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# The amphibians *Xenopus laevis* and *Silurana tropicalis* possess a family of activating KIR-related Immunoglobulin-like receptors

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## Abstract

In this study, we searched the amphibian species *Xenopus laevis* and *Silurana (Xenopus) tropicalis* for the presence of genes homologous to mammalian *KIRs* and avian *CHIRs* (KRIR family). By experimental and computational procedures, we identified four related *ILR* (Ig-like receptors) genes in *S. tropicalis* and three in *X. laevis*. *ILR*s encode type I transmembrane receptors with 3–4 Ig-like extracellular domains. All predicted ILR proteins appear to be activating receptors. *ILRs* have a broad expression pattern, the gene transcripts were found in both lymphoid and non-lymphoid tissues. Phylogenetic analysis shows that the amphibian KRIR family receptors evolved independently from their mammalian and avian counterparts. The only conserved structural element of tetrapod KRIRs is the NxxR motif-containing transmembrane domain that facilitates association with FcR subunit. Our findings suggest that if KRIRs of various vertebrates have any common function at all, such a function is activating rather than inhibitory.

### Keywords

genome mining; IgSF; ITAM; paired receptors; evolution; missing self recognition



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**Supplementary data.** Phylogenetic analysis of the Ig-like domains of mammalian, avian and amphibian KRIR family proteins. Phylogenetic analysis was performed with the MEGA3 software [30] using nucleotide sequences of exons and amino acid sequences of domains after alignment with the CLUSTAL option. Phylogenetic trees were constructed using the bootstrap and interior branch tests of the Neighbor-joining (NJ) method with p-distances (proportion of differences).

Appendix A Supplementary data
Refer to Web version for supplementary material.

## 1. Introduction

Regulation of the immune response in mammals is mediated by various receptors on the surface of the leukocytes. Killer Immunoglobulin-like Receptors or KIRs are among key players in this regulatory network. KIRs have been first discovered as crucial components of the missing self recognition by human NK cells [1]. It has been later demonstrated that mammalian genomes have a large group of genes that are structurally related to the *KIR* genes but are functionally different from them. In humans, genes of this family, which we will refer to as KIR-Related Ig-like Receptor (KRIR) family, lay within the Leukocyte Receptor Complex (LRC) on chromosome 19. In addition to the *KIR* genes, there is a large subfamily of genes known as *LILR* (also named *ILT, LIR, MIR*), and several singleton genes, such as *LAIR-1/2, OSCAR, FCAR, GPVI, NKp46* [2,3]. Except to LAIR2 that is a secreted protein, all members of the family are cell surface receptors. In the KRIR family, we may find pairs of receptors with similar extracellular regions but triggering opposing signaling pathways: either activating or inhibitory. Inhibitory forms of these so-called paired receptors possess ITIM motifs in their cytoplasmic tails and activating forms associate with signaling subunits containing activating ITAM motifs [4].

A variety of functions have been described for the mammalian KRIR family members. The major role of inhibitory KIRs in regulation of immune responses is to couple with MHC class I molecules on target cells and to protect these cells from NK-mediated lysis [1,2]. It has been speculated that activating KIRs may enhance cytotoxic response to cells with unusual or non-self peptides mounted on HLA I molecules [5]. Binding to classical and non-classical MHC class I antigens has been also demonstrated for human inhibitory receptors LILRB1 and LILRB2, along with their mouse counterpart PIR-B [5-8]. LILR and PIR receptors modulate immune response on various cell types [9-12]; NKp46 has been shown to recognize membrane associated heparan sulfate proteoglycans [13]; Human Fc R is a receptor for IgA [14]; GPVI and LAIR1 interact with collagen [15,16]. In addition, some of the family members have been found to recognize pathogen determinants. For example, NKp46 binds influenza virus hemagglutinin [5], whereas LILRB and PIR-B are able to recognize S*taphylococcus aureus* [12].

Which of these functions is the most ancient for this structural subset of the immunoglobulin superfamily remains unknown. It should be stressed that, in rodents, the function of MHC class I-specific recognition on NK cells is carried out by the Ly49 receptor family that belongs to the C-type lectin superfamily [17,18]. According to recent evidence, marine pinnipeds may use yet another group of molecules for this purpose. In this mammalian species, lineage Ly49 and KIR subfamilies are each represented by single gene that exhibit little polymorphism [19].

More than a hundred KRIR family genes named *CHIRs* have been identified in chicken [20-23]. Like their mammalian counterparts, CHIRs are subdivided into activating and inhibitory classes. Thus far, the only function known for some of these receptors is to bind to immunoglobulin class Y (IgY) molecules [24,25]. In the bony fish channel catfish (*Ictalurus punctatus*), Leukocyte Immune-Type Receptors (LITRs) were found to contain Ig-like domains with weak similarity both to the KRIR and the FcR family receptors [26]. MHC class I binding function was proposed for LITRs [27], but no experimental data supporting this assumption are available and LITR ligands remain yet unknown.

This study aimed to get a deeper insight into the structural and functional evolution of KRIRs by analysis of the family genes in the amphibians *Xenopus laevis* and *Silurana tropicalis.* The results obtained show that *Xenoponidae* KRIRs evolved separately from those of birds and mammals. Only activating receptors were found in the family.

## 2. Materials and methods

### Experimental Animals

Adult outbred *Xenopus laevis* and *Silurana tropicalis* were obtained from the *X. laevis* Research Resource for Immunobiology at the University of Rochester Medical Center (www.urmc.rochester.edu/smd/mbi/xenopus/index.htm). All animals were handled under strict laboratory and UCAR regulations. Animals were euthanized with 0.5% Tricaine methanesulfonate (TMS).

### RNA extraction, cDNA synthesis, RT-PCR amplification and cloning

Tissue samples were homogenized in 0.8 mL of Trizol reagent (Invitrogen). Total RNA was extracted according to the manufacturer's protocol. A sample RNA pellet was resuspended in RNase free water and quantified with SmartSpec spectrophotometer (BioRad). 500 ng of quantified total RNA were used to synthesize cDNA with iScript first strand cDNA synthesis kit (BioRad) according to the manufacturer's protocol. cDNA samples were diluted three times to a final volume of 60 μl before proceeding to PCR amplification. For each PCR reaction (30 μl total volume) 3 μl of 2 mM dNTPs, 3 μl of 10x PCR buffer, 10 pmol of each primer, 2 U of Taq DNA polymerase (Life Technologies), and 1 μl of cDNA were used. Then tubes were set for 30–40 cycles: 45 sec at 95°C, 45 sec at 60–64°C and 30–90 sec at 72°C. Negative controls (without RT) were also performed with same primers to control for genomic DNA contamination. Bands of our interest were re-amplifed with Pfu polymerase (Sibenzyme), cloned into pBluescript (Stratagene) vector and sequenced as described below. The following primers for different exons were used: *Xenopus laevis* xILR2.1 5′-GACTCTGAATTAAGTGACATGAT-3′ and 5′-GCAGGTTCCCGCTGGATCTCC-3′; xILR2.2 5′-ACTCTAAATTAAGTGACGTCGTG -3′ and 5′-GCAGGTTCATGCTGCATCTGA-3′; *Silurana tropicalis* sILR1 5′-GAATAGGAGGATCCTGGCTTCT-3′ and 5′-TGGCTCTTCTTGGTATGGCAGT-3′; sILR2 5′-CATCATGCTCAGAGCCTAGTGA-3′ and 5′-TGATGTTGCCTGTGGTGTGATC-3′; sILR3 5′-CCAGCAGGTTCCTACTAGAATG-3′ and 5′-CAAACGGATCTGTGCAGACTC-3′; sILR4 5′-CATGAGTGGCACATACACCTGT-3′ and 5′-AGGATTGCACTGACAGTAACAAC-3′.

### cDNA clones and sequencing

*S .tropicalis* sILR1 L12, L15, M6, M17, M18, H2, H3, H7 and sILR4 B1 clones were obtained through cloning as described above. *X. laevis* EST cDNA clones IMAGE:4962918, IMAGE: 8548181 and IMAGE:8074306 were purchased from the I.M.A.G.E. Consortium [28] through ATCC (USA) or Research Genetics Inc (USA). Both cloned and EST cDNAs were sequenced using an automated fluorescent sequencer ABI-Prizm 3130xl (Applied Biosystems) and submitted to GenBank. Following accession numbers were assigned to: *X. laevis* xILR1 - AY297107, xILR2.1 - EF431888, xILR2.2 - EF431889; *S. tropicalis* sILR1 - FJ716717-FJ716724, sILR4 - FJ716725.

### Southern blot analysis

Genomic DNA from *Xenopus laevis* erythrocytes was isolated as described by Sambrook [29] and digested to completion with restriction endonuclease HindIII or PvuII. The digested DNA (10 μg/lane) was separated on 1% agarose gel and transferred onto Zeta-probe nylon membranes (BioRad Laboratories) by the vacuum blotting technique in 0.4 M NaOH. Hybridizations with $^{32}$P-labeled probes were performed following the membrane manufacturer's recommendations in non-stringent conditions (55°C). The probes were PCR amplified fragments coding for the first domain either *X. laevis* xILR1 (257 bp) or xILR2.1 (210 bp). Following primers were used for probe amplification: xILR1 5′-

CATTGCAGATCTTGGCTG-3′ and 5′-TGCGGTCTACTTTCCTCA-3′; xILR2.1 5′-CGACACAATCAGCTTTTTCT-3′ and 5′-CGAACATAGATGTGTTCAGG-3′.

### Bioinformatics tools

Nucleotide and amino acid sequences were analyzed using utilities at the NCBI (www.ncbi.nlm.nih.gov), EMBL (www.ebi.ac.uk) and BCM (searchlauncher.bcm.tmc.edu) web sites. Amino acid sequences were aligned using Clustal utilities in the MEGA3 software [30] and shaded manually according to Timberlake classification of aminoacids [31]. The nucleotide and amino acid sequences of known genes were retrieved from GenBank using ENTREZ at the NCBI. The genomic sequences were retrieved from JGI web site (genome.jgi-psf.org). Similarity searches were performed using TBLASTN and BLASTP programs at the NCBI and EMBL sites. The GeneScan program (http://genes.mit.edu/GENSCAN.html) [32] and the Webgene program package (http://www.itb.cnr.it/sun/webgene/) [33] were used for the automated gene structure prediction. The ILR-surrounding genes were identified using the Ensembl (www.ensembl.org) and JGI utilities and were verified by reciprocal sequence comparisons at the NCBI website using the BLASTP program. Phylogenetic analysis was performed with the MEGA3 software [30] using nucleotide sequences of exons and amino acid sequences of domains after alignment with the CLUSTAL option. In certain cases, the CLUSTAL generated alignments were manually corrected. Phylogenetic trees were constructed using the bootstrap and interior branch tests of the Neighbor-joining (NJ) method with p-distances (proportion of differences). Minimum Evolution (ME) trees were essentially the same as the NJ trees in the major branching patterns.

### Constructions, transfections and flow cytometry

cDNA regions encoding either an extracellular or EC-TM-Cyt part (with stop-codon at the end) of *X. laevis* xILR1 [Genbank: AY297107] were cloned using primers containing XmaI and SalI sites and ligated into the pDisplay vector (Invitrogen). The cDNA portion used was 100–900 bp and 100–1090 bp, respectively. Both constructions encoded proteins fused with the N-terminal hemagglutinin (HA)-tag and the first one - also with the C-terminal PDGFR transmembrane. Complete coding region of *X. laevis* FcRγ [GenBank: AF499689] cDNA was cloned using primers with NheI and ApaI sites and ligated into the pAP-Tag5 vector (GenHunter). The latter construction encoded protein fused with C-terminal c-myc epitope. Eukaryotic 293T cells were transiently transfected with all obtained constructions using Unifectin 56 (IBCH, Moscow, Russia) according to the manufacturer's protocol. Seventy two hours after they were transfected, the cells were harvested and used for immunocytochemistry and cytometric analysis. For surface staining, transfected cells were washed twice with Wash Buffer (PBS, 1% FCS and 0.1% $NaN_3$). The cells were first incubated with mouse monoclonal 12CA5 anti-HA antibodies (Abcam) in Wash Buffer for 30 min on ice. Cells were then washed three times with cold Wash Buffer and incubated with goat anti-mouse Ig-FITC (BD Bioscience) in Wash Buffer for 30 min on ice. The cells were washed three times with Wash Buffer and analyzed using a microscope Axioscop 2 plus (Carl Zeiss) and FACSAria cytometer (BD Bioscience). For intracellular staining, transfected cells were smeared on glass slides, fixed with acetone and stained for c-myc tagged subunits with anti-c-myc 9E10 monoclonal antibodies (Abcam) and goat anti-mouse IgG-TexasRed (Molecular Probes).

## 3. Results and discussion

### 3.1. The amphibian X. laevis possesses a small family of KRIR-related genes - ILR family

The African clawed frog *X. laevis* is one of the most thoroughly studied non-mammalian immunological model organisms [34]. Abundant molecular data are available for this species - almost 700 thousand cDNA sequences are stored in the EST database (dbEST). We searched

this collection using TBLASTN analysis with sequences of various distantly related members of the KRIR family (i.e. KIRs, LILRs, PIRs and CHIRs). As a result, we identified 7 cDNAs similar to KRIR sequences. This similarity was rather weak and it ranged from 25 to 32% identical residues at the amino acid level. However, reciprocal BLASTP search in protein databases invariably indicated CHIRs and mammalian KRIR family proteins as the closest structural homologs of the predicted *X. laevis* amino-acid sequences. According to their similarity to each other, the identified cDNAs were subdivided into three clusters and a representative of each cluster was obtained from IMAGE consortium and sequenced. The cDNAs appeared to be products of three distinct genes encoding type I transmembrane proteins with similar structure: leader peptide, three Ig-like domains, transmembrane and short cytoplasmic tail. Based on the sequence comparisons, we designated these genes *xILR1* (*xenopus laevis* Immunoglobulin-Like Receptor 1), *xILR2.1* and *xILR2.2*. The deduced amino acid sequence of xILR1 showed less than 20% similarity with other two proteins. xILR2.1 and xILR2.2 share about 70% amino acid residues (Fig. 1).

To estimate the size of the *xILR* family, we performed Southern blot analysis of *X. laevis* genomic DNA. Hybridization with a probe encoding the xILR1 domain 1 (xILR1 d1) under non-stringent conditions gave a single strong band in all six individual samples of digested genomic DNA (Fig. 2). Two weak bands were also detected on the same blot. These may be explained either by cross-hybridization with *xILR1* d2 and d3 sequences or by the presence of 1–2 *xILR1*-like functional gene(s) or pseudogenes. The latter possibility is favored by the fact that *X. laevis* is an allotetraploid species. The probe corresponding to xILR2.1 d1 (86% of similarity with xILR2.2 d1) detected 4–5 hybridizing bands under non-stringent conditions (Fig. 2). Although exact number of *xILR* genes is to be established, dbEST and Southern blot analyses suggest that the *ILR* family *in X. laevis* is relatively small and most probably includes just a few genes per haploid genome.

### 3.2. The genome of S. tropicalis contains four ILR-related genes

To gain additional information about structure of xILR receptors and their phylogenetic relationships, we studied genome of the diploid amphibian *Silurana tropicalis* available at JGI [http://genome.jgi-psf.org] and Ensembl [http://www.ensembl.org] sites. The genomic sequences of *S. tropicalis* were searched for the presence of the KRIR family genes using TBLASTN analysis with sequences of mammalian, chicken and *X. laevis* KRIR family receptors as probes. In this genomic search, we used a strategy described in our previous analysis of the *Xenopus* FcR-like genes [35]. Two scaffolds, 381 and 1635, containing exons for Ig-like domains structurally related to those of xILRs, CHIRs, and LILRs were identified in the JGI genome assembly 4.1. Further analysis showed that scaffold 381 contains also *TTYH1*- and *LRC8 (LENG8)*-like genes. These two genes are located in the Leukocyte Receptor Complex (LRC) and closely linked to the KRIR family genes in the human and some other mammalian genomes [18].

Automatic and manual gene prediction analyses showed that scaffold 1635 contains a gene highly similar to *xILR1*. We designated this gene *sILR1* (*silurana tropicalis* Immunoglobulin-Like Receptor 1). *sILR1* contains five exons: two mini-exons for the leader peptide (LP), two exons for two extracellular (EC) Ig-like domains and one exon for both the transmembrane (TM) and cytoplasmic (Cyt) parts. The predicted protein lacked one of the Ig-like domains compared to xILR1 but shared with the latter about 70% identity in overlapped parts. To confirm the structure of *sILR1*, we performed RT-RCR of *S. tropicalis* spleen total RNA using primers for the second and the last exons of this gene. The RT-PCR yielded three fragments of different size. The largest encoded a transmembrane receptor with 3 EC domains. The exon corresponding to the second domain was absent in all available versions of the *S. tropicalis* genome assembly (Fig. 3 A). Cloning and sequencing of the smaller size PCR products showed

that they correspond to alternative transcripts from the same *sILR1* gene. These transcripts encode receptors with either two or one Ig-like domain (Fig. 3 B). Thus, *S. tropicalis* have one xILR1-similar receptor with three EC domains. However, additional diversity for this receptor may be generated by alternative splicing of the mRNA.

Apart from *sILR1*, the scaffold 381 was found to contain two more xILR-related genes. The *TTYH*-proximal gene was denoted as *sILR2* due to its high similarity to *X. laevis xILR2.1* and *xILR2.2* (70% identity at the protein level). The *TTYH*-distal gene was designated *sILR3* as its protein product shared only 42% identical residues with that of *sILR2. sILR2* and *sILR3* genes have genomic organization similar to that of *sILR1*. They both consist of 6 exons: one for 5′ untranslated region (5′UTR) and the first half of LP, one for the second half of LP, three for Ig-like domains and one for TM, Cyt and 3′UTR (Fig. 3, untranslated regions are not indicated). The predicted structure of *sILR2* and *sILR3* genes was confirmed by comparison with available *S. tropicalis* EST sequences. It should be noted that the second exon encoding the leader peptide of sILR1, sILR2 or sILR3, is 36 bp long. This is a common feature of the KRIR family genes.

Furthermore, the scaffolds 381 and 1635 were found to contain at their ends orphan exons with nucleotide sequences slightly similar to those of *sILR2* and *sILR3*. We postulated that these two scaffolds include adjacent genomic regions and that the orphan exons are parts of the same gene. We confirmed this by RT-PCR analysis of total RNA from *S. tropicalis* spleen using a series of primers matching exons from the two scaffolds. The amplification produced a single DNA fragment of 1068 bp, which encoded a protein with four extracellular domains, TM and a long cytoplasmic tail. The exon encoding the second EC domain is absent in all available versions of the *S. tropicalis* genome assembly. The other parts were completely identical to the sequences found in scaffolds 381 and 1635. We designated this gene as *sILR4*.

No other KRIR family genes were detected in the *S. tropicalis* genome. While the initial TBLASTN search produced some additional hits, they were attributed to other subsets of IgSF after subsequent examination. Scaffolds 1009 and 7950 were found to contain two exons completely identical to those of sILR1 (exons encoding d3 domain and TM-Cyt portion). Scaffold 381 contains an additional exon identical to that for the N-terminal domain of sILR4. We suppose that these exons may be either pseudoexons or results of assembly errors. The fact that the sequence identity between all mentioned exons is extended to the flanking non-coding regions and the absence of larger sILR4 cDNA fragments in RT-PCR are in favor of the latter possibility. Partial misassembling is inevitable at the current level of genome sequencing technology. Indeed, additional analysis demonstrated the presence of nucleotide sequences corresponding to the ‘missed’ exons for sILR1 d2 and sILR4 d2 in the Genbank trace archive containing raw data from *S. tropicalis* genome sequencing project. No sequences were found that could indicate the presence of additional *ILR* genes. This finding supports our gene models.

Comparisons of deduced amino acid sequences of sILRs showed their relatively weak homology to each other. The highest level of similarity, 45% identical residues, was found between the sILR2 and sILR3 extracellular parts (40–49% when comparing separate domains of homologous types). The sILR4 d1, d2 and d3.1 domains share 35–42% identical amino acid residues with corresponding d1-d3 domains of sILR2 and sILR3. The sILR4 d3.2 domain stands apart with only 21–26% of similarity with sILR4 d3.2 and sILR2/sILR3 d3 domains. The most distant member of the family is sILR1. Its extracellular domains show no more than 26% of identity with those of other sILRs. At the same time, comparisons of xILR1 with sILR1 or xILR2 with sILR2 (*X. laevis* vs. *S. tropicalis* homologs) show significantly higher similarity - 68–70% identical residues (Fig. 4).

### 3.3. ILR transcripts have a broad tissue distribution

To examine the tissue distribution of *sILR* transcripts, we performed RT-PCR with primers specific to different exons (L2 and TM-Cyt for *sILR1*; d1 and TM-Cyt for *sILR2*, *sILR3* and *sILR4*). *sILR1-3* products were detected in all examined *S. tropicalis* tissues: skin, kidney, testis, brain, intestine, spleen, liver and muscle. Expression of *sILR4* was found only in testis, brain, intestine and spleen. RT-PCR showed differential expression and the presence of alternative transcripts for *sILR1* and *sILR3* (Fig. 5 A). The broad tissue distribution of *xILR* transcripts was confirmed by RT-PCR analysis of *X. laevis* tissues (Fig. 5 B). All eight studied *X. laevis* tissues were found to express *xILR2.1*, whereas *xILR2.2* expression wasn't detected in kidney and skin (Fig. 5 B). The results of our analyses show that expression and by consequence the functional role of *Xenopoidae* KRIR family receptors is not restricted to lymphoid tissues. Therefore, it is unlikely that ILR receptors may be functionally equivalent to human KIR and mouse Ly-49 receptors, which are predominantly expressed on NK and NK/T cells.

### 3.4. X. laevis and S. tropicalis KRIR family receptors have features of activating receptors

The TM regions of all identified xILR and sILR proteins are homologous to TMs of FcRγ-associating members of the KRIR family, such as NKp46, Fc R, OSCAR, PIR-A, and LILR-A (Fig. 6 A). The characteristic feature of this evolutionary conserved TM domain is the presence of a NxxR motif. Such TM subtype has been recently found in the family of *Xenopus* FcR-like receptors (XFLs) [35] and in chicken KRIR family receptor ggFcR [36]. Like in mammals [3,37-41], this domain promotes association of frog and chicken proteins with FcRγ [35,36].

Another feature that ILRs share with activating receptors is the use of a single exon for both TM and cytoplasmic tail. This is true even for sILR4 whose intracellular portion includes 80 residues. The presence of tyrosine-based motifs in the cytoplasmic tails of *S. tropicalis* sILR2 and sILR4 does not contradict the assignment of these proteins as activating receptors. The sILR4 tail contains four tyrosine-based motifs separated by 8–9 residues (Fig. 4). The structural characteristics of these motifs, such as the presence of negatively charged residue at position −3 relative to tyrosine residues resemble those of the ITAM consensus [4]. sILR2 contains a motif similar to the consensus T/SxYxxV/I of the so called ITSM motif [42,43]. In mammals, the latter is known to promote either inhibitory or activating signals depending on various circumstances including the presence of adapter proteins SAP and EAT-2.

To assess the capacity of xILRs to interact with ITAM-bearing subunit, we made constructs encoding *X. laevis* HA-tagged xILR1 receptor either with its original TM or TM substituted with that of PDGFR. Both HA-xILR1 and HA-xILR1-PDGFRTM receptors were found on the surface of transfected cells in the absence of signal subunits. The Fluorescence Intensity Median for HA-xILR1 was taken as 1.0, and for HA-xILR1-PDGFRTM it reached 1.24. The addition of *X. laevis* c-myc-tagged FcRγ subunit caused a slight increase in the level of HA-xILR1 surface expression (FIM = 1.21), which nearly reached the level of HA-xILR1-PDGFRTM surface expression (Fig. 7). The experiment was repeated several times and the results were reproducible. These data show that xILR1 doesn't require FcRγ for surface transport, but the presence of the subunit makes such transport more efficient. The ability to be expressed as monomers is not rare among receptors with NxxR-containing TMs [44-46]. It appears that such ability provide an additional functional flexibility especially in the case of receptors which may possess their own signaling capacities.

### 3.5. Xenoponidae KRIR family receptors evolved in a species-specific manner

To infer phylogenetic relationships of IRLs with each other and with mammalian and chicken counterparts, we aligned amino acid sequences of their extracellular domains and generated

phylogenetic trees using MEGA3 software package. A schematic tree is presented in Fig. 8, for complete tree see Supplementary data. In accordance with the results of sequence comparisons, ILR1 domains form a separate clade with branching pattern suggesting generation of three-domain extracellular region by two consecutive duplications of a single ancestral domain. The second duplication occurred in a species-specific manner after radiation of *X.laevis* and *S.tropicalis*. The extracellular regions of ILR2, ILR3 and ILR4 contain three domain subtypes forming three separate clades. In all of them, the xILR 2.1 and 2.2 domains group together demonstrating that these two genes emerged by a *X. laevis*-specific duplication event. The tree shows that, despite its diverged primary sequence, the sILR4d 3.2 domain clearly belongs to the same subtype as sILR4 d3.1 and sILR2–3 d3. The mammalian and avian KRIR family domains form independent clusters that do not contain ILR domains (Fig. 8 and Supplementary data). Such topology is in agreement with that demonstrated by Nei and collegues [47]. Thus, phylogenetic analysis shows that the evolution of the KRIR family genes in amphibians has been lineage-specific. Separation of *ILRs* from other known KRIR family genes seems to have occurred early in evolution. This notion is supported by very weak similarity among domains composing extracellular regions of ILRs. The distances among them are comparable to those among ILR domains and their counterparts from ectothermic vertebrates.

We also inferred phylogenetic relationships of the TM domains of the *Xenoponidae* and mammalian KRIR and FcR family molecules. The generated tree shows that the ILR TMs are closer to those of NKP46, OSCAR and Fc R than to the TM domains of XFLs. However, NxxR-containing TMs group in a statistically supported monophyletic clade separated from TMs of classical FcRs and human KIRs (Fig. 6 B). Altogether these results provide further support for a common origin of the FcR and KRIR families, and a subsequent divergent mode in their evolution. Importantly, the phylogenetic data show that NxxR-containing TM domain has been the most conserved structural element of the FcR/KRIR family during its evolution in tetrapods. This is in disagreement with a model proposing that activating receptors are recurrently generated from inhibitory receptors. Although such model appears to be accurate for KIRs and CHIRs in homeothermic vertebrates [22,48], it should not be accepted as a common rule for the evolution of paired receptors.

## 4. Concluding remarks

Comparison of the immune systems of primates and rodents has demonstrated that the principle of functional analogy does not always apply to structurally-related immune genes [49]. The fact that missing-self recognition in human and mouse is performed by proteins belonging to the Ig and C-type lectin superfamilies, respectively, has been one of the first observations of this kind. With progress in studies of non-mammalian species, it is becoming evident that immune-related genes have even larger degree of flexibility and variability than previously thought [50-52]. Our data do not support the view that KRIR's function was originally related to missing-self recognition through MHC class I binding. Phylogenetic analysis shows that evolution of amphibian, avian and mammalian KRIRs has been completely independent. Furthermore, considering the dramatic differences in the family size and the high degree of divergence of the receptor extracellular regions, one can ask whether KRIRs of various tetrapod lineages have any common function. If the answer is positive, such a function is likely to be determined by the ability to transmit activating signals. Indeed, all identified *Xenoponidae* ILR proteins have features of activating receptors. The NxxR-containing TM, which is responsible for FcRγ association, is the only conserved structural element of KRIRs in tetrapods. It cannot be excluded, however, that functional evolution of KRIR receptors was independent and that these receptors fulfill various roles in different vertebrate lineages.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations and symbols used (in alphabetical order)

| | |
|---|---|
| α | alpha |
| γ | gamma |
| BLAST | Basic Local Alignment Search Tool |
| CHIR | Chicken Immunoglobilin-like Receptor |
| Cyt | cytoplasmic (tail) |
| dbEST | Expressed Sequence Tags Data Base |
| EC | extracellular (domain) |
| HA | Hemagglutinin |
| IgSF | Immunoglobulin Superfamily |
| ITAM | Immunoreceptor tyrosine-based activating motif |
| ITIM | Immunoreceptor tyrosine-based inhibitory motif |
| KIR | Killer-cell Immunoglobulin-like Receptor |
| KRIR | KIR-Related Immunoglobilin-like Receptor |
| LILR | Leukocyte Immunoglobilin-like Receptor |
| LITR | Leukocyte Immune-Type Receptors |
| LP | Leader Peptide |
| MHC | Major Histocompatibility Complex |
| PDGFR | Platelet Derived Growth Factor Receptor |
| PIR | Paired Immunoglobilin-like Receptor |
| TM | transmembrane (domain) |
| ILR | Immunoglobilin-like Receptor |

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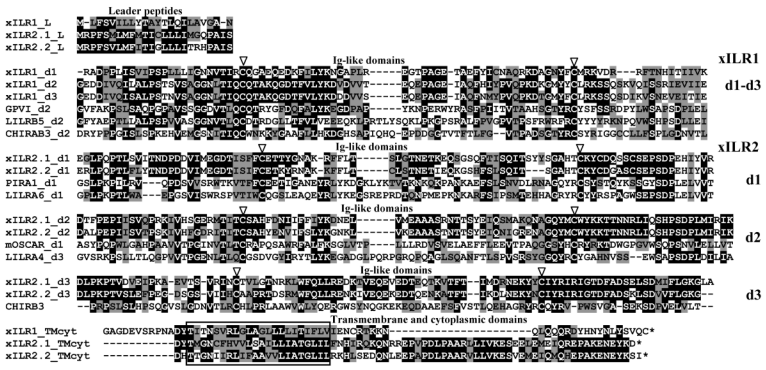


**Fig. 1.**

Alignment of *Xenopus laevis* xILR predicted proteins. Alignment of the KRIR family proteins includes chicken CHIR, human LILRs and GPVI, mouse PIR and OSCAR. Dashes represent gaps introduced for maximal similarity. Conserved and similar amino acid residues are denoted by white letter on the black background and by black letters on grey background, respectively. Similar residues were determined based on amino acid radical polarity and charge [31]. Asterisks designate stop-codons, triangles - conserved cysteine residues. TM domains are boxed.

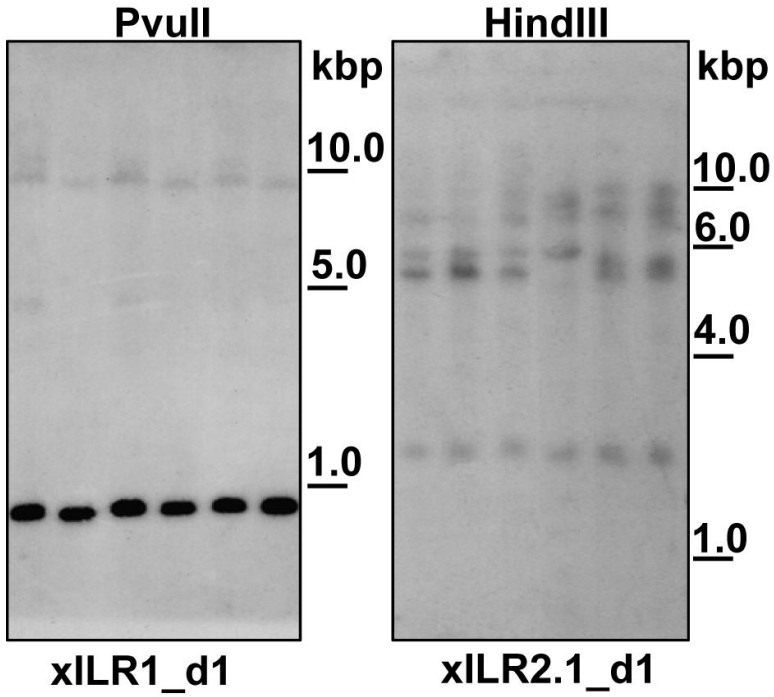


**Fig. 2.**

Southern blot analysis of *X. laevis* genomic DNA digested with HindIII or PvuII, and hybridized with xILR1- or xILR2-specific probes. Six individual samples of genomic DNA were used in each analysis. Molecular weight markers are shown at the right in kilobase pairs.

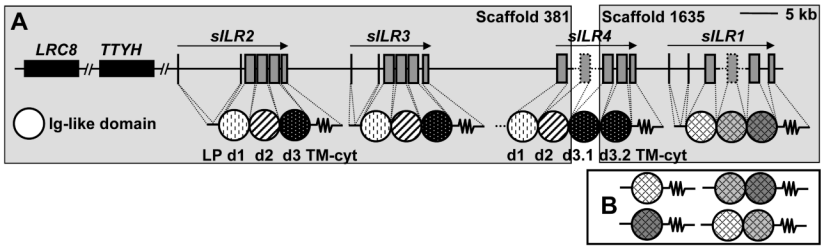


**Fig. 3.**

Genomic organization of *Silurana tropicalis sILR* genes. (A) *sILR* exons encoding Ig-like domains or TM-Cyt portions are designated by grey rectangles, exons for leader peptide (LP) - by vertical lines. The scale for intronic distances is shown at the upper right corner. Dashed lines correspond to sequences missed in the scaffolds but present in raw data files and cDNAs. Ig-like domains of the same phylogenetic type are filled with the identical pattern. Arrows show gene transcription direction. Adjacent non-*sILR* genes are indicated as black rectangles. Exons encoding sILR4 leader peptide remain unpredicted. (B) Alternative architectures of *sILR1*-encoded receptors.

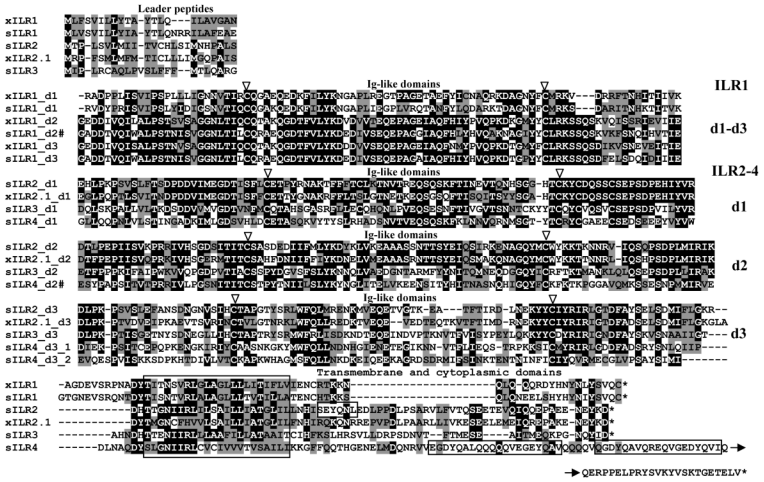


**Fig. 4.**

Alignment of *X. laevis* (xILR) and *S. tropicalis* (sILR) predicted KRIR family proteins. *S. tropicalis* amino acid sequences were deduced from genomic sequences and confirmed by EST or cloned cDNAs. Sequences absent in the genomic scaffolds are marked by # sign. Dashes represent gaps introduced for maximal similarity. Conserved and similar amino acid residues are denoted by white letter on the black background and by black letters on grey background, respectively. Similar residues were determined based on radical polarity and charge [31]. Asterisks designate stop codons, triangles - conserved cysteine residues. TMs, switch and ITAM motifs are boxed. *X. laevis* xILR2.2 was intentionally removed from the alignment due to its high similarity to xILR2.1.

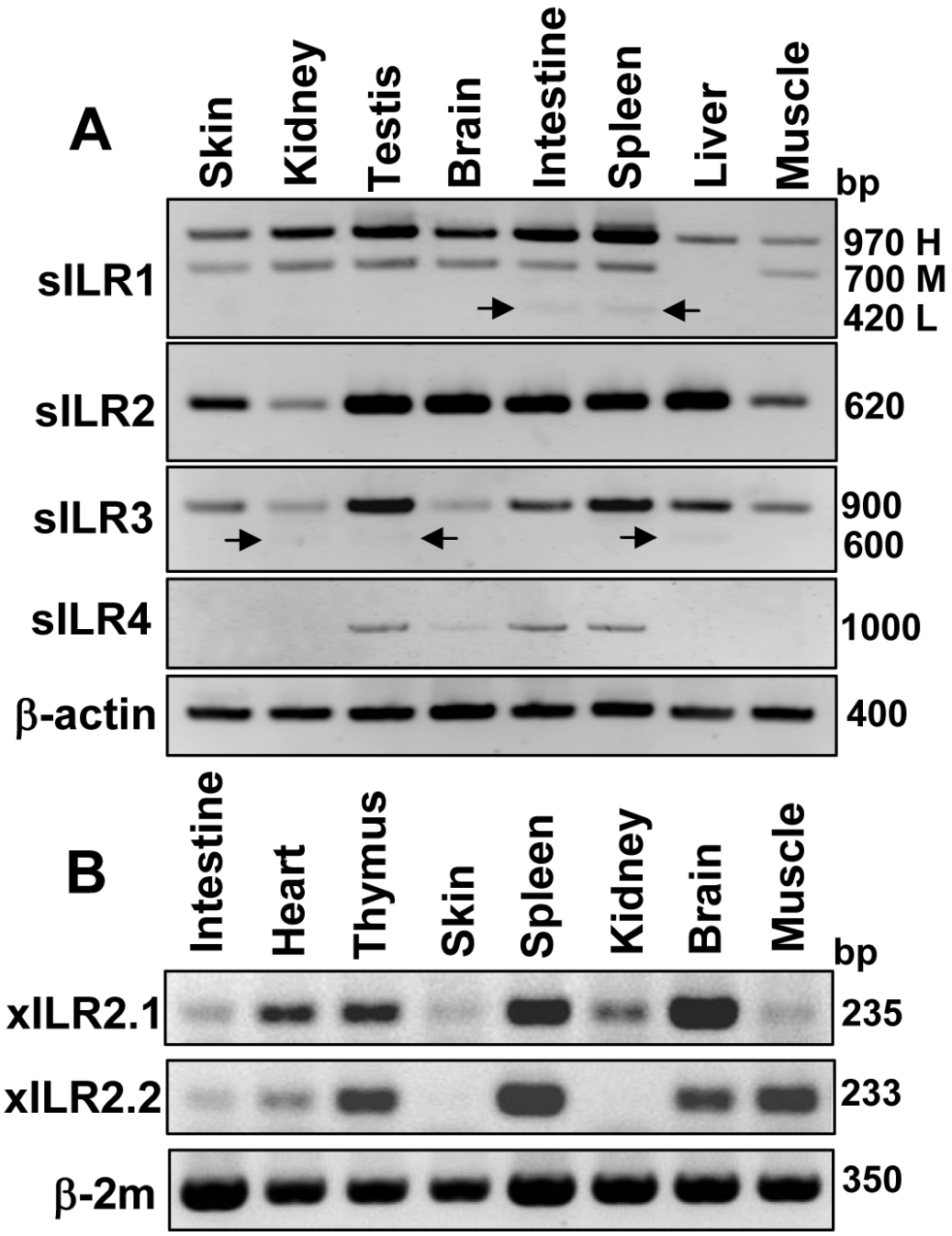


**Fig. 5.**

RT-PCR analysis of *S. tropicalis sILRs* (A) and *X. laevis xILR2* (B) expression. Molecular weights are shown at the right edge of gel pictures. Arrows point at weak signals corresponding to alternative splicing variants.

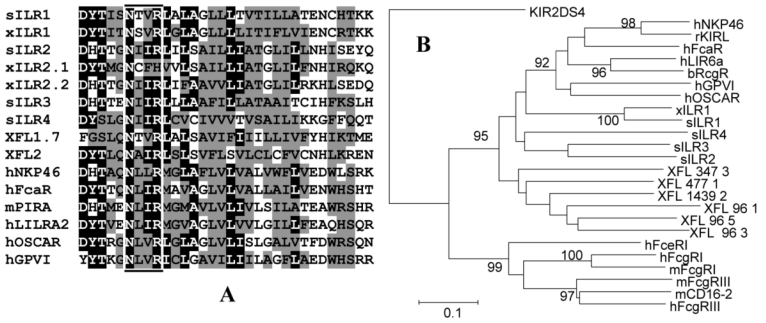


**Fig. 6.**

Alignment of the TM domains belonging to human, *Xenopus laevis* (xILR) and *Silurana tropicalis* (sILR) KRIR family receptors. Transmembranes of murine PIR-A and *X. laevis* XFL receptors are also shown in the alignment. Conserved and similar amino acid residues are denoted by white letter on the black background and by black letters on grey background, respectively. Similar residues were determined based on amino acid radical polarity and charge [31]. NxxR motif is boxed.

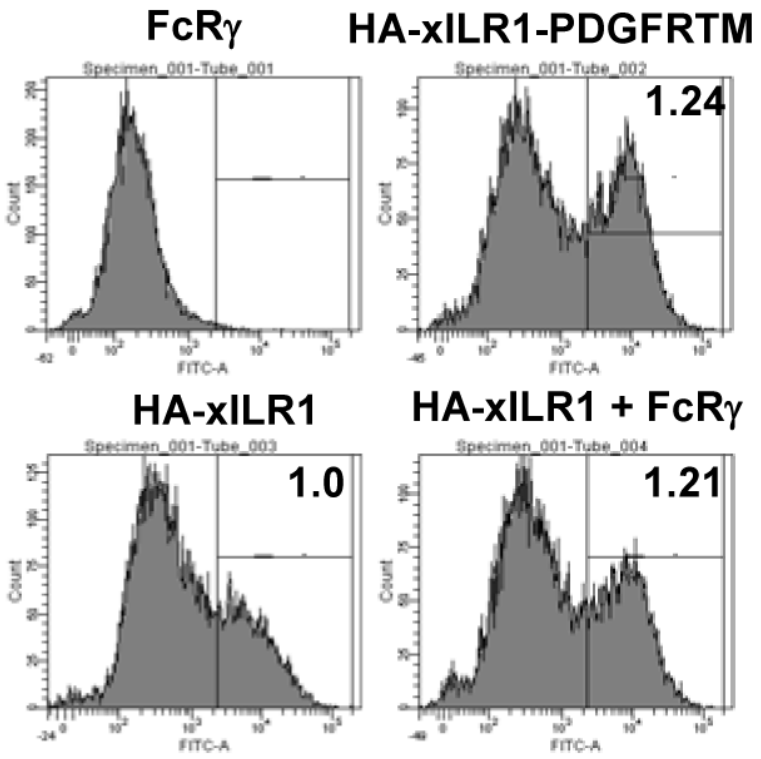


**Fig. 7.**

Flow cytometry (FACS) analysis of co-expression of *X. laevis* HA-xILR1 and FcRγ in 293T eukaryotic cells with α-HA Abs. HA-xILR1 was expressed with original TM domain or with TM substituted to that of PDFGR. FACS results were gated and the Fluorescence Intensity Medians were calculated. xILR1 FIM was taken as 1.0 and relative Medians for other experiments are shown at the top right corner of respective histograms.

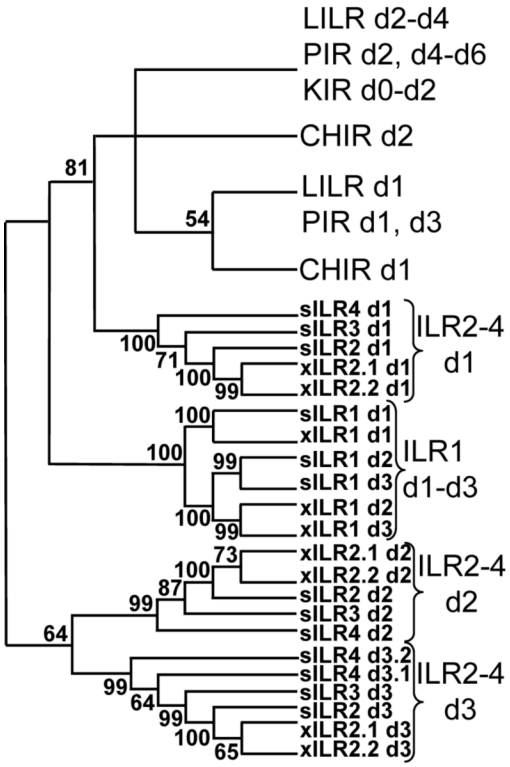


**Fig. 8.**

Phylogenetic analysis of the Ig-like domains of mammalian, avian and amphibian KRIR family proteins. Schematic bootstrapped NJ tree is shown. For the detailed tree see Supplementary data.