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Evolution and function of galectins in *Xenopus laevis*: Comparison with mammals and new perspectives

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ABSTRACT

Galectins are metal-independent sugar-binding proteins that recognize galactose (the β-galactoside structure) and regulate the cross-linking of sugar chains between cells and the extracellular matrix. Their specificity for galactose is attributed to their highly conserved carbohydrate recognition domain. Galectins participate in biological processes across species, including development, differentiation, morphogenesis, tumor progression, metastasis, immunity, and apoptosis. However, the relationship between the binding of galectin to sugar chains (glycans) and their biological functions remains unclear. Thus, a comprehensive functional analysis of galectins is required to better characterize their evolutionarily conserved and unique functions. We have previously identified and characterized 12 *Xenopus laevis* galectins (xgalectins), the only non-mammalian vertebrate species in which galectins have been comprehensively characterized to date. In this review, we present the latest findings on the types and functions of xgalectins and discuss prospects for elucidating their diverse functions from an evolutionary perspective through comparisons with mammalian galectins.

1. Introduction

Our vertebrate ancestors are considered to have undergone a whole-genome duplication (WGD) event approximately 500 million years ago, during which all genes were duplicated at least twice (Fig. 1) [1,2]. The doubling of genome sets creates a redundancy such that if a lethal mutation occurs in a duplicated gene, the other can maintain normal function and survival. Changes in the function of duplicated genes ultimately lead to the acquisition of novel genes, markedly impacting species diversity and differentiation [3,4]. In addition to these WGD events, partial duplication, such as tandem duplication, is also an important process for acquiring new genes. Gene tandem duplication can lead to multiple gene copies within the same species. It also plays a major role in the molecular evolution of galectins, such as the creation of proto-type galectins from tandem repeat-type galectins, as described below [5]. Notably, galectins appear to have expanded significantly through WGD and tandem duplication [6,7].

However, genome duplication does not always have a positive effect on evolution. Redundant copies of genes that are essential for maintaining basic cellular functions, such as housekeeping genes, increase the risk of accumulating harmful mutations. Therefore, housekeeping genes tend to be highly conserved during evolution with their function and expression patterns being protected [8]. This means that most housekeeping genes are under strong selection pressure and tend to maintain only a single functional gene copy. While certain genes undergo duplication, others are conserved and follow an evolutionary path. Gene number duplication promotes the evolution of new gene functions, significantly affecting species diversity and adaptive capacity [9]. Nevertheless, tetraploid organisms resulting from genome doubling are predicted to eventually revert to diploidy through a process known as rediploidization. In teleost fish, an additional WGD, known as Ts3R, occurred 320 million years ago (Fig. 1). This event initially doubled the number of genes to 40,000, but the number has since returned to approximately 20,000 [10,11]. This suggests these fish have evolved by acquiring new genes while losing unnecessary or redundant genes. Comparative genomic analysis of fish and mammals has traditionally aimed to clarify how fish and mammals evolved from a common ancestor through WGD. However, such studies have been challenging owing to factors such as the large genome size of lungfish (which are the closest relatives to tetrapods), the similarity of duplicated genes, and the

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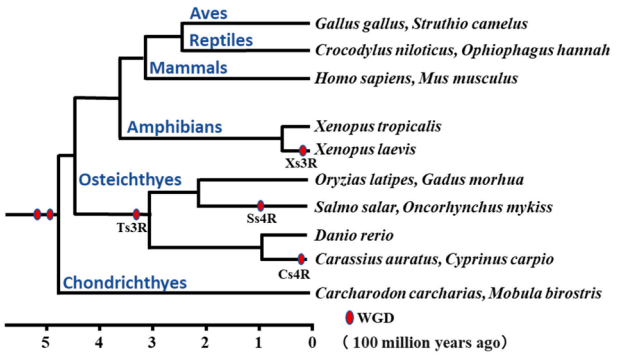

Fig. 1. Phylogenetic tree of vertebrates with whole genome duplication events. Estimated timing of whole genome duplication (WGD) events in vertebrate phylogeny and evolution. The phylogenetic tree is shown with divergent ages of taxa and representative animals listed on the right. WGD events (indicated by asterisks) occurred twice in the common ancestor of vertebrates approximately 500 million years ago. In teleost fish, the third WGD (Ts3R) occurred approximately 320 million years ago, followed by a forth WGD about 100 and 15 million years ago in the salmonidae lineage (Ss4R) and cyprinidae lineage (Cs4R). In amphibians, the third WGD occurred 18 million years ago in the lineage of *Xenopus laevis* (Xs3R).

evolutionary distance between bony fish and mammals. Notably, recent advancements in genome analysis technologies, coupled with the concerted efforts of numerous researchers, have led to significant progress. Specifically, genome analyses of coelacanths and lungfish have provided valuable insights into the mechanisms underlying the evolution from fish to tetrapods [12–14].

Polyploidy was initially believed to be limited to amphibians and certain reptiles among tetrapods and not tolerated in birds and mammals. However, recent research has demonstrated that polyploidy is not completely incompatible with birds and mammals [15]. In birds, triploids have been found in some individuals of *Gallus domesticus* [16] and in one individual of the blue-and-yellow macaw, *Ara ararauna* [17]. In mammals, *Tympanoctomys barrerae* and *Pipanacoctomys aureus* were found to be tetraploids [18,19]. These discoveries suggest that WGD may be broadly related to the evolution of tetrapods. WGD has also been observed in human cancers; however, it is a temporary state that promotes aneuploidy and increases genetic diversity and subsequent adaptive capacity. In other words, the occurrence of WGD does not appear to be random; instead, it is correlated with notable changes in the environment, such as exposure to stressful environments. Although polyploidy is a frequent phenomenon, the number of WGD that become established over the long term is relatively small. This is likely due to the disruptions in sex determination and development caused by polyploidy [20]. In contrast, the prevalence of polyploidy among amphibians is speculated to be related to regenerative ability, although direct evidence has not been provided.

Glycans, which are major components of higher organisms, participate in myriad physiological pathways, often by specifically binding lectins. Galectins are members of the soluble lectin family and bind specifically to the β-galactoside structure of glycans via their carbohydrate recognition domain (CRD) [21,22]. Considering that galectins are expressed in many species, including fish, amphibians, and mammals, they are expected to be involved in various phenomena as regulators of glycan functions. Additionally, glycans that are frequently expressed differ among species, suggesting that galectin molecules evolve gradually over time. However, several amino acids involved in carbohydrate recognition are conserved, raising the question of how galectins adapt their functions across different species.

Similar to animal galectins, plant lectins from the Fabaceae and Brassicaceae families also appear to have diversified and expanded through WGD and tandem duplication. Plant lectins are involved in stress responses and play an important role in the plant immune system. Some plant lectins have kinase domains, indicating that they have evolved differently from animal lectins [23]. Plant lectins, similar to animal galectin, exhibit a high degree of conservation in their sugar recognition sites. The expression of lectins is stimulated by the invasion of pathogenic bacteria. It is hypothesized that both animal galectins and plant lectins are evidence of the evolutionary history of animals and plants defending against pathogenic microorganisms with diverse types of carbohydrate chains [24]. Specifically, each organism appears to employ lectins to distinguish between self and non-self by utilizing sugar chains as a critical factor. Additionally, lectins adapt their sugar chain binding properties according to the needs of the organism, thereby contributing to survival. These similarities indicate that sugar-binding proteins are extremely important in the evolution of multicellular organisms, and that there has been pressure for convergent evolution in different biological lineages. This insight could provide a foundation for understanding the fundamental biological principles shared by both plants and animals.

Hence, the comparative classification of these evolutionarily conserved galectin functions across a wide range of species and their relationship to glycans is an attractive research area. In this review, we summarize the structure, distribution and function of galectins in the African clawed frog (*Xenopus laevis*), a species that has undergone WGD and is in the process of gene selection.

2. African clawed frog (*Xenopus laevis*)

The scientific name of the African clawed frog is derived from the Latin word, *Xenopus* for strange feet and *laevis* for smooth. *Xenopus laevis* (*X. laevis*) has three sharp black claws and muscular hind limbs, and its body surface is covered with mucus. This allotetraploid species arose during the Cenozoic era (approximately 18 million years ago) through the WGD of two relatively close ancestral *X. laevis* species (L and S) that interbred (Xs3R) (Fig. 1) [25]. Although these ancestral species are extinct, their genomes coexist in the genome of *X. laevis*, which has a total of 36 chromosomes. In such allotetraploids, it is believed that beneficial genes are selected from the duplicated genes, while the unnecessary ones are lost, as observed in teleost fish. However, owing to the relatively short time elapsed since the WGD event, *X. laevis* remains a heterogeneous tetraploid with significant allotetraploidy remnants; in other words, it is still undergoing the gene selection process [26].

In contrast, the third WGD event (autotetraploid) unique to teleost fishes (Ts3R), which occurred approximately 300 million years ago, resulted in the rapid loss of 70–80 % of the initially doubled genes (40,000) over the next 60 million years. This was followed by a slow return to the original gene number of 20,000 over the following 250 million years [11]. Additionally, the WGD (Ss4R) that occurred in the common ancestor of the salmon family approximately 100 million years ago was also autotetraploidization (i.e., direct genome doubling). Notably, the genes that remained as duplicate genes in Ts3R did not persist after Ss4R [27,28] (Fig. 1). The probability of independent gene retention suggests that this process is complex and that most of the genes after WGD are neutral.

Recently, it has been suggested that the first of the two WGD that occurred in the ancestors of vertebrates was an autotetraploidization, and the second was an allotetraploidization (i.e., genome duplication following interspecific hybridization) [29]. This second WGD is believed to have resulted from heterologous polyploidization due to interspecific hybridization in fish, although >450 million years have elapsed since the gene duplication event. As many species have diverged since the second WGD, it is important to investigate the molecular evolutionary processes of various proteins after heterologous polyploidization. Other than *X. laevis*, the most studied allopolyploid species are the carp and goldfish, which underwent WGD 15 million years ago (Cs4R). There is limited information on the changes that occur in duplicate genes after allotetraploid in vertebrates. As *X. laevis* has been the subject of extensive research on its developmental morphology, it is considered the

optimal model for understanding these changes.

The complete genome sequence of the diploid western clawed frog (*Xenopus tropicalis*), a close relative of the ancestral species of *X. laevis*, was revealed in 2010 [30], followed by the complete genome sequence of *X. laevis* in 2016 [25]. In general, loss of duplicated genes in polyploid species, such as plants and yeast, occurs in both genomes, albeit unevenly, while typically retaining "gene clusters" which are functionally related neighboring genes [31–33]. In *X. laevis*, gene loss, and chromosomal rearrangements are predominantly concentrated within the genome of the ancestral species of S origin [34]. However, in the allotetraploid carp and goldfish, no extensive gene loss was observed in either subgenome [35]. Although the expression of homeologous genes is dominant in one subgenome, they are almost coexpressed; it is of considerable interest to investigate whether this difference is due to the species.

Xenopus laevis is a representative vertebrate experimental model organism known for its high hormone sensitivity and production of large eggs, which is characteristic of amphibians. Furthermore, when maintained at a water temperature of 20 °C, they spawn year-round. *Xenopus laevis* have made major contributions to developmental biology research owing to the simplicity of embryo manipulation and their organ shapes and arrangements, which are more similar to those of humans than fish. The nucleus of a small intestinal epithelial cell of *X. laevis* was demonstrated to grow as an individual when the nucleus was extracted and transplanted into a fertilized egg from which the nucleus had been removed. This was the first somatic cloning of a vertebrate in the world, and the subject of the 2012 Nobel Prize in Physiology or Medicine [36]. Unlike mammals, amphibians can often regenerate even if they lose the retina of an eye or a limb. Although *X. laevis* has a high regenerative capacity as a tadpole, it is unable to regenerate its limbs as an adult. It was recently demonstrated that *X. laevis* may be able to regenerate amputated legs with appropriate treatment [37]. This report of a species that cannot normally regenerate but can regenerate under the right conditions could advance regenerative medicine in mammals. Elucidation of the mechanism of this high regenerative ability is expected to attract considerable attention in the field of regenerative medicine.

The physiological functions and response systems of most proteins are highly conserved in *X. laevis*, and several human disease models, such as congenital kidney and heart disease, have already been established using *X. laevis* [38–40]. Furthermore, the relatively clear mammalian counterparts of genes resulting from WGD in *X. laevis* compared to teleost fish make them excellent model organisms for studying gene evolution and functional differentiation. Studies on *X. laevis* have also contributed to elucidating the mechanisms of adaptation to environmental changes. For example, studies on gene expression changes and stress responses under different environmental conditions have improved our understanding of the ability of an organism to adapt to its environment. This high capacity to adapt to the environment is believed to be attributed to the relatively recent third WGD [41]. A comparison of the genomes of the heterozygous tetraploid *X. laevis* and the diploid *X. tropicalis* is expected to provide a good model for investigating how genome duplication affects evolution and adaptive changes in organisms.

3. Galectins

Galectins were first isolated from the electric eel, calf lung, and heart, and chick embryo muscle as β-galactoside structure-binding proteins with erythrocyte aggregation activity and molecular weights of approximately 14–16 kDa [42]. Some galectins were named according to their animal species, such as electrolectin, derived from the electric organ of the electric eel (*Electrophorus electricus*) [43]. Subsequently, identical molecular species were purified from various mammalian organs, and their amino acid sequences were determined. Mammalian galectins are assigned the formal name "galectin" followed by a number in the order of their discovery. Galectins are metal-independent, soluble lectins with glycan recognition functions that are widely expressed in all metazoans and are highly conserved throughout evolution. The interaction between galectins and glycans is mediated by an evolutionarily conserved CRD [44].

Galectins are classified into three subtypes based on their structure: (1) proto-type galectins comprising only CRDs; (2) chimera-type galectins that combine an approximately 120 amino acids N-terminal unrelated region with a CRD; and (3) tandem-repeat-type galectins in which two different CRDs, N-terminal CRD (N—CRD) and C-terminal CRD (C—CRD), are connected by a linker (Fig. 2). These structures support the diverse biological functions of galectins [21].

Whole-genome analyses have progressed in various organisms, and genes potentially encoding galectin proteins with different structural forms have been annotated in genome databases for various organisms, including *Caenorhabditis elegans, Drosophila melanogaster*, M*us musculus, Homo sapiens, X. tropicalis*, and *X. laevis* [25,30]. In recent years, the establishment of large-scale genome sequencing initiatives, such as the Earth BioGenome Project, the Vertebrate Genome Project and others, have provided the complete genome sequences of several hundred non-model species, greatly facilitating the identification of the full repertoire of galectin genes in many species, such as *Loxodonta Africana* and *Vicugna pacos* [45].

The origin of galectins can be traced back to sponges, where they were first identified; however, the diverse galectin family evolved only within Bilateria [7]. The evolution of vertebrate galectins is believed to have originated with the tandem repeat galectins of the oral-pharyngeal network, which were expanded to four members following two rounds of WGD (galectins-4, −8, −9, and −12). Moreover, additional small gene duplications (tandem duplications) may have led to the formation of proto-type galectins. These prototypes consist of either an F3CRD, in which the tandem repeat N—CRD has evolved, or an F4CRD, in which the C—CRD, defined by a short intermediate exon, has evolved (Fig. 2) [5].

To date, 15 galectin genes (galectins 1–16) have been identified in mammals, each with different tissue distribution and sugar-binding properties [22]. Among these, galectin-11 and galectin-15 refer to the same gene. The research group that first discovered it proposed naming it galectin-11 [46]. Subsequently, another group, which proved its lectin activity, suggested renaming it galectin-15 [47]. Currently, both names are in use. Thus, although mammalian galectins are numbered up to sixteen, there are actually fifteen different genes. However, some of these are species- or taxon-specific, including galectins-5, −6, −10, −11/−15, −13, −14, and −16, and have been identified in rats

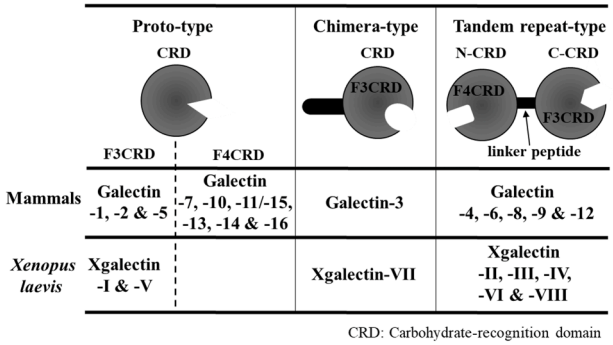

Fig. 2. Classification of mammalian and *Xenopus laevis* galectins based on structure. Proto- and chimera-type galectins contain one carbohydrate recognition domain (CRD), whereas tandem-repeat galectins possess two CRDs. Each CRD comprises approximately 140 amino acids and is classified as either F3CRD or F4CRD. The 7 amino acid residues important for sugar recognition are conserved in most galectins across species. Proto- and chimera-type galectins have only one CRD but can bind to multiple carbohydrate chains by dimer or polymer formation, depending on the molecular species of galectin. N—CRD, N-terminal CRD; C—CRD, C-terminal CRD.

(galectin-5), mice (galectin-6), humans (galectin-10), ruminants (galectin-11/−15), and primates (galectin-13, −14, −16) [48–50]. Notably, all these species-specific galectins, except for the tandem repeat-type galectin-6, are proto-type galectins.

The amino acid sequences of galectins are highly conserved across species with few major deletions or insertions. However, the seven amino acids directly involved in β-galactoside binding are conserved across many species [5], reflecting the important roles of galectins in glycan recognition. Furthermore, the carbohydrate-binding properties of galectins are regulated by the length of the loop around the carbohydrate recognition site.

Galectin expression can vary in a developmental time-specific manner depending on the species. Notably, while *galectin-1* knockout mice do not exhibit significant morphological abnormalities or differences in reproductive performance compared to normal mice, they develop an abnormal olfactory nervous system [51,52]. Additionally, as extracellular galectin-1 contributes to axon growth and repair, *galectin-1-knockout* mice also exhibit memory deficits [53,54]. Notably, the simultaneous knockout of *galectin-1* and *galectin-3*, both expressed during implantation, does not result in significant abnormalities. This is partially attributed to the redundant functions of other galectins. For instance, galectin-5 is also expressed during blastocyst implantation [55].

Galectins bind glycoproteins and glycolipids on the cell surface of various tissues, contributing to embryogenesis, epithelial function, cell differentiation, innate immune responses, tumorigenesis, and cancer metastasis. Although previous studies have suggested that many biological functions of galectins are based on direct interactions with glycans, limited evidence is available to support this hypothesis [56–58]. Nevertheless, galectins serve as targets in cancer therapy and immunotherapy owing to their involvement in cancer cell proliferation and metastasis. For example, galectin-3 is important in cancer cell adhesion and migration, and its inhibitors have been proposed as potential anticancer agents [59]. Galectin-1 also exerts immunosuppressive properties and has been studied as a potential target for cancer immunotherapy [60]. Continued research on the functions of galectins is expected to promote their use in fields from basic to applied and clinical research.

4. Amphibian galectin

Amphibians were the first group of vertebrates to adapt to terrestrial life, emerging approximately 360 million years ago. Owing to their unique ecological and physiological characteristics, amphibians depend on aquatic and terrestrial environments. They are classified into the Anura, Caudata, and Gymnophiona groups. Amphibian galectins have been isolated from several species, including *X. laevis*, Argentine toad (*Rhinella arenarum*), Bullfrog (*Aquarana catesbeiana*), Tiger frog (*Hoplobatrachus tigerinus*), and Mexican axolotl (*Ambystoma mexicanum*) [61–65]. A recent whole-genome sequencing of members of the order Gymnophiona, commonly called caecilians, such as *Rhinatrema bivittatum*, has identified galectin-related genes. Given that caecilians have an evolutionary lineage that extends over 275 million years, in-depth genomic analyses can provide critical insights into amphibian biology and evolutionary adaptations.

Although functional analyses of galectins have been performed in some species, most studies have focused on the common mammalian galectin-1 homolog. Their broad role in innate and acquired immunity is becoming clear. Specifically, galectins are suggested to be important pattern recognition receptors. They can recognize and bind to pathogen-associated molecular patterns of microbial pathogens, such as bacteria, fungi, and viruses, by interacting with β-galactoside [66]. Galectin-1 homolog has also been isolated from the Chinese giant salamander (*Andrias davidianus*), the largest extant amphibian species. This galectin exhibits binding activity against various gram-positive and gram-negative bacteria, strongly agglutinating them [67]. Moreover, galectins are important as antimicrobial molecules and non-self-recognition molecules in aquatic organisms [68]. Amphibian skin secretions contain many biologically active substances, such as antibacterials and toxins, and have been well studied. Typical biologically active substances from the skin of clawed frogs are peptides composed of a few amino acids, such as magainin and caerulein [69,70]. The venom secreted from the skin shows a wide range of predator adaptations and usually contains various toxins. However, when attacked by predators, some amphibians secrete a viscous fluid from their skin that quickly hardens into a sticky mass. The adhesive and frictional properties of this glue dramatically increase the energy cost of handling prey. Some amphibian glues are also toxic, whereas others have lost their toxicity, suggesting that “stickiness” is a suitable alternative to chemical defense mechanisms. Recently, galectins have been demonstrated to be involved in the formation of highly adhesive skin secretions (glues), which support the defense mechanism of a new frog species, the Madagascar tomato frog (*Dyscophus guineti*) [71]. This galectin is a proto-type galectin that is highly expressed in the skin but exhibits no homology to other proto-type galectins; instead, it has unique properties with very high similarity to galectin-9 N—CRD. This galectin has also been found in the West African savannah frog (*Phrynomantis microps*), where it interacts with highly glycosylated PRIT (Protein with Repeated IgGFcBD in Tandem) to form a powerful adhesion device. The galectin isolated from the skin secretion of tree frog species, the Mozambique rain frog (*Breviceps mossambicus*), is a typical galectin-1 homolog. This suggests that the *Dyscophus guineti* galectin is a relatively recent development. Additionally, the proto-type galectin highly expressed in the skin of the *X. laevis* is not homologous to galectins from either species suggesting that each species independently modifies its galectins for use in biological defense.

Our research group has identified and characterized 12 novel *X. laevis* galectins (xgalectin-Ia to xgalectin-VIIIa) whose expression is regulated throughout the life cycle, from the egg to the adult stages (Fig. 2) [72,73]. Considering the inherent differences between mammalian and *X. laevis* galectins, Roman numerals were assigned when naming xgalectins based on the order of their discovery.

Comparison of the amino acid sequences and expression patterns of *X. laevis* and mammalian galectins identifies several orthologous sequence pairs, while other pairs likely evolved independently. Furthermore, even among apparently orthologous pairs, the amino acid sequence similarity only reaches about 50 % (Table 1), which is lower than that typically shown for other orthologous pairs, e.g., housekeeping genes. However, as mentioned above, the sequence similarity of the glycan recognition sites is much higher, suggesting that galectins may have followed a different trajectory from other proteins, including their ability to respond to different glycans in a species-specific manner [74].

Studies on the evolutionary origin and functional differentiation of *X. laevis* galectin genes have also provided important insights into its evolutionary biology and comparative genomics with other vertebrates. The classification of xgalectins identified few proto-type and many tandem-repeat-type galectins in *X. laevis* (Fig. 2). Although many proto-type galectins have been reported in mammals, few have been identified in *X. laevis* and its ancestral relative, *X. tropicalis*. This suggests that the structure of galectins diverged during evolution and may provide insights into the differences in galectins between amphibians and mammals. Furthermore, certain proto-type galectins arose from tandem-repeat-type duplication [5]. Therefore, we speculate that some proto-type galectins found predominantly in mammals likely evolved after the divergence of amphibians and mammals. This is supported by the large number of proto-type galectins unique to mammals, notably galectin-5, −7, −10, −11/−15, −13, −14, and −16. Overall, the clawed frog is widely used as a model organism across various research fields. An analysis of xgalectins could help characterize the evolution and functional diversity of galectins through comparative studies with other vertebrates.

The role of glycans is a key area where future advancements are expected in elucidating the molecular mechanisms underlying animal

Table 1
Correspondence between Xgalectin and Human galectin.

Type	*Xenopus laevis*	Chromosome number	Human	Identity with human galectin (%)
Proto	Xgalectin-Ia	4S	Galectin-1	48.9
	Xgalectin-Ib	4L		49.6
Tandem repeat	Xgalectin-IIa	8L	Galectin-4	51
	Xgalectin-IIb	8S		52.9
Tandem repeat	Xgalectin-IIIa	2S	Galectin-9	42.5
	Xgalectin-IIIb	2L		44.6
Tandem repeat	Xgalectin-IVa	8L	missing	–
Proto	Xgalectin-Va	4L	None	–
	Xgalectin-Vb	4S		–
Tandem repeat	Xgalectin-VIa	8L	missing	–
Chimera	Xgalectin-VIIa	8L	Galectin-3	50
	Xgalectin-VIIb	8S		41.1
Tandem repeat	Xgalectin-VIIIa	5S	Galectin-8	53.6

embryo development. The analysis of glycans requires their extraction and purification, and the large size of the embryo makes it an advantageous model organism. In this respect, *X. laevis* is particularly indispensable for research into early development. A previous study has comprehensively analyzed the expression of N-glycans during early *Xenopus* development [75]. Along with such research, our ongoing lectin research is a promising avenue for significantly contributing to elucidating the role of expressed glycans. Future studies will help elucidate the complex functions of xgalectins and glycans and expand their potential medical applications.

5. *X. laevis* proto-type galectins

Marschal *et al.* reported that a 16-kDa proto-type galectin in skin secretions of *X. laevis* was similar to mammalian galectin-1 [61]. Subsequently, a galectin-1-like protein was reported in the ovaries of *Rhinella arenarum* (Argentine toad) [76]. Among amphibian anurans, the genera *Rhinella* and *Bufo* are relatively new in Neobatrachia, in contrast to very early genera such as *Xenopus* and *Bombina*, classified as Archeobatrachia. As the ovary galectin of *R. arenarum* exhibits features more similar to bovine galectin-1 than to the *X. laevis* skin galectin, the evolutionary aspects of galectins were presumed to have likely differed between *Rhinella* and *Xenopus* [77]. However, the discovery of two other galectin-1-like proteins in *X. laevis* tissues suggested otherwise [72]. The *X. laevis* skin 16-kDa galectin was named xgalectin-Va, while the other skin galectin, which is considered to be its isoform, was named xgalectin-Vb (Table 1) [73]. Additionally, the two galectin-1-like ubiquitous proteins were named xgalectin-Ia and -Ib. It has become clear that xgalectin-Va and -Vb are homeologs of each other (i.e. counterparts resulting from polyploidization), as are xgalectin-Ia and -Ib. Isolation and purification of galectins from adult *X. laevis* identified xgalectin-Ib and xgalectin-Va as the most abundant homeologs in the stomach and skin, respectively (unpublished). Notably, xgalectin-Ib and -Va are derived from ancestral L species.

The amino acid sequence alignments of representative mammalian and amphibian proto-type galectins are presented in Fig. 3. We have

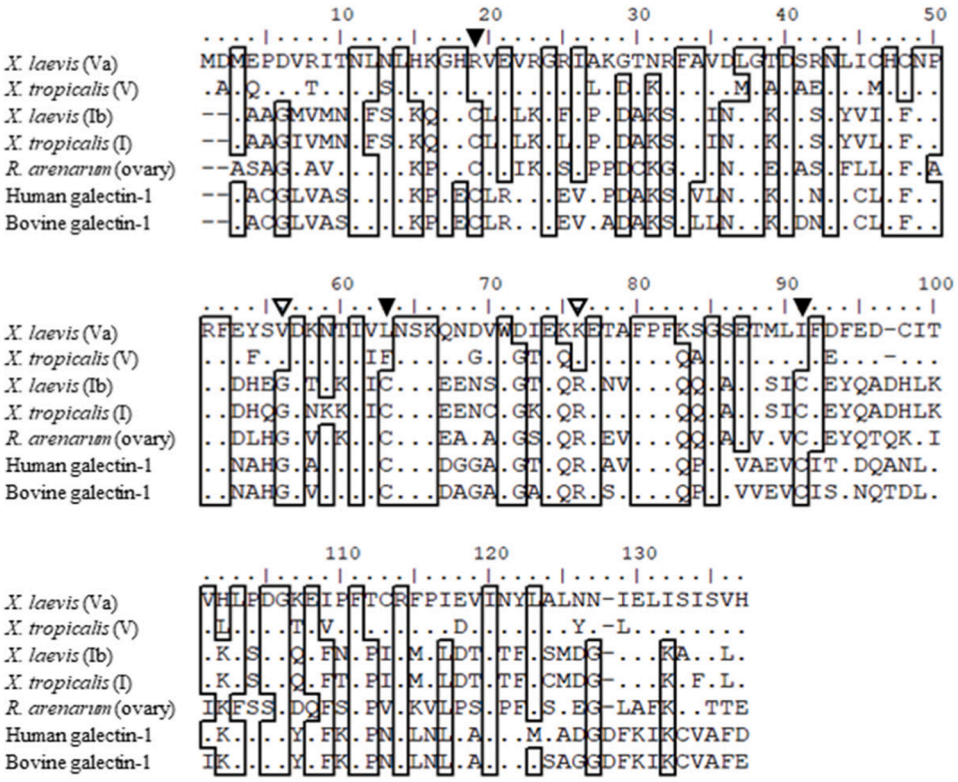

Fig. 3. Amino acid sequence alignment of proto-type galectins. Sequences were aligned using ClustalW (gap opening penalty was 10, gap extension penalty was 0.05, and Gonet protein weight matrix was used). Amino acid residues conserved in five or more sequences are highlighted with frames. Amino acids conserved in xgalectin-Va are shown as dots. Cysteine residues conserved in galectin-1 homologs are marked by closed triangles. Amino acids mentioned in the manuscript as being involved in ligand selectivity are marked by open triangles.

recently cloned *X. tropicalis* galectins, one of which is similar to xgalectin-Ia/Ib (*X. tropicalis* galectin-I), and another is similar to xgalectin-Va/Vb (*X. tropicalis* galectin-V). *Rhinella arenarum* ovary galectin, xgalectin-Ia/Ib, and *X. tropicalis* galectin-I share higher similarity with mammalian galectin-1 than with xgalectin-Va/Vb. For example, three cysteine residues conserved in both mammalian and amphibian galectins (Fig. 3, closed triangles) are substituted in xgalectin-Va/Vb. This similarity between mammalian galectin-1 and xgalectin-Ib is further supported by sequence similarity (Table 2). These data also suggest that xgalectin-Ia/Ib, rather than xgalectin-Va/Vb, should be the homologs of galectin-1.

Mammalian galectin-1, *R. arenarum* ovary galectin, and xgalectin-Ib have high affinity for Galβ1–4GlcNAc, a glycan structure commonly found on the cell surface, but low affinity for Galβ1–3GalNAc found in O-glycans [61,76,78,79]. Notably, xgalectin-Va is unique in that it exhibits a high affinity for Galβ1–3GalNAc and a low affinity for Galβ1–4GlcNAc. Several residues on the concave surface are involved in the selectivity of these sugars (Fig. 4) [80]. Of these, Gly54 and Arg74 are conserved in galectin-1 homologs, but not in xgalectin-Va (Fig. 3, open triangles). Additionally, the quaternary structure of xgalectin-Va also differs from that of galectin-1 (Fig. 5) [78,81]. Both mammalian and amphibian galectin-1 homologs form a homodimer via two intermolecular β-sheets [82,83]. Xgalectin-Va forms a homotetramer through the association of two galectin-1-like homodimers, and this tetramer structure is unique to *Xenopus* skin galectins. A marine sponge (*Cinachyrella* sp.) proto-type galectin also forms a tetramer composed of galectin-1-like homodimers, but the interface is different [84].

As described above, xgalectin-Va, -Vb, and *X. tropicalis* galectin-V have been suggested to be functionally distinct from galectin-1. However, the amino acid sequences of these skin galectins and galectin-1 are somewhat similar. Molecular phylogenetic analyses confirmed that the *Xenopus* skin galectins arose through divergence from the vertebrate galectin-1 group [5,78]. The xgalectin-Va and -Ib genes are both on chromosome 4 L, as are xgalectin-Vb and -Ia (chromosome 4S), and *X. tropicalis* galectin-V and -I (chromosome 4). Therefore, the ancestor of these skin galectins may have resulted from a gene duplication of galectin-1. Thus far, no protein homologous to xgalectin-Va has been reported for species other than the genus *Xenopus*.

Its abundance in skin secretions and its binding ability to the core of O-glycan suggest that xgalectin-Va may play a role in biological defense, such as binding to mucins and microorganisms, although the details remain unknown. As described above, proto-type galectins are also expressed in the skin secretions of several frogs other than *Xenopus*. These galectins arose independently of xgalectin-V, but they are believed to function in biological defense. For example, in fish, congerin is one of the proto-type galectins expressed in the skin mucus of conger eel [85]. Congerin is also considered to have diverged from galectin-1 and is known to agglutinate certain types of marine bacteria [86,87]. In mammals, galectin-7, another proto-type galectin, is expressed specifically in keratinocytes, which are involved in wound healing [88]. Each galectin likely plays a role in skin functions specific to its species. Further elucidation of skin galectins is expected to deepen our understanding of the diverse biological roles and evolutionary processes of galectins.

Table 2
Amino-acid-sequence identity among galectin-1-like proteins.

	X. laevis galectin-Ib	*R. arenarum* ovary galectin	Bovine galectin-1	Human galectin-1
X. laevis galectin-Va	39.0 %	36.8 %	38.0 %	38.7 %
X. laevis galectin-Ib		55.2 %	47.4 %	49.6 %
R. arenarum ovary galectin			48.1 %	44.4 %
Bovine galectin-1				86.7 %

Mammalian galectin-2 is also a proto-type galectin and is evolutionarily close to galectin-1. Galectin-2 is highly expressed in the skin of salamanders (*Andrias davidianus*), as revealed through a proteomic analysis [89]. Genes considered homologous to galectin-2 are also present in the *X. laevis* genome. However, we could not detect its mRNA or protein in our previous study, and it is unclear whether the genes are functional.

6. *X. laevis* chimera-type galectins

Xgalectin-VIIa, the only chimera-type galectin, and homolog of galectin-3 in *X. laevis*, is highly expressed, particularly during embryonic development. Xgalectin-VIIa, similarly to xgalectin-Ib and -Va, arose from the ancestral species L. Its mRNA is expressed in a tissue-specific manner and localizes primarily in the epidermal cells of early embryos [90]. Affinity chromatography using soluble extracts of tail-bud stage embryos has revealed that xgalectin-VIIa binds to a member of another lectin family, the *Xenopus laevis* egg surface grain-derived lectin (*X. laevis* cortical granule lectin; xCGL) [91] and xCGL2. XCGL and xCGL2 are N-glycosylated. Considering that xgalectin-VIIa cannot bind to N-glycanase-treated xCGL, it must recognize N-glycans. The structures of sugar chains absorbed by the xgalectin-VIIa column were analyzed using the two-dimensional-sugar map method, identifying poly-N-acetyllactosamine type N-glycans. Furthermore, the lectin activity of xCGL is similar to that of galectin in that it recognizes galactosides; however, the sugar-binding properties of the two differ in that xCGL binds to the monosaccharide portion of galactose [92]. This finding suggests the existence of an interaction network among lectins in *X. laevis*. However, further studies on its functional significance are required. Additionally, xgalectin-VIIa has two important amino acid substitutions compared to human galectin-3, around the carbohydrate-binding site. Lys176 and Asn180 in human galectin-3 are substituted with Met and Arg in xgalectin-VIIa, respectively (K176 M and N180R, Fig. 6 closed triangles). These substitutions suggest that xgalectin-VIIa may exhibit a carbohydrate specificity that is different from that of human galectin-3 [93]. Furthermore, Lys176 in human galectin-3 is also replaced with Met in our newly cloned galectin-3 homolog from *X. tropicalis*, and Asn180 is substituted with Lys, which has the same basicity as Arg (N180 K) (unpublished). These results suggest that in clawed frogs, galectin-3 homologs interact with xCGL by binding to glycans, forming a network of different lectin families. Considering that human galectin-3 is involved in cancer cell adhesion and migration, as well as the timing of its expression, xgalectin-VIIa may participate in embryonic development and tissue formation through the lectin network in the clawed frog. Moreover, galectins participate in adaptive and innate immune responses. This lectin network may play a role in regulating the immune system. However, more in-depth analyses are required to elucidate this role [94].

Xgalectin-VIIb, a homeolog of xgalectin-VIIa, is also registered in the Xenbase database (http://www.xenbase.org) [40]. This galectin is derived from the ancestral species S. The two amino acid substitutions described above occurred in xgalectin-VIIb in the same way as in *X. tropicalis* (Fig. 6, K176 M and N180 K). Moreover, a remarkable characteristic observed in the structure of xgalectin-VIIb was that the N-terminal region of xgalectin-VIIb is considerably longer than those of xgalectin-VIIa and human galectin-3. This region includes a structure of at least eight times repeated peptides consisting of 10 amino acids (PGFPAPGQ/HGQ/H), which was classified into type III repeat units according to Cooper [95] (red underlined in Fig. 6). The N-terminal region of the galectin-3 subfamily includes a unique intrinsically disordered region, which has been demonstrated to allow the oligomerization of galectin-3 [96–98]. Furthermore, an increasing number of reports are suggesting that glycan-bound galectin-3 undergoes liquid-liquid phase separation (LLPS) through oligomerization in a manner dependent on the intrinsically disordered region at N-terminus

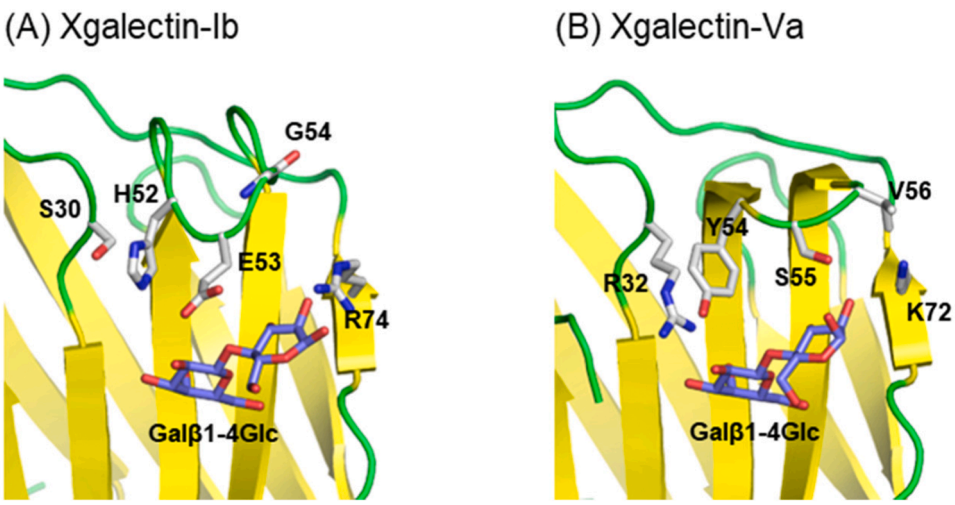

Fig. 4. Amino-acid residues involved in carbohydrate binding selectivity. Residues involved in ligand selectivity and ligand molecules are represented as stick models. Although Gly54 (Val56) is distant from the ligand, it affects the conformation of the loop region. (A) Structure of xgalectin-Ib/lactose complex. (B) Structure of galectin-Va/lactose complex.

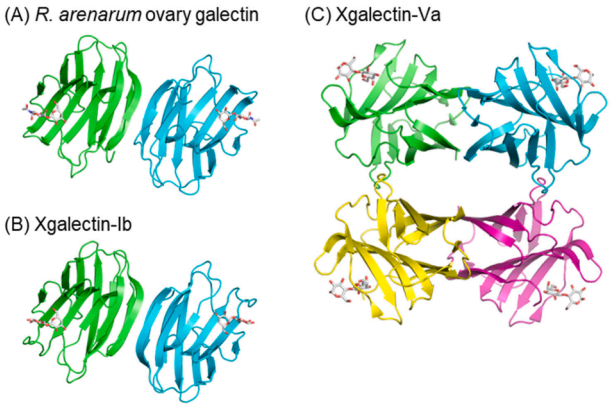

Fig. 5. Crystal structures of *Rhinella arenarum* and *Xenopus laevis* prototype galectins (A) Structure of *R. arenarum* ovary galectin dimer. (B) Structure of xgalectin-Ib dimer, which resembles the dimer structures of toad and mammalian galectin-1s. (C) Structure of xgalectin-Va tetramer. Two xgalectin-1-like dimers (green/cyan and magenta/yellow) assemble into a tetramer.

[99–104]. It would be valuable to examine if there is any difference in oligomerization and/or LLPS between xgalectin-VIIb and xgalectin-VIIa. Although xgalectin-VIIb expression has not been detected, at sufficient levels, it is expected to perform important functions given its conserved amino acid sequences involved in carbohydrate chain binding.

7. *X. laevis* tandem-repeat-type galectins

Several tandem-repeat-type galectins (xgalectin-II, -III, -IV, -VI, and -VIII) have been isolated from *X. laevis*. In particular, xgalectin-II, -III, and -VIII have high sequence similarities to mammalian galectin-4, −9, and −8, respectively, and are, thus considered homologs. Among them, homeologs (-IIa/-IIb, -IIIa/-IIIb) exist for xgalectin-II and -III, while one was lost for xgalectin-VIII (Table 1) [74]. Of particular interest are the mammalian galectin-4 homologs, xgalectin-IIa and -IIb. We are currently analyzing the distribution of galectin-4 homologs in the digestive tract of *X. laevis*. We found that both galectin-4 homologs from both ancestral species are expressed in the digestive tract of *X. laevis*. Furthermore, through further distributional analysis, we found that xgalectin-IIb from the ancestral species S is highly expressed in the upper digestive tract, and xgalectin-IIa from the ancestral species L is highly expressed in the lower digestive tract (in preparation). In mammals, galectin-4 is highly expressed in gastrointestinal epithelial cells, playing an important role in cell adhesion and intercellular signal transduction. Furthermore, galectin-4 induces apoptosis of mucosal T cells and decreases the secretion of pro-inflammatory cytokines. Thus, galectin-4 helps regulate immune and inflammatory responses in the intestine and may be associated with intestinal diseases. Cytoplasmic galectin-4 in the enchained daughter bacteria restricts their intracellular movement by directly binding to the lipopolysaccharide O-antigen. This enhances inflammasome activation in intestinal epithelial cells [105]. Moreover, human galectin-4 and galectin-8 target and kill pathogenic *Escherichia coli* expressing blood group B-type glycans [56]. Although the *X. laevis* homolog of human galectin-8 has been confirmed at the mRNA level, its protein expression remains undetectable. Hence, despite confirmation in the Xenbase database, the galectin-8 gene from the ancestral species L was lost, while xgalectin-VIIIa from ancestral species S was retained. This suggests that xgalectin-II, a galectin-4 homolog, is primarily responsible for resistance to pathogenic *E. coli* in *X. laevis*. In fact, xgalectin-II exhibits enhanced binding affinity to glycans, conferring resistance to parasites and pathogenic bacteria than human galectin-4 (study in preparation). Although slight differences were observed in the carbohydrate-binding properties of xgalectin-IIa and -IIb, their functions may be enhanced by altering their subcellular localization and tissue distribution. The functions of these galectins are currently under investigation.

Xgalectin-IVa and xgalectin-VIa have no clear mammalian homologs and are considered unique to clawed frogs. Although the analogous relationship between these galectins is unclear, both have high sequence similarity with xgalectin-IIa and -IIb and may, therefore, represent functionally important molecules. Notably, xgalectin-VIa is highly expressed during the transient formation of cement glands in amphibian embryos [73,106]. Cement glands are mucus-secreting neural organs located at the anterior end of the neural plate. Moreover, xgalectin-VIa expression is repressed by Wnt signaling and enhanced by Wnt inhibition [107]. The anterior-posterior axis pattern of the *X. laevis* embryo is determined during protoderm formation and is shaped by Wnt, fibroblast growth factor, and retinoic acid concentration gradients. Classical Wnt signaling is inhibited in the anterior region of the neuroectoderm, where the head develops. Hence, increased expression of xgalectin-VIa due to Wnt inhibition is a useful marker for cement glands in the head region during development [107]. Thus, xgalectin-VIa expression is closely associated with amphibian-specific developmental processes. Further analysis of these clawed frog-specific galectins may reveal novel

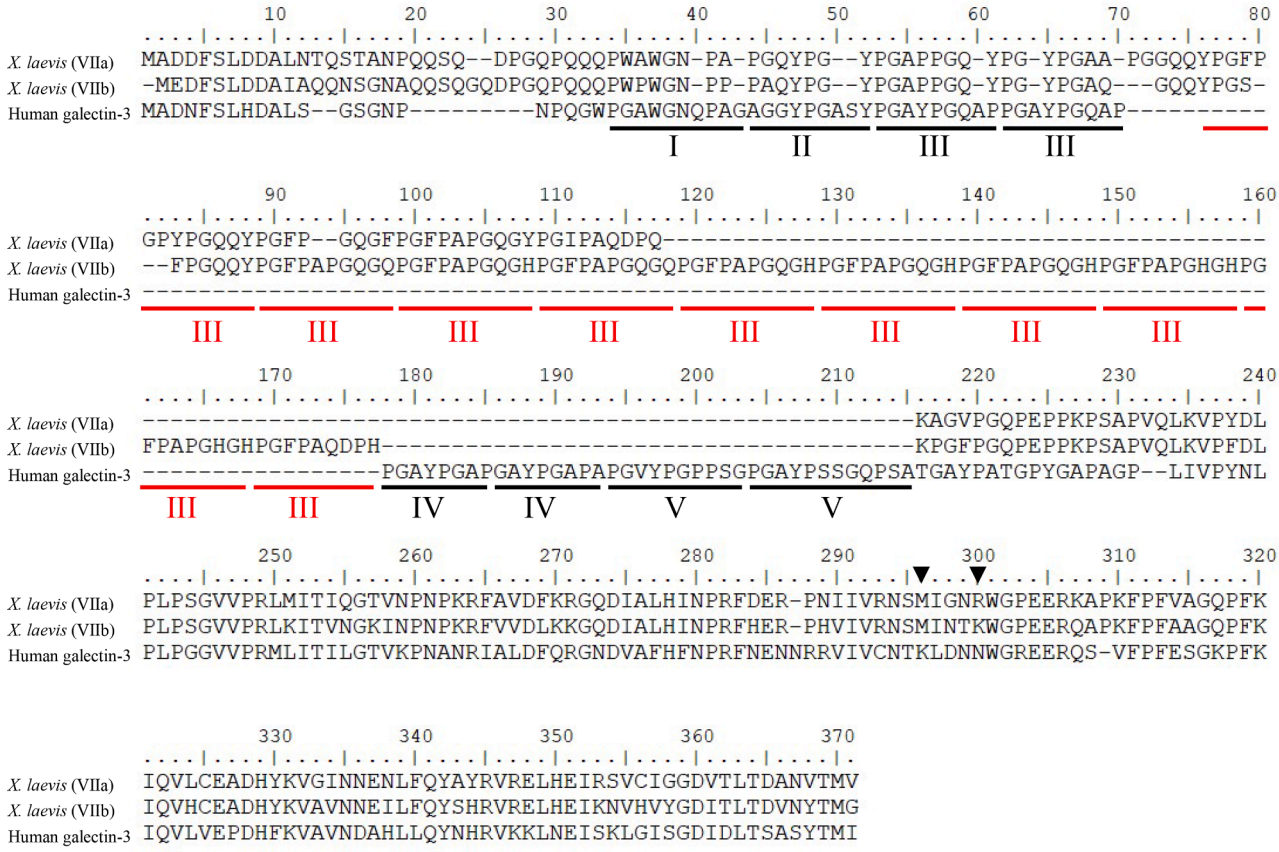

Fig. 6. Amino acid sequence alignment of xgalectin-VIIa, -VIIb, and human galectin-3. Sequences were aligned and the repeat units (I-V) are underlined according to cooper et al., [93]. The black line indicates the human repeating unit, and the red line indicates the repeating unit characteristic of xgalectin-VIIb. Gaps are represented by hyphens. Two amino acid substitutions are indicated with arrow heads (K176 M and N180 K).

species-specific functions and the molecular mechanisms acquired during evolution, demonstrating the evolutionary diversity of galectins and their adaptive roles in different species.

8. Conclusion

Although many aspects of *X. laevis* galectin research remain unexplored, these galectins, similar to those in mammals, are suggested to perform different functions in a tissue-specific and time-dependent manner. We have previously identified and characterized two groups of galectins: one conserved in mammals and the other unique to clawed frogs. The associated data provide a useful resource for studying how these galectins have diverged and acquired new functions during evolution. Amphibians are evolutionarily positioned between fish and mammals. *Xenopus* is a rare allotetraploid vertebrate and a potential source of new insights from both evolutionary and molecular selection perspectives. Furthermore, *Xenopus* is divided into *X. laevis*, which underwent a third WGD (Xs3R), and *X. tropicalis*, which did not, making it an ideal model for comparative studies.

In *X. laevis*, many galectins from ancestral species L have been detected, with galectins (homeologs) from the ancestral species S expressed in certain organs. The simultaneous presence of homeologs from different ancestral species with unique functions is of great interest from an evolutionary biology perspective. Clarifying the significance of the expression of galectins derived from the ancestral species S is key to understanding how galectins have acquired new functions during evolution and their contributions to species adaptation. Analysis of the interactions between different galectins and the temporal and spatial regulation of their expression may also contribute to elucidating the diverse biological roles of galectins.

Further, we have successfully detected and cloned galectins in the diploid *X. tropicalis*. We are currently in the process of comparatively analyzing their evolutionarily conserved and specialized functions. Analyzing galectins from two species offers many advantages as experimental animals. As the environments in which they thrive differ, it is anticipated that the distribution of pathogenic microorganisms will also vary. In the future, we aim to elucidate the evolutionary process by which galectins have acquired function characteristics of each organism through a comparative analysis of galectins and glycans expressed by *X. laevis* and *X. tropicalis*. This will reveal the differences between galectins that have retained specific functions during evolution and those that have acquired new functions. Such an analysis would provide important insights into the galectin functional diversity in vertebrates. Additionally, considering that galectins can bind various pathogenic bacteria and parasites via glycans, we will also analyze their role in biological defense using various galectin-knockout *Xenopus* and mouse models. Through these studies, the importance of glycans will be demonstrated from the perspective of galectins.

Glossary

WGD, Whole-genome duplication; CRD, Carbohydrate recognition domain; Xgalectin, Xenopus laevis galectin

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CRediT authorship contribution statement

Takashi Ogawa: Writing – review & editing, Writing – original draft, Investigation. **Yasuhiro Nonaka:** Writing – review & editing, Writing – original draft, Investigation. **Hiroki Shoji:** Writing – review & editing, Writing – original draft, Investigation. **Takanori Nakamura:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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