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Review

Comparative biology needs *de novo* transcriptome assemblies: drawing attention towards amphibians

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Abstract. The study of transcriptomics across amphibians opens a window to understand how species have adapted to and cope with their environment, diseases, and new challenges. Transcriptomics can accelerate comparative studies across the amphibian tree of life because they capture diverse biological information at a fraction of the cost of genomics. Currently, 337 amphibians (3.82% of the described species) have transcriptomic data available, and 60 of them (0.68% of the described amphibian species) have reconstructed *de novo* assemblies readily accessible on public repositories. Here, we summarise taxonomic gaps for amphibian transcriptomics, highlighting studies that have used these resources in a multi-species comparative framework to uncover the genetic variation and gene expression patterns that underlie phenotypes across different aspects of amphibian biology. Given the particularities of amphibians, including their complex life cycles, we provide some guidelines to generate reference transcriptomes while identifying challenges that researchers might encounter. We explore the developmental and tissue-specific transcriptome divergence across the three amphibian orders to aid in identifying suitable target samples for reference transcriptomes (e.g., developmental stages, brain, kidney, reproductive tissues). Since annotations for amphibians are very limited, we recommend researchers to be critical of annotations assigned through homology. We encourage the availability of transcriptome assemblies in public repositories, sparing computational efforts and costs to advance multi-species research. Comparative studies should expand taxonomic and ecological breadth to unveil the molecular bases of evolution, adaptation, and resilience mechanisms for one of the most imperilled groups of vertebrates.

Keywords: Amphibians, Anura, Caudata, gene expression, Gymnophiona, molecular evolution, RNA-Seq, transcriptomics.

Introduction

Understanding life requires the study of natural variation. This idea underpins the need to compare patterns and processes across all biological levels, from genes to species and communities. Comparative biology investigates shared and distinctive biological features and can integrate evolutionary histories accounting for similarities of phylogenetically related taxa (Sanford, Lutterschmidt, and Hutchison, 2002). For instance, comparative studies can provide an in-depth understanding of adaptations by correlating trait variation with ecological factors and species relatedness (Felsenstein, 1985; Harvey and Purvis, 1991). Comparative frameworks and ideas pre-date the advent of evolutionary theory and the rise of comparative phylogenetics. Historically, some comparative studies, in morphology and physiology, have followed the Krogh principle, in which one or a few chosen species (model organisms) provide insights about biological processes applicable to others due to their similarities or dissimilarities (Krebs, 1975; Sanford, Lutterschmidt, and Hutchison, 2002; Green et al., 2018). While the study of these organisms has provided undeniable scientific breakthroughs, their limitations result in a simplified explanation of life's complexity through generalisations that are not representative of the wide natural diversity. These shortcomings can be bridged by integrating different methodological approaches and broadening biodiversity inclusion in comparative studies (Sanford, Lutterschmidt, and Hutchison, 2002; Pottier et al., 2024).

Many biological disciplines have benefited from the rapid improvement of high-throughput sequencing technologies. These advancements can enhance diversity in comparative studies thanks to the increasing number of species with sequence information available (Bertile et al., 2023). Genome sequencing efforts have shed light on the evolution of many groups, such as mammals (Genereux et al., 2020; Christmas et al., 2023) and birds (Stiller et al., 2024), for which numerous genomes have been sequenced, covering a significant part of the diversity of their trees of life. Despite the major advances in genome sequencing, other groups continue to lag behind in the generation of high-quality reference genomes (e.g., gymnosperms). This is the case for amphibians, whose large, repetitive genomes are still costly to sequence and assemble (Kosch et al., 2024). Compared to the diversity of amphibian species, the current number of sequenced genomes limits our ability to conduct large-scale comparative genomic analyses. Transcriptomic data, however, can be generated much quicker and at a lower cost than whole-genome sequencing, primarily due to their smaller size and the simpler assembly requirements compared to genomes. Consequently, transcriptomic data can serve as an important resource for comparative studies. Additionally, transcriptomes offer not only gene sequence data but also gene expression data, expanding the scope for comparative analyses (Tirosh, Bilu, and Barkai, 2007). Here, we review the availability of amphibian transcriptomic data to date and highlight exemplary studies that have applied a multi-species comparative transcriptomic framework to study amphibians. We also provide best practices for generating amphibian reference transcriptomes and identify challenges and future directions for comparative studies in this field. This review aims to draw attention towards the utility of amphibian transcriptomics. A broader representation of reference transcriptomes can help unravel the molecular bases of amphibian evolution and their resilience to diseases and environmental perturbations, which are alarmingly threatening amphibian populations worldwide (Scheele et al., 2019; Luedtke et al., 2023).

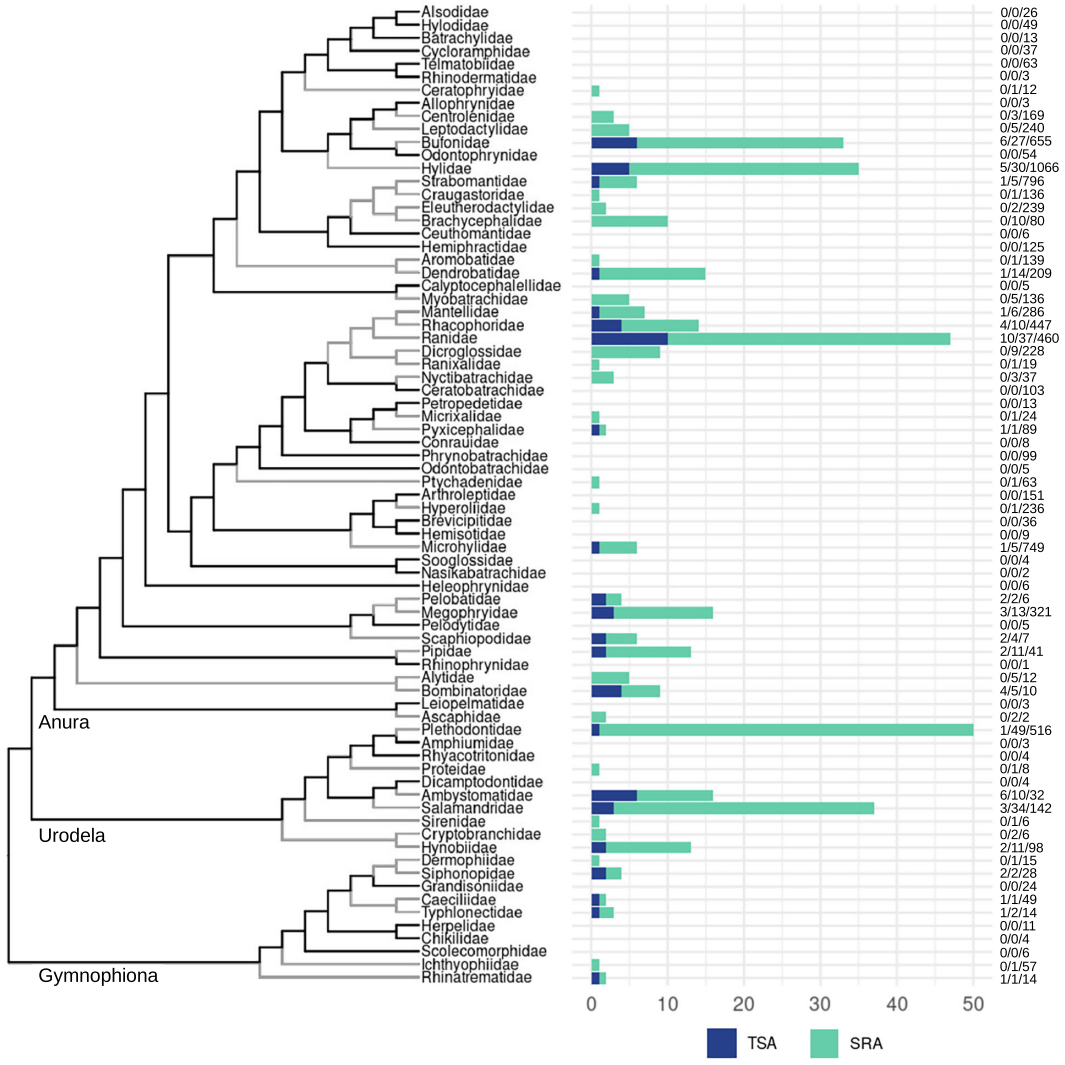

Figure 1. Taxonomic gaps in amphibian transcriptomics. Phylogenetic tree including 75 amphibian families (subset tree from Jetz and Pyron, 2018) and highlighting the families without transcriptomic data in black branches (the two families missing from the tree, Caligophrynidae and Neblinaphrynidae, also lack sequenced transcriptomes). Transcriptomic data of each family per species are represented by stacked bars colour-coded to distinguish between de novo assemblies from TSA and raw transcriptome sequences from SRA. Numbers to the right of the bar plot refer to the total number of TSA files, SRA files, and described extant species per amphibian family respectively.

Available de novo transcriptome assemblies for amphibians

Transcriptomic data is available for 337 amphibian species (NCBI SRA database was accessed on 2 September 2024; records were filtered by selecting TRANSCRIPTOMIC as Library Source and RNA-Seq as Library Strategy, and entries per species were counted using the Scientific Name column; see Supplementary File S1). These species belong to 43 of the 77 described families (see fig. 1; taxonomic data consulted on AmphibiaWeb, https://amphibiaweb.org/). To date, the families Plethodontidae and Ranidae have the highest number of species with sequenced transcriptomes (49 and 37 species, respectively), followed by Salamandridae, Hylidae, and Bufonidae (34, 30,

and 27 species, respectively). When accounting for the total number of described species, only eight families have some trancriptomic record for more than 25% of their species (2 out of 2 species for Ascaphidae: 100%, 4 out of 7 for Scaphiopodidae: 57.1%, 5 out of 10 for Bombinatoridae: 50%, 5 out of 12 for Alytidae: 41.7%, 2 out of 6 for Cryptobranchidae and Pelobatidae: 33.3%, 10 out of 32 for Ambystomatidae: 31.3%, and 11 out of 41 for Pipidae: 26.8%; see fig. 1). As is the case for reference genome assemblies, transcriptomes are critically absent for multiple evolutionarily distinct families across all three amphibian orders (Kosch et al., 2024); for example Anura: Leiopelmatidae, Gymnophiona: Scolecomorphidae, Urodela: Rhyacotritonidae (measures not presented here but calculated following Redding and Mooers (2006) using a subset tree from Jetz and Pyron (2018); see fig. 1). Despite the taxonomic gaps, transcriptomes are advancing our knowledge of amphibians. This is particularly relevant for 16 families (12 Anura, 2 Gymnophiona, and 2 Urodela) without any reference genomes at the time of writing (Kosch et al., 2022), but with transcriptomic data for 60 species, including for example 10 species of the family Brachycephalidae.

A small fraction of the amphibian transcriptomes from the SRA NCBI dataset are reconstructed and readily accessible for comparative studies (NCBI TSA database records were accessed on 2nd September 2024; see fig. 1 and Supplementary File S2). The 79 de novo transcriptome assemblies publicly accessible belong to 60 amphibian species from 22 different families (43 species from 14 families of Anura, 5 species from 4 families of Gymnophiona, and 12 species from 4 families of Urodela). These assemblies were built using different numbers and types of tissues (mainly skin and liver) from mostly adult individuals (see next section for information about comparative studies where some of these assemblies have been explored). The majority of samples were sequenced with Illumina sequencing technology (HiSeq, NextSeq, and NovaSeq; fig. 2B). De novo transcriptomes were assembled from raw reads using different software, with Trinity (Haas et al., 2013) being the most frequently used (fig. 2C). Assembly lengths and N50 values, which represent the length of the shortest contig at 50% of the total assembly length, vary across amphibian transcriptome assemblies (metrics computed using BBMap stats tool (https://sourceforge.net/projects/bbmap), fig. 2A). While, such length metrics can be used to score quality, helping to identify fragmented transcriptomes, they are limited (especially N50, see Raghaven et al. (2022)) by the lack of information about transcript length range and transcriptome complexity.

Coding and non-coding RNAs in multi-species comparative studies on amphibians

Transcriptomes can be explored in a comparative framework to investigate a breadth of topics through sequence homology and sequence expression analyses. In this section, we highlight a few examples of how transcriptomic approaches have been applied to uncover the basis of adaptation and evolutionary processes in amphibians. Given the pervasive transcription of eukaryotic genomes (Kapranov et al., 2007), we categorise the study examples regarding the RNA molecule of focus and provide a brief definition of coding and non-coding RNAs.

Despite constituting just a small fraction of the entire transcriptome, most research conducted in amphibians has investigated RNAs that encode proteins (messenger RNAs or mRNAs). Sequence similarity studies apply phylotranscriptomics and molecular evolution analyses to disentangle the relationships among amphibian species, identify genes subject to selection during diversification, and unravel the evolution of gene families of interest. Unresolved relationships within the amphibian tree of life can greatly benefit from the addition

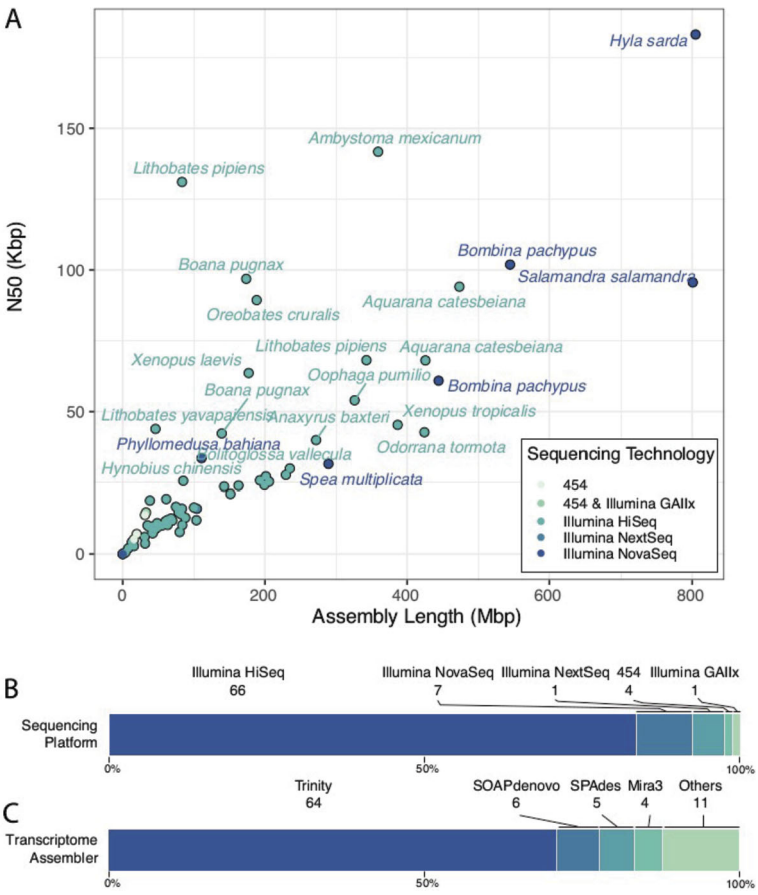

Figure 2. Available de novo transcriptomes assemblies for amphibians. (A) Scatter plot representing length metrics for the 79 TSA de novo transcriptome assemblies with data points coloured-coded by sequencing platform. (B, C) Bar plots with the percentage of sequencing platform and the assembling program used to generate the transcriptomes.

of new genetic markers through transcriptomic data. For example, Siu-Ting et al. (2019) combined transcriptomic and genomic data to investigate the evolutionary relationships among the three main lineages of Lissamphibia, supporting the most accepted hypothesis (Batrachia instead of Procera) while revealing that orthology and inadvertent paralogy influenced phylogenomic signals and impacted time-tree estimates. Similarly, studies such as Rancilhac et al. (2021) have uncovered introgression among salamander lineages in the Salamandridae family, highlighting unresolved relationships within several groups. When phylogenetic relationships are not in question, molecular evolution analyses of complete transcriptomic datasets allow the identification of candidate genes under selection that can potentially unveil molecular mechanisms of adaptation during amphibian diversification. This approach has provided candidate genes involved in the adaptation to high-elevation of one of the most specious vertebrate genera, the *Pristimantis* frogs (Christodoulides et al., 2024), or revealed genes associated with the colonization of soil layers in caecilian amphibians (Torres-Sánchez et al., 2019b). Some of these candidate genes can be more thoroughly studied, broadening taxa diversity. For instance, the opsin gene family has been studied in more than a hundred frog species representing almost 60%

of the anuran families (34 of 57), documenting both gene duplications and gene losses, and shifts in selection in relation to habitat and life history (Schott et al., 2024).

On the other hand, many studies of coding RNAs have focused on describing gene expression patterns across multiple species during the same biological process. For instance, gene expression profiles have revealed the molecular bases of the developmental plasticity of different species of spadefoot toads (Liedtke, Harney, and Gomez-Mestre, 2021; Isdaner, Levis, and Pfennig, 2023). Disease response, regeneration, tissue specificity, and toxin production have also been explored in multi-species comparative transcriptomic frameworks to elucidate common and distinctive gene expression changes (Ellison et al., 2015; Dwaraka et al., 2019; Torres-Sánchez et al., 2020; Firneno et al., 2022). A special case of comparative transcriptomics concerning amphibians is the study of their symbionts. Amphibians, like other animals, have been evolving with symbiotic microorganisms (their microbiome) since their origin (McFall-Ngai et al., 2013). These microorganisms inhabit different parts of their bodies and have a remarkable influence on their metabolism and adaptive ability that amphibian evolution could not be holistically understood without focusing on the holobiont (host plus symbionts; Lynch and Hsiao, 2019; Fontaine and Kohl, 2023; Woodhams et al., 2023). Following the principle of the dual RNA-Seq strategy (transcriptome profiles for hosts and symbionts usually sorted using reference genomes), the functional role of amphibian symbionts can be revealed through trancriptomics (Westermann, Gorski, and Vogel, 2012). This technique has been applied to describe expression variation and phylogenetic relationships of the multihost fungal pathogen *Batrachochytrium dendrobatidis* and the symbiotic *Oophila* algae across several amphibian species (Torres-Sánchez et al., 2022; Vences et al., 2024).

Non-coding RNAs (ncRNAs) are a significant yet understudied portion of the transcriptome, with amphibians providing valuable insights into their roles in evolution and adaptation. ncRNAs are broadly categorized by length into short (sncRNAs, 19-30 nt) and long (lncRNAs, >200 nt) molecules, many of them derived from transposable elements (TE). The primary function of short regulatory sncRNAs, including microRNAs (miRNAs), piwi-interacting RNAs (piRNAs), and small interfering RNAs (siRNAs), is gene expression regulation, whereas housekeeping sncRNAs, such as small nucleolar RNAs (snoRNAs), guide RNA modification (Hombach and Kretz, 2016). Notably, *Xenopus tropicalis* has revealed species-specific snoRNAs, challenging the assumption of evolutionary conservation in closely related vertebrates (Deryusheva, Talhouarne, and Gall, 2020). lncRNAs are pivotal in processes such as cell differentiation, development, and responses to environmental stress (Mattick et al., 2023). In amphibians, most ncRNA studies have focused on model organisms (e.g. *Xenopus*, *Ambystoma*), advancing the knowledge in a vertebrate comparative framework. Recent studies have supplied new sequencing data from non-model species such as *Pelobates cultripes* (The RNAcentral Consortium, 2019; Liedtke et al., 2022). Both conserved and non-conserved ncRNAs have been identified in many adaptation processes (Schrader and Schmitz, 2019), such as anoxia and cold tolerance across vertebrates including several amphibian species (Riggs et al., 2018; Cai et al., 2023). In *Pleurodeles waltl*, microRNAs and TEs are co-expressed during limb regeneration, highlighting their regulatory role (Brito, 2018). Similarly, cold stress in *Polypedates megacephalus* involves mitochondrial lncRNAs (Cai et al., 2023), while miRNAs in freeze-tolerant species like *Rana sylvatica* regulate apoptosis and metabolism during freezing (Biggar and Storey, 2011). Amphibians are also highly sensitive to environmental stressors, with endocrine-disrupting chemicals such as atrazine

altering lncRNA expression and contributing to testicular dedifferentiation in *Xenopus laevis* (Sai et al., 2019). These findings underscore the importance of ncRNA-guided processes in amphibian adaptation and resilience.

Guidelines for amphibian reference transcriptomes

Developmental stages, sex, and seasonal and environmental variation

Amphibians, particularly those with complex life cycles (Liedtke, Wiens, and Gomez-Mestre, 2022), exhibit unique gene expression profiles at different developmental stages (Yanai et al., 2011). Tadpoles and adults have vastly different morphologies and ecologies, with metamorphosis involving significant physiological and morphological changes (Brown and Cai, 2007). Sampling only one stage may overlook genes that are transiently expressed and essential for developmental processes. For example, during limb regeneration, Mahapatra et al. (2023) show differential gene expression between tadpoles and metamorphosed froglets of *Polypedates maculatus*. While some of these genes may be constantly expressed at low levels, they are typically more detectable at stages where they play active roles. When building a reference transcriptome, including individuals from different developmental stages increases the representation of functional elements (see fig. 3A-C for gene expression comparison among developmental stages and adult samples of *X. laevis*).

The transcriptome of any organism is highly dynamic and varies across seasons being influenced by the environment through biotic and abiotic interactions. For instance, it is important to consider the impact of brumation on temperate species, a period during which many genes involved in processes such as reproduction, digestion, and the immune response may be downregulated or even inactive (Fan et al., 2022). The transcriptome during brumation reflects a unique, low-energy physiological state that may not be detectable during active periods, due to a shift aimed at conserving energy while also being capable of responding to potential physical stressors (Niu et al., 2024). Consequently, including brumation samples across different life stages is critical for capturing genes related to metabolic suppression, stress tolerance, and cold adaptation. More broadly, many amphibians demonstrate dimorphic seasonal differences in gene expression which lead to the development of sex-related structures, usually involved in mating. Such structures include the spines in male *Leptobrachium boringii* frogs (Li et al., 2019) and male nuptial pads on the forelimbs of many other amphibians to facilitate amplexus (Sever and Staub, 2011). In some species, these differences may be indicated by extreme and rapid changes in colouration which assist in attracting potential mates (Spaethe, Sztatecsny, and Hödl, 2008; Kindermann and Hero, 2016). Whereas females are likely to express genes related to egg production, ovulation, and spawning. Outside of this period, these genes may be dormant or minimally expressed, making them hard to detect in a transcriptome unless sampled during the reproductive season or cycle. This reflects the dynamic pattern of transcriptomes.

Many other biotic and abiotic interactions can modify amphibian gene expression. One prime example is host-pathogen interactions. Transcriptomics has emerged as a potent tool for elucidating the intricate molecular responses to *Batrachochytrium dendrobatidis* infection since many amphibians change their gene expression during this process (Zamudio, McDonald, and Belasen, 2020). Novel miRNAs have also been discovered in the model *X. laevis* while investigating transcription responses to viral stimulation of Ranavirus (Todd, Bui-Marinos, and Katzenback, 2021).

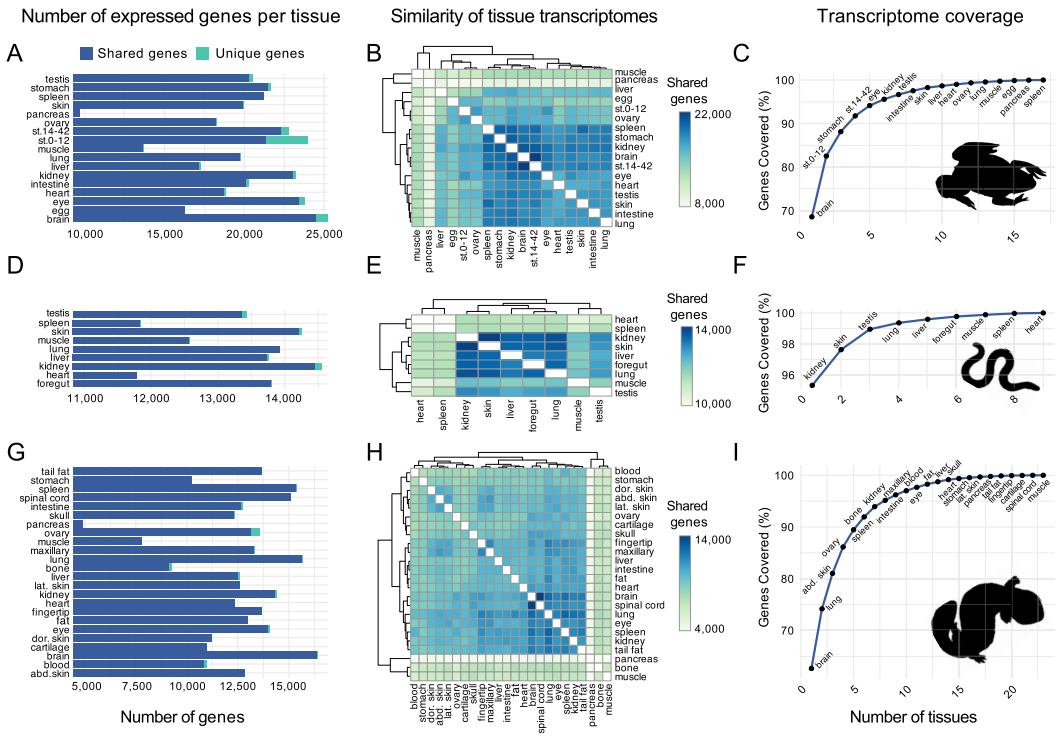

Figure 3. Transcriptomic diversity across tissues in the three amphibian orders. (A, D, G) Number of shared and unique genes expressed in each tissue or developmental stage. (B, E, H) Similarity across tissues and developmental stage-specific transcriptomes, represented by the number of expressed genes shared between tissue or developmental stage pairs. (C, F, I) Cumulative transcriptome coverage as a function of number of tissues sequenced. (A-C) African clawed frog (*Xenopus laevis*) RNA-Seq data downloaded from Xenbase (www.xenbase.org). All reads were aligned to the *X. laevis* genome assembly (v10.1) and transcripts per million (TPM) values were used. Genes with an averaged TPM $>$ 1 across samples were considered expressed. Embryonic tissues are grouped into two developmental stage categories: st.0-12 (unfertilized egg to NF stage 12: "medium yolk plug", early neurulation) and st.14-42 (stages NF14-42: "neural plate", mid-neurulation, to free swimming tadpole). All other tissues were sampled from adults. (D-F) Binary presence/absence data combining homolog genes expressed by adult tissues across five caecilian species (*Caecilia tentaculata*, *Typhlonectes compressicauda*, *Microcaecilia unicolor*, *M. dermatophaga*, *Rhinatrema bivittatum*) from Torres-Sánchez et al. (2019) was used (see reference for details about number of samples per tissue). (G-I) Expression data (FPKM) for adult tissues of the Chinese giant salamander (*Andrias davidianus*) from Geng et al. (2017). Genes with FPKM $>$1 were considered expressed.

Tissue selection and RNA extraction

Tissues used to build a reference transcriptome should aim to capture most of the transcripts of an amphibian species, by targeting different tissues throughout life stages, including any seasonal/environmental changes that may occur as outlined above. For tissue selection, it should be considered that tissue expression profiles can overlap substantially. Thus, to optimize sampling and sequencing, complementary tissues should be prioritized. Figure 3 exemplifies how tissue-specific transcriptomes overlap in *X. laevis* (information obtained through www.xenbase.org), *Andrias davidianus* (Geng et al., 2019), and five species of caecilians (Torres-Sánchez et al., 2019a). In adults, the brain and kidney are particularly transcript-rich tissues, and harbour transcripts not found in other sampled tissues (fig. 3). These tissues should thus be prioritised for de novo multi-tissue transcriptome projects. Pancreas, on the other hand, yielded the lowest number of transcripts both in *X. laevis* and *A. davidianus*. Testes and ovaries, unsurprisingly, provided unique transcripts in all three groups, and should therefore

be included in transcriptome projects. In the *X. laevis* dataset, which contained both embryonic and adult tissues, the necessity of using various developmental stages to obtain a complete transcriptome is evident, as embryo stages harbour the highest number of uniquely expressed genes (fig. 3A). The number and type of tissues used to produce a transcriptome are highly dependent on the species, samples available, and the experimental conditions. High sequencing depth and biological replicates of the same tissue type can somewhat compensate for a low number of tissue types used or the lack of certain developmental stages (e.g. Montero-Mendieta et al., 2017; Torres-Sánchez et al., 2019a; Ceschin et al., 2020). Based on these three examples, we revealed some trends of tissue-specific transcriptome similarities across independent studies and in the three different amphibians orders. Accordingly, when designing an experiment aiming to assemble a species' complete transcriptome, we suggest using multiple adult and larval tissues selected based on their respective transcriptome divergence.

Alternatively, when financial resources or biological materials are scarce, multiple tissues, whole organisms (especially tadpoles), or tissues across multiple animals, sexes, and life stages can be pooled before sequencing. However, unless absolutely necessary, we advise against this, because tissue-specific expression information (transcripts only expressed in specific cell types such as claws; see Carron et al., 2024), and weakly expressed transcripts are lost with this approach (Li et al., 2014). Many amphibians are very small, and smaller still are their embryos and larvae, posing challenges for tissue dissections. Regardless, if a tissue-specific transcriptome of a specific stage is required, it is possible to perform a laser-capture microdissection (Espina et al., 2006). This method, however, requires schooled personnel and the corresponding expensive equipment, which makes this approach costly and should be considered only if a specific research aim requires it.

Tissue samples for RNA extraction can easily be collected by flash freezing in liquid nitrogen or by soaking in RNA stabilizing reagents such as RNA-later, DNA/RNA shield stabilization solution, or Trizol. RNA-later and DNA/RNA shield solutions have the advantage in that they can be used in the field at ambient temperatures whereas Trizol needs to be handled on ice and requires a hood for a safe work procedure. If RNA-later or a similar storage buffer is used, the amount of solution should be adjusted to the size and type of tissue to avoid dilution of RNA and preserve yield. Tissue thickness should also be considered when preserving tissues or samples (e.g., tadpoles), which might need to be minced to allow effective buffer penetration. Once tissues are sampled, the first step in RNA extraction protocols is homogenization and cell disruption. Amphibians can pose a unique challenge for the homogenization, particularly due to the complex structure of their skin (Akat Çömden, Yenmiş, and Çakır, 2023), thus it is recommended to test different methods for the homogenization of particular tissues. Homogenization methods include but are not limited to mechanical disruption via bead mills or pestle and mortar, enzymatic digestion or ultrasonication. RNA-extraction methods such as Trizol-based RNA extraction (Chomczynski, 1993), column-based extraction (for specific tissue or sample types), or a combination of both could be followed after homogenization.

RNA sequencing and transcriptome assembly and annotation

Before library preparation, one needs to assess which sequencing method is the best fitting for obtaining a comprehensive reference transcriptome. Current main options include short-read sequencing (usually with the Illumina platform) using paired-end strategy, as well as long-read sequencing (such as those offered by Oxford Nanopore Technologies or Pacific Biosciences, PacBio). We herein provide a recommendation about the sequencing methods, which are not exclusive to amphibians.

For any species without previous sequencing data, paired-end sequencing provides both forward and reverse reads for each transcript, which allows a structural reconstruction of a transcriptome (Williams et al., 2014). In general, long-read sequencing has several advantages over short-read sequencing with the ability to sequence full-length transcripts, leading to accurate identification of gene isoforms and complex splicing events with increasing accuracy in the base calling (Wenger et al., 2019; Wu et al., 2023; Wijeratne et al., 2024). However, data throughput remains a disadvantage when compared to short-read technologies (Pearman, Freed, and Silander, 2020). Besides the sequencing strategy, the sequencing depth should also be considered, as it is influenced by the available financial resources. To capture a comprehensive transcriptome with high resolution 150 million to 200 million short reads per sample should be the target (Sims et al., 2014). As for long reads, for example, one PacBio Revio machine single-molecule real-time (SMRT) cell provides 40 million reads and 5 to 10 million could be enough per sample (see provider's webpage, https://www.pacb.com/revio/). Finally, libraries should ideally include coding and non-coding RNAs encompassing the most complete collection of functional elements as possible. The most common mRNA libraries are performed by poly-A selection (i.e., enrichment of poly-A RNAs) and therefore contain lncRNAs since many of them contain poly-A tails (other molecule selection methods are available, including depletion of ribosomal RNAs (rRNAs)), being part of the sequencing library (Mattick et al., 2023).

De novo assemblies can be achieved through several bioinformatic steps without a reference genome (for a detailed guide to transcriptome assembly and annotation, see Raghavan et al., (2022)). These steps are not exclusive to amphibians and the consensus recommendations of the scientific community is to apply multiple assemblers and multiple parameters strategies. For the assembly of short-read transcriptomes, sequence files can be *in silico* normalised with Trinity program and subsequently assembled with Trinity, SPAdes, SOAPdenovo-trans, TransABySS, or Velvet/Oases (Zerbino and Birney, 2008; Robertson et al., 2010; Schulz et al., 2012; Haas et al., 2013; Xie et al., 2014; Bushmanova et al., 2019). Pipelines such as TransPi (Rivera-Vicéns et al., 2022) facilitate running multiple assemblers, with individual assemblies combined into non-redundant consensus assemblies. For the assembly of long-read transcriptomes, the reference-free tools RATTLE, RNA-Bloom2, and StringTie2 can be used (Kovaka et al., 2019; de la Rubia et al., 2022; Nip et al., 2023). To balance gene representation and transcript redundancy, assemblies can be clustered and functionally classified with, for example, CD-HIT and EviGene programs (Fu et al., 2012; Gilbert, 2019).

Annotation of the reference transcriptome is a critical and complicated step for functional inference allowing researchers to assess transcriptome completeness and draw biologically relevant conclusions from transcriptomic data (Stein, 2001). The functional annotation process can be divided into four broad categories: RNA classification, transcript identification, sequence feature annotation and functional category assignments. Best practices to achieve each of these are not always clear-cut, but the associated challenges are not necessarily unique to amphibian transcriptomics (see Raghavan et al., 2022).

Here, we focus on one of the core problems faced by amphibian researchers during the annotation process: the dependency on homology transfers from divergent model species. The underlying principle here is that genes that are descendants from the same ancestral sequence are likely to have similar functions (Langschied et al., 2024). For amphibian researchers, however, the options for reference annotations are very limited. Currently, the Ensembl genome database has only two curated amphibian proteomes to draw annotations from:

Xenopus tropicalis and *Leptobrachium leishanense*, both Anura (Harrison et al., 2024). The more comprehensive UniProt database includes peptides of nine amphibian proteomes; seven anurans and two caecilians, but no urodeles (UniProt Consortium, 2023). The development and regeneration model, *Ambystoma mexicanum*, has some well developed resources but at the moment these tend to be dispersed (e.g. https://www.axolotl-omics.org/). The amphibian clade is estimated to be over 300 mya old (Siu-Ting et al., 2019) and thus restricting homology searches to only this handful of proteomes may be limiting for many species. Moreover, most amphibian annotations available on UniProt, like those for *Pelobates cultripes* for example, originated through homology searches on the *X. tropicalis* genomes (Liedtke et al., 2022), and thus the broader taxonomic representation may be misleading. Quality check methodologies based on annotations, such as the BUSCO method, face the same problems with available BUSCO reference datasets being not specific for amphibians (Manni et al., 2021). The utility of homology-derived annotations for non-model systems is nonetheless unquestionable, especially because few alternatives currently exist. However, we urge that the implications of often tens or hundreds of millions of years of independent sequence evolution between reference and query be considered critically. Moreover, particularly in cases where transcripts are left unannotated, the rapid development of deep learning and protein language models (e.g. Martínez-Redondo et al., 2024) are promising alternatives for predicting transcript function.

Annotation also involves the de novo identification of ncRNAs from both total RNA and small RNA libraries. For this task, there are specialised pipelines such as Annocript for lncRNAs, which relies on transcript length, lack of similarity with proteins or domains, absence of short ncRNA matches from Rfam and rRNAs, longest open reading frame (ORF) <100 amino acids, and a statistical non-coding probability $>$ 0.95 (Musacchia et al., 2015). Platforms like Compsra can help to describe sncRNA diversity (Li et al., 2020). Another tool, sRNAfrag, allows the identification of ncRNAs and the detection of fragments derived from small RNAs (Nakatsu et al., 2024). The improvement of machine learning algorithms such as FEELnc for example, used to annotate lncRNAs of the toad *Rhinella arenarum* (Wucher et al., 2017; Ceschin et al., 2020), are expected to help overcome challenges due to the limited information in current databases. As for protein-coding genes, online reference databases such as the RNAcentral database only includes a small number of amphibian species and notably, the majority of amphibian entries are sourced from a single study on the Iberian spadefoot toad, *Pelobates cultripes* (The RNAcentral Consortium, 2019; Liedtke et al., 2022). For the limitations exposed in these last two paragraphs, we encourage amphibian researchers to be critical with annotations assigned through homology, keeping in mind the evolutionary divergences between queries and references.

Data availability and storage

Before sacrificing a specimen, phenotypic and geographic data should be collected and uploaded to a public repository to associate with the raw sequencing data (i.e., NCBI SRA and SRA metadata). Raw reads can be immediately deposited in NCBI with an embargo period until research publication. We also strongly advise making the de novo transcriptome assemblies available, uploading them to the NCBI TSA repository and including related information in a permanent DOI associated with the publication. In contrast to genome assemblies, transcriptome reconstructions are rarely available (less than 20% of the amphibian species with transcriptomic data in NCBI SRA has an associated de novo assembly in NCBI TSA; see Section 2 of this review for further details). For species with reference genomes, generated transcriptomic data can be mapped directly to

the genomic reference without de novo transcriptome assembly reconstruction, and it is expected that with increased genomic sequencing, the number of genome-guided transcriptome assemblies will increase. Despite this, at the moment, the long-term archive of reference transcriptomes, even if they are study-specific, is vital to scientific reproducibility, efficiency, and advancement of amphibian genomics and transcriptomics as a research field.

Challenges

In the pursuit of amphibian reference transcriptomes, researchers might face several challenges. While many of these are not exclusive to amphibians, we want to bring awareness to some limitations that scientists might encounter when studying amphibian comparative transcriptomics. For generating a new reference transcriptome, the first step requires finding individuals of the species of interest and ideally collecting specimens from various developmental stages, both sexes, and different seasonal and environmental conditions. This process can be particularly problematic for secretive species, for which much of their biology, such as their life cycle, remains unknown. After collecting the specimens, extracting tissues, and isolating and sequencing RNAs, the availability of computational resources for bioinformatic analyses often represents the biggest limitation in transcriptomic research.

High-Performance Computing (HPC) is a pivotal resource for carrying out bioinformatic analyses, particularly with large datasets such as transcriptomics. HPC significantly speeds up the processing and analysis of large-scale biological data, such as those generated by high-throughput sequencing technologies, making it possible to handle terabytes of data efficiently (Carrier et al., 2015; Castrignanò et al., 2020). HPC allows the application of advanced computational methods, including de novo transcriptome assemblies. These systems can scale to thousands of cores, eliminating memory bottlenecks and accelerating workflows, such as those involved in RNA-Seq data analysis (Raghavan et al., 2022). Assembly tools often require more RAM than are generally available on personal computers, depending on the size of the transcriptome and the depth of sequencing. These resources, however, are not evenly available to researchers, impairing mainly early career researchers, when changing, for example, institutions and losing access to HPC resources, and those researchers from lower GDP countries. In the tropics, amphibian taxa are still being described at a high rate while also experiencing the greatest risk of extinction (Collins and Halliday, 2005; Button and Borzée, 2021). Therefore, these regions should be the focus of a commensurate amount of research with access to HPC. Instead, we observe the opposite trend in regard to computational resources, which are more accessible in wealthier countries, while other parts of the world lack the necessary infrastructure (Gitler, Gomes, and Nesmachnow, 2020; Okanda, 2023). Disparities in access to HPC resources hinder progress and limit the ability to formulate transcriptomic-based hypotheses in underfunded research groups and countries. To make amphibian transcriptomic research more equitable, institutions that possess advanced computational resources should take a proactive role in providing access to local researchers working on underrepresented taxa, such as amphibians (Zhong, Zhang, and Su, 2001; Vicens and Bourne, 2007). The adoption of cloud-based computing platforms and the establishment of regional HPC centres can further democratise access to these resources, stimulating independence and expertise in underserved regions (Abiona et al., 2011; Langmead and Nellore, 2018; Alvarez, Mariño-Ramírez, and Landsman, 2021). Finally, to promote open science, reduce redundancy, and promote environmentally sustainable computational science

(Lannelongue et al., 2023), we strongly encourage that all researchers generating transcriptomes upload their de novo assemblies to public repositories. Also, we recommend to Editors and Reviewers to require it to authors. This practice will promote comparative transcriptomic studies in amphibians.

Future directions

Most transcriptomic studies lack adequate representation across taxonomic and ecological breadth, hampering comparative studies that could elucidate processes of evolution and adaptation across the amphibian tree of life. A broader representation of reference transcriptomes can also help identify the molecular bases of resilience to diseases and environmental perturbations, such as habitat degradation, pollution, or climate change, that are alarmingly threatening amphibian populations worldwide (Scheele et al., 2019; Luedtke et al., 2023). Amphibian microbiomes play an important role in their response to these challenges (Fontaine and Kohl, 2023; Buttimer et al., 2024; Eterovick et al., 2024). Besides increasing the transcriptomic information about amphibian species, from individuals of both sexes at different developmental stages with varying responses to amphibian biodiversity threats, future studies could integrate host and microbiome gene expression. This integration would enhance our understanding of the mechanisms connecting alterations in the microbiomes and could reveal biomarkers indicative of amphibian health (Campbell et al., 2018). Filling the taxonomic gaps in amphibian transcriptomics will improve our understanding of functional genomics in a comparative framework to enhance amphibian research and conservation.

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