgH genes of crocodilians were identified; the results indicated that there are multiple μ genes and that IgM subclasses can be expressed through class-switch recombination. The crocodilian α genes are the first IgA-encoding genes identified in reptiles and suggested that reptiles and birds share a common ancestral organization [56, 57].

Crocodilians are the closest phylogenetic group to birds, and they all come from a group known as archosaurs. However, little is known about the IgL locus of crocodilians. Although a previous study suggested that two distinct light chain types were present in alligator [48], the isotypes and the genomic organization of their encoding genes are still not known [39]. In this study, we present the phylogeny, genomic organization and expression of the Igκ/λ of the Alligator sinensis and provide insight into understanding the crocodilian immune system and the evolution of immunoglobulin in vertebrates.

Materials and Methods
Sample collection, DNA and RNA extract
Blood samples of Alligator sinensis were collected from the Beijing Zoo. Genomic DNA was extracted from the blood following the standard protocol. Total RNA was extracted from the blood using a TRIzol kit (TIANGEN BIOTECH, Beijing) following the manufacturer’s instructions. Our studies were approved by the Animal Care and Use Committee of the China Agricultural University.

BAC library
An Alligator sinensis genomic BAC library was constructed using a service provided by Bioestablish Biotechnology Co., Ltd. (Beijing, China) and was stored in our laboratory [56].

BAC screening and sequencing
Based on sequences derived from Gallus gallus and other related species, we designed degenerate primers for the Igκ/λ. We ascertained the identities of the PCR-generated product sequences by BLAST against the NCBI GenBank, and then designed specific primers for the Igκ/λ genes based on the determined sequences (S1 Table). BAC clones containing Igκ/λ genes were rescreened from the BAC library using PCR. The positive BAC clones were sequenced from both ends, and the end sequences were used to design primers to determine overlapping BAC clones and to obtain the extended segments in the next round of screening (S1 Table).
The positive BAC clones were then sequenced by shotgun sequencing and assembled with the next generation sequencing platform by BGI (Beijing, China).

Cloning of expressed *Alligator sinensis* Igλ and Igκ light chain genes at the cDNA level

Expressed *Alligator sinensis* Igλ and Igκ chains were amplified using the 5’ RACE System kit (Invitrogen, Beijing). The gene-specific primers for the Igλ chain are as follows: IgLC138L18, 5’-CAT TAG GGA GAT ACT ACA-3’; IgLC133L21, 5’-CAG GGA TCC CAG CTC TCT ACT-3’; IgLC129L21, 5’-AGG GTC TTC ATG CTC TTC-3’; IgLC129L21, 5’-GCT GGC CAT GTA CTT GTT GTC-3’. The sequences of these primers are conserved in the *Alligator sinensis* Cλ gene. Gene-specific primers for the Igκ chain are as follows: IgLCx319L18, 5’-ATA AAG AAA GCA TAA GAA-3’; IgLCx236L21, 5’-GCT ACA CTC GGT CCT TCT GAA-3’; IgLCx121L21, 5’-CTG CTC TTG CTG TAC GTG TTG-3’, which are conserved in the *Alligator sinensis* Cκ gene.

All PCR amplifications were performed using a proofreading enzyme Pyrobest DNA polymerase (TaKaRa, Dalian). The PCR products were cloned into the pMD-19 T vector (TaKaRa, Dalian) and sequenced.

Southern blotting

Genomic DNA was digested with restriction endonuclease and was loaded into a 0.9% agarose gel, electrophoresed for 6 h, and transferred to a positively charged nylon membrane (Roche, Germany) for hybridization. The restriction endonucleases Bgl II, Nco I, Hind III and Sph I were used to digest genomic DNA to identify Igλ. Genomic DNA was digested with restriction endonucleases Kpn I, Nde I and Xho I to validate Igκ. The single exon of the Cλ/Cκ probe was labeled using a PCR digoxigenin probe synthesis kit (Roche, Germany). The primers used to amplify the Cλ/Cκ exon probes were as follows: LC-F, 5’-ACA GCC AAA GGC CTC TCT TC-3’; LC-R, 5’-CAG TCT CTT CAG GGT CTT CTC-3’; KC-F, 5’-AAA GGG GGA AGA GCC ACC-3’; KC-R, 5’-TAC ACT CGG CTC TCT TGA-3’. The hybridization and detection were performed following the manufacturer’s instructions.

Construction of phylogenetic trees

The phylogenetic trees were constructed using MrBayes3.1.2 [58] and were viewed in TREEVIEW [59]. Furthermore, in order to validate the topologies of the phylogenetic trees, we also used MEGA6.0 and Phylip3.695 [60] to build all the phylogenetic trees [59]. Multiple amino-acid alignments for the tree construction were performed using ClustalW. Each Vλ/κ subgroup was represented with one family per species chosen at random. The accession numbers of sequences used for variable regions are as follows: *Heterodontus francisci* σ (ABO64185); *Heterodontus francisci* type I (CAA33375); *Heterodontus francisci* type II (AAA59379); *Heterodontus francisci* type III (AAA59373); *Ginglymostoma cirratum* NS5 (AAV34678); *Ginglymostoma cirratum* σ (ABO64187); *Danio rerio* type I (AAG31721); *Danio rerio* type II (AAG31729); *Danio rerio* type III (AAG31698); *X. laevis* type III V1 (AAL40100); *X. laevis* type III V2 (AAL40101); *X. laevis* type III V3 (AAL40102); *X. laevis* type III V4 (AAL40103); *X. laevis* type III V5 (AAL40097); *X. laevis* type III V6 (AAL40093); *X. laevis* σ (NP_001087883); *X. laevis* ρ (AAH68859); *Gallus gallus* IGλV (BAB71862); *Anas platyrhynchos* IGλV (AAA403006); *Anolis carolinensis* IGκV (ACB45832); *Anolis carolinensis* IGκV1 (XP_008115579); *Anolis carolinensis* IGκV2 (XP_008115579); *Anolis carolinensis* IGκV3 (XP_008115579); *Anolis carolinensis* IGκV4 (XP_008115579); *Anolis carolinensis* IGκV5 (XP_008115579); *Mus musculus* IGκV1-132 (CAB46115); *Mus musculus* IGκV2-112 (AAA39032); *Mus musculus* IGκV3-4.
are as follows:

were derived in this study. The accession numbers of sequences used for constant regions from an
chromosome) genomic library, which was constructed using the peripheral blood leucocytes
of 2.1 × 10⁵ clones with an average insert size of ~100 kb, representing ~9 × genomic coverage.

were analyzed using the online program FUZZNUC (http://embossgui.sourceforge.net/demo/fuzznuc.html).

using the IMGT numbering system [62]. The RSSs for the V and J gene segments were ana-
sequences used for constant regions are as follows: Gallus gallus λ (AAA48862); Anas platyrhynchos λ (AAA03009); Ornithorhynchus λ (AAO16062); Homo sapiens λ (AAA59107); Mus musculus λ (AAA39089); Bos taurus λ (AAI46273); Didelphimorphia λ (AAL37214); Orictolagus cuniculus λ (AAA31660); Anolis carolinensis λ (XP_008115579); Sus scrofa λ (AAA03572); Chelonias mydas λ (XP_007055069.1); Chrysemys pictabellii λ (XM_008167097.1); X. laevis type III (AAL40101); X. tropicalis type III (AAL66944); Mus musculus κ (CAA24185); Homo sapiens κ (AAY24201); Bos taurus κ (AAI51501); X. laevis ρ (AAA49880); X. laevis σ (NP_001087883.1); X. tropicalis σ (AAI67133); X. tropicalis ρ (AAI58339); Oryctolagus cuniculus κ (AAI10920); Sus scrofa κ (AHB17990); Didelphimorphia κ (AAL17681); Ornithorhynchus κ (AAO4649); Chelonia mydas κ (EMP6807.1); Chrysemys pictabellii κ (XM_008167924.1); Ginglymostoma cirratum NS5 (AAV34681); Ginglymostoma cirratum NS4 (A49633); Ginglymostoma cirratum NS3 (Ref. [61]); Ginglymostoma cirratum σ (AB064188); Danio rerio IGIC1 (AAG31721); Danio rerio IGIC2 (XP_009298120); Heterodontus francisci type I (CAA33376); Heterodontus francisci type II (CAA33375); Heterodontus francisci type III (AA59373); Heterodontus francisci σ (AB064185); Salmo salar IGIC1 (AA18364); Salmo salar IGIC2 (AAG37201); Salmo salar IGIC3 (AAG97962). All other sequences were derived in this study.

Sequence computations

DNA and protein sequence editing, alignments and comparisons were performed with the MegAlign software (DNASTAR). The EquCab2 assembly in Ensembl database (http://www.ensembl.org/index.html) was used to retrieve the genomic contig that contained the Alligator sinensis IgL/Igκ chain sequences. IgBLAST (http://www.ncbi.nlm.nih.gov/igblast/) was used to predict the V₃₅/κ segments. Germline V₅ and V₆ gene segments were grouped into families using the IMGT numbering system [62]. The RSSs for the V and J gene segments were analyzed using the online program FUZZNUC (http://embossgui.sourceforge.net/demo/fuzznuc.html).

Results

Genomic organization of IgL chain gene loci in Alligator sinensis

According to the IgL chain isotypes in Anolis carolinensis, the genomic organization of the Igλ gene locus in the Alligator sinensis was analyzed. An Alligator sinensis BAC (bacterial artificial chromosome) genomic library, which was constructed using the peripheral blood leucocytes from an Alligator sinensis and stored in our laboratory, was employed. The library is composed of 2.1 × 10⁵ clones with an average insert size of ~100 kb, representing ~9 × genomic coverage (Alligator sinensis genome size of ~2.5 Gb). Using a PCR-based approach and sequencing, we identified four Igλ gene-positive BAC clones (Y21003, Y47P24, Y147P18 and Y127H24) (S1 Table). An ~331 Kb genomic sequence was obtained and was found to contain four λ chain C genes (C₅₄, C₁₂, C₂₃ and C₄₄) and four λ chain J genes (J₅₄, J₅₂, J₁₃ and J₁₄) in front of each C
gene, spanning approximately 12 kb DNA, there are potentially 37 functional \( \lambda \) chain V genes, 32 \( \lambda \) chain V pseudogenes and one ORF. Furthermore, using the available genomic database of the Alligator sinensis (http://www.ncbi.nlm.nih.gov/), a genomic contig (AVPB01119656.1) was identified by BLAST; three \( \lambda \) chain C genes were identified in the contig: one is identical with \( C_\lambda 4 \) in the ~331 kb genomic sequence, and one appears to be a pseudogene because it contains an in-frame stop codon. Furthermore, three \( J \) genes were found in the contig. There are six \( \lambda \) chain C genes (\( C_\lambda 1, C_\lambda 2, C_\lambda 3, C_\lambda 4, \Psi C_\lambda 1 \) and \( C_\lambda 5 \)) and seven \( \lambda \) chain \( J \) genes (\( J_\lambda 1, J_\lambda 2, J_\lambda 3, J_\lambda 4, J_\lambda 5, J_\lambda 6 \) and \( J_\lambda 7 \)) (Fig 1 and S1 Fig). All of the \( C_\lambda \) genes share at least 84.1% amino acid sequence identity, of which the amino acid sequence identities between \( C_\lambda 1 \) and \( C_\lambda 2, C_\lambda 3 \) and \( C_\lambda 4 \) are greater than 90.7%. Each \( C_\lambda \) gene was preceded by a single \( J \) gene segment that was 5' flanked by conserved RSS (nonamer and heptamer) with a 12 bp nucleotide spacer, resembling the genomic organization of the \( V \) gene segments.

We performed a BLAST search against the Alligator sinensis whole-genome shotgun sequence (WGS) assembly deposited in the Ensemble database. Seven genomic contigs (KE698600.1, AVPB01102472.1, KE698001.1, KE698031.1, KE697531.1, KE697662.1 and KE695978.1) were found to contain \( \lambda \) chain V segments (S2 Table). Each \( V_\lambda \) gene, which is 3' flanked by a conserved RSS (heptamer and nonamer) with 23 bp nucleotide spacer, was identified, resembling the genomic organization of the \( V_\lambda \) chain gene loci in mammals. In summary, a total of 86 potentially functional \( V_\lambda \) segments (Fig 1B and S1 Appendix), two ORFs and 67 \( V_\lambda \) pseudogenes were identified upstream from the (\( J_\lambda \)-\( C_\lambda \))\(_n\) segments (S1 Fig), and 67 \( V_\lambda \) that either contain in-frame stop codons or lack a leading peptide appear to be pseudogenes (S2 Appendix). According to the sequence identity (> 75% sequence identity within a single family) and phylogenetic analysis, the potentially functional \( V_\lambda \) genes can be classified into at least 19 families (Fig 3; S3 and S4 Figs; S3 Appendix). In addition, there may be more \( V_\lambda \) segments unidentified in the Alligator sinensis based on the gaps in the contig and incomplete genomic data.

A similar approach was used to identify the \( C_\kappa \) from the genome of the Alligator sinensis. Using a PCR-based approach and sequencing, we obtained 5 \( \kappa \) gene-positive BAC clones (Y146M9, Y65C14, Y77E6, Y329F14 and Y146B4) (S1 Table). An ~484 kb genomic sequence was found to contain a single copy of the Alligator sinensis \( C_\kappa \) gene, which showed homology to several mammalian species, six \( J_\kappa \) gene segments and 66 \( V_\kappa \) gene segments, including 29 \( V_\kappa \) pseudogenes. We performed a BLAST search against the Alligator sinensis whole-genome shotgun sequence (WGS) assembly deposited in the Ensemble database. Seventeen DNA contigs (AVPB01013186.1, AVPB01053098.1, AVPB01130521.1, AVPB01143799.1, KE695928.1, KE697554.1, KE697644.1, KE698008.1, KE698055.1, KE698081.1, KE698096.1, KE698098.1, KE698149.1, KE698335.1, KE698356.1, KE698428.1, and KE698585.1) comprise a leash of \( V_\kappa \) genes that is variable in number from 1 to 15 (S3 Table). At least 62 potentially functional \( V_\kappa \) gene segments (Fig 1B and S4 Appendix); 56 \( V_\kappa \) pseudogenes, which either contain in-frame stop codons or lack a leading peptide (S5 Appendix); and 4 partial \( V_\kappa \) genes were identified from the Alligator sinensis genomic sequence (S5 Fig).
The C\(\kappa\) gene as a single copy in the genome was subjected to confirmation by Southern blotting. We designed a pair of degenerate primers for the C\(\kappa\) gene based on the conserved C\(\kappa\) sequences of the *Alligator sinensis*. Only a single band was observed in *Kpn I*, *Nde I* and *Xba I* digested genomic DNA, which supported the C\(\kappa\) gene as a single copy present in the genome (Fig 2). Upstream of the single copy of the C\(\kappa\) gene, six functional J\(\kappa\)s (J\(\kappa\)1-J\(\kappa\)6) gene segments with RSS interrupted by a 23 bp nucleotide spacer at their 5' ends were identified (S6A Fig). An amino acid sequence alignment of the C\(\kappa\) gene in the *Alligator sinensis* with other species suggested homology to the Ig\(\kappa\) chains of several vertebrates, including the *Homo sapiens*, *Mus musculus*, *Didelphimorphia*, *Ornithorhynchus*, *Anolis carolinensis*, *X. laevis* and *X. tropicalis* (S6B Fig). The C\(\kappa\) protein sequence contained three cysteines, among which the third one at the carboxyl terminal was assumed to link heavy chains (S6B Fig).

Almost all V\(\kappa\) genes were flanked on the 3' end by RSS and were separated by a 12 bp nucleotide spacer to conform the 12–23 rules (S5 Appendix). All V\(\kappa\) genes showed the same transcriptional orientation as (J\(\kappa\))\(n\) C\(\kappa\), with the exception of pseudogene V\(\kappa\)46. The 62 potentially functional V\(\kappa\) genes can be integrated into 12 families based on the phylogenetic analysis and the rule that V\(\kappa\) members in one family share at least 75% identity at the nucleotide level (Fig 4; S7 and S8 Figs; S6 Appendix). Because gaps exist in the contigs and the genomic data are incomplete, it is possible that more V\(\kappa\) genes present in the *Alligator sinensis* genome were not found.

**Phylogenetic analysis of the Alligator sinensis Ig light chain gene segments**

Using the amino acid sequences of IGLV- and IGLC- encoded genes from different jawed vertebrates, we constructed V and C phylogenetic trees, respectively. The trees were constructed...
using protein sequences without CDR3. The phylogenetic trees, based on both the C domains and the V domains, support the fact that there are three major groups of IgL genes in jawed vertebrates: $\kappa$, $\lambda$, and $\sigma$ (including $\sigma$-cart), and Alligator sinensis $\kappa$ and $\lambda$ clearly fall into their own respective groups, suggesting that the Alligator sinensis has only two IgL isotypes: $\kappa$ and $\lambda$.

Fig 2. Southern blotting detection of the Alligator sinensis Ig light chain C gene segments. Genomic DNA was digested with restriction endonucleases, which are indicated above each lane, and hybridized with probes for C$\lambda$ and C$\kappa$, respectively.

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Fig 3. Phylogenetic tree analysis of the 86 *Alligator sinensis* V_\lambda_ genes. A phylogenetic tree of the nucleotides of 86 *Alligator sinensis* V_\lambda_ segments was constructed. The 19 V_\lambda_ gene families are labeled with numbers on the right. Phylogenetic trees were constructed using MrBayes3.1.2 [58] and viewed in TREEVIEW [59].

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Fig 4. Phylogenetic tree analysis of the 62 *Alligator sinensis* Vκ genes. A phylogenetic tree of the nucleotides of the *Alligator sinensis* Vκ segments was constructed. The 12 Vκ gene families are labeled with numbers on the right. Phylogenetic trees were constructed using MrBayes3.1.2 [58] and viewed in TREEVIEW [59].

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(Figs 5 and 6; S9, S10, S11 and S12 Figs). The results reveal that the ρ gene of X. tropicalis, teleost L1 and L3 and cartilaginous fish type III/NS4 is located in the κ group, which also includes the κ genes of the Crocodilians, lizards and mammals. Teleost L2, cartilaginous fish type II/NS3, and X. tropicalis type III all belong to λ groups, including the λ genes of the Crocodilians, lizards, birds and mammals. The σ genes are only found in cartilaginous fish, teleost and amphibians. Taken together with our shared synteny of the κ and λ locus in the Alligator sinensis and the phylogenetic analysis, these data provide convincing evidence that the Alligator sinensis expresses two IgL isotypes: κ and λ. From the phylogenetic analysis, it is not difficult to obtain the relationships between the Alligator sinensis and other species’ V families. Alligator sinensis families Vκ,10 and Vκ,11 are clustered with Anolis carolinensis Vκ. Alligator sinensis family Vκ,7 is clustered with X. laevis ρ; and the same phylogenetic analysis was also performed for Vλ. As shown in Fig 6, Alligator sinensis families Vλ,9 and Vλ,19 are clustered with X. laevis type III V5 and Mus musculus families 1 and 3; Alligator sinensis families Vλ,1-Vλ,8 are related to the Anolis carolinensis Vλ,1, Vλ,3, Gallus gallus and Anas platyrhynchos Vλ; and X. laevis type III V4; and Alligator sinensis families Vλ,11 is clustered with X. laevis type III V6. The V genes were orthologous in different isotypes of IgL. We found no relations between the remaining Vλ genes and other jawed vertebrate species, suggesting that Vλ genes exhibit more abundant diversity in the Alligator sinensis.

Syntenic analysis of Igλ and Igκ chain loci in tetrapods

To determine the identified genes belonging to the λ lineage, we analyzed the chromosomal location relative to the flanking genes of the available genomic data containing the Igλ loci in tetrapods. GNZA (guanine nucleotide-binding protein, αz subunit) and RTDR1 (rhabdoid tumor deletion region gene 1), MRPL40 (mitochondrial ribosomal protein L40) and HIRA (histone cell cycle regulation defective homologue A) located on, respectively, the two sides of the λ locus in Homo sapiens were selected as markers to provide evidence for the gene. An available genomic contig (NW_005841940) containing the Igλ locus of the Alligator sinensis was used for analysis. The results showed three situations in which the λ genes had the same transcriptional orientation: first, the Igλ locus was flanked downstream by MRPL40 and HIRA and upstream by GNZA and RTDR1, as in Homo sapiens and X. tropicalis; second, the opposite situation existed, with the Igλ locus flanked downstream by GNZA and RTDR1 and upstream by MRPL40 and HIRA, as in Gallus gallus, which can occur via intrachromosomal gene conversion; and third, the Igλ locus was only flanked upstream by MRPL40 and HIRA, as in Mus musculus and Anolis carolinensis. In the third situation, GNZA and RTDR1 were identified on chromosome 10, which does not contain IGL in Mus musculus, and in Anolis carolinensis, the chromosomal position of GNZ was identified in contig (NW_003341094.1). However, no IGL gene was found in this contig, and the RTDR1 gene was not identified in Anolis carolinensis. In Mus musculus, the chromosome was recombined, leading to GNZ and RTDR1 being separated from the Igλ locus and located on another chromosome, whereas in Anolis carolinensis, the position of GNZ could not be confirmed because of limited genomic data. The Igλ locus of the Alligator sinensis was also flanked upstream by GNZA and RTDR1 (Fig 7), whereas MRPL40 and HIRA were located in another Alligator sinensis genomic contig (NW_005841997.1), which could not be identified as an IGL gene. We cannot confirm that the two contigs of Alligator sinensis assembled together due to the preliminary nature of the genome assembly. The results suggested that the position of the Igλ locus on the chromosome in the Alligator sinensis was syntenic to that in Homo sapiens and X. tropicalis. In the other species, the flanking genes of the Igλ locus have changed in different ways, including possible intrachromosomal gene conversion (e.g., Gallus gallus), chromosome recombination (e.g., Mus...
musculus), and others that are not confirmed because of limited genomic data (e.g., Anolis carolinensis). All taxa studied showed the same flanking genes on one side or both sides of the Igλ locus. These data provide convincing evidence that the identified genes originated from the same ancestral gene as the λ gene in tetrapods and originated from the same ancestral gene as the type III light chain gene in X. tropicalis. The position of the Igλ locus on chromosome in X. tropicalis may be the oldest form in tetrapods.

Similarly, to determine the identified κ genes in the Alligator sinensis belonging to the κ lineage, we performed a syntenic analysis of the κ genes using the data available for tetrapods,

**Fig 5.** Phylogenetic analysis of the IgL chain C genes in jawed vertebrates. The phylogenetic tree was constructed using C domains. The scale shown as a bar represents the genetic distance (number of nucleotide changes at the given scale). The credibility value for each node is shown. Phylogenetic trees were constructed using MrBayes3.1.2 [58] and viewed in TREEVIEW [59].

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Fig 6. Phylogenetic analysis of the IgL chain V genes in jawed vertebrates. The phylogenetic tree was constructed using V domains. The scale shown as a bar represents the genetic distance (number of nucleotide changes at the given scale). The credibility value for each node is shown. Phylogenetic trees were constructed using MrBayes3.1.2 [58] and viewed in TREEVIEW [59].

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including *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Anolis carolinensis* and *X. tropicalis*. We used the available long genomic contig (NW_005843366.1) containing the Igκ locus of the *Alligator sinensis* to compare with the chromosomal location relative to the flanking genes of the κ gene in other species. The Igκ loci in all analyzed species, except the *Gallus gallus*, were flanked on the 5′ side by *RPIA* (ribose-5-phosphate isomerase A) and *EIF2AK3* (eukaryotic translation initiation factor 2-α kinase 3) encoding genes (Fig 8), revealing that the Igκ locus of the *Alligator sinensis* was syntenic to the *Homo sapiens*, *Mus musculus*, *Anolis carolinensis* and *X. tropicalis*. We also searched for relevant genes upstream of the Igκ locus in the analyzed species and found some gene families that were located far from the Igκ locus, including *SCL* (solute carrier family 4, sodium borate transporter) and *RP* (ribosomal protein). In the analyzed species, either one or two of these gene families were located in the same chromosome with the Igκ locus, except for the *Alligator sinensis* and *X. tropicalis*, which lack a complete genomic sequence. Similar to the Igκ locus, we found intrachromosomal gene conversion, as in *Homo sapiens*, *Anolis carolinensis* and *Gallus gallus*, and chromosome recombination leading to lost genes, as in *Mus musculus*. The preservation of the precise order of genes near the Igκ locus on the chromosome suggested that the Igκ of the *Homo sapiens*, *Mus musculus*, *Anolis carolinensis* and *Alligator sinensis* and the p of *X. tropicalis* was passed down from a common ancestor. However, we did not find any light chain gene located together with the *RPIA* and *EIF2AK3*,

Fig 7. Chromosomal locations of the λ genes in different species and type III genes in *X. tropicalis*. Arrows indicate the transcriptional orientation of the genes. Chr: chromosome; IGLC: immunoglobulin λ chain constant region gene; GNAZ: guanine nucleotide-binding protein, α z subunit; HIRA: histone cell cycle regulation defective homologue A; MRPL40: mitochondrial ribosomal protein L40; RTDR1: rhabdoid tumor deletion region gene 1. The figure was modified from Ref. [31].

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but SUCGL1 (succinate-CoA ligase, GDP-forming, α subunit) was located on the 5’ side of RPIA and EIF2AK3 in the *Gallus gallus*. SUCGL1 was located downstream from the same chromosome and far from RPIA and EIF2AK3 in the *Homo sapiens* (~4.0 Mb) and *Mus musculus* (~2.4 Mb), suggesting intrachromosomal gene conversion, such as Igλ in the *Gallus gallus*. During this process, the *Gallus gallus* Igκ locus was lost. In *Anolis carolinensis*, SUCGL1 is located on chromosome 5 rather than on chromosome 6, on which the Igκ locus is located. In *X. tropicalis*, gene EIF2AK3 was not identified with confidence. We also could not identify the gene SUCGL1 in the *Alligator sinensis*.

**IgL loci functionality and V-J junction diversity in *Alligator sinensis***

Using 5’RACE, we cloned and sequenced 402 amplified cDNA fragments from the blood of *Alligator sinensis* which was the same *Alligator sinensis* to construct genomic BAC library, generating 181 clones that exhibited unique V-J junctions. The sequences were somewhat different from the corresponding genome sequence of the EquCab2 assembly. Among these 181 clones, 56 clones contained a Cλ1, 44 clones contained a Cλ2, 32 clones contained a Cλ3, 3 clones contained a Cλ4, and 37 clones contained another Cλ chain that slightly differed from the identified Cλ4 gene and shared at least 97.3% sequence identity with Cλ4, suggesting the existence of an allelic variant of Cλ4. In addition, two new Cλ genes were found in clones LV6-51 and LV61, which were distinct and shared at least 92.7% sequence identity with Cλ3, Cλ2, Cλ3, Cλ4 and Cλ5. However, in the rest of the C region, clones exhibited chimeras: clone LV2-11 and clone LV-14 are Cλ3-Cλ2 and Cλ4-Cλ1 chimeras, respectively; clones LV11, LV2-38 and LV56 are Cλ3-Cλ1 chimeras (*S7 Appendix*). All chimeras most likely indicated PCR artifacts. The results of the usage of Cλ genes and the genomic organization of the Igλ chain gene locus suggested the existence of additional Cλ genes in the Igλ locus of *Alligator sinensis*. Furthermore, we could not amplify Jλ6-Cλ5 in the *Alligator sinensis* due to its low expression level.
As expected, Jκ1, Jκ2, Jκ3 and Jκ4 were co-expressed with their respective Cκ genes in most cases. However, in some cases, Jκ segments were not co-expressed with their respective Cκ genes, such as one Jκ2-Cκ1 in clone LV25, one Jκ3-Cκ2 in clone LV109, one Jκ1-Cκ3 in clone LV5-51 and one Jκ4-Cκ3 in clone LV6-82, which were generated by template jumping during PCR amplification. Furthermore, two additional Cκ genes were not found in the genome and were co-expressed with Jκ1 and Jκ2 in clones LV6-51 and LV61, respectively. By alignment, the amino acid sequence identities of the two Cκ genes were 97.6% and 98.8% with Cκ1 and Cκ2, respectively, suggesting that the two Cκ genes in clone LV6-51 and LV61 might be two allelic genes with Cκ1 and Cκ2 genes. All three clones containing Cκ4 and the other 37 clones, which contain an allelic variant of Cκ4, were co-expressed with Jκ4, indicating the existence of a Cκ4 allelic gene. Moreover, we analyzed the Jκ genes in 7 chimeras of Cκ genes; clones LV2-11 (C2 +C1) and LV6-91 (C2+C1) included Jκ2, clone LV14 (C4+C1) included Jκ4, and clones LV2-8 (C3+C2), LV11 (C3+C1), LV2-38 (C3+C1) and LV56 (C3+C1) contained Jκ3. All of these products most likely represented PCR artifacts or were generated by template jumping during PCR amplification. We did not find Jκ5, Jκ6, Jκ7 or any other Jκ in the unique 181 clones because of their low expression. We did not find any other Cκ genes in our study, although an isolated Jκ7 was located in the present genomic sequence. It is possible that more Cκ genes were not found because of the incomplete genomic data for the *Alligator sinensis*.

Of the 181 cDNA clones described above, 115 had an identifiable Vκ gene, which provided 63 uniquely recombined V-J junctions (S8 Appendix), and were chosen for analysis and revealed a biased usage pattern of Vκ (Fig 9). The results showed that Vκ segments family 7 was the most frequently used, which accounted for roughly one-third of the expressed Vκ repertoire (45/115). Family 1, family 6 and family 9 were more frequently used segments (Fig 9). Vκ segments from families 2, 8, 11, 12 and 17 were less frequently used. The Vκ segments of other families were not observed in the cDNA clones of the *Alligator sinensis*. In these 63 uniquely recombined V-J junctions, 30% of the clones (35/115) had insertion of N and P nucleotides, generally one to two nucleotides, but there were some exceptions. For example, clone LV6-73 had seven N and P nucleotides in its junction; clone LV5-13 and clone LV5-34 had six and five N and P nucleotides in their junctions, respectively; clones LV6-51 and LV6-8 had four N and P nucleotides in their junctions; and clone LV2-8 had three N and P nucleotides in its junction. On average, the length of the N + P nucleotides in these clones was 0.6 ± 1.2 nucleotides. More than 85% of the clones (98/115) had exonuclease removals at the 3′ end of Vκ. Compared with Vκ, fewer nucleotides were removed at the 5′ end of Jκ (67/115) by the exonuclease activity (Vκ 3.1 ± 2.2 vs. Jκ 1.6 ± 1.8). The average length of the CDR3 in these λ gene clones was 10.6 ± 0.9 (S8 Appendix). The results above demonstrated the abundant diversity of the Vκ genes in the *Alligator sinensis*.

We cloned and sequenced 237 cDNA fragments from the *Alligator sinensis* using 5′ RACE to analyze the use of Jκ and Vκ segments in the expressed κ chain, among which KV-4 has a stop codon in the leading peptide. After the removal of redundant clones, 124 clones that showed unique V-J junctions were obtained for analysis. All six functional Jκ segments were used in these clones: 51 clones contained Jκ1; 22 clones contained Jκ2; 18 and 21 clones contained Jκ3 and Jκ4, respectively; 10 clones contained Jκ6; and Jκ5 was only employed in clone KV-47. In addition, another Jκ that was not found in the genome occurred only once in clone KV2-67, suggesting the existence of another Jκ in the genome or an allelic variant of Jκ. The results revealed a preferential Jκ segment with Jκ1 as the first preferential usage. The usage frequencies of Jκ5 and Jκ6 were lower, with Jκ5 being the lowest.

We chose 91 clones from the above mentioned 124 clones that had identifiable Vκ genes for analysis, revealing a preferential Vκ usage pattern (Fig 10). The results showed that Vκ segments family 1 and family 5 demonstrated obvious advantages, which accounted for 51% and
40% of the expressed V$_\kappa$ repertoire, respectively. V$_\kappa$ segments from families 2, 3, 6 and 8 were less frequently used. The V$_\kappa$ of other families were not observed in the cDNA clones of the *Alligator sinensis* probably because these families contained only one or two members and their expression levels were low. These 91 clones represented 59 uniquely recombined V-J junctions
Four isotype groups: type I (NS5), type II (NS3), type III (NS5) and type IV (NS5). Mammals have more similar to type I, whereas a single Cκ locus is present in cartilaginous and bony fishes, with a clear phylogenetic relationship, and in tetrapods, with the exception of birds, which are present in a cluster and are generally followed by a single Cκ gene. In our recent study, two IgL loci were duplicated after speciation [7, 31]. Because the κ isotype is present in cartilaginous and bony fishes, with a clear phylogenetic relationship, and in tetrapods, with the exception of Gallus gallus, it is believed to be the oldest and most evolutionarily conserved isotype [13]. Unlike Igκ, the λ gene locus often contains several pairs of Jκ-Cκ, which are also present in different numbers in different species, located downstream from the V segments [34]. Previous studies found that multiple Jκ-Cκ were duplicated after speciation [7, 31].

In our recent study, two IgL loci λ and κ were identified in another reptile, the Alligator sinensis, using an available genomic database and sequencing of the Alligator sinensis genomic BACs, which contain IgL genes. In addition, using the X. tropicalis Cα as a template [31], we performed a BLAST search against the Alligator sinensis whole-genome shotgun sequence assembly. No similar sequence was identified (data not shown). The results are consistent with those for Anolis carolinensis, revealing only λ and κ isotypes in reptiles. We sketched the map of the genomic organization of the Igκ and Igκ gene loci of the Alligator sinensis (Fig 1; S1 and S5 Figs). As in other species, each Cκ gene is preceded by a single Jκ gene segment (Fig 1A and S1 Fig), whereas a single Cκ gene follows a cluster of Jκ gene segments (Fig 1B and S5 Fig). To analyze the structure of the RSS elements flanking the IGLV and IGLJ genes, the rule of the

(S9 Appendix). More functional Vκ genes that were not found in the genome were expressed in 33 clones, suggesting more Vκ genes in the Alligator sinensis that have not been identified because of gaps in contigs and incomplete genomic data. The majority of V-J junctions in uniquely recombined κ chain clones lack N and P nucleotide additions. In 59 uniquely rearranged clones, 10 clones show putative N or P nucleotides, and the number of N and P nucleotides is 1 or 2, with an average of 0.16 ± 0.47 bp per clone. The exonuclease removals at the 3’ end of Vκ and the 5’ end of Jκ were 2.1 ± 1.5 and 1.3 ± 1.7 nucleotides. The average length of the CDR3 was 8.8 ± 0.5 nucleotides, and 89% the expressed κ V-J junctions might be formed by microhomology (S9 Appendix).

Discussion

Reptilian is comprised of Aves and non-avian reptilia (Crocodilia, Testudines and Squamata) [63, 64]. Immunoglobulin genes have been studied in non-avian reptilia of Testudines species [50, 51] and Squamata species [45, 46, 65–67]. Crocodilians are thought to be the closest relatives of birds, and they are believed to have strong immune systems [52–55]. Recently, the IgH gene of crocodilians was identified [56, 57]. An interesting feature of the crocodilian IgH constant loci is the presence of a number of duplicated genes encoding five Ig classes [57]. In addition, an investigation of the crocodilian α genes suggested that reptiles and birds share a common ancestral organization [56, 57]. To better understand the immune system of crocodilians, to provide a more complete data of crocodilians Igs, and to obtain more information about immunoglobulin evolution in mammals, birds and reptiles, we identified the Alligator sinensis IgL gene repertoire based on the genome sequence and genomic BAC library.

Previous studies suggested that different IgL genes of jawed vertebrates were classified into four isotype groups: λ, κ, σ and σ-cart. To date, all four isotypes are present only in cartilaginous fishes: type I (NS5), type II (NS3), type III (NS5) and σ [13]. Type III is clearly κ, type II is more similar to λ [15, 16], type I is classified as σ-cart [13], and σ is orthologous to the σ isotype in amphibians [13]. Three IgL isotypes exist in amphibians, including λ, κ, and σ [27–31], whereas most other tetrapods, including reptiles, have two IgL isotypes (λ and κ) [5, 7, 32–36, 44]. Birds and snakes have only the λ isotype [39, 42, 45]. The different IgL isotypes are located in different genomic regions. The genomic organizations of these regions are also different [13]. In the κ locus, multiple Jκ genes, which are present in different numbers in different species, are present in a cluster and are generally followed by a single Cκ [5]. Because the κ isotype is present in cartilaginous and bony fishes, with a clear phylogenetic relationship, and in tetrapods, with the exception of Gallus gallus, it is believed to be the oldest and most evolutionarily conserved isotype [13]. Unlike Igκ, the λ gene locus often contains several pairs of Jκ-Cκ, which are also present in different numbers in different species, located downstream from the V segments [34]. Previous studies found that multiple Jκ-Cκ were duplicated after speciation [7, 31].
heptamer-12 bp spacer-nonamer and the nonamer-23 bp spacer-heptamer, which is a universal rule of IGLV and IGLL gene in all species, is demonstrated. The results reveal that the genomic organization of Igκ in the Alligator sinensis is similar to that in X. tropicalis, lizards, birds and mammals, whereas Igλ is similar to that in X. tropicalis, lizards and mammals because the κ gene has been lost in birds. We found six Cκ genes and seven Jκ genes from the genomic DNA sequence, and the Cκ5 gene and Jκ5–7 were not found to be expressed, likely because of their low expression levels. Generally, Jκ-Cκ pairs are located in the genome. In our study, an isolated Jκ7 was located on the 3’ end of the Igκ locus without following a corresponding Cκ gene. This result suggested that more Cκ genes might be located in the Igκ locus in the Alligator sinensis, which was supported by the Southern blotting results.

Our study also found multiple germline Vκ and Vλ in the Alligator sinensis. A total of 155 Vλ and 118 Vκ gene segments were identified, which contain 69 Vλ pseudogenes and 56 Vκ pseudogenes, respectively. All Vλ genes are oriented in the same transcriptional orientation as the Cλ gene and are upstream of the (Jλ-Cλ)n or (Jλ)n. The multiple functional V genes can increase the antibody diversity and enhance the immune response of antigen recognition and binding. The ratio of functional Vλ and Vκ varies significantly in different species [5, 32–36]. It has been proposed that the number of V gene segments may be connected to the preferential use of light chain isotypes at the protein level [68].

Alligator sinensis showed that a large number of V genes were employed in both λ and κ isotype. Additionally, there is a large number of pseudogenes in the Vλ and Vκ loci. We question whether these pseudogenes are functional as those in birds for use as donors of uniquely combined functional V genes in gene conversion [43]. These pseudogenes were likely involved in generating Ig diversity. The diversification of IgLs in the Alligator sinensis is similar to that in most tetrapods but is different from that in the Gallus gallus. A total of 142 potentially functional Vλ genes (Vλ and Vκ) are classified into 31 families in the Alligator sinensis: 19 families in Vλ and 12 families in Vκ (Figs 3 and 5; S3, S4, S7 and S8 Figs, S3 and S6 Appendices). For other species, 177 functional Vλ genes (Vλ and Vκ) are classified into 23 families in Mus musculus (http://www.imgt.org/IMGTrepertoire/), 148 functional Vλ genes (Vλ and Vκ) are classified into 23 families in Homo sapiens (http://www.imgt.org/IMGTrepertoire/), 51 functional VL genes (Vλ and Vκ) are classified into 11 families in Anolis carolinensis [7], and only one Vκ gene (or one family) is present in Gallus gallus [42]. The diversity of the IgL chain is generated by V-J recombination, somatic hypermutation, and the polymorphism of the VL genes, including the number of VL genes and families (classifying family according to the similarity of sequence). Our results reveal that the Alligator sinensis possesses at least 142 functional VL genes (possibly more) and 31 VL gene families, although the number of VL genes in the Alligator sinensis is not the most plentiful in the tetrapods. However, the number of VL gene families is the greatest. The phylogenetic analyses show that many Vλ gene families in the Alligator sinensis are orthologous with other species, but the remaining Vλ gene families are characteristic of the Alligator sinensis. The Alligator sinensis also possesses a large number (68) of DH gene segments and multiple μ genes in the IgH locus, suggesting that the DH segments may contribute significantly to antibody diversity in crocodilians and that IgM subclasses can be expressed through class-switch recombination in the IgH gene locus [36]. These results reveal the vast diversity of Ig in the Alligator sinensis, suggesting that crocodilians have a strong immune system.

We compared IgL chains between two reptiles: the Alligator sinensis and Anolis carolinensis. We found more abundant VL genes in the Alligator sinensis than in Anolis carolinensis, including functional VL genes and pseudogenes. The analysis of the expressed Vλ and Vκ in the Alligator sinensis showed that a large number of V genes were employed in both λ and κ,
suggesting that somatic V-J recombination can contribute to the *Alligator sinensis* antibody diversity, as in *Anolis carolinensis* [7]. Additionally, the occurrence of N or P nucleotide additions at V-J junctions is increased in the *Alligator sinensis* compared to the paucity of N or P nucleotide additions in the V-J junctions in *Anolis carolinensis*, suggesting that crocodilians have more V-J combinatorial diversity than lizards.

We analyzed the preserved co-localization of genes on the Igλ and Igκ loci in different species. First, we identified a syntenic relationship between two conserved gene clusters the GNZA and RTDRI1 cluster and the MRPL40 and HIRA cluster with the Igλ gene on the chromosome in the *Alligator sinensis* and other species, including *Homo sapiens, Mus musculus, Gallus gallo*

lus, *Anolis carolinensis* and *X. tropicalis* (Fig 7). All species retained either one or two gene clusters beside the Igλ locus, although two gene clusters reversed their position in *Gallus gallus* and one gene cluster was lost in *Mus musculus*, suggesting that the location of Igλ locus was conserved in tetrapods, including crocodilians. The oldest form was found in *X. tropicalis* and *Homo sapiens* and possibly in *Alligator sinensis*. We also found a syntenic relationship of the Igκ gene on the chromosome in different species. The results showed that conserved genes RPIA and EIF2AK3 were flanked on the 3’ side of Igκ in all species, except in *Gallus gallus* (Fig 8). The two gene families, SCL and RPL, were located far upstream of the Igκ locus. The results suggested that likely intrachromosomal gene conversion occurred in *Gallus gallus* and *Homo sapiens* or *Anolis carolinensis* during speciation, leading to *Gallus gallus* Igλ and Igκ loci changes. The flanking genes of Igλ were reversed and were lost, and the positions of SCL and RPL were reversed in *Homo sapiens* and *Anolis carolinensis*. Either *Homo sapiens* or *Anolis carolinensis* retained the oldest Igκ locus in the genome.

The results of the phylogenetic tree based on the C domain revealed that isotypes were grouped first, and then species were grouped (Fig 5; S9 and S10 Figs). The phylogenetic tree of V genes also showed the same result (Fig 6; S11 and S12 Figs), suggesting that IgL isotypes were individually orthologous. The phylogenetic analyses showed that the σ gene was only present in cartilaginous fish, bony fish and amphibians and was absent in reptiles, birds and mammals [13, 24, 31, 39]. The κ gene existed in all vertebrates except birds [13, 39–41]. Therefore, the σ gene was lost in other vertebrates after their divergence from amphibians [13, 31], and the κ gene was lost in birds [39–41]. Phylogenetic analysis of the IGLV gene, including all 19 Vλ families and 12 Vκ families in the *Alligator sinensis, Alligator sinensis* families Vλ1-Vλ8 are related to the *Anolis carolinensis* Vλ1, Vλ3, *Gallus gallus* and *Anas platyrhynchos* Vλ3, and X. *laevis* type III V4 (Fig 6; S11 and S12 Figs), which suggested that during the evolution of the λ locus, there was an ancestral locus shared by birds, reptilia and Salientia [7]. *Alligator sinensis* families Vκ11 is clustered with X. *laevis* type III V6; *Alligator sinensis* families Vκ11 and Vκ10 are clustered with *Anolis carolinensis* Vκ7 and *Alligator sinensis* family Vκ7 is clustered with X. *laevis* ρ (Fig 6; S11 and S12 Figs), which indicated that reptilia and amphibians shared some Vλ and Vκ families and originated from descendants of a common ancestor. Crocodilians possess more Vλ families than frogs, lizards and mammals, and there is more abundant diversity of the V gene in crocodilians. Taken together, the results strongly suggest that we have identified two IgL loci in *Alligator sinensis* that belong to the κ and λ lineages. We present evidence that the σ was lost in early reptilians, avian and mammalian after their divergence from amphibians [13, 31], and the κ gene was absent in birds after their divergence from reptilians, similar to the δ gene [39–41].

This study investigated the genomic organization of *Alligator sinensis* IgL genes. The organizations and structures of IgL genes are similar to those of other jawed vertebrates. The study of the *Alligator sinensis* λ and κ loci revealed a diverse and complex repertoire of IgL in crocodilians; the information provides key insights into the evolution of IgL genes in jawed vertebrates.
Supporting Information

S1 Appendix. Multiple sequence alignment of *Alligator sinensis* V\_\(\lambda\) genes. (DOCX)

S2 Appendix. The *Alligator sinensis* V\_\(\lambda\) gene DNA segment in contigs. (DOCX)

S3 Appendix. The alignment of the deduced amino acid sequence of 86 functional V\_\(\lambda\) genes in the *Alligator sinensis*. (DOCX)

S4 Appendix. Multiple sequence alignment of *Alligator sinensis* V\_\(\kappa\) genes. (DOCX)

S5 Appendix. The *Alligator sinensis* V\_\(\kappa\) gene DNA segment in contigs. (DOCX)

S6 Appendix. The alignment of the deduced amino acid sequence of 62 functional V\_\(\kappa\) genes in the *Alligator sinensis*. (DOCX)

S7 Appendix. Sequence of the C region chimeras in the cDNA clones. (DOCX)

S8 Appendix. V-J junctions of the \(\lambda\) chain genes. The letter in the middle indicates N/P nucleotides. The column “N+P” indicates the total nucleotide length of the N and P nucleotides, and the column “CDR3” indicates the codon numbers. The column “Deletions in 3’ end of V\_\(\lambda\)” indicates the number of nucleotides deleted by exonuclease activity at the 3’ end of V\_\(\lambda\), and the column “Deletions in 5’ end of V\_\(\lambda\)” indicates the number of nucleotides deleted by exonuclease activity at the 5’ end of J\_\(\lambda\). Germline sequences of each V\_\(\lambda\) gene segment are shown above the cDNA clones in bold, and the CDR3 is also underlined. (DOCX)

S9 Appendix. V-J junctions of the \(\kappa\) chain genes. The letter in the middle indicates N/P nucleotides. The column “N+P” indicates the total nucleotide length of the N and P nucleotides, and the column “CDR3” indicates the codon numbers. The column “Deletions in 3’ end of V\_\(\kappa\)” indicates the number of nucleotides deleted by exonuclease activity at the 3’ end of V\_\(\kappa\), and the column “Deletions in 5’ end of J\_\(\kappa\)” indicates the number of nucleotides deleted by exonuclease activity at the 5’ end of J\_\(\kappa\). Germline sequences of each V\_\(\kappa\) gene segment are shown above the cDNA clones in bold, and the CDR3 is also underlined. (DOCX)

S1 Fig. The genomic organization of the *Alligator sinensis* immunoglobulin \(\lambda\) gene locus. V: variable gene segments; \(\Psi V\): pseudo-variable gene segments; ORF: variable gene segments with open reading frames but with defects in splicing sites, RSS and/or regulatory elements, and/or changing the conserved amino acids, which have been suggested to lead to incorrect folding [69]; J: joining gene segments; C: constant region gene; \(\Psi C\): pseudo-constant region gene. Gaps between contigs are indicated by a dotted black line, and the sequences from BAC are indicated by a bold line. (TIF)

S2 Fig. Sequences of the *Alligator sinensis* J\_\(\lambda\) and C\_\(\lambda\). (A) Nucleotide and amino acid sequences of the seven *Alligator sinensis* J\_\(\lambda\) segments. (B) Sequence comparison of the six *Alligator sinensis* C\_\(\lambda\) genes with their counterparts in the *Homo sapiens*, *Mus musculus*, *Gallus gallus*, and *Anas*.
platyrhynchos and Anolis carolinensis. In the alignment, dots indicate identical amino acids and A-G over the lines represent potential IgSF strands. The cysteine (C) and tryptophan (W) residues are shaded.

S3 Fig. Phylogenetic trees based on 1000 bootstraps for the Alligator sinensis Vλ gene segments. The phylogenetic tree was constructed using Phylip3.695 [60] and viewed in TREEVIEW [59].

S4 Fig. Phylogenetic analysis of the Alligator sinensis Vλ gene segments. The tree is made by Neighbor-joining P-distance and pairwise deletions using MEGA6.0.

S5 Fig. Genomic organization of the Alligator sinensis immunoglobulin κ gene locus. V: variable gene segments; ΨV: pseudo-variable gene segments; ORF: variable gene segments with open reading frames but with defects in splicing sites, RSS and/or regulatory elements, and/or changing the conserved amino acids, which have been suggested to lead to incorrect folding [69]; J: joining gene segments; C: constant region gene. Gaps between contigs are indicated by a dotted black line, and the sequences from BAC are indicated by a bold line.

S6 Fig. Sequences of the Alligator sinensis Jκ and Cκ. (A) Nucleotide and amino acid sequences of the six Alligator sinensis Jκ segments. (B) Sequence comparison of the Alligator sinensis Cκ genes with their counterparts in Homo sapiens, Mus musculus, Didelphimorphia, Ornithorhynchus, X. laevis, X. tropicalis, and Anolis carolinensis. In the alignment, dots indicate identical amino acids and A-G over the lines represent the potential IgSF strands. The cysteine (C) and tryptophan (W) residues are shaded.

S7 Fig. Phylogenetic trees based on 1000 bootstraps for the Alligator sinensis Vκ gene segments. The phylogenetic tree was constructed using Phylip3.695 [60] and viewed in TREEVIEW [59].

S8 Fig. Phylogenetic analysis of the Alligator sinensis Vκ gene segments. The tree is made by Neighbor-joining P-distance and pairwise deletions using MEGA6.0.

S9 Fig. Phylogenetic trees based on 1000 bootstraps for the IgL chain C genes in jawed vertebrates. The phylogenetic tree was constructed using Phylip3.695 [60] and viewed in TREEVIEW [59].

S10 Fig. Phylogenetic analysis of the IgL chain C genes in jawed vertebrates. The phylogenetic tree was constructed using C domains, and by Neighbor-joining P-distance and pairwise deletions using MEGA6.0.

S11 Fig. Phylogenetic trees based on 1000 bootstraps for the IgL chain V genes in jawed vertebrates. The phylogenetic tree was constructed using V domains. Each V subgroup is represented with one sequence per species chosen at random among the functional genes. The scale shown as a bar represents the genetic distance (number of nucleotide changes in the given
The credibility value for each node is shown. The phylogenetic tree was constructed using Phylip3.695 [60] and viewed in TREEVIEW [59].

**S12 Fig. Phylogenetic analysis of the IgL chain V genes in jawed vertebrates.** The phylogenetic tree was constructed using V domains, and by Neighbor-joining P-distance and pairwise deletions using MEGA6.0.

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**Author Contributions**

Conceived and designed the experiments: LR YZ HW GC. Performed the experiments: XW GC. Analyzed the data: LR XW GC. Contributed reagents/materials/analysis tools: YL CZ XW. Wrote the paper: LR YZ XW GC. Sample collection: YL CZ XW.

**References**

1. Litman GW, Anderson MK, Rast JP. Evolution of antigen binding receptors. Annual review of immunology. 1999; 17:109–47. PMID:10358755
2. Chothia C, Lesk AM, Tramontano A, Levitt M, Smith-Gill SJ, Air G, et al. Conformations of immunoglobulin hypervariable regions. Nature. 1989; 342(6252):877–83. PMID:2687698
3. Hamers-Casterman C, Atarhouch T, Muyldermans S, Robinson G, Hammers C, Songa EB, et al. Naturally occurring antibodies devoid of light chains. Nature. 1993; 363(6428):446–8. PMID:8502296
4. Greenberg AS, Avila D, Hughes M, Hughes A, McKinney EC, Flajnik MF. A new antigen receptor gene family that undergoes rearrangement and extensive somatic diversification in sharks. Nature. 1995; 374(6518):168–73. PMID:7877689
5. Kawasaki K, Minoshima S, Nakato E, Shibuya K, Shintani A, Asakawa S, et al. Evolutionary dynamics of the human immunoglobulin κ locus and the germline repertoire of the Vκ genes. Eur J Immunol 2001; 31(4):1017–28. PMID:11298326
6. Gerdes T, Wabi M. Physical map of the mouse lambda light chain and related loci. Immunogenetics. 2002; 54(1):62–5. PMID:11976793
7. Wu Q, Wei Z, Yang Z, Wang T, Ren L, Hu X, et al. Phylogeny, genomic organization and expression of lambda and kappa immunoglobulin light chain genes in a reptile, Anolis carolinensis. Developmental and comparative immunology. 2010; 34(5):579–89. doi: 10.1016/j.dci.2009.12.019 PMID:20056120
8. Akira S, Okazaki F, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science. 1987; 238(4830):1134–8. PMID:3120312
9. Gu H, Förster I, Rajewsky K. Sequence homologies, N sequence insertion and JH gene utilization in VHDJH joining: implications for the joining mechanism and the ontogenetic timing of Ly1 B cell and B-CLL progenitor generation. EMBO J. 1990; 9(7):2133. PMID:2113468
10. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V (D) J recombination. Cell. 2002; 109(2):S45–S55.
11. McBlane JF, van Gent DC, Ramsden DA, Romeo C, Cuomo CA, Gellert M, et al. Cleavage at a V (D) J recombination signal requires only RAG1 and RAG2 proteins and occurs in two steps. Cell. 1995; 83 (3):387–95. PMID: 8521468
12. Fugmann SD, Lee AI, Shockett PE, Villey IJ, Schatz DG. The RAG proteins and V (D) J recombination: complexes, ends, and transposition. Annu Rev Immunol. 2000; 18(1):495–527.
13. Criscitiello MF, Flajnik MF. Four primordial immunoglobulin light chain isotypes, including lambda and kappa, identified in the most primitive living jawed vertebrates. European journal of immunology. 2007; 37(10):2883–94. PMID: 1799545
14. Jung D, Giallourakis C, Mostoslavsky R, Alt FW. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annual review of immunology. 2006; 24:541–70. PMID: 16551259
15. Lee SS, Fitch D, Flajnik MF, Hsu E. Rearrangement of immunoglobulin genes in shark germ cells. J Exp Med. 2000; 191(10):1637–48. PMID: 10811858
16. Dooley H, Flajnik MF. Antibody repertoire development in cartilaginous fish. Developmental and comparative immunology. 2006; 30(1–2):43–56. PMID: 16146649
17. Edholm ES, Wilson M, Bengten E. Immunoglobulin light (IgL) chains in ectothermic vertebrates. Developmental and comparative immunology. 2011; 35(9):906–15. doi: 10.1016/j.devimm.2011.01.012 PMID: 21258661
18. Hikima J, Jung TS, Aoki T. Immunoglobulin genes and their transcriptional control in teleosts. Developmental and comparative immunology. 2011; 35(9):924–36. doi: 10.1016/j.devimm.2010.10.011 PMID: 21078341
19. Daggfeldt A, Bengtén E, Pilström L. A cluster type organization of the loci of the immunoglobulin light chain in the Siberian sturgeon (Acipenser baeri). J Immunol. 1993; 151(12):6900–12. PMID: 8258698
20. Ghaffari SH, Lobb CJ. Structure and genomic organization of immunoglobulin light chain in the channel catfish. An unusual genomic organizational pattern of segmental genes. J Immunol. 1993; 151(4):1544–50. PMID: 8631390
21. Lundqvist M, Bengtén E, Strömberg S, Pilström L. Ig light chain gene in the Siberian sturgeon (Acipenser baeri). J Immunol. 1996; 157(5):2031–8. PMID: 8757324
22. Partula S, Schwager J, Timmus S, Pilström L, Charlemagne J. A second immunoglobulin light chain isotype in the rainbow trout. Immunogenetics. 1996; 45(1):44–51. PMID: 8881036
23. Ghaffari SH, Lobb CJ. Structure and genomic organization of a second class of immunoglobulin light chain genes in the channel catfish. J Immunol. 1997; 159(1):250–8. PMID: 9200461
24. Haire RN, Rast JP, Litman RT, Litman GW. Characterization of three isotypes of immunoglobulin light chains and T-cell antigen receptor α in zebrafish. Immunogenetics. 2000; 51(11):915–23. PMID: 11003385
25. Edholm ES, Wilson M, Sahoo M, Miller NW, Pilstrom L, Wermenstam NE, et al. Identification of Igsigma and Iglambda in channel catfish, Ictalurus punctatus, and Iglambda in Atlantic cod, Gadus morhua. Immunogenetics. 2009; 61(5):353–70. doi: 10.1007/s00251-009-0365-z PMID: 19333591
26. Bao Y, Wang T, Guo Y, Zhao Z, Li N, Zhao Y. The immunoglobulin gene loci in the teleost Gasterosteus aculeatus. Fish Shellfish Immunol. 2010; 28(1):40–8. doi: 10.1016/j.fsi.2009.09.014 PMID: 19782140
27. Schwager J, Bürckert N, Schwager M, Wilson M. Evolution of immunoglobulin light chain genes: analysis of Xenopus IgL isotypes and their contribution to antibody diversity. EMBO J. 1991; 10(3):505. PMID: 1705882
28. Zezza DJ, Mikoryak C, Schwager J, Steiner L. Sequence of C region of L chains from Xenopus laevis Ig. J Immunol 1991; 146(11):4041–7. PMID: 1903418
29. Zezza D, Steward S, Steiner L. Genes encoding Xenopus laevis Ig L chains. Implications for the evolution of kappa and lambda chains. J Immunol 1992; 149(12):3968–77. PMID: 1460285
30. Haire R, Ota T, Rast J, Litman R, Chan F, Zon L, et al. A third Ig light chain gene isotype in Xenopus laevis consists of six distinct VL families and is related to mammalian lambda genes. J Immunol 1996; 157(4):1544–50. PMID: 8759737
31. Qin T, Ren L, Hu X, Guo Y, Fei J, Zhu Q, et al. Genomic organization of the immunoglobulin light chain gene loci in Xenopus tropicalis: evolutionary implications. Developmental and comparative immunology. 2008; 32(2):156–65. PMID: 17624429
32. Butler J. Immunoglobulin gene organization and the mechanism of repertoire development. Scand J Immunol. 1997; 45(5):455–62. PMID: 9160087
33. Bengten E, Wilson M, Miller N, Clem L, Pilström L, Warn G. Immunoglobulin isotypes: structure, function, and genetics. Curr Top Microbiol Immunol: Springer; 2000. p. 189–219.
34. Gerdes T, Wabl M. Physical map of the mouse λ light chain and related loci. Immunogenetics. 2002; 54 (1):62–5. PMID: 11976793
35. Pilstrom L. The mysterious immunoglobulin light chain. Developmental and comparative immunology. 2002; 26(2):207–15. PMID: 11696386
36. Butler JE, Sun J, Wertz N, Sinkora M. Antibody repertoire development in swine. Developmental and comparative immunology. 2006; 30(1):199–221.
37. Sanders BG, Travis JC. Evidence for one immunoglobulin light-chain type in chickens: absence of a blocked N-terminal light-chain type. Biochemical genetics. 1975; 13(11–12):779–82. PMID: 812481
38. Magor KE, Higgins DA, Middleton DL, Warr GW. cDNA sequence and organization of the immunoglobulin light chain gene of the duck, Anas platyrhynchos. Developmental and comparative immunology. 1994; 18(6):523–31. PMID: 7768317
39. Das S, Nikolaidis N, Klein J, Nei M. Evolutionary redefinition of immunoglobulin light chain isotypes in tetrapods using molecular markers. Proceedings of the National Academy of Sciences of the United States of America. 2008; 105(43):16647–52. doi: 10.1073/pnas.080800105 PMID: 18940927
40. Huang T, Zhang M, Wei Z, Wang P, Sun Y, Hu X, et al. Analysis of immunoglobulin transcripts in the ostrich Struthio camelus, a primitive avian species. PloS one. 2012; 7(3):e34346. doi: 10.1371/journal.pone.0034346 PMID: 22479606
41. Das S, Mohameda U, Hirano M, Nei M, Nikolaidis N. Analysis of the immunoglobulin light chain genes in zebra finch: evolutionary implications. Mol Biol Evol. 2010; 27(1):113–20. doi: 10.1093/molbev/msp212 PMID: 19744999
42. Olivieri DN, von Haeften B, Sanchez-Espinel C, Faro J, Gambon-Deza F. Genomic V exons from whole genome shotgun data in reptiles. Immunogenetics. 2014; 66(7–8):479–92. doi: 10.1007/s00251-014-0784-3 PMID: 24893587
43. Magadan-Mompo S, Sanchez-Espinel C, Gambon-Deza F, Sanchez-Espinel C, Faro J, Gambon-Deza F. Genomic V exons from whole genome shotgun data in reptiles. Immunogenetics. 2014; 66(7–8):479–92. doi: 10.1007/s00251-014-0784-3 PMID: 24893587
44. Saluk PH, Krauss J, Clem LW. The presence of two antigenetically distinct light chains (and) in alligator immunoglobulin. Proc Soc Exp Biol Med. 1970; 133:365–9.
45. Xu Z, Wang GL, Nie P, Igm, IgD and IgY and their expression pattern in the Chinese soft-shelled turtle Pelodiscus sinensis. Molecular immunology. 2009; 46(10):2124–32. doi: 10.1016/j.molimm.2009.03.026 PMID: 19414198
46. Li L, Wang T, Sun Y, Cheng G, Li L, Cao Z, et al. Evidence of IgY subclass diversification in snakes: evolutionary implications. J Immunol. 2012; 189(7):3557–85. PMID: 22393626
47. Sun Y, Wei Z, Hammanstrom L, Zhao Y. The immunoglobulin delta gene in jawed vertebrates: a comparative overview. Developmental and comparative immunology. 2011; 35(9):975–81. doi: 10.1016/j.dci.2010.12.010 PMID: 21182659
48. Saluk PH, Krauss J, Clem LW. The presence of two antigenetically distinct light chains (and) in alligator immunoglobulin. Proc Soc Exp Biol Med. 1970; 133:365–9.
49. Xue Z, Wang GL, Nie P, Igm, IgD and IgY and their expression pattern in the Chinese soft-shelled turtle Pelodiscus sinensis. Molecular immunology. 2009; 46(10):2124–32. doi: 10.1016/j.molimm.2009.03.026 PMID: 19414198
50. Li L, Wang T, Sun Y, Cheng G, Yang H, Wei Z, et al. Extensive diversification of IgD-, IgY-, and truncated IgY(deltaFc)-encoding genes in the red-eared turtle (Trachemys scripta elegans). J Immunol. 2012; 189(8):3995–4004. doi: 10.4049/jimmunol.1200188 PMID: 22972932
51. Magadan-Mompo S, Sanchez-Espinel C, Gambon-Deza F. Immunoglobulin genes of the turtles. Immunogenetics. 2013; 65(3):227–37. doi: 10.1007/s00251-012-0672-7 PMID: 23208582
52. Janke A, Arnason U. The complete mitochondrial genome of Alligator mississippiensis and the separation between recent archosaurs (birds and crocodiles). Mol Biol Evol. 1997; 14(12):1266–72. PMID: 9402737
53. Kumar S, Hedges SB. A molecular timescale for vertebrate evolution. Nature. 1998; 392(6679):917–20. PMID: 9582070
54. Merchant ME, Leger N, Jerkins E, Mills K, Pallansch MB, Paulman RL, et al. Broad spectrum antimicrobial activity of leukocyte extracts from the American alligator (Alligator mississippiensis). Vet Immunol Immunopathol. 2006; 110(3):221–8.
56. Cheng G, Gao Y, Wang T, Sun Y, Wei Z, Li L, et al. Extensive diversification of IgH subclass-encoding genes and IgM subclass switching in crocodilians. Nature communications. 2013; 4:1337. doi: 10.1038/ncomms2317 PMID: 23299887
57. Magadan-Mompo S, Sanchez-Espinel C, Gambon-Deza F. IgH loci of American alligator and saltwater crocodile shed light on IgA evolution. Immunogenetics. 2013; 65(7):531–41. doi: 10.1007/s00251-013-0692-y PMID: 23558556
58. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics. 2003; 19(12):1572–4. PMID: 12912839
59. Page RD. TreeView: an application to display phylogenetic trees on personal computers. Computer applications in the biosciences: CABIOS. 1996; 12(4):357–8. PMID: 8902363
60. Retief JD. Phylogenetic analysis using PHYLIP. Methods in molecular biology. 2000; 132:243–58. PMID: 10547839
61. Lee SS, Tranchina D, Ohta Y, Flajnik MF, Hsu E. Hypermutation in shark immunoglobulin light chain genes results in contiguous substitutions. Immunity. 2002; 16(4):571–82. PMID: 11970880
62. Lefranc MP. IMGT, the international ImMunoGeneTics information system: a standardized approach for immunogenetics and immunoinformatics. Immune research. 2005; 1:3. PMID: 16305737
63. Modesto SP, Anderson JS. The phylogenetic definition of reptilia. Systematic biology. 2004; 53(5):815–21. PMID: 15545258
64. Shedlock AM, Botka CW, Zhao S, Shetty J, Zhang T, Liu JS, et al. Phylogenomics of nonavian reptiles and the structure of the ancestral amniote genome. Proceedings of the National Academy of Sciences of the United States of America. 2007; 104(8):2767–72. PMID: 17307893
65. Wei Z, Wu Q, Ren L, Hu X, Guo Y, Warr GW, et al. Expression of IgM, IgD, and IgY in a reptile, Anolis carolinensis. J Immunol. 2009; 183(6):3858–64. doi: 10.4049/jimmunol.0803251 PMID: 19717516
66. Gambon-Deza F, Espinel CS. IgD in the reptile leopard gecko. Molecular immunology. 2008; 45(12):3470–6. doi: 10.1016/j.molimm.2008.02.027 PMID: 18486212
67. Gambon Deza F, Sanchez Espinel C, Magadan Mompo S. The immunoglobulin heavy chain locus in the reptile Anolis carolinensis. Molecular immunology. 2009; 46(8–9):1679–87. doi: 10.1016/j.molimm.2009.02.019 PMID: 19299020
68. Almagro JC, Hernandez I, Ramirez MC, Vargas-Madrazo E. Structural differences between the repertoires of mouse and human germline genes and their evolutionary implications. Immunogenetics. 1998; 47(5):355–63. PMID: 9510553
69. Lefranc MP. IMGT (ImMunoGeneTics) locus on focus. A new section of Experimental and Clinical Immunogenetics. Experimental and clinical immunogenetics. 1998; 15(1):1–7. PMID: 9619395