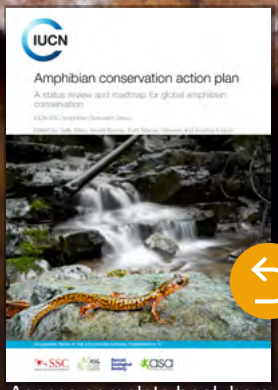
Chapter 13



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Only eight amphibian genomes are fully annotated, including the two banded caecilian (*Rhinatrema bivittatum*), which is classified as Least Concern on the Red List. © Pedro Peloso

Chapter 13

Genomics: using genomics approaches in amphibian conservation

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Abstract

Amphibians are the most threatened major group of vertebrates worldwide and yet they are lagging behind other taxa in genomic resources that could aid in their conservation management. Here, we provide a status update on genomics technologies, how they have been used in amphibian research, and an outlook on how these approaches could inform future conservation planning and management strategies. Overall, amphibians lag far behind other vertebrates in the number of sequenced genomes. Both transcriptome and reduced representation sequencing have become popular tools for understanding amphibian physiology and population dynamics. Environmental DNA sequencing and epigenomics are also becoming useful tools for amphibian biology, although their adoption by the community has been slower. In addition to summarising technologies, their applications, and their challenges, we also provide case studies on how these approaches have been used for amphibian conservation projects. We focus on projects aimed at increasing pathogen resistance, informing captive breeding programmes, and biocontrol of invasive species, although we acknowledge that many more unpublished projects are progressing our understanding of amphibian biology and conservation. Our outlook includes pressing needs for increasing whole genome assemblies across the amphibian phylogeny, providing more bioinformatics training opportunities for conservation biologists, and increasing genomics accessibility to researchers in countries that hold most of the amphibian diversity on the planet.

Introduction

Genetic diversity is critical for natural selection and the continued survival and fitness of species in a rapidly changing environment. The ability to generate genomic data for any species has progressed in technological approaches, accessibility through declining prices, and more widespread computational resources. However, the adoption of sequencing has been slow in amphibian research,

including whole genome assembly, expressed transcripts, genomic markers, and epigenetic modifications. This is surprising given how quickly amphibian species are declining, and these technologies would be useful for rapid responses in establishing conservation strategies. Others have recently reviewed the state of amphibian genomes (Sun, Zhang & Wang, 2020) and their application to understanding amphibian behaviour, physiology, and evolution (Funk, Zamudio & Crawford, 2018; Shaffer et al., 2015; Walls & Gabor, 2019). Here we bring together the fields of genomics and conservation to provide a status update on sequencing technologies and their use for amphibian genomics and conservation projects. As genetic diversity is often used as a predictor of the long-term survival of populations, genomics is a toolkit that is broadly useful for amphibian conservation projects.

Many different genomics approaches have been used to study amphibian biology, although its application is not well distributed across species and geographic regions, which creates many challenges for amphibian conservation. While genomics research in amphibians is more advanced than non-avian reptiles, it lags far behind birds and mammals (Figure 13.1a). Most genomic research in amphibians has been conducted on IUCN Least Concern taxa, but among the threatened categories, the Critically Endangered species have received proportionately more attention (Figure 13.1b). Moreover, there is a geographic bias with respect to the percentage of species with genomics data in the Sequence Read Archive (SRA), where regions with more amphibian species have less genomic data (Figure 13.2). As we move forward with utilising genomics technologies for a greater understanding of amphibian biodiversity, we need to address the inequity in access to training and sequencing platforms in both instrumentation and the cost of data collection, especially in regions of the world that hold the greatest amphibian biodiversity. With equal

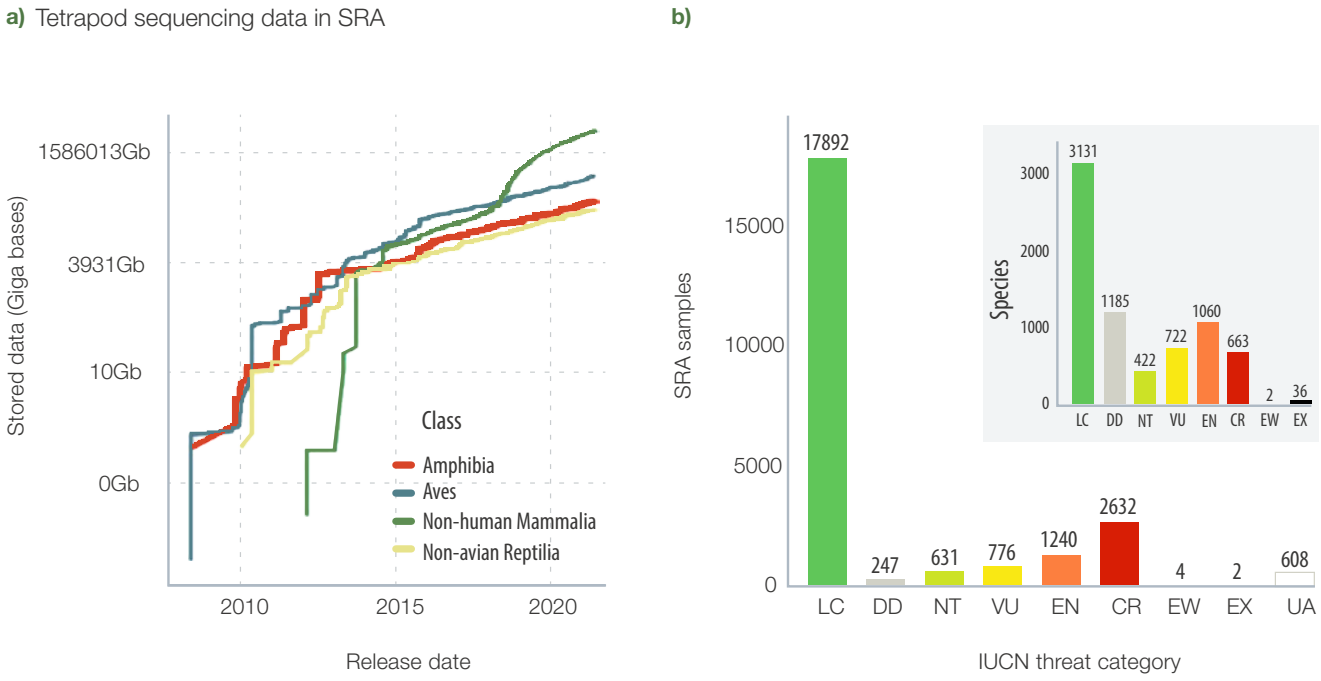

Figure 13.1: Genomic sequencing efforts in amphibians compared to other tetrapods. **a)** Cumulative sum, in logarithmic scale, of high-throughput sequencing data stored in the Sequence Read Archive (SRA) for four main tetrapod groups. **b)** Distribution of amphibian biosamples (equivalent to individuals) stored in SRA for each threat category in The IUCN Red List of Threatened Species™ (UA: unassessed), the inset shows the number of species in each threat category. Source: Data from SRA (www.ncbi.nlm.nih.gov/sra, accessed in January 2021) and the Red List (www.iucnredlist.org, accessed in January 2021).

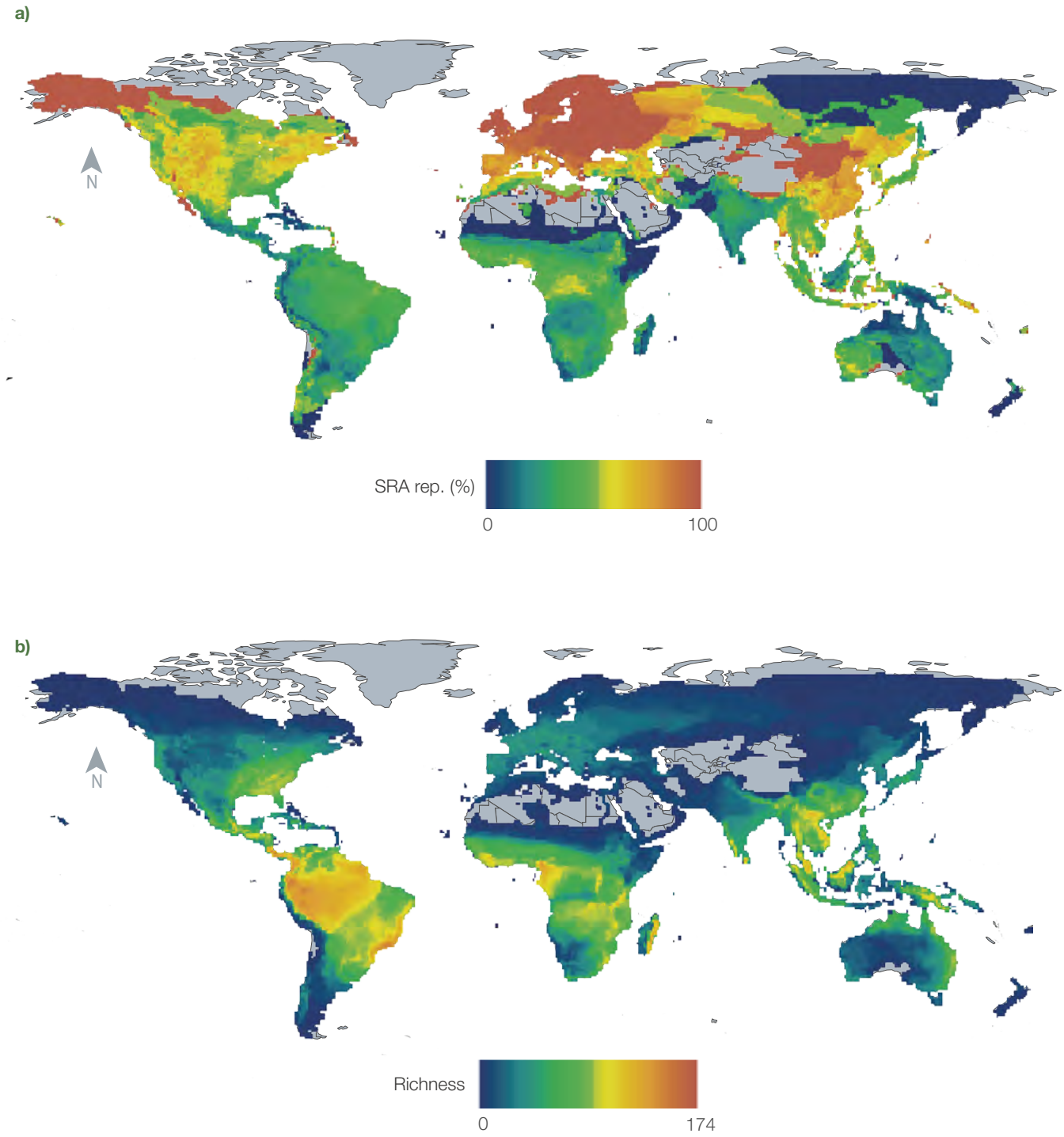

Figure 13.2: Biased geographic distribution of high-throughput sequencing effort. **a)** Percentage of amphibian species sequenced and **b)** amphibian species richness. Distribution polygons from the Red List and SRA records were spatially joined at ~10km resolution in ArcGIS® software (ESRI, Redlands, CA) to estimate the species richness and the percentage of occurring species with high-throughput sequence information. Source: Data from SRA (www.ncbi.nlm.nih.gov/sra, accessed in January 2021) and the Red List (www.iucnredlist.org/, accessed in January 2021).

access to training and technologies, amphibian conservation is poised to utilise genomics technologies in assessing species biodiversity and resilience to environmental stressors to inform conservation priorities, captive breeding programmes, reintroduction surveillance, and management planning.

Table 13.1: Popular genomics approaches for amphibians**.** Advantages and disadvantages of each approach are summarised. Cost range estimates in US$ refer to the direct sequencing cost (library preparation and sequencing). These cost estimates represent the authors' experience (in January 2021) and are provided as guidance, actual price quotes should be obtained from providers

Advantages	Disadvantages	Costs
Whole Genome Sequencing		
• Most comprehensive, genome-wide representation. • Broad taxonomic and biological applicability. • Provides detailed reference for the study of the target species and close relatives.	• **Cost**: Medium to High depending on coverage and genome size. • **Practicality**: Limited by the cost of sequencing (re-sequencing), assembly and annotation. • **Particularities**: Repetitive regions in some amphibian genomes make assembly difficult.	US$ 10K – 100K depending on genome size. Assembly and annotation are additional costs.
Transcriptomics		
• Broad taxonomic and biological applicability. • Provides reference information for development of genomic markers for diverse applications. • Provides information on coding and limited non-coding genomic regions. • Functionally interpretable results that may provide genomic insights into the mechanisms underlying phenotypic variation and adaptation.	• **Cost**: Medium • **Practicality**: Restricted (RNA instability prevents its application to museum samples). • **Particularities**: **a)** Variability in gene expression at cell, tissue, organ, and individual levels; **b)** Sub-optimal *de-novo* assemblies can affect downstream results; **c)** Transcriptome annotation and construction of gene-to-transcript models can be challenging without a reference genome; **d)** Misses most non-coding features of the genome	US$ 170 – 1,000 per sample (library prep. and sequencing). Price varies according to target exome size and desired depth.
Reduced Representation Libraries		
• Reduced genome-wide representation at a relatively low cost. • Provides sufficient genotypic information for informative population genetic analyses. • Capture assays targeting conserved regions have broad applicability in terms of sampling and taxonomic scope.	• **Cost**: Low • **Practicality:** Restricted sampling and scalability (except for targeted capture protocols that can be applied to museum samples across many species). • **Particularities**: **a)** Design of the capture probes or selection of restriction enzyme is critical; **b)** Strategies for loci selection can affect genotype calling in RADSeq assays; **c)** Functional interpretation of results are limited without a reference genome.	US$ 8.5 – 100 per sample (price varies depending on the amount of data, desired depth, and protocol)

Metagenomics		
• A cost-effective approach that can target specific genome regions to assess a wide variety of fields, including systematics, ecology, and conservation. • May be developed in the field or laboratory with portable devices. • Accessible worldwide with standardised protocols that can improve the robustness of results	• **Cost:** Low • **Practicality:** Restricted field availability of reagents, high variation in cost. • **Particularity:** **a)** Studies on a single species need specific primers and risk amplification of non-target sequences; **b)** Bias from primer mismatches, bioinformatic issues, molecule and consensus accuracy, contamination, under sampling or incomplete databases.	US$ 10 – 100 per sample (price varies depending on technology, target, desired depth, and protocol)
Epigenetics		
• Can quickly provide genome-wide estimates of epigenetic modification patterns related to adverse environmental changes for rapid screening purposes. • May be used as biomarkers for population stress vs. health.	• **Cost:** Low to Medium. Costs of different methods are reviewed in Eirin-Lopez and Putnam (2019). • **Practicality:** More affordable methods give genome-wide resolution, more expensive approaches have more specific modifications in specific loci or proteins. • **Particularity:** More research is needed as to which type of epigenetic modification and which genes modified are indicative of different stressors.	From US$ 10 for mass spectrometry or gel-based assessment of global methylation to US$ 1,000 per sample for whole genome bisulfite sequencing.

Genomics: status update

Genomics encompasses many approaches, including whole genome sequencing (WGS), RNA sequencing (RNASeq and IsoSeq), reduced representation sequencing (RRL), metagenomics, and epigenetic sequencing. Different approaches have been used depending on the scientific question and there are advantages and disadvantages of each approach (Table 13.1).

A large taxonomic bias in sequencing effort exists in NCBI's Sequence Read Archive (SRA), where a limited number of amphibian families with few species are represented, including Caudata (Cryptobranchidae) and Archeobatrachian Anura (Ascaphidae, Pelobatidae, Pelodytidae, and Rhinophrynidae). Most amphibian families, however, are underrepresented with 23% of extant families having less than 5% of their species diversity represented in SRA.

Amphibian genomes

- **Whole genome approaches**

  There are several amphibian genomes currently available, and they vary greatly in size and quality. The western clawed frog (*Xenopus tropicalis*) was the first amphibian species with a whole genome

assembly (Hellsten et al., 2010). The African clawed frog (*Xenopus laevis*) was later sequenced at the chromosome level using high-throughput sequencing, chromatin conformation capture and chromosome FISH (Session et al., 2016). XenBase (https://www.xenbase.org) is the central resource for *Xenopus* genomic data and phenotyping information. Available genomes of 19 amphibian species are summarised in a recent review (Sun et al., 2020) and genomes of 22 species are currently (as of January 2021) deposited in the NCBI genome database (*see* Figure 13.3). Two additional species, the common toad (*Bufo bufo*) and the hourglass treefrog (*Dendropsophus ebraccatus*), are available through the GenomeArk of the Vertebrate Genome Project (https://vgp.github.io/genomeark/), and a third, the rufous grassfrog (*Leptodactylus fuscus*) was made available more recently (Mohammadi et al., 2021). Gene annotations are critical for these genomes to be widely useful to the community, and yet only eight amphibian genomes are fully annotated (*X. laevis, X. tropicalis, Nanorana parkeri, Bufo bufo*, *Rana temporaria*, and three caecilians *Microcaecilia unicolor, Geotrypetes seraphini* and *Rhinatrema bivittatum*). UniProt (https://www.uniprot.org) is a broad resource for annotated genes and its current version (2021_01) contains five amphibian species (Anura: *X. laevis*, *X. tropicalis*, *L. catesbeianus*; Gymnophiona: *M. unicolor*, *G. seraphini*).

Genome assembly and annotation can be difficult due to the large size and repetitive elements of many amphibian genomes, especially in Caudata (Figure 13.4). For example, the 30 Gb haploid genome size of the axolotl (*Ambystoma mexicanum*) is about 10 times larger than the human genome (Nowoshilow et al., 2018; Smith et al., 2019). In Anura, some of the existing assemblies are also larger than the human genome: 5.8 Gb in *Lithobates catesbeianus* (Hammond et al., 2017), 6.76 Gb in *Oophaga pumilio* (Rogers et al., 2018), and 4.55 Gb in *Bufo gargarizans* (Lu et al., 2021). Nevertheless, some anuran genomes are much smaller, like the 1.7 Gb genome of *X. tropicalis* and the 1.1 Gb genome of *Platyplectrum ornatum* (Lamichhaney et al., 2021).

- **Whole genome challenges**

The assembly of amphibian genomes remains challenging due to their large size and the vast amount of repeat elements (Rogers et al., 2018). The quality of available amphibian genomes ranges from near-complete chromosomal-scale genomes to fragmented contigs, and future efforts should focus on improving contiguity and completeness of these reference assemblies (Rhie et al., 2020). There are numerous threatened species with moderate genome sizes that we suggest be prioritised for sequencing (Table 13.2). Obtaining good estimates of genome sizes should be considered a top priority for threatened species, as this information is crucial for sequencing prioritisation. Data on genome size and chromosome numbers can be found at the phylogenetically aware database, GoaT (Genomes on a Tree; https://goat.genomehubs.org/). Even smaller genomes require sufficient computational resources, analytical expertise, and time to complete assembly and annotation. High repeat content necessitates that genome assemblers incorporate a variety of data types, including long reads (PacBio HiFi or Oxford Nanopore platforms), medium-range linked reads (Hi-C approaches by Dovetail or Arima Genomics), and optical mapping of genetic markers on whole chromosomes (e.g. BioNano platform; Formenti et al., 2021; Nowoshilow et al., 2018; Rhie et al., 2020; Session et al., 2016). Dense genetic maps of F1 progenies can contribute to finalising chromosome-scale genome assembly (Mitros et al., 2019; Smith et al., 2019), and light-coverage sequencing of parental genomes can resolve a diploid genome assembly into its two component haploid genomes (Koren et al., 2018).

A central resource for amphibian genomic data (outside of *Xenopus*) with a standard procedure for annotation is critically needed. Amphibase (https://amphibase.gitlab.io/) was established to organise transcriptome resources with a unified gene annotation procedure, but more community effort is required for this to become a comprehensive resource. A database with diverse species is critically needed, as other sequence databases

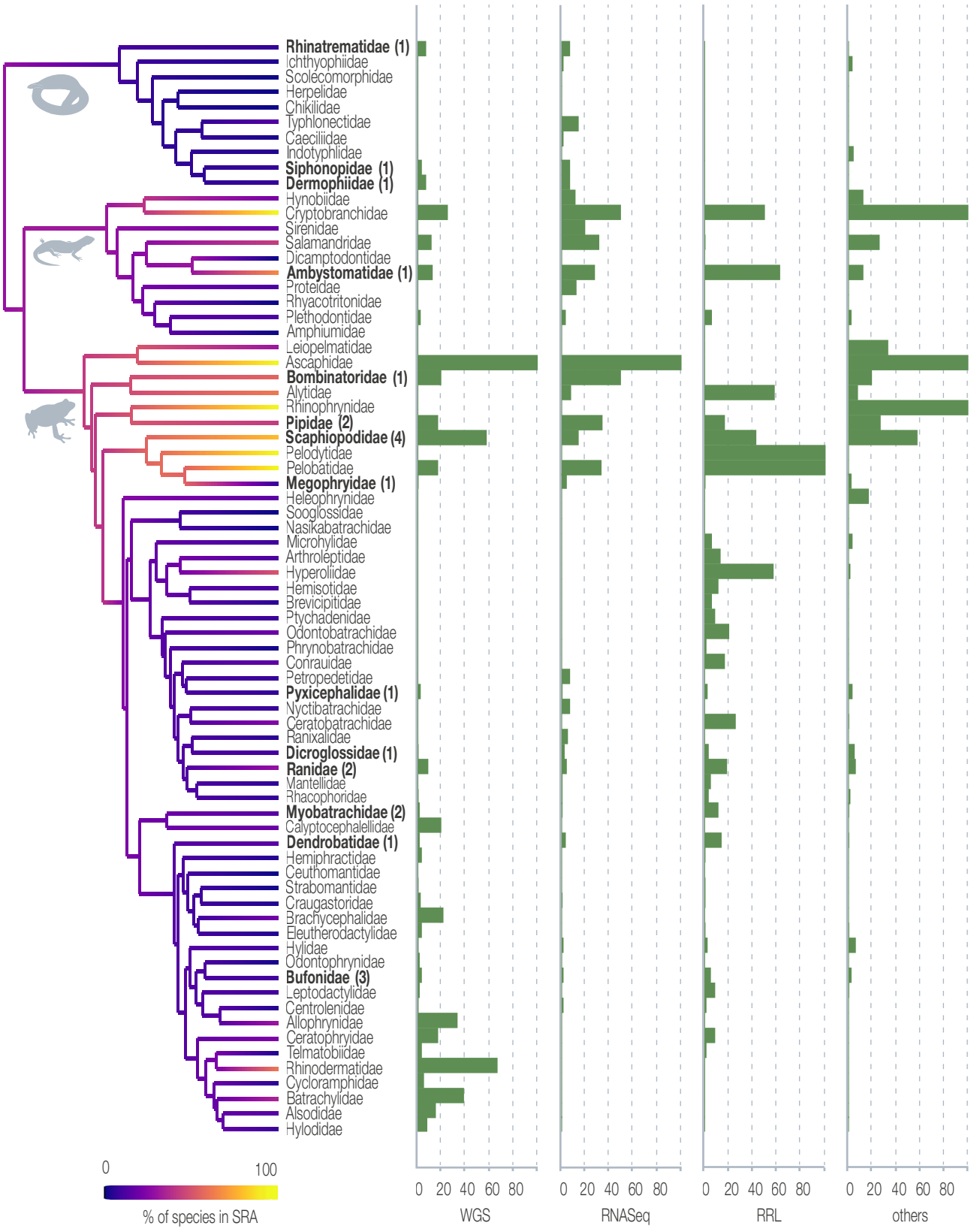

Figure 13.3: Taxonomic representation of amphibians in the Sequence Read Archive (SRA: www.ncbi.nlm.nih.gov/sra, accessed in January 2021). The percentage of species in each family is displayed on the amphibian phylogeny (*sensu* Jetz & Pyron, 2018), pruned to family level), with bar plots on the right representing the percentage for each of the following SRA assay categories: Whole genome sequencing (WGS), RNA sequencing (RNASeq), Reduced Representation Libraries (RRL), and all other assays (includes other approaches such as the sequencing of amplicons, transposase-accessible chromatin, bisulfite modifications, microRNA, and many others). Families with available reference genomes (as per the NCBI Genomes database, accessed in April 2021) are highlighted in bold with the number of genomes in parentheses. Source: Sequence Read Archive, accessed in January 2021.

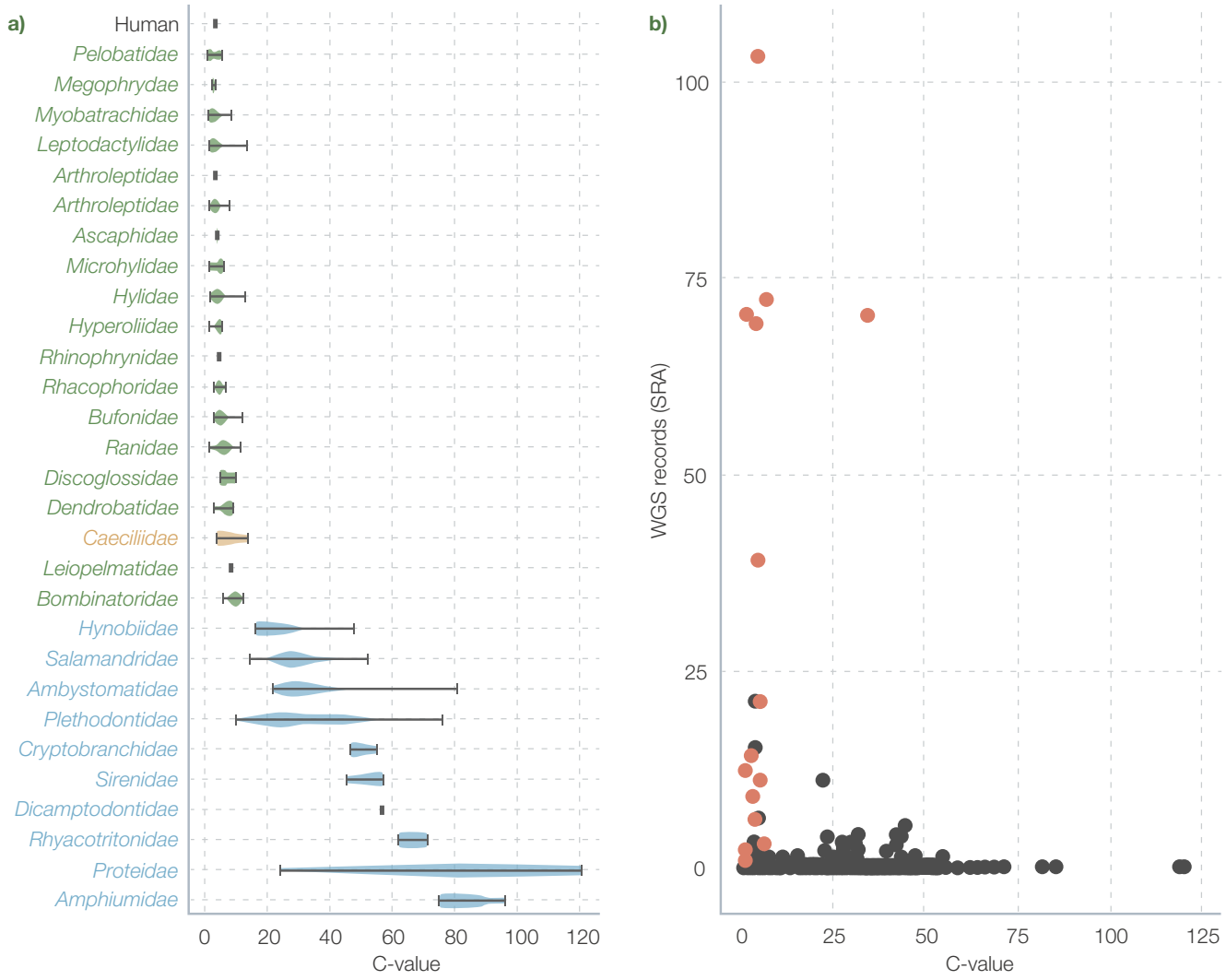

Figure 13.4: Genome size distribution across amphibian families and whole-genome sequencing (WGS) projects. **a)** Genome size estimates (C-value, coloured by order with anurans in grey-blue, caecilians in light blue, and salamanders in green) vary widely by family. Human genome size is displayed at the top as a point of reference. **b)** The relationship between genome sizes and submissions (WGS) per species is shown with assembled genomes marked by orange dots. Source: C-values from Liedtke et al. (2018) and WGS records from NCBI SRA (www.ncbi.nlm.nih.gov/sra, accessed January 2021).

like UniProt are derived from very few amphibian species, which hinders our understanding of amphibian genome diversity.

Overall, whole genome sequencing has not yet become a widespread tool for amphibian conservation. For example, a chromosome-scale reference genome is a valuable resource for understanding genetic diversity, although additional genomic samples are needed to estimate species genetic variation. We expect with decreased sequencing costs and more widely available annotation tools, whole genome sequencing will become a valuable conservation tool in the near future.

Transcriptomics

- **Transcriptomics approaches**

Messenger RNA sequencing (RNASeq) is a method that sequences the expressed fraction of the genome. The assembled coding sequences of mRNAs can be compared with orthologous

sequences in reference protein databases to infer and annotate their function. Transcript coding sequences could be used to design targeted enrichment probes and, along with the non-coding mRNA regions, can be used to develop microsatellite markers or genotyping panels for population genetic studies. The possibilities presented by the ability to quantify functional (presumptive amino acid sequence) variation without a reference genome makes this technique appealing for studying many molecular processes linked to conservation biology. Reference transcriptomes from 40 amphibian species are currently deposited in the NCBI Transcriptome Sequence Archive (TSA), a database of transcriptomes representing a fraction of the 222 species in SRA Database.

Best approaches for generating a transcriptome vary depending on the research question, and factors such as age, sex, and tissue type should be considered. For species with no reference genome assembly, transcriptomic data need to be assembled de novo into transcripts. Accurate annotation of the reference is also important for functional interpretation of downstream results (Hart et al., 2020; Musacchia et al., 2015) and several pipelines are now available for transcriptome assembly, annotation, and analyses (Cabau et al., 2017; Conesa et al., 2016; MacManes, 2018; McKenna et al., 2010; Smith-Unna et al., 2016; Van Den Berge et al., 2019). Although not currently widespread, transcriptomics studies are expected to benefit from long-read sequencing platforms (e.g. PacBio Iso-Seq, Oxford Nanopore Tech) for increased assembly contiguity and resolution of alternative splicing variants. However, the deep sequencing provided by short-read Illumina platforms may provide better depth, thus detecting rare transcripts useful for annotation.

- **Transcriptomics challenges**

RNA sequencing is starting to be more widely applied to amphibian conservation projects and the current challenges are mostly associated with limited taxonomic diversity, as 76% of extant families have less than 5% of their species diversity represented by transcriptomic data (*see* Figure 13.3). In addition to identifying differentially

Table 13.2: Threatened species with moderate genome sizes that should receive priority in future genome sequencing projects. This list is not exhaustive and should be expanded as genome size estimates of more species become available

Species	Genome size (C-value)	Lineage	Red List category
Leptopelis vermiculatus	3.1	Anura, Arthroleptidae	Endangered
Conraua goliath	3.1	Anura, Conrauidae	Endangered
Quasipaa boulengeri	3.1	Anura, Dicroglossidae	Endangered
Boulengerula taitana	2.9	Gymnophiona, Herpelidae	Endangered
Osteopilus vastus	2.5	Anura, Hylidae	Vulnerable
Phrynobatrachus krefftii	1.7	Anura, Phrynobatrachidae	Endangered
Buergeria oxycephala	1.6	Anura, Rhacophoridae	Vulnerable
Sooglossus sechellensis	1.8	Anura, Sooglossidae	Endangered
Telmatobius culeus	2.1	Anura, Telmatobiidae	Endangered

Source: Estimates of genome size from Liedtke et al. (2018), Red List categories from IUCN (2021)

expressed genes, RNA sequencing can also be used to study a range of important phenotypes linked to conservation planning. For example, these data can be used to identify a large set of SNPs to study signatures of selection in imperilled amphibian species. This information would be helpful to identify genotypes associated with adaptive polygenic traits like thermal tolerance, habitat preference, or disease resistance (Spurr et al., 2020). Finally, co-expression network analyses could be used to identify networks of genes with similar expression patterns across samples and how these vary under different conditions (Serin et al., 2016; van Dam et al., 2018). Combining gene co-expression networks with time series analyses in species experiencing drastic environmental challenges has the potential to uncover modules of co-expressed genes and changes in their interactions associated with a challenge of interest. This approach could pinpoint gene modules as markers for resilience or vulnerability, thus providing crucial information for implementing effective conservation measures.

Reduced representation library (RRL) sequencing

- **RRL approaches**

Reduced Representation Libraries (RRL) are designed to focus sequencing on a subset of the genome. Restriction-site associated DNA sequencing (RADseq) and the targeted capture and sequencing of specific genomic regions are the two most common approaches currently used in amphibian genomics. RADseq was designed by Miller et al. (2007) and further modified into genotyping-by-sequencing (GBS; Elshire et al., 2011), double-digest RADseq (ddRAD, two restriction enzymes are used; Peterson et al., 2012), triple-digest RADseq (3RAD, three restriction enzymes are used; Bayona-Vásquez et al., 2019), and Diversity Arrays Technology DArTseq (Lambert, Skelly & Ezaz, 2016). There are also multiple methods of targeted capture such as Ultra Conserved Elements (UCE; Faircloth et al., 2012; Mccormack et al., 2012), Anchored Hybrid Enriched (AHE) loci (Lemmon, Emme & Lemmon, 2012). Restriction enzyme digestion and sequence capture probes can also be combined, as in the RADcap protocol (Hoffberg et al., 2016), and is exceptional at sequencing hundreds of specific loci across hundreds of individuals.

RRL methods provide hundreds to thousands of loci that allow for fine-scale analysis of population structure and genetic diversity. These methods can even be applied to samples having low DNA quality like museum specimens, and thus RRL methods have important implications in conservation recommendations. Consequently, RRL techniques are useful for understanding reproductive isolation and gene flow as well as estimating hybridisation rates, species delimitation, and the identification of cryptic species (Dufresnes & Martínez-Solano, 2020; Dufresnes et al., 2018a; Guillory et al., 2019; Homola et al., 2019). Within species, population structure and demography are equally important, as gene flow and inbreeding depression influence adaptive potential and resilience to environmental change. For these questions, one of the most important parameters to quantify is effective population size, which can be used to study demographic history and extinction risk of populations. For example, RAD sequencing has been used with *Ambystoma* salamanders to determine effective population size, which could prove useful for population monitoring and management planning (Nunziata et al., 2017; Nunziata & Weisrock, 2018).

RRL data has also been used for improving whole genome assembly methods by sequencing specific chromosomes (also known as ChromSeq; Iannucci et al., 2021). This approach resolved the assembly of the sex chromosomes of *X. tropicalis* (Seifertova et al., 2013) and *Amolops mantzorum* (Luo et al., 2020), and helped to assemble the large genomes of *A. mexicanum* (Keinath et al., 2015; Smith et al., 2019) and *Notophthalmus viridescens* (Keinath et al., 2017). In addition, RRL sequencing has enabled the identification of important genome features, such as sex-related markers (Cauret et al., 2020; Lambert et al., 2016) or candidate genes linked to conservation relevant traits (Guo et al., 2016).

- **RRL challenges**

RRL approaches are likely to remain popular tools for informing amphibian conservation given their cost-effectiveness, especially for large amphibian genomes. However, a biased taxonomic distribution of RRL sequencing effort is noticeable (*see* Figure 13.3), as there is currently no data for Gymnophiona and multiple families of Anura (mostly Neobatrachians) and Caudata. Most families are underrepresented and only Pelobatidae and Pelodytidae have all their species sequenced with RRL assays. Although public datasets may accelerate the improvement of specimen samplings, combining different RRL datasets may be very challenging, especially when they resulted from non-targeted genome-subsampling methods. As the data produced by RADseq are randomly sampled across the genome, the sequences recovered in different experiments are not necessarily the same, even if the same restriction enzymes are used. Another challenge of RRL is that functional interpretations can be limited without a reference genome.

Metabarcoding and metagenomics

- **Metabarcoding and metagenomics approaches**

Emerging from DNA barcoding (Hebert et al., 2003), metabarcoding focuses on the amplification and sequencing of specific genetic markers from multiple individuals while metagenomics corresponds to the study of genetic material from many individuals within an environment. Both approaches have broad applicability in taxonomy, ecology, population dynamics, evolution, and biogeography, all of which are essential contributors to amphibian conservation biology (Ficetola, Manenti & Taberlet, 2019). Metabarcoding and metagenomics, along with RNA sequencing, are also being used to profile microbial and parasitic communities of amphibians (Shakya, Lo & Chain, 2019). Successful examples include profiling parasites in the eastern dwarf tree frog (*Litoria fallax*; Ortiz-Baez et al., 2020) and poison frogs (Dendrobatidae; Santos et al., 2018).

The use of environmental DNA (eDNA) metabarcoding has been applied to survey amphibian communities in threatened ecosystems (Lopes et al., 2017; Sasso et al., 2017), rediscover "extinct" or "rare" species (Goldberg, Strickler & Fremier, 2018; Lopes et al., 2021), detect invasive species (Bento et al., 2021; Dufresnes et al., 2017; Dufresnes et al., 2018b; Dufresnes et al., 2019; Secondi et al., 2016), identify emerging diseases (Romero-Zambrano et al., 2021), and develop strategies in accordance with the *Amphibian Conservation Action Plan* (Wren et al., 2015). For example, this approach has successfully been used to monitor the distribution of the threatened great crested newt (*Triturus cristatus*) and detect invasive species associated with population declines (Harper et al., 2019).

- **Metabarcoding and metagenomics challenges**

The success of metabarcoding studies for amphibian conservation is dependent on representative reference sequences within these databases. Metabarcoding and metagenomics facilitate the identification of relevant taxa from high-throughput sequencing data (Wilson, Sing & Jaturas, 2019; Xu et al., 2015) and rely on reference sequences in public databases like BOLD (www.boldsystems.org), ENA (www.ebi.ac.uk/ena), GenBank (www.ncbi.nlm.nih.gov/genbank), and Silva (www.arb-silva.de), among others. BOLD, for example, contains reference sequences for only 3,247 species of amphibians (39% of described species) with Anura (2,728 spp., 37% of total species diversity) and Gymnophiona (84 spp., 39%) taxa being less well represented than those in Caudata (435 spp., 57%). Therefore, efforts toward reducing taxonomic gaps in reference databases are encouraged.

Epigenetics

- **Epigenetics approaches**

Epigenetics describes genome-wide patterns of DNA modifications and structures that impact

gene regulation. These can be inherited somatically or through the germline without altering the DNA sequence (Rando & Verstrepen, 2007). Such modifications can serve as stress biomarkers predicting population persistence in unstable environments (Rey et al., 2020). In this context, whole genome bisulfite-sequencing (WGBS) can be used, which relies on the conversion of cysteines into thymines by sodium bisulfite. Activity levels of methylation-inducing genes can then be measured using qPCR (Hudson et al. 2008) or DNA methylation-specific antibodies (Hawkins & Storey, 2018; Zhang, Hawkins & Storey, 2020). For example, temperature-related research in amphibians includes studies of expression of genes whose products have gene silencing functions in striped burrowing frogs (*Cyclorana alboguttata;* Hudson et al., 2008), changes in methylation patterns linked to the freeze-thaw cycle in wood frogs (*Rana sylvatica*; Hawkins & Storey, 2018; Hudson et al., 2008), and histone modifications linked to the onset of metamorphosis in *L. catesbeianus* (Mochizuki et al., 2012).

Epigenetic modifications can change under other environmental stressors such as endocrine disrupting chemicals (Jacobs, Marczylo & Guerrero-Bosagna, 2017) or radiation. For example, exposure of *X. laevis* to the pesticide atrazine causes disturbances in steroidogenesis via epigenetic modifications (Hayes et al., 2002). Japanese tree frogs (*Dryophytes japonicus*) sampled two years after the Fukushima nuclear accident show genome-wide increases in methylation patterns (Gombeau et al., 2020). These connections highlight the importance of epigenetic modifications as stress biomarkers and the untapped potential of this tool for amphibian conservation.

- **Epigenetics challenges**

This approach requires a high-quality reference genome and extensive sequencing depth, which is expensive at present but likely to decrease in cost in the future. Once epigenome markers are identified (Thorson et al., 2020), other more cost-effective methods may be used to assess their modification (reviewed in Eirin-Lopez & Putnam, 2019). To reliably relate epigenetic changes with environmental stressors, baseline research is needed to identify which external variables influence gene methylation (Mochizuki et al., 2012; Rey et al., 2020). There is also a need for understanding the role of long-term acclimatisation in reintroduction efforts given the longevity of epigenetic modifications across generations (van Oppen et al., 2015). Including epigenetics in conservation planning (conservation epigenetics *sensu* Rey et al., 2020) would ensure that recent ecological history and phenotypic plasticity are considered.

Case studies on applying genomics approaches to amphibian conservation

The recent revolution in genomics technologies means that many projects are underway for which the successes and failures are not yet known. Here, we look at specific conservation projects that have successfully used genomics technologies to inform conservation approaches to disease resistance, captive breeding, and biocontrol of invasive species.

- **Understanding and increasing chytridiomycosis resistance**

Understanding the genetic contribution to chytridiomycosis susceptibility caused by *Batrachochytrium dendrobatidis* (Bd) infection is critical for prioritising species for conservation efforts and producing species capable of surviving the disease through captive breeding programmes. Most efforts to identify genetic regions associated with Bd immunity have involved targeted studies of immune genes or gene expression comparisons between infected and uninfected frogs (Table 13.3). The majority of Bd genetic association studies have targeted the major histocompatibility complex (MHC), which have detected correlations between MHC variation and Bd resistance (Table 13.3). One of the best examples comes from lowland leopard

Table 13.3: Bd immunity studies using genetic/genomic approaches

Species	Experimental Design	Gene Region	Reference
Bufo calamita	Field study	MHCII	(May, Zeisset & Beebee, 2011)
Lithobates yavapaiensis	Laboratory challenge	MHCII	(Savage & Zamudio, 2011)
Multiple sp.	Field study and laboratory challenge	MHCII	(Bataille et al., 2015)
Lithobates yavapaiensis	Field study	MHCII	(Savage & Zamudio, 2016)
Physalaemus pustulosus	Field study	MHCII	(Kosch et al., 2016)
Lithobates chiricahuensis	Field study	MHCII	(Savage et al. 2018)
Thoropa taophora	Field study	MHCII	(Belasen et al., 2019)
Lithobates pipiens	Field study	MHCII	(Trujillo et al., 2021)
Japanese Rana spp.	Field study	TLRs	(Lau et al., 2018)
Xenopus tropicalis	Laboratory challenge	Transcriptome	(Rosenblum et al., 2009)
Lithobates muscosa, L. sierrae	Laboratory challenge	Transcriptome	(Rosenblum et al., 2012)
Atelopus zeteki	Laboratory challenge	Transcriptome	(Ellison, et al., 2014a)
Agalychnis callidryas, Atelopus glyphus, Atelopus zeteki, Craugastor fitzingeri	Laboratory challenge	Transcriptome	(Ellison, et al., 2014b)
Rana temporaria	Laboratory challenge	Transcriptome	(Price et al., 2015)
Rhinella marinus, Anaxyrus boreas	Laboratory challenge	Transcriptome	(Poorten & Rosenblum, 2016)
Lithobates sylvatica, L. catesbeianus	Laboratory challenge	Transcriptome	(Eskew et al., 2018)
Litoria verreauxii alpina	Laboratory challenge	Transcriptome	(Grogan et al., 2018; Savage et al., 2020)
Lithobates yavapaiensis	Laboratory challenge	Transcriptome	(Savage et al., 2020)
Pseudophryne corroboree	Laboratory challenge	Genome-wide SNPs, MHCI	(Kosch et al., 2019)
Atelopus varius, A. zeteki	Field study	Exome	(Byrne et al., 2021)

frogs (*Lithobates yavapaiensis*), where an MHC allele (the Q-allele) predicts increased survival (Savage & Zamudio, 2011; Sommer, 2005). RNA sequencing approaches have identified many immune genes that are differentially expressed in response to Bd infection including the MHC, B-cells, complement, and chitinase (Table 13.3). These studies also found that Bd suppresses lymphocyte expression (Ellison

et al., 2014a), more resistant populations exhibit robust early immune response (Grogan et al., 2018), and dysregulation of immune genes is associated with susceptibility (Grogan et al., 2018; Savage et al., 2020). Although these approaches have identified many candidate resistance genes for future study, their design does not permit testing the link between gene expression differences and Bd survival given study animals were euthanised for tissue sampling.

A thorough understanding of the genes underlying chytrid immunity and their effect size is critical for managing amphibians threatened by Bd. To date, only two studies have used genome approaches to investigate Bd resistance: a genome-wide association study in southern corroboree frogs (*see* Box 13.1; Kosch et al., 2019) and targeted exome sequencing in harlequin frogs (Byrne et al., 2021). Although pioneering in their approaches, these studies lack the robust statistical power recommended before use in management. With the rapid development of genomics technologies in recent years, and the increasing availability of amphibian reference genomes, such investigations are now possible in many species. Future efforts should apply genomics approaches discussed in this Status Update to better understand genetic contributions to Bd resistance.

Box 13.1: Developing methods to increase Bd-resistance in southern corroboree frogs

Southern corroboree frogs (*Pseudophryne corroboree*) – an Australian alpine endemic species – have been driven to functional extinction in the wild by chytridiomycosis (Hunter et al., 2010) and their continued survival is dependent on captive breeding and reintroduction (Box Figure 13.1). Although a successful breeding programme has been in place for over a decade, self-sustaining populations have yet to be established in the wild (Kosch et al., 2019). One of the challenges of re-establishing this species is that it co-occurs with Bd-tolerant reservoir species *Crinia signifera* (Scheele et al., 2017). As culling the reservoir host is not a desirable option, Bd-resistance will have to be increased to allow this species to survive along with the Bd pathogen.

Research is underway to understand the genetic basis of Bd-resistance and develop methods to enhance it in currently susceptible species (Kosch et al., 2022; Skerratt, 2019). The southern corroboree frog restoration project consists of a multi-institutional group of academics, threatened species managers, and zoo practitioners dedicated to restoring this species in the wild (University of Melbourne: Lee Skerratt, Lee Berger, and Tiffany Kosch; Zoos Victoria: Deon Gilbert; NSW Department of Planning, Industry, and Environment: David Hunter; Taronga Conservation Society: Michael McFadden; University of Rochester: Jacques Robert; and James Cook University: Kyall Zenger). As genetic intervention is a long-term endeavour requiring decades before animals are fit for release, participants have committed to proceeding cautiously, involving all stakeholders, and vetting the safety and efficacy of each step before proceeding. The programme consists of multiple stages: **1)** understanding the genetic basis of immunity to Bd, **2)** developing genetic tools to increase resistance, **3)** testing effectiveness of genetic intervention by Bd-challenge in the lab and field enclosures, **4)** testing for off-target effects in the lab and the field, **5)** release into the wild, and **6)** long-term monitoring to evaluate success. Such methods, if successful, can be used as a proof of concept for other threatened amphibians worldwide.

One of the biggest challenges for this project has been developing genetic resources for *P. corroboree*. However, current efforts to sequence a reference genome and develop gene editing and transgenesis tools should help alleviate this problem. Pilot studies have been conducted to sequence immune genes, develop genome-wide DArT-seq markers, and begin to understand the genetic architecture of resistance (Kosch et al., 2017, 2019). Future work will involve testing other genotyping technologies such as targeted

sequence capture and low-pass sequencing to increase genotyping coverage and performing well-powered genome-wide association studies with increased sample size. There are also plans to expand the standard phenotypes used to measure Bd-resistance by including molecular phenotypes and longitudinal gene expression data to better understand genetic architecture and identify putative Bd-resistance variants.

Box Figure 13.1: Southern corroboree frogs (*Pseudophryne corroboree*) are conservation-reliant due to their susceptibility to Bd. A captive-bred *P. corroboree* frog (left, photograph by Corey Doughty), *P. corroboree* breeding facility at the Melbourne Zoo (middle, photograph by Mikaeylah Davidson), and outdoor enclosures maintained by the Australian National Parks and Wildlife Service (right, photograph by Michael McFadden).

- **Genomic approaches for invasive amphibian biocontrol**

Invasive species are linked to approximately one-third of amphibian extinctions and threaten 16% of extant amphibian species (Blackburn, Bellard & Ricciardi, 2019). These effects occur primarily through habitat alteration, predation, competition, hybridisation, and disease spread (Falaschi et al., 2020; Nunes et al., 2019). The use of genomic approaches for understanding and managing invasions has rapidly increased in recent years (McCartney, Mallez & Gohl, 2019), but is only beginning to be applied to amphibian systems (*see* Box 13.2).

Genomic tools offer powerful methods to study invasive-native hybridisation. For example, hybridisation with invasive tiger salamanders (*Ambystoma tigrinum mavortium*) threatens the endemic California tiger salamander (*Ambystoma californiense*; Mccartney-Melstad & Shaffer, 2015), where hybrids outcompete and cannibalise pure natives and prey upon other amphibians in the community (Ryan, Johnson & Fitzpatrick, 2009). Preservation of the native species requires introgression prevention, and genomic scans have been used to track the movement of non-native alleles (Shaffer et al., 2015). Moreover, genome regions associated with traits critical to population viability are candidates that may indicate to managers which populations have the strongest potential to further spread non-native alleles (Shaffer et al., 2015). For example, genomic regions associated with metamorphosis were identified using RRL sequencing (Voss et al., 2012) and genes promoting thermal tolerance have been identified using RNA sequencing (Cooper & Shaffer, 2021). Thus, genomics approaches are critical tools for understanding invasive-native population dynamics and can inform conservation management practices (Dufresnes & Dubey, 2020).

Genomic tools also offer new perspectives into invader-mediated population declines. Invasive cane toads (*Rhinella marina*) in Australia increase parasitic infections in native amphibians (Kelehear, Brown & Shine, 2013) that can be fatal (Pizzatto & Shine, 2011). RNA sequencing of invasive Australian cane toad livers revealed a novel virus at high prevalence (Russo et al., 2018), while follow up studies showed that native range cane

toads contained a diversity of viruses (Russo et al., 2021). This suggests an "enemy release", where viruses left behind in the native range may serve as effective control agents due to evolutionary distance (Russo et al., 2021). Although biocontrol through pathogenic agents has been suggested, selection of a suitable agent would require careful investigation due to the risk of infecting native frog species.

Cane toads also carry lethal toxins that lead to population-level declines in Australian predators (Shine, 2010), as well as shifts in behavioural traits of some predator populations (Pettit, Ward-Fear & Shine, 2021). Gene editing in cane toads using CRISPR has been used to knock-out a toxin hydrolase that converts toad toxin from its storage form to a lethal active form (Cooper et al., 2020). Other genes that may enhance the toad's invasion success may also serve as future knockout candidates using these protocols. However, this approach requires caution due to the potential risk of gene-edited toads being inadvertently introduced back to the native South America range through human translocation.

Box 13.2: Genomics of the cane toad invasion

Originally sourced from native South American populations, cane toads (*Rhinella marina*) were introduced to Puerto Rico in 1920, then to Hawaii in 1932, and finally to north-eastern Australia in 1935 (Turvey, 2013) (Box Figure 13.2). The cane toad invasion has since garnered much attention in Australia due to its ecological effects on a diversity of native taxa (Shine, 2010).

The collection of genomic data on invasive cane toads is relatively recent, enabled by the development of a multi-tissue reference transcriptome (Richardson et al., 2018) and draft genome assembly (Edwards et al., 2018). These tools have been critical for elucidating genetic changes that occur as the toads disperse across northern Australia to the arid western regions. Population genetics studies using RNA-Seq (Selechnik et al., 2019a) and RADSeq (Trumbo et al., 2016) have characterised population structure and identified two genetic clusters separated at a continental divide marked by an abrupt change in rainfall and temperature. Candidate genes involved in heat and dehydration resistance (Selechnik et al., 2019a) and those involved in metabolism and stress responses (Rollins, Richardson & Shine, 2015) have been identified that may underlie the successful range expansion. Differential expression analyses on the RNA-Seq dataset suggest that environment-driven gene expression follows a similar pattern across the continental divide (Selechnik et al., 2019b).

The application of genomic techniques to the cane toad system has allowed for the investigation of invasion from novel perspectives. Analyses using 16S rRNA sequencing data to characterise colon microbiota in toads from each side of the continental divide revealed differences in both microbial compositional and functional variation (Zhou et al., 2020). Furthermore, behavioural traits were linked to microbial functional variation while infection prevalence of lungworm parasites was linked to both compositional and functional variation (Zhou et al., 2020). Further exploration of the relationships between gut microbiota, endoparasites, and invasive behaviours may cultivate new management strategies.

The role of epigenetics in shaping the cane toad invasion has also been investigated. Reduced representation bisulfite sequencing on common garden-bred cane toad tadpoles exposed to conspecific alarm cues revealed differential changes to DNA methylation in lineages from each side of the continental divide (Sarma et al., 2020). Further, these alarm cue-exposed individuals exhibited an induced defence mechanism and this defence was shown to be transferred to the next generation (Sarma et al., 2021).

These are among the first studies to demonstrate a potential role for epigenetics in rapid evolution during invasion and suggest that such effects should be considered in future biocontrol studies.

Box Figure 13.2: The invasive Australian cane toad (*Rhinella marina*). Photograph taken by Dr. Matt Greenlees.

Discussion

Amphibians are less intensively researched than mammals or birds (Figure 13.1) and most genomic sequencing efforts in amphibians have concentrated on Least Concern taxa. Being the tetrapod group with the most threatened species, a boost on genome sequencing projects in threatened amphibian species is urgent. Although the lack of high-quality reference genomes may preclude some genomic applications, the use of reduced genome representation techniques (e.g. RNA-Seq, RAD-Seq, and Targeted Capture assays) are a viable alternative to genome-based approaches and should be more extensively applied to imperilled amphibian species. We strongly suggest that Red List assessments incorporate genomics approaches for estimating genetic diversity and species delimitation in biodiverse regions. We can now envision a future where genomic-informed interventions in translocations, genomic rescue, and disease prevention and mitigation are part of our toolkit for ensuring the long-term preservation of amphibian biodiversity.

Many approaches have been successfully used to conserve threatened amphibians including habitat conservation, restoration, and supplementation (Cook, 2010; Woodhams et al., 2011). Unfortunately, these approaches are not always effective for threats that are hard to mitigate such as disease, climate change, and invasive species, thus requiring the development of novel approaches to increase survival. If the goal of a conservation programme is to establish self-sustaining populations in the wild, then genomic methods that promote survival alongside the threat should be considered. Measurement of genetic diversity is critical for assessing inbreeding and outbreeding depression prior to population augmentation or captive breeding strategies and genomics is currently the simplest way of tackling this problem (Byrne & Silla, 2020; Frankham et al., 2011). Although more complex and drastic, genetic intervention is also a

promising approach for establishing self-sustaining populations of amphibians that can survive alongside key threats. Genetic intervention methods can include genetic rescue, CRISPR gene editing, and genomic selection, all of which rely on genomics technologies and reference genomes. Of these, only genetic rescue has been used for conservation purposes (but *see* Newhouse & Powell, 2021; van Oppen & Oakeshott, 2020; and Box 13.1). However, the widespread success of gene editing and/or genomic selection methods in medicine and agriculture (Meuwissen, Hayes & Goddard, 2016; Piaggio et al., 2017) suggests these methods should be considered. Genetic intervention in wildlife is controversial (Kardos & Shafer, 2018; Redford et al., 2019) and should be performed with utmost caution along with careful testing to ensure that manipulated animals pose no environmental risk and are fit for release. Another challenge of applying genetic intervention methods in amphibians is the lack of fundamental genomic understanding of key survival traits, but this should increase as more genomic resources become available.

This Genomics Status Update has highlighted several critical needs for the amphibian conservation community, including equity in training and technology access, data resource management and transparency, and the involvement of stakeholders and conservation practitioners in genomics analyses. There is a clear geographic bias in the origins of genomics data compared to amphibian biodiversity hotspots (Figure 13.2). We call for more equity in training opportunities and access to genomics technologies for researchers from Central and South America, Africa, and Southeast Asia. Cheap and portable sequencing platforms are one promising avenue, coupled with bioinformatics training and decolonisation of field-based genomic studies. Data transparency and accessibility is another community challenge, as annotation and genomic resource management often lack funding but are critical for rapid progress. Additionally, transparency in data and sequencing should be a requirement of any funded project, including rapid public release of sequence data prior to publications that may take years to appear. Finally, there is a clear need to involve stakeholders and conservation practitioners in genomics research. This could include community driven annotation or metadata necessary for genome usability as well as “plug and play” platforms coupled with free online bioinformatics training opportunities that make these approaches more accessible in concept and in practice. Portable high throughput nanopore MinION sequencers are now being used directly in the field to generate genomic data for rapid biodiversity assessments, thus strengthening local capacities for monitoring and conservation (Pomerantz et al., 2018). The ability to conduct massively parallel DNA sequencing studies in situ can also alleviate the need to export genetic material or digital sequence information on genetic resources (DSI), two key components of the Convention on Biological Diversity (CBD) and the Nagoya Protocol (https://www.cbd.int/dsi-gr/). Portable devices with quick high-throughput sequencing and analysis capabilities can boost data accessibility for decision-makers, researchers, and local government officials to improve amphibian management decisions. Genomics can make an important contribution to global amphibian conservation, but only if access to its power is equitable for all people involved.

In summary, we recommend the following actions:

» Research

- The genomes of threatened amphibian species should be sequenced to create genomics tools that inform conservation practices.
- Genetic intervention methods like genetic rescue, CRISPR gene editing, and genomic selection, should developed as tools for conservation, with careful consideration of ecological risks.
- Tools to measure genetic diversity, such as reduced genome representation sequencing, should more widely applied to assess inbreeding and outbreeding depression prior to population augmentation or captive breeding strategies.

Management

- Red List assessments should incorporate genomics approaches for estimating genetic diversity and species delimitation in biodiverse regions.
- Stakeholders and conservation practitioners should be involved in genomics research and have online bioinformatics training opportunities.

Community

- Training opportunities and access to genomics technologies for researchers from Central and South America, Africa, and Southeast Asia should be prioritised.
- Researchers should consider rapidly releasing sequence data on public archives prior to publication, especially for threatened amphibian species.

Box 13.3: Glossary

Chromatin conformation capture: a method to analyse the spatial organisation of chromatin in a cell.

Chromosome FISH: a method to identify the physical location of a piece of DNA on a chromosome by fluorescence in situ hybridisation.

Contigs: a DNA sequence reconstructed from a series of overlapping DNA fragments.

CRISPR gene editing: a method for engineering genetic elements of an organism derived from the prokaryotic antiviral system with clustered regularly interspaced short palindromic repeats (CRISPR).

DNA barcoding: a method of identifying species by sequencing a short segment of DNA that is conserved across distantly related species.

Environmental DNA (eDNA): DNA collected from environmental samples (e.g. water, faeces, soil) rather than directly from the organism.

Epigenetic sequencing: a method to analyse the gene activity changes caused by mechanisms other than DNA sequence changes, such as histone modification and DNA methylation.

Expressed transcripts: RNAs actively transcribed from DNA.

Genome annotations: a process to identify functional elements on the genome, such as genes, pseudogenes, promoters, and repeats.}

Gene editing: techniques that modify DNA sequence.

Genetic rescue: method for increasing genetic diversity by facilitating immigration and gene flow into an isolated population.

Genome: an organism's complete genetic sequence information.

Genome assembly: creation of a contiguous genome sequence by piecing together smaller DNA sequence fragments decoded experimentally.

Genomic selection: a selective breeding method that predicts phenotypes of prospective breeding stock using impacts of genome-wide markers evaluated from a reference population.

Genetic markers: the physical location and sequence of DNA within a genome used to track genetic inheritance.

Genotype-by-sequencing (GBS): a method to genotype samples by identifying genetic variants after aligning their sequences against a reference genome.

Genome-wide association study (GWAS): analysis of the associations between traits and genetic variants across individuals and populations.

High-throughput sequencing: technology that sequences millions of DNA and RNA fragments simultaneously, also known as next-generation sequencing (NGS).

Metagenomics: a collection of genetic material from a mixed community of organisms.

Optical mapping: a method to order the single molecule of DNA to construct a high-resolution map of restriction enzyme recognition sites.

Reduced representation sequencing: an umbrella term for many technological approaches that centre on obtaining genetic information for an organism by sequencing small portions of the genome.

Restriction-site associated DNA sequencing (RADseq): a method for obtaining genotype data throughout the genome of an organism by sequencing small fragments generated by restriction enzymes.

Transcriptome: a collection of RNAs transcribed from DNA, including messenger RNAs, long non-coding RNAs, microRNAs, transfer RNAs, ribosomal RNAs.

Whole Genome Sequencing (WGS): various methods for sequencing the entire genome of an organism by iterative sequencing of smaller fragments. Methods include Illumina short read, PacBio Hifi, and Oxford nanopore.

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The South American cane toad (*Rhinella marina*) is classified as Least Concern on the Red List. © Daniel Shaykevich

The South American cane toad is invasive in many parts of the world, and has been the subject of numerous genomic approaches. One study reported the use of CRISPR gene editing to knock-out a toxin hydrolase that converts toad toxin from its storage form to a lethal active form to reduce the impact on predators of cane toads in Australia (Cooper et al., 2020). © Daniel Shaykevich