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Clustered *Xenopus* keratin genes: A genomic, transcriptomic, and proteomic analysis



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ABSTRACT

Keratin genes belong to the intermediate filament superfamily and their expression is altered following morphological and physiological changes in vertebrate epithelial cells. Keratin genes are divided into two groups, type I and II, and are clustered on vertebrate genomes, including those of *Xenopus* species. Various keratin genes have been identified and characterized by their unique expression patterns throughout ontogeny in *Xenopus laevis*; however, compilation of previously reported and newly identified keratin genes in two *Xenopus* species is required for our further understanding of keratin gene evolution, not only in amphibians but also in all terrestrial vertebrates. In this study, 120 putative type I and II keratin genes in total were identified based on the genome data from two *Xenopus* species. We revealed that most of these genes are highly clustered on two homeologous chromosomes, XLA9_10 and XLA2 in *X. laevis*, and XTR10 and XTR2 in *X. tropicalis*, which are orthologous to those of human, showing conserved synteny among tetrapods. RNA-Seq data from various embryonic stages and adult tissues highlighted the unique expression profiles of orthologous and homeologous keratin genes in developmental stage- and tissue-specific manners. Moreover, we identified dozens of epidermal keratin proteins from the whole embryo, larval skin, tail, and adult skin using shotgun proteomics. In light of our results, we discuss the radiation, diversification, and unique expression of the clustered keratin genes, which are closely related to epidermal development and terrestrial adaptation during amphibian evolution, including *Xenopus* speciation.

1. Introduction

Two clawed frogs, *Xenopus laevis* and *X. tropicalis* are widely used to study cell and developmental biology, because of their unique, excellent properties as experimental animals (Harland and Grainger, 2011). *X. tropicalis* is diploid and *X. laevis* is allotetraploid carrying two subgenomes, L and S. Since both the genomes have been sequenced (Hellsten et al., 2010; Session et al., 2016), researchers can easily retrieve genome information from the two clawed frogs for various purposes; gene cloning, transcriptomic analysis, epigenetic analysis, and the functional analysis of genes of interest using genome editing. Thus, this genome information makes these species useful experimental animals in life science research.

Keratin is a member of the intermediate filament superfamily and forms the cytoskeleton in vertebrate epithelial cells. Keratins are

divided into two groups, which are referred to as type I and II, based on their biochemical characteristics such as isoelectric points and molecular weights (Moll et al., 1982). The obligate heterodimerization of type I and II keratins leads to the formation of 10-nm cytoskeletal filaments by self-assembly. Differential keratin expression resulting from changes in physiological strength and motility produces various epithelial cells in vertebrates. Therefore, their genes are strictly regulated in a cell-type specific manner: their expression profiles are closely related to cell differentiation and transformation in organogenesis and pathogenesis (Bragulla and Homberger, 2009; Moll et al., 2008). Over 60 keratin genes exist in the human genome and their type I and II keratin genes are clustered on chromosomes 17 and 12, respectively (Hesse et al., 2001; Schweizer et al., 2006). Other model vertebrates including the mouse, chicken, zebrafish, and western clawed frog (*Xenopus tropicalis*) also have numerous type I and II

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keratin genes separately clustered on their genomes (Greenwold et al., 2014; Hesse et al., 2004; Vandebergh and Bossuyt, 2012; Zimek and Weber, 2005, 2006). Vertebrate keratin genes have been extensively studied to elucidate the molecular mechanisms underlying epithelial cell differentiation and transformation.

To date, fifteen *X. laevis* keratin genes have been identified and characterized according to their unique expression changes and functions in oogenesis, embryogenesis, metamorphosis, and regeneration, in which drastic cell differentiation and transformation occur (Klymkowsky et al., 1991, 1992; Mathisen and Miller, 1987; Miyatani et al., 1986; Suzuki et al., 2009, 2010; Tazaki et al., 2005; Watanabe et al., 2001, 2002). Notably, switching of various epidermal keratin genes from the larval- to adult-type occurs during metamorphosis (Mukhi et al., 2010; Suzuki et al., 2009; Watanabe et al., 2001, 2002). In organ regeneration, several keratin genes are either highly induced or down-regulated in the wound epidermis of amputated limb buds and tails (Tazaki et al., 2005; Tazawa et al., 2006). There have been some reports regarding *X. laevis* transcriptional regulation of epidermal keratin genes in embryogenesis and metamorphosis (Jonas et al., 1989; Suzuki et al., 2009, 2010; Warshawsky and Miller, 1995; Watanabe et al., 2002). However, developmental expression and regulation of *Xenopus* keratin genes remain unclear.

In this study, based on the latest *X. laevis* and *X. tropicalis* genome data, we revealed all of the *Xenopus* clustered keratin genes. We found numerous putative type I and II keratin genes, most of which are highly clustered on two homeologous chromosomes, XLA9_10 (L and S) and XLA2 (L and S) in *X. laevis* and XTR10 and XTR2 in *X. tropicalis*. In both *Xenopus* species, each of the keratin genes exhibited differential expression patterns in tissue-specific and developmental stage-specific manners throughout ontogeny. Intriguingly, some homeologous keratin genes had subgenome-specific expression profiles in *X. laevis*, suggesting the possibility of their subfunctionalization. We also discovered that eight adult-type epidermal keratin genes exist as singletons in the allotetraploid genome of *X. laevis* by asymmetrically being lost from S chromosomes. We further discuss the evolution of the clustered keratin genes in *Xenopus* speciation and their unique expression changes that are closely related to the epidermal development adapting environmental change throughout ontogeny.

2. Materials and methods

2.1. Animals

Xenopus laevis, the inbred J strain, embryos, tadpoles, and adults were maintained in house (provided by Dr. Yumi Izutsu, Niigata University). *Xenopus tropicalis*, the Golden strain, embryos, tadpoles, and adults were obtained from the National BioResource Project (NBRP) *Xenopus tropicalis*, Hiroshima University (Igawa et al., 2015). Embryos and tadpoles were staged according to NF staging (Nieuwkoop and Faber, 1994). Animals were anesthetized with MS-222 (Sigma-Aldrich) prior to being euthanized. Each experimental group used in RNA-Seq was independently collected twice (Session et al., 2016). The animals were handled in accordance with the Hiroshima University guidelines for the use and care of experimental animals and this study was approved by the committee (approval number: G23-1).

2.2. Gene models, phylogenetic tree, and expression profiles

Xenopus keratin genes were screened from gene models of *X. laevis* annotation v1.8 and *X. tropicalis* annotation v8.0 and 9.0 with Blastp using known *Xenopus* and human keratin peptide sequences as queries (Altschul et al., 1990). Gene model sequence errors were corrected manually using genome assemblies in GenomeMatcher (Ohtsubo et al., 2008). Because a type I keratin cluster was separated in the *X. tropicalis* genome assembly ver. 9, the gene order in this study was

according to *X. tropicalis* assembly ver. 8. Gene positions on the *Xenopus* genome are described in Supplementary Table 1.

A phylogenetic tree of the keratin genes was generated in MEGA 6 (Tamura et al., 2013). *Xenopus* and human keratin genes were aligned in ClustalW (Thompson et al., 1994) and trimmed manually as 174 peptides including core peptide sequences (Supplementary Data 1–3). Maximum-likelihood method was performed with 500 bootstraps, a parameter model was estimated in MEGA 6 and using LG with a gamma-distributed model, the inference option was a nearest-neighbor-interchange method on a neighbor-joining (NJ) tree. *Xenopus* expression profiles were obtained from RNA-Seq data (Session et al., 2016). Expression levels were indicated as transcripts per million of mapped reads (TPM), which were obtained by mapping paired-end RNA-Seq reads to predicted full-length cDNA. The *X. tropicalis* expression profile was re-measured from RNA-Seq data by Tan et al. (2013). All RNA-Seq data are supplied in Supplementary Tables 2–4. *X. tropicalis* sampling stages that differed from that of *X. laevis* are indicated in parentheses in the figures.

For the transcriptome correlation analysis, we analyzed data sets from 14 *X. laevis* embryonic stages. Any gene whose TPM value was < 1 for all samples was removed from the analysis to reduce noise. Correlations of expression profiles between homeologs were examined using Pearson's correlation and Student's paired *t*-test on log₂-transformed data [log₂(TPM+1)] as previously described (Berthelot et al., 2014; Session et al., 2016). The homeolog pairs were categorized into four groups based on (1) correlation (HC: high correlation, $p \leq 0.05$; NC: no correlation, $p > 0.05$, Pearson's correlation test) and (2) expression levels (SE: Same expression levels, $p > 0.05$; DE: Different expression levels, $p \leq 0.05$, Student's paired *t*-test).

2.3. Cytoskeletal fraction, SDS-PAGE, and in-gel digestion

Cytoskeletal fraction of embryo, larval body skin, tail, and adult skin tissues were prepared according to previous reports with some modifications (Ellison et al., 1985; Suzuki et al., 2001). The cytoskeletal fractions were dissolved by 2× SDS-PAGE sample buffer containing 10 mM DTT and iodoacetamide according to the manufacturer's protocol (Sample Buffer Kit, APRO Science). After denaturation, the samples were subjected to 15% SDS-PAGE and the gels were stained with Coomassie Brilliant Blue. Bands corresponding to keratins (50–70 kDa) were excised with a disposable blade and then digested with an XL-Tryp Kit (APRO Science) at 37 °C overnight. Tryptic peptides were extracted from supernatants and gels twice by 50% acetonitrile containing 0.1% formic acid. Once dried, the peptides were again dissolved by in 0.1% formic acid and purified by C18 ZipTip pipette tips according to the manufacturer's instructions (Millipore). The purified peptides were then dissolved in 0.1% formic acid.

2.4. Shotgun proteomics using nanoLC-ESI MS/MS

The tryptic peptide mixtures of each sample in 0.1% formic acid were automatically applied to an LTQ Orbitrap XL mass spectrometer through an UltiMate 3000 RSLCnano System (Thermo Fisher Scientific). The peptides were bound on a C18 PepMap100 trap column (300 $\mu\text{m} \times 5 \text{ mm}$, Thermo Fisher Scientific) with 0.1% TFA at a flow rate of 30 $\mu\text{l/min}$. The peptides were then separated on a C18 Nano HPLC capillary tip column (75 $\mu\text{m} \times 120 \text{ mm}$, Nikkyo Technos) at a flow rate of 200 nl/min. A binary solvent system consisting of solvent A (0.1% formic acid in ultra-pure water) and solvent B (0.1% formic acid in acetonitrile) with the following gradient was used; 4–35% for 35 min and then 35–90% for 10 min. For electrospray ionization (ESI), spray voltage was applied at 1.5 kV. The transfer capillary temperature was 200 °C. With this method, full MS1 spectra were obtained in the positive ion mode (the mass range; 300–2000 *m/z*) in an Orbitrap analyzer. The top five intense precursor ions were fragmented by collision-induced dissociation and subjected to MS2 analysis in a linear

iontrap analyzer.

2.5. Proteomics data analysis and database search

To identify proteins, all acquired MS/MS spectra were analyzed by MASCOT server (Matrix Science) and SEQUEST (Thermo Fisher Scientific) through Proteome Discoverer 1.4 (Thermo Fisher Scientific). The predicted protein sequence data sets were based on the *X. laevis* annotation v1.8 and *X. tropicalis* annotation v8.0 from Xenbase (<http://www.xenbase.org>; Karpinka et al., 2015). We replaced the legacy *krt* names and sequences with the new counterparts in this study in these FASTA formatted data sets. The parameters for MS analysis are as follows; miscleavage site, 1; fix modification, carbamidomethylation in Cys; variable modification, oxidation in Met. The MS1 and MS2 mass tolerances were defined as 10 ppm and 0.8 Da, respectively. The false discovery rate (FDR) was < 0.01 in this search engine with Percolator.

3. Results

3.1. Genomic organization of clustered *Xenopus* keratin genes

We found 76 and 44 predicted keratin genes in *X. laevis* and *X. tropicalis*, respectively, based on the genome data from both *Xenopus* species (Hellsten et al., 2010; Session et al., 2016; Fig. 1). Most of the keratin genes were divided into two subfamilies, referred to as type I and type II keratin. A total of 43 and 26 type I keratin genes were identified in *X. laevis* and *X. tropicalis*, respectively. These type I keratin genes were highly clustered on the two subgenomes, XLA9_10L and S chromosomes in *X. laevis* and XTR10 in *X. tropicalis*. Furthermore, 28 and 15 type II keratin genes were also identified in *X. laevis* and *X. tropicalis*, respectively. These type II keratin genes were also highly clustered on the two subgenomes, XLA2L and S in *X. laevis* and XTR2 in *X. tropicalis*. *Smrce1/efl1* and *faim2/efl4b* were located on the flank of clusters I and II in both *Xenopus* and human, suggesting syntenic equivalency with those of human and other vertebrates, which is in accordance with previous reports (Greenwold et al., 2014; Hesse et al., 2004; Vandeborgh and Bossuyt, 2012; Zimek and Weber, 2005, 2006). According to the rules of keratin nomenclature in other species and *Xenopus* nomenclature (Schweizer et al., 2006; <http://www.xenbase.org/gene/static/geneNomenclature.jsp>), we have given all of the clustered keratin gene names and symbols based on their synteny and one-to-one relationship between both *Xenopus* species (Fig. 1A). In the clustered type I keratin, although most of the genes were retained between the two *Xenopus* species, eight singleton genes were found on the *X. laevis* L subgenome. A singleton occurs when a counterpart of a duplicated gene is retained by selection after whole genome duplication. Notably, *krt51.S*–*krt57.S*, except for *krt53.S*, were locally lost, suggesting selection pressure of some kind during *Xenopus* evolution. Only *krt58.L* was most likely a pseudogene because it had a point mutation at the 1st ATG, which became GTG, and some deletion and insertion mutations provoking a frame-shift in the predicted exon1. In the clustered type II keratin, most of the genes and organizations were retained between the two *Xenopus* species. Similar to type I keratin, four singleton genes were only seen on the *X. laevis* L subgenome.

In *X. laevis*, 15 keratin genes have thus far been identified and well-characterized; endoB, CK1/8, XK81A2, XK81B1, XAK (*Xenopus* adult keratin)-A/B/C, XLK (*Xenopus* larval keratin), XLK2, FGK (Fin gill keratin), 51 kDa keratin, XK70, CK55/56 (55 kDa), and 63 kDa keratin (pUF23, pUF164), most of which are differentially expressed as epidermal keratin genes in a cell-specific manner at the embryo, tadpole, and adult stages (Fouquet et al., 1988; Franz and Franke, 1986; Hoffmann et al., 1985, 1988; Jonas et al., 1985; LaFlamme et al., 1988; Miyatani et al., 1986; Suzuki et al., 2010; Tazawa et al., 2006; Watanabe et al., 2001, 2002; Winkles et al., 1985). All of the 15 keratin

genes were confirmed in the predicted gene models based on the *X. laevis* genomic data (Fig. 1A; Supplementary Table 5).

Phylogenetic analysis revealed a one-to-one relationship among all type I and II keratin genes in both *Xenopus* species (Fig. 1B). Intriguingly, *krt24*, *krt50*–*krt57*, *krt12.6*, and *krt78.2*–*krt78.9* formed two unique clades in the phylogenetic tree. As has been pointed out, some *X. tropicalis* *krt* genes corresponding to these two clades are predominantly expressed in adult skin (Vandeborgh and Bossuyt, 2012). We designated these two clades as adult epidermal keratin (AEK) subclusters according to their genomic organization and their traits described later. Genomic organization and phylogenetic analysis also indicated some evolutionary conservation of keratin genes between human and *Xenopus*. In particular, *Xenopus krt18.1* and *krt8.1* exhibited a close evolutionary relationship to Human *KRT18* and *KRT8*, the primordial type I and II keratins, respectively, in their conservation of amino acid sequences and synteny (Hesse et al., 2004; Vandeborgh and Bossuyt, 2012; Zimek and Weber, 2005).

3.2. Keratin transcript expression profiles in two *Xenopus* species during embryogenesis

Based on two transcriptome RNA-Seq data sets in *X. laevis* and *X. tropicalis* (Session et al., 2016; Tan et al., 2013), we found keratin genes that were expressed during embryogenesis (Fig. 2A). In both species, the *krt7*, *krt8.1*, *krt12.2*, *krt12.4*, *krt12.5*, *krt18.1*, *krt19*, and *krt70* transcripts increased noticeably after mid-blastula transition (MBT) at around NF10 and their expression levels gradually decreased until the swimming stage, NF40. The RNA-Seq data revealed that these homeologs except for *krt8.1* were differentially expressed in *X. laevis* embryo, and the temporal expression patterns of these genes were similar to those of *X. tropicalis*. In the case of *krt12.4*, there was no statistically significant difference between homeologs but *.L* gene expression was obviously decreased earlier than that of the *.S* gene, suggesting a subgenome-specific expression. These results imply that there is subfunctionalization between the homeologs of these seven gene pairs. Extremely low or no expression of *krt* genes on the AEK subclusters was observed in both *Xenopus* species during embryogenesis (green boxes in Fig. 2B).

3.3. *X. laevis* keratin transcript expression profiles in various adult tissues

We obtained information regarding keratin gene expression in a tissue-specific manner, by analyzing the RNA-Seq data sets from various adult *X. laevis* tissues (Fig. 3). Most of the keratin genes on the AEK subclusters were exclusively and highly expressed in adult skin and eye tissues including stratified epidermis, but not in other tissues (green boxes in Fig. 3A, B). Conversely, *krt7*, *krt8.1*, *krt18.1*, and *krt19* were predominantly expressed in all tissues including simple epithelium. These genes were also expressed in the stratified epidermis. In particular, *krt19*, *krt8.1*, and *krt18.1* are likely orthologous to human *KRT19*, *KRT8*, and *KRT18*, respectively, which are primary keratins of simple epithelial cells (Bragulla and Homberger, 2009; Moll et al., 2008). In many adult tissues, *krt* gene expression on the L subgenome exhibited higher levels than those on the S subgenome. There were a few exceptions, *krt19.S* expression levels in the liver and skin and *krt8.1.S* in the heart were four times higher than those of their L counterparts (see Supplementary Table 4).

3.4. Changes in keratin protein profiles are closely related to the transition of the epidermis throughout ontogeny

To analyze keratin expression changes in the transition of the epidermis throughout ontogeny in *X. laevis* (J strain) and *X. tropicalis* (Golden strain), we performed shotgun proteomics analysis of cytoskeletal fractions from whole embryo (NF34/35), larval body skin

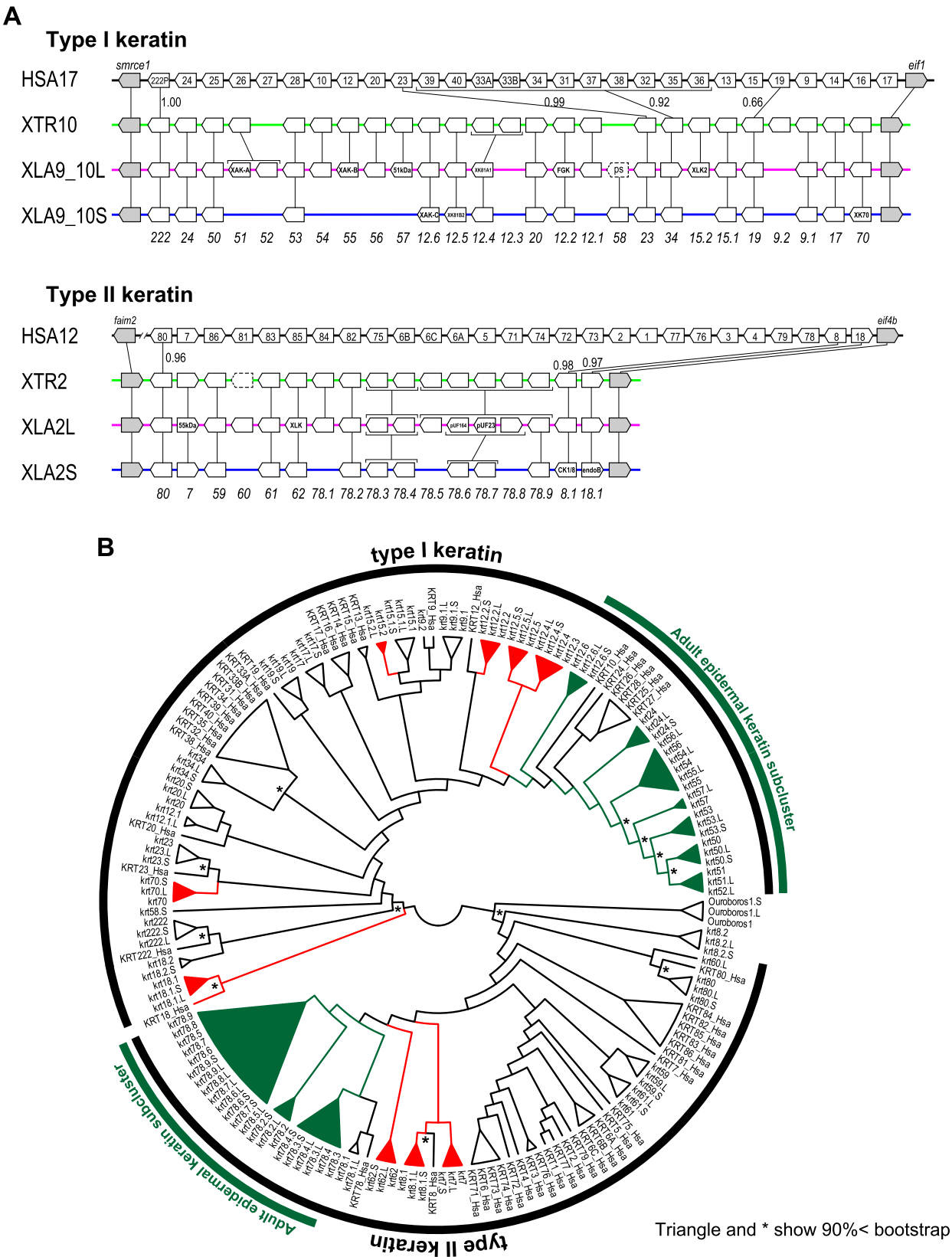


Fig. 1. Genomic organization of clustered keratin genes and conserved synteny between *Xenopus* and human. (A) Genomic organization of clustered type I and II keratins. Chromosome abbreviations are HSA, *H. sapiens*; XTR, *X. tropicalis*; XLA_L and XLA_S, *X. laevis* subgenome L and S. Symbols represent the position and direction of each gene. Gray symbols show the syntenic flanking genes. Suffix numbers of the *Xenopus* keratin genes are shown below each cluster diagram. Gene names of human and *Xenopus* gene synonyms are shown in the symbols. Orthologous and homeologous relationships obtained from phylogenetic analysis are indicated by lines between the genes, and numbers between HSA and XTR show bootstrap values. *X. laevis* *krt58.L* was a pseudogene and *X. tropicalis* *krt60* was not constructed because of N-gaps (shown by dotted symbols). (B) Phylogenetic tree of human and *Xenopus* keratin genes. The tree indicates 174 keratin proteins including 76 *X. laevis*, 44 *X. tropicalis*, and 54 *Homo sapiens* genes. Upper and lower arcs show type I and II keratins, respectively. Ouroboros, *krt8.2* and *krt18.2* genes do not belong to the two clusters. Triangles and asterisks indicate bootstrap values > 90%. Green and red indicate adult and embryonic expression from RNA-Seq data, respectively. Adult epidermal keratin subclusters are highlighted by green curves.

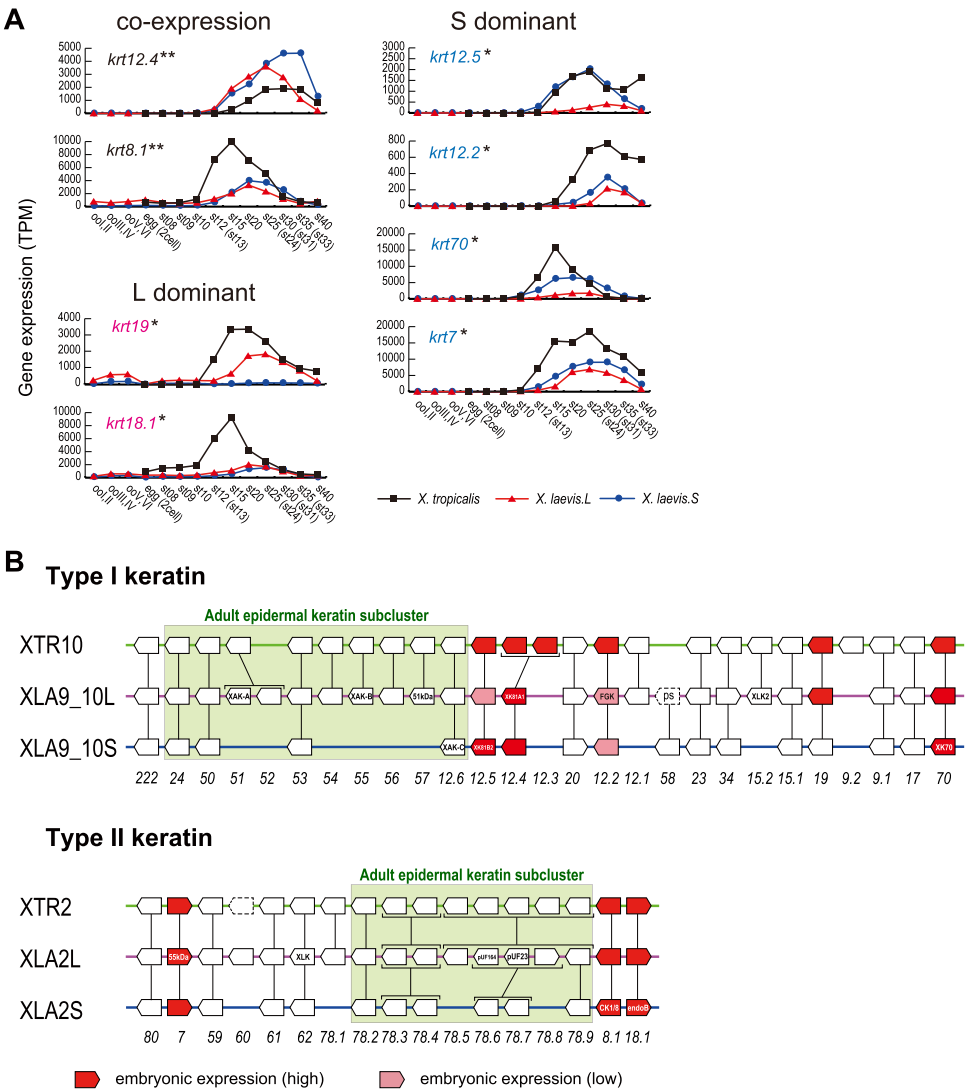


Fig. 2. Keratin transcript expression profiles in two *Xenopus* species during embryogenesis. (A) Keratin gene expression profiles from RNA-Seq data. Expression levels of eight *krt* orthologs and homeologs are shown at the indicated developmental stage (NF stage). *X. tropicalis* sampling stages that differed from those of *X. laevis* are indicated in parentheses. Gene expression levels are shown by TPM (transcripts per million of mapped read) of each *krt* gene in both *Xenopus* species. Black, *X. tropicalis*; red, *X. laevis* L; blue, *X. laevis* S. The *krt8.1* and *krt12.4* homeologs were concomitantly expressed, whereas *krt7.S*, *krt18.1.L*, *krt12.2.S*, *krt12.5.S*, *krt19.L*, and *krt70.S* were preferentially expressed during *X. laevis* embryogenesis, estimated by statistical analysis, *HCDE and **HCSE (see Materials and Methods). (B) A schematic summary of *krt* gene expression during embryogenesis. Symbols show the position and direction of each gene. Shades of red in symbols reflect high or low expression during embryogenesis. The basal and high/low cut off values were 199 and 682 TPM, which were the average of the data set of embryonic stages and the average of the maximum value of each gene in the data set, respectively. Chromosome abbreviations are XTR, *X. tropicalis*; XLA_L and XLA_S, *X. laevis* subgenome L and S, respectively. Note that extremely low or no expression of *krt* genes on the adult epidermal keratin (AEK) subclusters was observed in both *Xenopus* species during embryogenesis (Green boxes in B).

(NF54), tail (NF54), and adult skin using LC-MS/MS (Fig. 4). A total of 3463 peptides from these tissues were assigned to the predicted keratin protein sequences based on the *X. laevis* and *X. tropicalis* genome data. In *X. laevis*, we eventually identified 25 type I and 14 type II keratin proteins from 1067 unique peptides (Fig. 4A; Supplementary Table 6). A unique peptide is defined as follows: a tryptic peptide sequence can be found from only one protein and is non-redundant in a protein data set. To distinguish between *X. laevis* L and S subgenome Krt proteins, we counted unique peptides in each gene and evaluated their expression at the protein level. Notably, a large number of unique peptides on the two AEK subclusters were identified from adult skin (green boxes in Fig. 4A, B). Among them, the unique peptides of Krt12.6.L, Krt57.L, Krt78.2.S, and Krt78.6.L were also detected in tadpole body skin, reflecting the evidence that the progenitor cells of adult epidermis exist in the tadpole body skin but not in the tail (Suzuki et al., 2009; Watanabe et al., 2002). It is noteworthy that the unique peptides of Krt15.2.L, Krt62.L, Krt62.S, and Krt78.1.L were exclusive or and abundant in tadpole body skin and tail, the skin of which

consists of larval-type epidermal cells; apical, skein, and basal cells (Suzuki et al., 2009; Watanabe et al., 2002). Therefore, these *krt* genes can be defined as larval-specific keratins. The unique peptides of Krt12.4.S, Krt12.5.L, Krt12.5.S, and Krt70.S, were abundant in embryos, larval body skin and tail, but not in adult skin. Generally, the keratin protein expression patterns in *X. tropicalis* were similar to those of *X. laevis*: we eventually identified 16 type I and 9 type II keratin proteins from 597 unique peptides.

4. Discussion

Based on the latest *X. laevis* and *X. tropicalis* genome data, genomic organization, and phylogenetic analysis, we present a complete picture of all clustered keratin genes in two *Xenopus* species. Transcriptomics and proteomics analyses by RNA-Seq and LC-MS/MS comprehensively elucidated their expression in both *Xenopus* species throughout ontogeny. Moreover, the existence of singleton keratin genes and subgenome-specific expression represent asymmetric evolu-

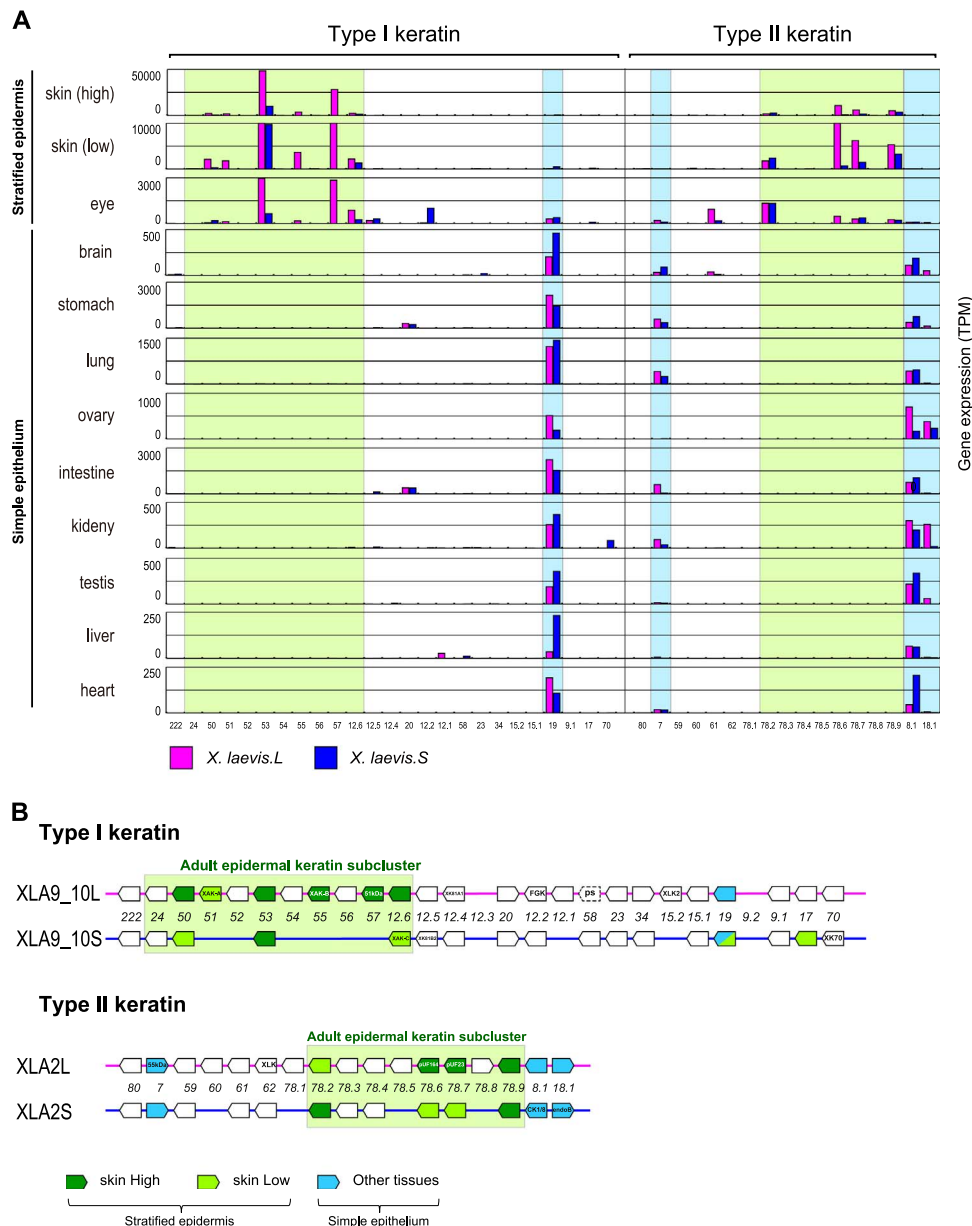


Fig. 3. Comparison of keratin transcript expression levels in various *X. laevis* adult tissues. (A) *krt* gene expression profiles from RNA-Seq data in various adult tissues. Gene expression levels are shown by TPM (transcripts per million of mapped read) of each *krt* gene in two and nine adult tissues containing stratified epidermis and simple epithelium, respectively. Magenta, *X. laevis. L*; blue, *X. laevis. S*. We defined the two subclusters on which *krt* genes were highly expressed in skin and eye containing epidermis as adult epidermal keratin (AEK) subclusters. AEK subclusters are highlighted in the green boxes. *krt7*, *krt8.1*, *krt18.1*, and *krt19* that were predominantly expressed in the tissues containing simple epithelium, are highlighted in the blue boxes. (B) A schematic summary of *krt* gene expression in various adult tissues. Symbols show the position and direction of each gene. Shades of green in symbols reflect either high or low *krt* gene expression in adult skin. The basal and high/low cut off values were 176 and 2010 TPM, which were the average of the data set of adult tissues and the average of the maximum value of each gene in the data set, respectively. Blue symbols indicate *krt* gene expression in the tissues containing simple epithelium. AEK subclusters are highlighted in the green boxes. Chromosome abbreviations are XLA_L and XLA_S, *X. laevis* subgenome L and S, respectively.

tion of particular genes during *Xenopus* evolution after allopolyploidization. Finally, we discuss the unique expression patterns and evolution of the clustered keratin genes accompanied by the epidermal development and terrestrial adaptation in *Xenopus*.

Thirty-six keratin genes have been identified based on previous *X. tropicalis* v4.1 genome data (Vandebergh and Bossuyt, 2012). In the present study, 43 type I and 28 type II keratin genes were identified in *X. laevis*, while 26 type I and 15 type II keratin genes were found in *X. tropicalis*. The large number of *X. laevis krt* genes was caused by whole genome duplication with allopolyploidization 17–18 million years ago (Session et al., 2016). Similar to other vertebrates, type I and II keratin genes were clustered on each of the two chromosomes, and the clusters were flanked by *smrce1/eif1* and *faim2/eif4b*, respectively. Syntenic

equivalency of cluster keratin genes among other tetrapods has been previously reported (Greenwold et al., 2014; Hesse et al., 2004; Vandebergh and Bossuyt, 2012; Zimek and Weber, 2005). Despite the conserved synteny of the keratin clusters among mammals and *Xenopus*, a few *krt* genes exhibited a one-to-one orthologous relationship to mammalian keratin genes with high homologies. Among the exceptions, *krt8.1* and *krt18.1* corresponding to human *KRT8* and *KRT18* exhibited conserved synteny and sequence homologies. It is noteworthy that subclustered and massively expanded hair keratin genes, such as those found in mammals and birds, were not seen in either of *Xenopus* genomes, clearly reflecting that amphibians have no hair, feathers, scales, and the related appendages (Alibardi, 2003, 2009). Although Vandebergh and Bossuyt (2012) reported the presence

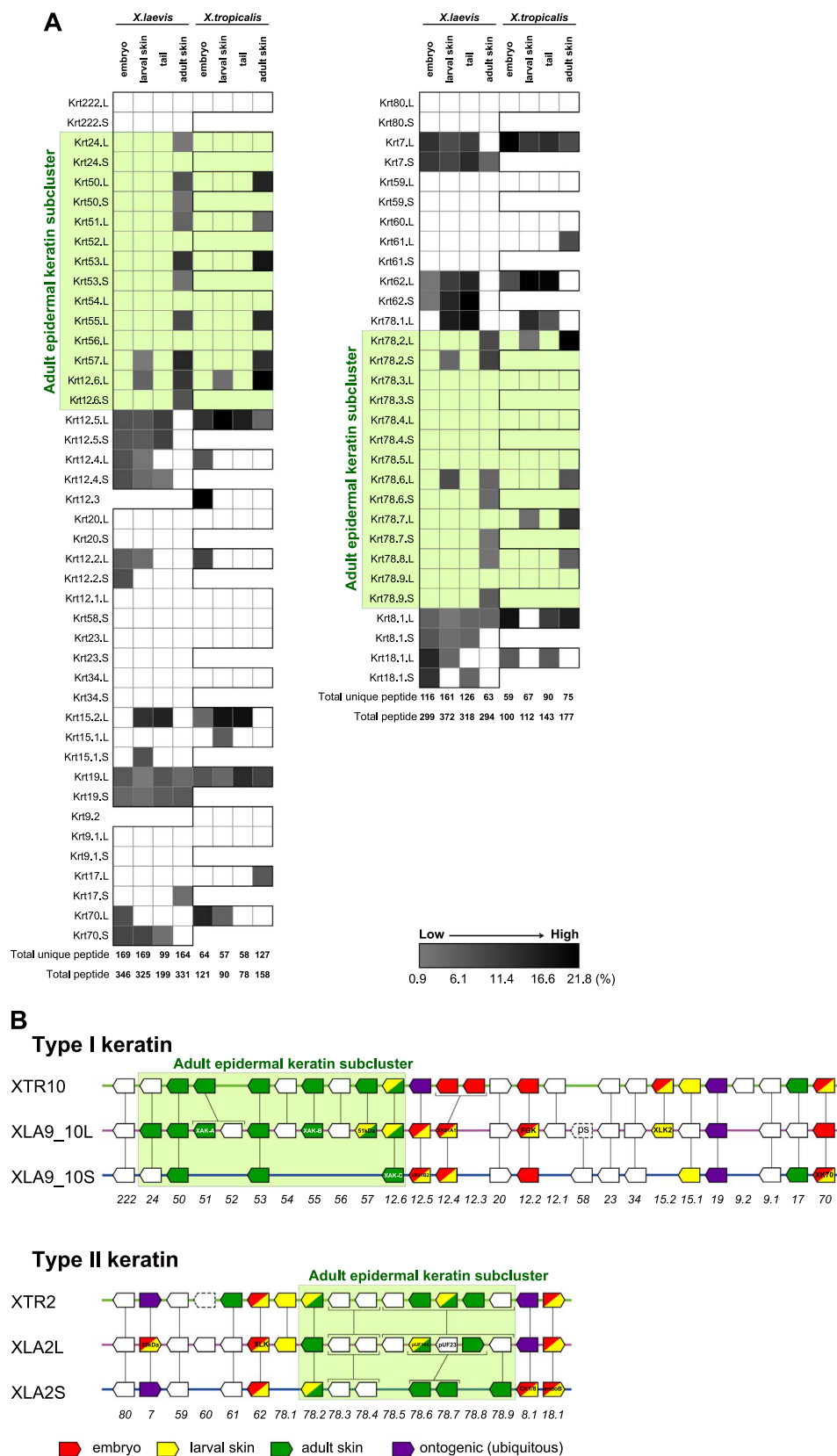


Fig. 4. Changes in epidermal keratin proteins in two *Xenopus* species throughout ontogeny. (A) Shotgun proteomics analysis of keratin proteins in whole embryo (NF34/35), larval body skin (NF54), tail (NF54), and adult skin. The results of type I and II keratin are shown in the left and right boxes, respectively. Shaded black represents their expression levels as judged by the percentage of unique peptides of each Krt per unique peptides of total Krt in each sample. The actual numbers at the bottom indicate the assigned unique and total peptides of Krt from each sample. (B) A schematic summary of Krt protein expression throughout ontogeny. Each colored symbol represents unique Krt expression at the indicated developmental stage; embryo, larva, adult, and ontogeny. Two colored symbols show Krt expression at both indicated stages. Adult epidermal keratin (AEK) subclusters are highlighted in the green boxes. Chromosome abbreviations are XTR, *X. tropicalis*; XLA_L and XLA_S, *X. laevis* subgenome L and S, respectively.

of hair keratin orthologs in the phylogenetic tree, further comparative and functional analysis of keratin genes in amphibians are required in the future. Moreover, except for *krt58*, a total of 12 singleton *krt* genes were only found on the *X. laevis* L subgenome. This suggests that homeologous *X. laevis* keratin genes evolved under selection pressure of some kind during the course of evolution following polyploidization (Hellsten et al., 2010; Session et al., 2016).

Switching of epidermal keratin expression reflects the drastic transition of the epidermis throughout ontogeny, adapting to environmental changes from water to land throughout amphibian life. Most of the identified keratin genes are exclusively expressed in the epidermis in cell type- and developmental stage-specific manners and can be largely classified into three types: embryonic-, larval-, and adult-type epidermal keratins.

According to the RNA-Seq and shotgun proteomics results, a total of 18 and 11 *krt* genes were expressed in *X. laevis* and *X. tropicalis* embryos, respectively. Among them, *krt12.2*, *krt12.4*, *krt12.5*, *krt19*, and *krt70* genes on the type I clusters exhibited subgenome-specific expression in *X. laevis*. *krt12.5.S*, *krt12.4.L*, and *krt70.S* correspond to XK81B2, XK81A1, and XK70, which are abundantly and specifically expressed in the *X. laevis* embryonic epidermis (Jamrich et al., 1987; Miyatani et al., 1986). Miyatani et al. proposed the possibility of XK81 (*krt12.4* and *krt12.5*) gene evolution in *X. laevis* as follows: the single ancestral XK81 gene was tandemly duplicated in XK81A (*krt12.4*) and XK81B (*krt12.5*), and these XK81 genes were then further duplicated by allotetraploidization. Consequently, two XK81A (*krt12.4.L* and *krt12.4.S*) and XK81B (*krt12.5.L* and *krt12.5.S*) homeolog pairs are established in *X. laevis* (Miyatani et al., 1986). Therefore, *krt12.4* and *krt12.5* provide us a unique model to analyze asymmetric gene evolution accompanied by both whole- and local-genome duplication. In *X. laevis*, keratin gene evolution is highly complicated because diversification of these genes arises from both whole-genome and local (tandem) duplication. Two models have been proposed to explain the occurrence of subfunctionalization in these keratin genes: DDC-model (duplication-degeneration-complementation) may be most appropriate in homeologous keratin genes on two subgenomes, and the specialization-model may be applicable to functionally diversified keratin genes followed by local (tandem) duplication (Hahn, 2009).

The *Xenopus* larval epidermis consists of three types of cells at the tadpole stage; apical, skein, and basal cells (Suzuki et al., 2009; Watanabe et al., 2001, 2002). The proteomics results showed that XLK (Krt62.L) and XLK2 (Krt15.2.L) proteins were highly abundant in the *X. laevis* tadpole epidermis, whereas these keratins were not expressed in the adult skin at all. These findings concur with previous reports regarding *xlk* and *xlk2*, which are abbreviations of *Xenopus* larval-specific keratin and exclusively expressed in skein and basal cells (Suzuki et al., 2009; Tazawa et al., 2006; Watanabe et al., 2001). Additionally, Krt78.1.L and Krt62.S were also abundant in tadpole body skin and tail, but not in the adult skin of either *Xenopus* species. Therefore, these keratins can be newly defined as larval-type keratins. It is remarkable that adult-type keratins, e.g., 51 kDa keratin (Krt57.L), Krt12.6.L, Krt78.2.S, and 63 kDa keratin/pUF164 (Krt78.6.L), were also detected in tadpole body skin but not in the tail at the protein level. These results imply that the progenitors of adult-type epidermal cells already exist in the larval body skin, but not in the tail of tadpoles in amphibian metamorphosis (Suzuki et al., 2001, 2002, 2009; Watanabe et al., 2002). Indeed, XAK-C (Krt12.6.S) and XLK (Krt62.L) are concomitantly expressed in basal cells of tadpole body skin (Suzuki et al., 2009; Watanabe et al., 2002). Alternatively, these keratins could be only expressed in other larval-type cells that are not the adult-progenitors.

Histological features of amphibian adult epidermis appear to be well-conserved with those of mammals. The stratified epidermis possessing a keratinized layer is an essential tissue to withstand drying and adapt to the terrestrial environment in tetrapods including *Xenopus* and other amphibians (Alibardi, 2003, 2009). The adult

epidermis consists of basal, granular, spinous, and cornified cells. Similar to mammals, differential expression of *krt* genes is also observed in the *X. laevis* adult epidermis. Adult-type keratin, e.g., 51 kDa (*krt57.L*), XAK-C (*krt12.6.S*), and 63 kDa (*krt78.6.L* or *krt78.7.L*), are specifically expressed in basal cells on the basement membrane in *X. laevis* adult skin (Kinoshita and Sasaki, 1994; Suzuki et al., 2009; Watanabe et al., 2002). Moreover, XAK-A (*krt51.L*), XAK-B (*krt55.L*), and 63 kDa (*krt78.6.L* or *krt78.7.L*) are predominantly expressed in terminally differentiated adult epidermal cells such as granular and spinous cells, which are derived from basal adult skin cells (Nishikawa et al., 1992; Suzuki et al., 2002; Watanabe et al., 2001). Our RNA-Seq and proteomics results showed expression profile of adult-type epidermal keratins in accordance with the previous reports. In addition, the proteomic profiles of *X. tropicalis* keratins were similar to those of *X. laevis* throughout ontogeny, strongly supporting conservation of keratin expression and epidermal transition between the two *Xenopus* species.

We found two unique subclusters on which these adult-type keratins were located and designated them as AEK subclusters. Previous reports have also suggested the possibility that the epidermal keratin gene radiation adapting to the terrestrial environment occurs in early amphibians (Vandebergh and Bossuyt, 2012). Intriguingly, the two AEK subclusters, *krt24*, *krt50–krt57*, *krt12.6*, and *krt78.2–krt78.9* also formed unique clades in the phylogenetic tree. These findings suggest the possibility that the *krt* genes on the AEK subclusters expanded and diversified from a common ancestral epidermal keratin gene after amphibious tetrapod ancestors had moved from an aquatic to a terrestrial habitat. It is noteworthy that out of 13 singleton *krt* genes, eight were found on the two AEK subclusters. We retrieved keratin genes from the *X. laevis* and *X. tropicalis* genome data again but in the end only identified non-cluster keratins in this research. This result suggests that translocation of these keratin genes did not occur during *Xenopus* evolution. Additionally, the intergenic regions from *krt50* to *krt57* (type I AEK) in the L and S chromosomes are almost unchanged in terms of length. Thus, we speculate that local loss of adult-type keratin occurred in the S subgenome during *X. laevis* speciation followed by allotetraploidization. It should have originally consisted of complete homeologous sets in an *X. laevis* ancestor just after polyploidization, because AEK gene loss in diploids probably causes severe defects in the adult-type epidermis resulting in low survival rates in nature. In fact, either loss or mutation of epidermal keratins corresponding to the AEK genes results in fragile skin and severe phenotypes in both mice and humans (Bragulla and Homberger, 2009; Moll et al., 2008). It is generally assumed that asymmetric gene retention/loss is caused by selection pressure of some kind. Given the fact that the *Xenopus* genus inhabits water, these findings lead to the hypothesis that asymmetric loss of these adult-type *krt* genes on their subgenomes may be the result of readaptation from a terrestrial life to an aquatic one during *Xenopus* evolution and speciation. This hypothesis needs to be verified in future research by comparing the *X. laevis* clustered keratin genes with those of other polyploid *Xenopus* species, such as *X. borealis* and *X. muelleri*.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ydbio.2016.10.018>.

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