Advancing Understanding of Amphibian Evolution, Ecology, Behavior, and Conservation with Massively Parallel Sequencing

W. Chris Funk, Kelly R. Zamudio, and Andrew J. Crawford

Abstract Genomics has great potential to advance understanding of amphibian evolution, ecology, and behavior, as well as to improve conservation of this highly imperiled class of vertebrates. However, application of new massively parallel sequencing technology to amphibians lags behind its application to other vertebrates, due in part to their large, repetitive genomes, making genome assembly challenging. The goal of our chapter is to outline ways in which population genomics – coupled with field biology, experiments, and modeling – can deepen our understanding of basic and applied questions in amphibian evolutionary ecology and conservation. We start by discussing potential applications of genomics to several long-standing questions in amphibian evolution, ecology, and behavior, including phylogenetic relationships, phylogeography, sex chromosome evolution, population structure and demography, local adaptation, and mating systems and sexual selection. We then highlight opportunities for improving amphibian conservation with genomics, focusing on hybridization, disease evolution and ecology, and captive breeding programs. Next, we provide strategies for moving amphibian genomics forward in the face of challenges such as few available reference genomes and large repetitive genomes, including a bold proposal for whole genome sequencing of a minimum of one species per amphibian family. We conclude by providing suggestions for maximizing the potential of genomics to advance understanding of amphibian evolutionary ecology and conservation and recommendations for getting started in genomics.

Keywords Amphibian · Local adaptation · Massively parallel sequencing · Population genomics

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1 Introduction

Massively parallel sequencing (MPS) has enormous yet largely untapped potential to advance understanding of the evolution, ecology, behavior, and conservation of amphibians, including frogs (Anura), salamanders (Caudata), and caecilians (Gymnophiona). Advances in sequencing technology and computational power have enabled the genomics era, in which vast quantities of DNA or RNA can be sequenced relatively quickly and cheaply to address questions in new, creative, and more powerful ways than was possible with traditional dye-termination sequencing technology like Sanger sequencing (Rokas and Abbot 2009; Allendorf et al. 2010). MPS platforms, such as Illumina's sequencing by synthesis technology, PacBio's single molecule real-time (SMRT) sequencing, or the Oxford Nanopore PromethION system, generate hundreds of millions of sequence reads – typically in the range of 100 to 10,000+ base pairs (bp) – from DNA or RNA (Glenn 2011). Depending on which library preparation protocol is used, the researcher can sequence the entire genome or target specific regions or loci (Andrews et al. 2016; Jones and Good 2016). By ligating unique individual barcodes to DNA libraries, it is possible to multiplex (pool) dozens to hundreds of individuals in a single MPS run, increasing efficiency and reducing costs (Baird et al. 2008). This flexibility in MPS has resulted in a plethora of different genomic techniques suited for addressing a wide variety of questions (Table 1).

Analysis of MPS data using population genomics – a subfield of genomics – is a particularly powerful framework for answering many open questions in amphibian evolution, ecology, behavior, and conservation. Population genomics is defined as the study of numerous loci (hundreds of genes to millions of polymorphisms) to understand the microevolutionary processes (mutation, genetic drift, gene flow, and selection) that influence genetic variation within and among populations (Black et al. 2001; Luikart et al. 2003). The advantage of population genomics over traditional population genetics, which involves the use of fewer loci (typically 1–20), is twofold. First, population genomics allows identification and analysis of loci under natural selection, providing a new window into patterns and genetic mechanisms of adaptation (Beaumont and Nichols 1996; Beaumont and Balding 2004; Foll and Gaggiotti 2008). Second, by allowing the identification and filtering of genetic variation under selection, population genomics allows unbiased inference of evolutionary history via accurate estimation of genome-wide, neutral demographic parameters such as effective population size (N_e) and gene flow (N_em), without the confounding effects of natural selection (Luikart et al. 2003).

Amphibians have many characteristics that make them an excellent taxonomic group for genomic studies. First, unlike mammals, most amphibians have nucleated red blood cells, allowing extraction of large quantities of high-quality DNA. Second, many amphibian species are abundant (Burton and Likens 1975) and often congregate in dense breeding associations, providing sufficient sample sizes for population genomic analyses. Third, most species of amphibians have exposed eggs that allow

Table 1 Massively parallel sequencing (MPS) methods used commonly for addressing questions in evolution, ecology, behavior, and conservation

Method name	Brief description	Amphibian applications	Amphibian examples
Whole-genome sequencing (WGS), assembly, and annotation (Pritchard 2011)	Sequencing of nearly all base pairs in the genome. Can range from reference-standard genome to sequencing at low depth (genome skimming)	Local adaptation (to map outliers and infer function); historical demography; phylogenomics (to identify independent markers); chromosome evolution	*Xenopus tropicalis* (Hellsten et al. 2010); *Nanorana parkeri* (Sun et al. 2015); *Xenopus laevis* (Session et al. 2016); *Rana catesbeiana* (Hammond et al. 2017)
Whole-genome resequencing (WGR; Fuentes-Pardo and Ruzzante 2017)	Short-read MPS sequencing nearly all base pairs in the genome from multiple individuals and assembling reads to a high-quality reference genome	Historical demography, speciation, hybridization, genomic bases of adaptation, speciation, and introgression	*Nanorana parkeri* (Wang et al. 2018)
Restriction site-associated DNA sequencing (RADseq; Andrews et al. 2016)	A suite of reduced representation methods that sequence and genotype loci adjacent to restriction sites	Population structure; test for signatures of local adaptation; markers for pedigrees; characterization of hybridization	*Rhinella marina* (Trumbo et al. 2016); *Bufo andrewsi* (Guo et al. 2016); *Ambystoma talpoideum*, *A. opacum* (Nunziata et al. 2017)
Targeted capture (Jones and Good 2016)	A reduced representation method that enriches for targeted regions of the genome using labeled oligonucleotides	Population structure; test for signatures of local adaptation; markers for pedigrees; characterization of hybridization; targeting functional genes; phylogenomics	Anura (Portik et al. 2016); *Ambystoma californiense*, *A. mavortium* (McCartney-Melstad et al. 2016)
Ultraconserved elements (UCE; Faircloth et al. 2012)	Highly conserved regions of the genome that can be used to generate sequence data at orthologous loci from evolutionarily distant taxa	Phylogenomics; phylogeography	*Kaloula* spp. (Alexander et al. 2017); *Plethodon serratus* (Newman and Austin 2016)
Anchored phylogenomics (Lemmon et al. 2012)	Sequencing and genotyping of libraries enriched for conserved, anonymous, and/or functional loci	Phylogenomics; phylogeography	Microhylidae (Peloso et al. 2016); Hylidae, Bufonidae, Ranidae (Barrow et al. 2018); Terraranae (Heinicke et al. 2018)

(continued)

Table 1 (continued)

Method name	Brief description	Amphibian applications	Amphibian examples
Transcriptomics (e.g., RNAseq; Wang et al. 2009)	Analysis of gene expression levels, usually conducted by sequencing cDNA from RNA (RNAseq); obtaining complete coding sequence of expressed genes	Local adaptation (e.g., gene expression in different populations); disease ecology (e.g., gene expression with and without pathogen); obtaining candidate genes (e.g., sex determination gene ID); scans for increased rates of non-synonymous substitutions	*Bufo viridis* (Gerchen et al. 2016); *Lithobates clamitans*, *Pseudacris regilla* (Robertson and Cornman 2014); *Andrias davidianus* (Che et al. 2014); *Rana chensinensis*, *R. kukunoris* (Yang et al. 2012)
Metabarcoding (Caporaso et al. 2011)	MPS of DNA barcode genes for species delineation	Characterization of amphibian skin or gut microbial diversity	*Lithobates pipiens*, *Pseudacris maculata*, *Ambystoma tigrinum* (McKenzie et al. 2012)
Environmental DNA (eDNA) metabarcoding (Taberlet et al. 2012)	MPS of DNA barcode from an environmental sample (e.g., skin sloughed off in water)	Monitoring of amphibian diversity in streams or ponds	*Bufo bufo*, *B. calamita*, *Hyla meridionalis*, *Pelobates cultripes*, *Pelodytes punctatus*, *Pelophylax* sp., *Rana dalmatina*, *Lissotriton helveticus*, *Salamandra salamandra*, *Triturus marmoratus* (Valentini et al. 2016); *Hylodes phyllodes*, *H. asper*, *Cycloramphus boraceiensis*, *Thoropa taophora*, *Vitreorana uranoscopa*, *Scinax trapicheiroi*, *Bokermannohyla* sp. aff. *circumdata*, *Bokermannohyla*, *Aplastodiscus eugenioi*, *Phasmahyla cruzi* (Lopes et al. 2017)

For each MPS method, we provide its name (and reference for method), a brief description, most appropriate uses for amphibian research, and examples of its use in amphibians

for application of gene-editing techniques such as CRISPR/Cas for gain or loss of function studies (Fei et al. 2014; Bhattacharya et al. 2015; Elewa et al. 2017).

At the same time, amphibians have posed some significant challenges for genomic studies. The main challenge is that because of the large size of most amphibian genomes, with median sizes of 4.1 gigabases (Gb) for frogs, 5.6 Gb for caecilians, and 32 Gb for salamanders (Gregory 2011; Liedtke et al. 2018), fewer amphibian genomes have been sequenced relative to other vertebrates (Table 2). Compounding the problem of large genomes is the highly repetitive structure of many amphibian genomes, making genome assembly computationally challenging. Having a reference genome available for aligning reads or contigs improves the accuracy of genotyping and facilitates determining the potential function of loci (Manel et al. 2016; Toews et al. 2016). A third challenge of population genomic studies of amphibians is that, since amphibians often have low gene flow and high population structure (Crawford 2003; Zeisset and Beebee 2008), it can be more challenging to identify loci with a genetic signature of divergent selection using genome scans (Francois et al. 2016). Nonetheless, these challenges of studying amphibian genomics – large genomes with repetitive elements and high population structure – are also some of the reasons that they are interesting taxa for genomic studies.

Although MPS and genomic analyses are powerful new tools for understanding amphibian biology, we argue that the best research is integrative, combining new genomic technology with tried-and-true approaches such as field observations, controlled experiments, and modeling. We (WCF, KRZ, and AJC) are field biologists in addition to evolutionary geneticists/genomicists. We are not proposing that genomics will replace classic approaches for studying amphibian biology. Rather, genomics will expand what is possible to know about these fascinating organisms by allowing us to characterize genetic variation across a much larger proportion of the genome to understand evolutionary and ecological processes more deeply than previously imaginable.

The goal of our chapter is to provide an overview of potential applications of MPS and genomics to advance studies of amphibian evolution, ecology, behavior, and conservation. A handful of recent reviews on amphibian genetics and genomics have been published, but none focuses exclusively on genomics and all focus on amphibian conservation (Storfer et al. 2009; McCartney-Melstad and Shaffer 2015; Shaffer et al. 2015). Here, we restrict our discussion to the potential of MPS and genomics to advance understanding of basic questions in evolution, ecology, and behavior, as well as applied questions in amphibian conservation and management. We first highlight opportunities for applying genomics to basic questions about amphibian evolutionary ecology, particularly in the areas of phylogenomics, phylogeography, chromosome evolution, population structure and demography, local adaptation, and mating systems. We then highlight opportunities for improving amphibian conservation with genomics, focusing on characterizing hybridization between invasive and native amphibian species, understanding disease dynamics, and designing captive breeding programs. Next, we discuss challenges of genomic studies of amphibians in more depth and provide possible solutions. Finally, we conclude by discussing how genomics can best be harnessed to advance

Table 2 List of target amphibian families for whole genome sequencing (WGS), with one suggested species for each taxonomic family

Order	Family	Genus	Species	Motive	Progress
Anura	Allophrynidae	*Allophryne*	*ruthveni*	Sister to Centrolenidae	None
Anura	Alsodidae	*Eupsophus*	*calcaratus*	PD	None
Anura	Alytidae	*Alytes*	*obstetricans*	Males carry fertilized eggs	Pending
Anura	Aromobatidae	*Rheobates*	*palmatus*	Adaptation to elevation	Pending
Anura	Arthroleptidae	*Arthroleptis*	*poecilonotus*	Direct development; long finger	Pending
Anura	Ascaphidae	*Ascaphus*	*truei*	Co-sister to all other anurans	In progress
Anura	Batrachylidae	*Batrachyla*	*leptopus*	PD	None
Anura	Bombinatoridae	*Bombina*	*variegata*	Hybrid zones	In progress
Anura	Brachycephalidae	*Brachycephalus*	*didactylus*	Very small tetrapod	None
Anura	Brevicipitidae	*Breviceps*	*macrops*	Direct development. Adapted to sand	None
Anura	Bufonidae	*Atelopus*	*zeteki*	Conservation	In progress
Anura	Bufonidae	*Rhinella*	*marina*	Invasive species	Published (Edwards et al. 2018)
Anura	Calyptocephalellidae	*Calyptocephalella*	*gayi*	PD	Pending
Anura	Centrolenidae	*Hyalinobatrachium*	*fleischmanni*	Parental care	In progress
Anura	Ceratobatrachidae	*Platymantis*	*montanus*	Direct development; speciation	None
Anura	Ceratophryidae	*Lepidobatrachus*	*laevis*	Small genome	In progress
Anura	Conrauidae	*Conraua*	*goliath*	World's biggest frog. Tadpole diet specialization	None
Anura	Craugastoridae	*Craugastor*	*fitzingeri*	Resistant to disease	RNAseq data
Anura	Cycloramphidae	*Cycloramphus*	*boraceiensis*	PD	None
Anura	Dendrobatidae	*Oophaga*	*pumilio*	Rapid color evolution	Published (Rogers et al. 2018)
Anura	Dicroglossidae (Dicroglossinae)	*Nanarana*	*parkeri*	Adaptation to high elevation	Published (Sun et al. 2015)
Anura	Eleutherodactylidae	*Eleutherodactylus*	*coqui*	Model of evodevo in frogs	In progress

Anura	*Geobatrachus* sedis incertis	*Geobatrachus*	*walkeri*	PD. Direct development	None
Anura	Heleophrynidae	*Hadromophryne*	*natalensis*	PD	None
Anura	Hemiphractidae	*Gastrotheca*	*cornuta*	Frog "placenta." Conservation	Pending
Anura	Hemisotidae	*Hemisus*	*marmoratus*	PD	None
Anura	Hylidae	*Hyla*	*arborea*	Well studied	In progress
Anura	Hylidae	*Pseudacris*	*feriarum*	Hybrid zones	In progress
Anura	Hylidae	*Dendropsophus*	*ebraccatus*	Phenotypic plasticity	In progress
Anura	Hylodidae	*Hylodes*	*japi*	PD	None
Anura	Hyperoliidae	*Hyperolius*	*riggenbachi*	Color pattern polymorphism	None
Anura	Leiopelmatidae	*Leiopelma*	*hochstetteri*	Co-sister to all other anurans	Pending
Anura	Leptodactylidae (Leiuperinae)	*Physalaemus*	*pustulosus*	Mate choice, sexual selection	In progress
Anura	Leptodactylidae (Leptodactylinae)	*Leptodactylus*	*pentadactylus*	Sex chromosome evolution	Pending. RNAseq data
Anura	Limnodynastidae	*Limnodynastes*	*dumerilii*	PD	In progress
Anura	Mantellidae	*Mantella*	*aurantiaca*	Convergent evolution. Breeds in captivity	In progress
Anura	Megophryidae	*Scutiger*	*boulengeri*	Highest elevation frog	RNAseq data
Anura	Micrixalidae	*Micrixalus*	*kottigeharensis*	PD	None
Anura	Microhylidae	*Microhyla*	*fissipes*	Thyroid hormone receptors and metamorphosis	RNAseq data
Anura	Myobatrachidae	*Platyplectrum*	*ornatum*	Very small amphibian genome	In progress
Anura	Myobatrachidae	*Rheobatrachus*	*silus*	Gastric brooder	None
Anura	Nasikabatrachidae	*Nasikabatrachus*	*sahyadrensis*	EDGE Top-100 Amphibian #04	None
Anura	Nyctibatrachidae	*Nyctibatrachus*	*humayuni*	PD	None
Anura	Odontobatrachidae	*Odontobatrachus*	*natator*	PD	None
Anura	Odontophrynidae	*Proceratophrys*	*moratoi*	PD	None

(continued)

Table 2 (continued)

Order	Family	Genus	Species	Motive	Progress
Anura	Pelobatidae	*Pelobates*	*cultripes*	Developmental plasticity	In progress
Anura	Pelodryadidae	*Ranoidea* (*Litoria*)	*alboguttata*	Cell metabolism and dormancy	RNAseq data
Anura	Pelodytidae	*Pelodytes*	*punctatus*	PD	Pending
Anura	Petropedetidae	*Petropedetes*	*parkeri*	Striking sexual dimorphism	None
Anura	Phrynobatrachidae	*Phrynobatrachus*	*auritus*	Ecological gradients	None
Anura	Phyllomedusidae	*Agalychnis*	*callidryas*	Hatchling plasticity	RNAseq data
Anura	Pipidae	*Hymenochirus*	*boettgeri*	Small genome	In progress
Anura	Pipidae	*Xenopus*	*laevis*	Model organism. Tetraploid	Published (Session et al. 2016)
Anura	Pipidae	*Xenopus*	*tropicalis*	Model organism	Published (Hellsten et al. 2010)
Anura	Ptychadenidae	*Ptychadena*	*pumilio*	PD	None
Anura	Pyxicephalidae	*Pyxicephalus*	*adspersus*	ZW sex chromosomes	Published (Denton et al. 2018)
Anura	Ranidae	*Pelophylax*	*lessonae*	hybrid species	RNAseq data
Anura	Ranidae	*Rana*	*mucosa*	Conservation	In progress
Anura	Ranidae	*Rana*	*temporaria*	Hybrid species	In progress
Anura	Ranidae	*Rana*	*sylvatica*	Freeze tolerance	In progress
Anura	Ranidae	*Rana*	*catesbeiana*	Invasive species	Published (Hammond et al. 2017)
Anura	Ranixalidae	*Indirana*	*gundia*	EDGE Top-100 Amphibian #88	None
Anura	Rhacophoridae	*Chiromantis*	*xerampelina*	Desiccation resistance	None
Anura	Rhinodermatidae	*Rhinoderma*	*darwinii*	Brooding pouch	None
Anura	Rhinophrynidae	*Rhinophrynus*	*dorsalis*	Deep PD	Pending
Anura	Scaphiopodidae	*Spea*	*bombifrons*	Phenotypic plasticity, speciation	In progress
Anura	Sooglossidae	*Sooglossus*	*sechellensis*	EDGE Top-100 Amphibian #70	None

Caudata	Ambystomatidae	*Ambystoma*	*mexicanum*	Model organism	Published (Nowoshilow et al. 2018)
Caudata	Amphiumidae	*Amphiuma*	*means*	Elongation. PD	None
Caudata	Cryptobranchidae	*Andrias*	*davidianus*	Biggest amphibian	RNAseq data
Caudata	Hynobiidae	*Hynobius*	*chinensis*	Salamander model	RNAseq data
Caudata	Plethodontidae	*Desmognathus*	*fuscus*	Reduced genome size	None
Caudata	Proteidae	*Necturus*	*alabamensis*	EDGE Top-100 Amphibian #27	None
Caudata	Rhyacotritonidae	*Rhyacotriton*	*olympicus*	PD	None
Caudata	Salamandridae	*Pleurodeles*	*waltl*	Limb regeneration	Published (Elewa et al. 2017)
Caudata	Salamandridae	*Salamandra*	*salamandra*	Viviparity	Pending
Caudata	Sirenidae	*Pseudobranchus*	*striatus*	PD	None
Gymnophiona	Siphonopidae	*Microcaecilia*	*unicolor*	PD	Pending
Gymnophiona	Chikilidae	*Chikila*	*fulleri*	PD	None
Gymnophiona	Dermophiidae	*Geotrypetes*	*seraphinii*	PD	In progress
Gymnophiona	Herpelidae	*Herpele*	*squalostoma*	PD	None
Gymnophiona	Ichthyophiidae	*Ichthyophis*	*bannanicus*	PD	Pending
Gymnophiona	Indotyphlidae	*Grandisonia*	*sechellensis*	PD	None
Gymnophiona	Rhinatrematidae	*Rhinatrema*	*bivittatum*	Co-sister to all other caecilians	Published[a]
Gymnophiona	Scolecomorphidae	*Crotaphatrema*	*tchabalmbaboensis*	PD	None
Gymnophiona	Siphonopidae	*Microcaecilia*	*dermatophaga*	PD	None
Gymnophiona	Typhlonectidae	*Typhlonectes*	*compressicauda*	PD	Pending

Some families are represented more than once since these have additional species with WGS projects already in progress or published. Species are recommended based on the following criteria: (1) preliminary data, (2) scientific interest or interesting biology, (3) conservation interest, and, by default, (4) phylogenetic diversity (PD). "EDGE" refers to rank among amphibian species based on evolutionarily distinct and globally endangered criteria (Isaac et al. 2012). While additional WGS projects are likely going on that we are unaware of, we attempted to indicate Progress as follows. "Published" includes BioRxiv or publicly available data. "In progress" implies a status between initial data collection and a complete but unpublished draft. "RNAseq data" indicates species with transcriptomic data posted in the Short Read Archive at NCBI. "Pending" implies there is a laboratory or research community in place with interest in starting a WGS effort

[a]Publication not yet available, but very high-quality assembly posted online at https://vgp.github.io/genomeark/Rhinatrema_bivittatum/

understanding of amphibians and providing advice for researchers considering applying genomics for their amphibian study species.

2 Opportunities for Advancing Understanding of Amphibian Evolution, Ecology, and Behavior with Genomics

2.1 Phylogenomics

Phylogenomics, the analysis of genomic data for phylogenetic inference, has grown rapidly in the last decade, providing increased resolution in the tree of life for many vertebrate lineages (Jarvis et al. 2014; Ruane et al. 2015; Streicher et al. 2018; Chakrabarty et al. 2017; Irisarri et al. 2017). The advances in phylogenomic data acquisition have been propelled primarily by sequence-capture methods that selectively capture previously identified genomic regions (Faircloth et al. 2012; Lemmon et al. 2012; McCormack and Faircloth 2013), by transcriptomic datasets based on expressed gene sequences (Irisarri et al. 2017), and by the availability of large-scale PCR-based nuclear protein-coding gene panels that can be sequenced with MPS (Shen et al. 2013). Compared to earlier multilocus methods that relied on a few to dozens of genes, these new methods now allow for analyses of hundreds of nuclear loci across a large sample of individuals, which has proven useful in resolving some of the most problematic nodes in the tree of life.

The application of phylogenomics to amphibians is rapidly growing. Recently, phylogenomic methods have helped resolve higher-level relationships among salamanders (Shen et al. 2013) and frogs (Feng et al. 2017). The salamander study addressed controversial relationships within Lissamphibia. The close relationship between frogs and salamanders (the clade Batrachia) is repeatedly recovered in molecular studies (Frost et al. 2006; Roelants et al. 2007; Zhang and Wake 2009; Pyron and Wiens 2011). However, one study based on 26 nuclear genes (Fong et al. 2012) supported a caecilian–salamander sister relationship. A phylogenomic analysis confirmed the monophyly of Batrachia and confirmed the monophyly of the internally fertilizing salamanders (Salamandroidea; all salamanders exclusive of Hynobiidae, Cryptobranchidae, and Sirenidae; Shen et al. 2013) in contrast to earlier studies based on a smaller number of markers (Frost et al. 2006). This newer study also provided a strongly supported phylogeny of all major frog lineages and estimated a much younger divergence time for frog lineages than inferred by earlier studies (Feng et al. 2017). In particular, divergence-time analyses indicated that three species-rich clades (Hyloidea, Microhylidae, and Natatanura), which comprise ~88% of extant anuran species, underwent simultaneous and rapid diversification at the Cretaceous–Paleogene (K–Pg) boundary. Thus, the K–Pg mass extinction may have triggered frog radiations by creating new ecological opportunities, as has been suggested for other animal groups (Feng et al. 2017).

An accurate inference of the amphibian tree of life provides the framework for important studies of macroevolution, diversification, and biogeography. These two recent studies demonstrate that phylogenomics has the potential to greatly increase resolution of our inferred topologies.

2.2 Genomic Data for Phylogeographic Inference

The field of phylogeography focuses on the evolutionary and ecological processes that shape the spatial distribution of genetic variation within species. At its inception, the field bridged microevolutionary processes within populations and macroevolutionary patterns at larger scales (Avise et al. 1987), providing a framework to examine the factors influencing population divergence, persistence, and change over time. This framework spurred a large number of comparative studies that elucidated common landscape barriers impeding gene flow, identified "suture zones" in regional faunas of diverse taxa, and detailed the historical spatial and demographic processes acting on populations (Soltis et al. 2006; Bell et al. 2012; Barrow et al. 2017). The focus on population histories placed phylogeography squarely between population genetics and systematics, and many of the earliest studies on amphibians relied on a combination of rapidly evolving loci, including mtDNA markers, some nuclear genes, and microsatellites (Crawford 2003; Zamudio and Savage 2003; Funk et al. 2007, 2008; García-R et al. 2012; Lemmon and Juenger 2017). Genomic-scale data derived from target capture of orthologous loci, either as sequences or SNPs, have lifted previous limitations on the availability of adequate markers, including for amphibians, which have large genomes compared to other tetrapods. Phylogeographic studies are now expanding their reach by incorporating genome-scale data, providing an unprecedented level of genetic detail, fostering new techniques for tests of divergence, and synergies with landscape genetics and population genetics (McCormack et al. 2013; Barrow et al. 2014; Bell et al. 2015; Garrick et al. 2015; Pie et al. 2018).

A primary goal of phylogeography is the test of concordance of divergence among species, based on the hypothesis that co-distributed organisms should exhibit a concerted response to the same historical processes. The recent study by Barrow et al. (2018) exemplifies the application of genomic data to the test of concordance. Using target capture, they compared orthologous loci across 36 populations of 4 frog species distributed across known biogeographic barriers in the southeastern USA. Target capture, combined with thorough population sampling, allowed for tests of concordance at various levels of variation: among sites within a locus, among multiple loci within a species, among multiple species within a region, and between established biogeographic provinces (Barrow et al. 2018). The study found similar patterns within species, but high discordance among species, with little correspondence of genetic patterns with putative biogeographic barriers.

Discordance in phylogeographic structure is in some ways a more interesting outcome, because it points to differences among species in traits that mediate their

response to landscape barriers (Bell et al. 2017; Barrow et al. 2018; Polato et al. 2018). This is an exciting area of active research and uncovering what those traits are, and identifying the genes that underlie them, has the potential to complete the links between selection on phenotypes, mechanisms of divergence, and species differences in phylogeographic structure and speciation (Crispo 2008; Zamudio et al. 2016a; Polato et al. 2017). This focus, which has been termed "trait-based phylogeography" (Paz et al. 2015), will become possible by including genomic-scale data across populations and the measurement of genetic variability in functional traits that accelerate or deter divergences within species.

2.3 Sex Chromosome Evolution

The evolution of sex chromosomes offers a valuable opportunity to study how genomes respond to changes in gene copy number. The process of gene duplication is fundamental to the origin of new genes and novel phenotypes, yet changes in amounts of gene product may also cause gene dose problems (Bachtrog 2006). The challenge of dosage compensation is faced by all species with heteromorphic sex chromosomes, where the two sexes have zero, one, or two copies of each sex chromosome. All mammals (except monotremes) have an XX/XY genetic sex-determining mechanism, and all birds have the opposite, ZZ/ZW system. Thus, these clades solved the dose problem early in their history and are recalcitrant to further change in sex chromosomes. Amphibians and nonavian reptiles, in contrast, have evolved and re-evolved sex chromosomes many times in their history, providing researchers with replicated potential case studies of the evolution of sex chromosomes (Hillis and Green 1990; Ezaz et al. 2009; Nakamura 2009; Abbott et al. 2017).

As of 2014, systems of sex determination had been resolved in 173 species of amphibians, including one caecilian, revealing 28 species with XY male heterogamety, 16 species with ZW female heterogamety, and 1 case of OW female heterogamety, implying at least 18 independent evolutionary transitions (Ashman et al. 2014). The neotropical frog, *Leptodactylus pentadactylus*, now holds the record for most sex chromosomes in a vertebrate, with six X and six Y, accompanied by just ten autosomes (Gazoni et al. 2018). Mechanisms of sex determination evolve quickly in amphibians, with multiple systems found within taxonomic families and genera, or even within a single species. Conspecific populations of the Japanese frog, *Rana rugosa*, have one of three sex-determining systems: XX/XY, ZZ/ZW, or homomorphic sex chromosomes (Miura et al. 1998). Sex is determined genetically in all amphibians, thus even species with homomorphic sex chromosomes likely have a heterogametic sex (male or female). Evolutionarily, transitions are equally common between homomorphic versus heteromorphic sex chromosomes and between male versus female heterogamety (Pennell et al. 2018). Many species of amphibians, therefore, may be found in some initial state of evolution from homomorphic to

heteromorphic sex chromosomes, or low amounts of recombination may prevent divergence between homomorphic sex chromosomes (Guerrero et al. 2012).

Roughly 95% of amphibian species still lack information on which is the heterogametic sex. Progress has been slow because only specialized laboratories possess the knowledge and the dedication to produce karyotypes to search for heteromorphic versus homomorphic sex chromosomes in amphibians (Schmid et al. 2010). As most amphibians fall in the latter category, determining which sex is the heterogametic sex requires genetic tools. Traditionally, sex-linked markers are obtained by creating linkage maps. These maps can be developed from molecular genotyping including MPS approaches, but most organisms are not readily amenable to crossing experiments.

Recently, MPS genotyping has been used to develop sex-linked markers directly from a collection of DNA samples from multiple individuals of known sex, without the need for linkage maps or cytogenetics. Gamble and Zarkower (2014) outline a workflow based on restriction site-associated DNA sequencing (RADseq), which generates SNP genotypes from anonymous loci throughout the genome (Table 1), and applied this method to anoles and geckos (Gamble et al. 2015). By performing standard RADseq experiments on males and females, sex-specific markers can be recovered, screened for false positives, and validated on additional samples using PCR and Sanger sequencing. The required density of markers will depend on the absolute size of the sex-specific region and its size relative to the pseudoautosomal region (PAR) of the homomorphic sex chromosome.

A similar approach to recover sex-linked markers from the North American green frog (*Rana clamitans melanota*) involved DArT complexity reduction combined with MPS in a proprietary technology called DArTseq™ (Lambert et al. 2016). This genotyping by sequencing (GBS)-type method is similar to RADseq except that single-nucleotide polymorphisms (SNPs) are obtained preferentially from gene-rich regions, avoiding repetitive regions which can be especially problematic in amphibians. The authors found 15 SNPs and 8 presence-absence markers that together established that this species shows an XY/XX or male-heterogametic system. This finding confirmed a much earlier study based on allozyme data (Elinson 1983).

MPS approaches provide more than just information on the genetic basis of sex determination. Lambert et al. (2016) also found that sex-linked markers had variable levels of female homozygosity and male heterozygosity, reflecting variation in distances to the putative sex locus on the otherwise homomorphic sex chromosomes. In other words, loci located between the PAR and the sex-determining genes may experience some recombination and show intermediate levels of sex linkage. Additionally, since RADseq and DArTseq methods provide a few hundred base pairs of DNA sequence, the marker sequences themselves can be compared to reference genomes. In the case of the green frog, one marker was a putative paralog of *DMRT1*, a gene related to sex determination in many metazoans [including in *Xenopus laevis*, but not in *X.* (*Silurana*) *tropicalis*; Lambert et al. 2016].

Studies of other vertebrates have yielded a few dozen candidate genes involved in sex determination and sex differentiation. These genes can be accessed in amphibians with no previous genetic information through the application of RNAseq

(Wang et al. 2009). Furthermore, the relative positions of these genes may potentially be inferred from their positions in published genomes. *Xenopus* and *Nanorana* genomes show remarkable levels of synteny despite 266 million years of divergence (Sun et al. 2015). Gerchen et al. (2016) took advantage of this conservation of gene function and gene order to obtain DNA sequences of candidate sex genes using RNAseq of a single individual of *Bufo viridis*. From the resulting transcriptome, they further developed microsatellite loci located in coding regions of these genes in *Xenopus*. Finally, sex linkage was confirmed in *Bufo viridis* by genotyping these variable markers in parents and their offspring of known sex.

Not only do sex-determining mechanisms evolve rapidly, including sex chromosome turnover (Miura et al. 1998), sex steroids and steroid mimics can override sex-determining genes and reverse the gonadal sex of adults of some amphibian species (Hayes 1998). Combining these unusual amphibian traits with genetic recombination between sex chromosomes (see above) should be a warning to researchers using MPS methods based on reduced representation to study sex chromosome evolution. Carefully designed experiments should sample numerous adults of each sex to minimize false positives and catch possible sex reversals. Surveying multiple populations may reveal environmental correlates of sex reversal, such as contamination by endocrine disruptors, or may reveal additional cases of sex chromosome turnover within species (Lambert et al. 2016).

Whole genome resequencing (WGR) is currently nontrivial in amphibians; thus, until cheaper and more powerful sequencing methods become available, the study of sex chromosome evolution will benefit from new MPS-based genome reduction methods, such as RADseq. While a reference genome is an invaluable tool for any evolutionary genetic study, polymorphism data are even more important to link phenotypic and gonadal sex of the individual with potential sex-determining genes. Sex-linked loci identified through RADseq approaches could be further screened in more individuals and species using exon capture; although without a reference genome, complete gene sequences would be difficult to obtain. Alternatively, RNAseq could provide complete coding sequences, but the genes obtained may depend on the tissue, ontogeny, condition, and environment of the donor animal. In the study by Gerchen et al. (2016), RNA was extracted from six tissues from one adult male toad, providing 37 candidates genes but some with only partial coverage. While RADseq-based approaches for finding sex-linked markers obviate the need for linkage maps generated from experimental crosses between individuals (Gamble and Zarkower 2014), such maps may be helpful in assembling large, repetitive genomes, as demonstrated for the 32 Gb axolotl genome (*Ambystoma mexicanum*; Smith et al. 2018).

2.4 Population Structure and Demography

The most common application of genetics to amphibians is the study of population structure, which involves characterizing the distribution of genetic variation within and among populations (Wright 1965; Allendorf and Phelps 1981), inferring the

evolutionary processes contributing to these patterns (primarily genetic drift and gene flow) (Slatkin 1981), and estimating important population genetic parameters such as effective population size (N_e) (Kimura and Crow 1963; Do et al. 2014). A related field is landscape genetics, which combines population genetics, landscape ecology, and spatial statistics to understand how complex landscapes affect patterns and rates of gene flow (Manel et al. 2003; Balkenhol et al. 2016). Understanding population structure and demography of amphibians is especially important in light of amphibian population declines, given that N_e and gene flow mold the distribution of genetic variation, which in turn influences inbreeding depression, adaptive potential, and population persistence (Allendorf et al. 2013).

Population genomics will provide greater accuracy and power than ever to characterize population structure and demography of amphibians. First, population genomics allows identification of loci with a signature of divergent selection (Hohenlohe et al. 2010). It has been shown that inclusion of these non-neutral loci can severely bias estimates of population structure, gene flow, and other demographic parameters (Luikart et al. 2003). Thus, identification and removal of these loci should increase accuracy of estimates. Second, population genomics simply provides more independent loci from a larger proportion of the genome with which to characterize population structure and estimate population genetic parameters, increasing precision of estimates, as well (Luikart et al. 2003).

Characterization of population structure and estimation of N_e, gene flow, and related demographic parameters requires neutral genetic markers. Thus, MPS approaches that provide neutral markers are required. SNP data generated from RADseq are appropriate for any analyses that require data from independent loci spread across the genome, such as characterizing population structure, estimating N_e, or testing for population bottlenecks. Moreover, if longer contigs are generated from paired-end RADseq libraries or other reduced representation approaches such as anchored phylogenomics (Lemmon et al. 2012), which provide haplotype blocks with multiple SNPs, then it is possible to use coalescent-based approaches to infer population divergence, gene flow, N_e, and changes in N_e through time simultaneously (Drummond et al. 2012).

Field approaches should ideally be combined with inference from population genomics to understand amphibian demography and dispersal. For example, field estimates of dispersal based on multistate capture-mark-recapture (CMR) analysis have been successfully combined with population genetic estimates of gene flow to more fully understand contemporary and historic patterns and rates of movements among amphibian populations (Funk et al. 2005; Lowe et al. 2006). Each approach has its strengths and limitations, but integrating both provides a more complete picture of movement across the landscape. CMR estimates of movement provide a detailed snapshot of contemporary movement for a limited number of populations, whereas genetic or genomic approaches are more useful for understanding deeper historic gene flow over a broader geographic sampling area.

A handful of studies have taken advantage of the power of MPS to address questions about demography and gene flow in amphibians. Nunziata et al. (2017) genotyped two species of salamanders (*Ambystoma talpoideum* and *A. opacum*)

with double-digest RADseq (ddRAD; Peterson et al. 2012) and tested whether coalescent-based analysis could detect changes in population sizes documented in the field. For both species, coalescent models largely agreed with CMR estimates of population declines or increases, demonstrating the utility of population genomics for detecting changes in population size over ecological time scales. Trumbo et al. (2016) also used a population genomic dataset consisting of over 20,000 SNPs to test the central marginal hypothesis (CMH) in the invasive range of *Rhinella marina* in Australia. The CMH predicts that genetic variation decreases from the core to edge of species' range, potentially limiting adaptation to new environmental conditions at the range margin (Eckert et al. 2008). They found support for the CMR in the southern portion of the species' range, but not in the northwestern or northeastern part of its range, which has important implications for management of this damaging, invasive species in Australia. These two studies provide evidence of the huge potential of MPS and population genomics to understand amphibian demography and population structure.

2.5 *Local Adaptation*

Amphibians occupy heterogeneous environments and have relatively low dispersal and gene flow compared to other vertebrates such as birds and mammals (Ward et al. 1992). This combination of high habitat heterogeneity and low gene flow suggests they will often be highly locally adapted. The fact that many amphibian species live in extreme environments (e.g., deserts, tree canopy, alpine ponds and lakes, high latitudes, caves) despite being ectothermic and having permeable skin speaks to their adaptive potential (Duellman and Trueb 1986). The observation that many amphibian species span dramatic environmental gradients also indicates adaptive divergence within species (Berven 1982; Palo et al. 2003; Funk et al. 2016). Understanding the ultimate environmental drivers and proximate genetic mechanisms of adaptive divergence is a fundamentally important question in evolutionary biology. Characterizing patterns of adaption across real-world landscapes is also of the utmost importance in conservation for assuring that the maximum amount of additive genetic variation is conserved (McKay and Latta 2002; Funk et al. 2012). Understanding patterns of adaptation is also critical for making sure that source populations for augmentation of declining populations are not adaptively divergent from the target population, which can lead to outbreeding depression rather than the desired outcome of genetic rescue (Edmands 2007; Frankham et al. 2011).

Multiple analytical approaches are available in the field of population genomics for studying local adaptation, and the best MPS approach depends on which of these analyses will be applied. One analytical approach is to identify loci under divergent selection using genome scans, for example, to detect locus-specific F_{ST} values significantly higher than the baseline genome-wide average F_{ST} value (Beaumont and Nichols 1996; Beaumont and Balding 2004). Genome scans can be performed using a variety of marker types, but since these methods are designed to identify loci

with values higher than those observed at neutral loci, they require that the majority of loci are not under directional selection (Luikart et al. 2003). Given this requirement, WGR (Table 1) or RADseq (and other related reduced representation restriction enzyme-based methods) are two appropriate choices, but each has its strengths and limitations. WGR provides complete or nearly complete coverage of the entire genome but requires a reference genome, which currently exist for few amphibian species (Table 2). However, this approached could theoretically allow detection of natural section across all types of variation (not just SNPs) including structural variants (Toews et al. 2016; Fuentes-Pardo and Ruzzante 2017). In perhaps the first-ever application of WGR to amphibians, Wang et al. (2018) took advantage of the published reference genome of the Tibetan frog (*Nanorana parkeri*; Sun et al. 2015) by resequencing 63 more frogs at a depth of 6- to 17-fold coverage and recovered almost 9 million SNPs to infer historical demography, speciation, hybridization, and potential genomic bases of adaptation to high elevation environments. For a given budget, however, complete coverage of the entire genome comes at the cost of fewer individuals that can be sequenced, potentially resulting in lower power to detect selection at any given locus. In contrast, since RADseq is a reduced representation approach, many more individuals can be genotyped for a given budget than with WGR but at the cost of no coverage of a sizable percent of the genome (Baird et al. 2008; Andrews et al. 2016). If a reference genome is available, it is possible to map RADseq loci to the genome so that linkage disequilibrium can be calculated and the protocol can be fine-tuned to make sure that marker density is high enough to detect most loci under selection (Lowry et al. 2017; Catchen et al. 2017).

Another genomic analysis available for dissecting the genetic basis of adaptation is genome-wide association studies (GWAS; Stinchcombe and Hoekstra 2008). The basic premise behind GWAS is to identify loci and alleles correlated with variation in phenotypes. If populations have different values of phenotypic traits that are hypothesized to be adaptive in their respective environments, GWAS can estimate the presence and strength of statistical correlations between phenotypic differences and the frequency of alternative alleles at a locus. A conceptually similar analytical framework is genotype-environment association (GEA) methods, which are designed to test for correlations between genotypic variants and environmental variation (e.g., Joost et al. 2007; Coop et al. 2010; Frichot et al. 2013). As with genome scans, the most important criterion for choosing an appropriate MPS approach for GWAS and GEA is that it provides high-density coverage of the genome so that most loci influencing the phenotype can be detected. Whole-genome resequencing and the family of RADseq methods both fit this bill but with the same pros and cons discussed above for genome scans.

Yet another genomic approach for studying adaptive divergence is transcriptomics based on RNAseq (including the Iso-Seq method implemented by PacBio). Experiments provide three types of information: DNA and inferred amino acid sequences, gene diversity such as duplications, and quantitative gene expression patterns such as differences among populations or different environmental conditions (Zhen et al. 2012). The strength of this approach is that it focuses exclusively on functional, expressed genes that might underlie adaptive phenotypic differences

(Ghalambor et al. 2015). Differences in gene expression patterns could be caused by environmental or genetic differences, or both; thus, inferring adaption can be challenging. On the other hand, RNAseq data can be used for comparative analyses of the adaptive basis of natural selection, such as dN/dS ratios, which may have more power than some population genetic tests of selection (Zhai et al. 2009). One example of this approach applied to frogs looked for accelerated rates of non-synonymous substitution across expressed genes in Himalayan versus lowland species of *Rana* (Yang et al. 2012). Combining transcriptomics with other tools such as GWAS can also be a powerful integrative approach for understanding adaptive divergence.

The study of adaptation is a prime example of an area of study that requires both classic field observations and experiments (Endler 1986) in addition to population genomics for robust inferences. The first criterion for local adaptation is that phenotypes differ in different environments, which cannot be determined with genomics. Secondly, evidence is required that these phenotypic differences are adaptive (increase fitness) in the local environment. The gold standard for testing this is a reciprocal transplant experiment (Claussen et al. 1948), which is feasible for some amphibian species but not others (Urban et al. 2017). If a reciprocal transplant experiment is not feasible for a given species, then the combination of fieldwork showing among-population phenotypic differences, genome scans identifying loci under divergent selection, and GWAS showing that some of these same loci are related to the observed phenotypic differences provides compelling evidence that the phenotypic differences are adaptive. The key for successful studies of adaptation in nature is to combine traditional field and cutting-edge genomic approaches in creative and well-designed ways.

The application of genomics to studies of adaptation is increasing dramatically but is still in its infancy in amphibians. For example, Richter-Boix et al. (2011) used genome scans to identify a locus under divergent selection among ponds associated with variation in tadpole life history characteristics (Ficetola and Bonin 2011). Since Richter-Boix et al.'s analysis was based on only 15 microsatellite loci, they almost certainly missed many other loci under divergent selection. In a more recent study, Guo et al. (2016) used genome-wide scans of over 15,000 SNP loci obtained using RADseq to test for loci with signatures of divergent selection between low and high elevation populations of *Bufo andrewsi*. They found many SNPs associated with differences in elevation, temperature, or both hypothesized to be involved in adaptation to high elevations. These studies pave the way for future work harnessing MPS and genomics to understand adaptation in amphibians.

2.6 Mating Systems and Sexual Selection

Amphibians have some of the most diverse and complex reproductive modes of all vertebrates, including eggs versus live birth, terrestrial versus aquatic oviposition sites and larval development, and, sometimes, parental care and even feeding of

offspring (Salthe and Duellman 1973). Although all amphibian orders show diversity of reproductive modes, the patterns of evolution in these modes have best been characterized in frogs (Anura). Two evident patterns in the evolution of these traits are the higher diversity of reproductive modes in the tropics and the apparent progression from aquatic to terrestrial reproduction, often attributed to higher fitness resulting from decreased predation on terrestrial eggs and tadpoles (Gomez-Mestre et al. 2012) or to reduced loss of fitness due to polyandry in terrestrial breeders (Zamudio et al. 2016b). Thus, reproductive modes of frogs offer an excellent opportunity to genetically characterize mating systems, measure reproductive fitness, and quantify the selective advantage of different traits or behaviors during reproductive events. To date, very few studies have taken advantage of the diversity in amphibian reproduction or used genetic or genomic techniques to characterize mating outcomes in species with different modes. The few studies that have done so have typically used few microsatellite markers for paternity assignment and estimates of relatedness; thus, the power of genomics has not yet been harnessed in this field. Nonetheless, every study that has genetically assessed reproductive outcomes in amphibians has found surprising results such as high degrees of multiple paternity (Laurila and Seppa 1998; Myers and Zamudio 2004; Kupfer et al. 2008; Adams et al. 2005), evidence for "good genes" and heritability of fitness traits (Welch et al. 1998), novel reproductive strategies and mate choice (Vieites et al. 2004; Ringler et al. 2012), and a high degree of parental care relative to parentage (Summers and Amos 1997; Chen et al. 2011; Muralidhar et al. 2014).

Data on individual relationships is essential to studies of the behavioral ecology of wild organisms. Advances in molecular and analytical techniques have enhanced our ability to test hypotheses about reproductive modes and mating systems by providing information on the genetic relationships among individuals (Hughes 1998; Avise et al. 2002; Griffith et al. 2002; Myers and Zamudio 2004; Thrasher et al. 2018). The application of genome-wide SNPs to analyses of parentage and relatedness has received greatest attention (Glaubitz et al. 2003; Thrasher et al. 2018). Studies in birds (Weinman et al. 2015; Kaiser et al. 2017), fish (Hauser et al. 2011), and domesticated species (Tokarska et al. 2009; Fernandez et al. 2013) have developed large SNP panels with power comparable to or higher than polymorphic microsatellites. Likewise, targeted amplicon resequencing of large panels of microsatellites also permits rapid and accurate genotyping (Andrés and Bogdanowicz 2011; Nali et al. 2014). Once polymorphic loci have been identified, each individual can be genotyped at >150 microsatellite loci using multiplex PCR reactions. Multiplexed loci are then pooled for each individual, barcoded, and sequenced on next-generation sequencing platforms. Targeted amplicon sequencing offers a couple of advantages. First, once the loci have been identified, this method is fast, allowing for genotyping of a large number of individuals, which is often required in amphibian parentage studies. Second, resequencing microsatellite loci allows for the identification of homoplasy caused by flanking mutations or reversals, thus reducing assignment error in parentage analyses. This method has been applied in a variety of taxa (Nali et al. 2014; D'Aloia et al. 2017) and provides paternity assignments with

high probabilities. MPS methods for parentage and relatedness assays, once applied to amphibians, have the potential to reveal the selective contexts and mechanisms leading to their unusually high diversity of reproductive modes.

3 Opportunities for Improving Amphibian Conservation with Genomics

3.1 Hybridization

Hybridization between species often results in offspring that are less fit than parental forms, which may result in selection for traits that enhance prezygotic barriers to gene flow ("reinforcement"). Alternatively, in the absence of reproductive barriers, hybridization may result in the "genetic swamping" of one of the parental forms, due to extensive introgression (Rhymer and Simberloff 1996). This duality in the nature of hybridization, potentially enhancing or decreasing biodiversity, has been a focus of much genetic work, especially in the context of conservation. Many studies have identified a role for interspecific hybridization in promoting the evolution of novel adaptive forms (Anderson and Stebbins 1954; Harrison 1993; Arnold 1997). Natural hybridization occurs relatively frequently among divergent populations of animal species (Barton and Bengtsson 1986; Grant and Grant 1992; Mallet 2005), and only a few hybridization events are needed to allow the exchange of advantageous alleles between species. The historical admixture of genomes has also contributed to speciation, especially in plants, but also in some animal taxa (Arnold 1997; Dowling and Secor 1997; Mavárez et al. 2006; Gompert et al. 2006; Grant and Grant 2008). Therefore, genomic studies of hybrid zones have the potential to inform not only the causes but also the consequences of hybridization.

In amphibians, genetic or genomic approaches have been used to identify the extent of hybridization between endangered and non-endangered species to guide conservation and management actions (Austin et al. 2011; Zamudio et al. 2010). For example, natural hybridization has been detected between endangered *Ambystoma tigrinum stebbinsi* and the widespread barred tiger salamander *A. t. mavortium* (Storfer et al. 2004), raising concern for the persistence of *A. t. stebbinsi* populations in Arizona. In some cases, anthropogenic translocation of one species outside its natural range causes population dynamics that can favor hybrids over pure parental forms, as is the case with the hybridogenetic frogs *Rana lessonae* and *R. ridibunda* in Europe. Hybridogenesis is an unusual form of reproduction in which a hybrid persists and spreads in populations with just one parent, with which it backcrosses over multiple generations (Beebee 2005). Introduced *R. ridibunda* have replaced *R. lessonae* in several areas of Western Europe in recent decades (Vorburger and Reyer 2003). Likewise, nonnative *A. t. mavortium* introduced into Central California in the 1950s have led to the formation of a hybrid swarm within the range of the federally protected California tiger salamander (*A. californiense*). Genomic analyses

show that a small fraction of superinvasive genes are introgressing more rapidly into the native species (Riley et al. 2003; Fitzpatrick et al. 2009, 2010). The hybrids have higher fitness than the native *A. californiense*, raising concerns of the possibility of genetic extinction of populations of the native species (Fitzpatrick and Shaffer 2007; Box 1).

Box 1 Anthropogenically Mediated Hybridization in the Critically Endangered *Ambystoma californiense*

A well-characterized example of hybridization is found in the California tiger salamander (*Ambystoma californiense*), an endangered species that hybridized with the more widespread barred tiger salamander (*Ambystoma tigrinum mavortium*), following anthropogenic introductions of the barred tiger salamander within the breeding range of the formerly allopatric California tiger salamander (Riley et al. 2003; Fitzpatrick and Shaffer 2007). The introduced species has spread and the hybrid swarm currently occurs throughout 25% of the native species' original range (Shaffer et al. 2015). The conservation challenge is exacerbated by the fact that hybrids seem to have higher fitness, especially in disturbed environments (Fitzpatrick and Shaffer 2007; Ryan et al. 2013). Using a panel of 64 genome-wide SNP markers and a survey of hybrid and pure populations, researchers found that 3 introduced alleles have largely displaced native alleles within the hybrid populations, likely due to strong selection favoring allelic fixation at those loci (Fitzpatrick et al. 2009, 2010). Introgression of a few, strongly selected introduced alleles may not directly affect the persistence of California tiger salamanders, but these patterns underscore how selection can rapidly promote introgression and pose challenges for maintaining pure populations of endangered species threatened by hybridization (Fitzpatrick et al. 2009).

Ambystoma californiense (Photo credit: Ian J. Wang)

Not all hybridization results in negative consequences for populations, of course. Hybrid-origin or allopolyploidization is an important mechanism of hybrid speciation with strong, although often incomplete, postzygotic reproductive barriers between the polyploid hybrid and its diploid parents (Ficetola and Stock 2016). In amphibians, allopolyploids often have novel phenotypes that differ from those of the

ancestral lineages, leading to the hypothesis that niche shifts could be one of the consequences of hybrid speciation. A recent review of hybridization in toads showed that some allopolyploids occupy intermediate niches to those of the diploid parental lineages, but in other cases, allopolyploids showed transgressive niche evolution; they inhabited environments that were more arid and with colder winters than either of their parental species (Ficetola and Stock 2016). This leads to the possibility that endangered species with hybrid genomes might show higher fitness in anthropogenically modified environments, suggesting that some degree of hybridization might provide greater adaptive potential with which to respond to environmental change (Zamudio et al. 2010). A fruitful avenue of research in conservation will be characterizing the genomic architecture and evolutionary potential of fit hybrids.

3.2 Disease Evolution and Ecology: Lessons from Chytridiomycosis

Genomic studies have been used effectively to disentangle host-pathogen interactions and disease dynamics in amphibians (Longo et al. 2014). The amphibian fungal disease, chytridiomycosis, which affects species across all continents, recently emerged as a major threat to biodiversity. The disease is caused by the chytrid fungus, *Batrachochytrium dendrobatidis*, [hereafter *Bd* (Longcore et al. 1999)], a generalist amphibian pathogen which has extirpated populations of diverse species from around the globe. *Bd* has now been reported from over 500 amphibian host species and has a cosmopolitan distribution (Olson et al. 2013; James et al. 2015). The emergence of *Bd* has shown that host-pathogen interactions can play a major role in species declines and even extinctions (Crawford et al. 2010). Genomic approaches have elucidated aspects of the biology and evolution of frogs (hosts) and the pathogen itself.

The first genetic assessments of pathogen diversity in this system showed a surprising absence of genetic variability, leading researchers to propose a recent emergence of *Bd* followed by a rapid spread of a Global Panzootic Lineage (*Bd*-GPL) around the world (Morgan et al. 2007; James et al. 2009). More recently, novel genotypes putatively endemic to the Cape of South Africa (*Bd*-Cape), Switzerland (*Bd*-CH), Brazil (*Bd*-Brazil), and Korea (*Bd*-Korea) were found, and whole genome sequencing showed that those lineages are basal divergences within the *Bd* phylogenetic tree (Farrer et al. 2011; Schloegel et al. 2012; Rosenblum et al. 2013; Bataille et al. 2013). By increasing the sampling of global *Bd* strains, it became apparent that the earlier perspective on low genetic diversity was due to biased sampling of only epizootic strains (James et al. 2015). The discovery of enzootic lineages of *Bd* that are more restricted in their distribution contrasts with the broad distribution and spread of the virulent genotype (*Bd*-GPL).

A recent study has probed the functional genomics of *Bd* virulence (Ellison et al. 2017). Using laser capture microdissection (LCM), which allows for analysis of

pathogen gene expression in infected tissues of living hosts, Ellison et al. (2017) performed the first in vivo functional assays of *Bd*-GPL infection in the amphibian skin. Using sequencing of *Bd* RNA from infected epidermal cells in two different hosts and in culture, that study identified more than 2,000 differentially expressed genes between *Bd* in tissues and in culture that included key *Bd* defense and host exploitation mechanisms. Significant enrichments of genes with increased expression in both host species compared to *Bd* cultures were those related to proteolysis and membrane transport activity, both important during the host invasion and infection process (Ellison et al. 2017). In contrast, variation in *Bd* transcriptomes from different amphibian hosts demonstrates shifts in pathogen resource allocation. *Bd* genes more highly expressed in *Atelopus zeteki* were those related to cilium organization and cilium morphogenesis, suggesting a greater investment in motile zoospore production. In contrast, *Bd* samples collected from *Hylomantis lemur* were predominantly enriched for biosynthetic and amino acid metabolic processes (Ellison et al. 2017). Earlier studies of *Bd* in culture show that abiotic conditions can significantly alter life history trade-offs in the pathogen (Woodhams et al. 2008). Different gene expression profiles in different hosts indicate that host species also influences the relative investment of *Bd* in growth and reproduction, likely as a response to the host's defensive capabilities (Ellison et al. 2015). Thus, the selective environment provided by different host species has a strong effect, and the pathogen may respond by shifts in resource allocation rather than evolutionary changes. This level of flexibility, revealed by functional genomic assays, is most likely part of the strategy ultimately underlying the success of *Bd* as a generalist pathogen.

In the field, hosts vary in their disease outcomes with some populations persisting after the arrival of *Bd*, while others go extinct. These variable outcomes suggest potential differences in host genotype and potential for evolution of resistance to *Bd*. Amphibians can rely on innate or adaptive immune responses to manage *Bd* infections, and at least some of these immune responses have a genetic basis (Savage and Zamudio 2011; Ellison et al. 2014), suggesting host genotypic variation may be an important factor explaining persistence or mortality. For instance, alleles of the major histocompatibility complex (MHC), a family of genes in the adaptive immune response, were significantly associated with resistance and survival in *Lithobates yavapaiensis* (Savage and Zamudio 2011) and *Litoria verreauxii* (Bataille et al. 2015). Various immunogenetic studies have reported either a strong or weak adaptive immune response post-*Bd* infection (Rosenblum et al. 2013; Ellison et al. 2014), underscoring variation among species in their potential for the evolution of resistance. A comparative study of amphibian functional immunogenomic responses to *Bd* provided some insights into key genetic mechanisms underlying variation in disease outcomes among amphibian species (Ellison et al. 2015). That study challenged four Central American frog species that vary in *Bd* susceptibility with a sympatric virulent strain of *Bd*. Comparison of host gene expression profiles showed that resistant species have reduced skin inflammatory responses and increased expression of genes involved in skin integrity. In contrast, only highly susceptible species exhibited suppression of splenic T-cell genes, likely in response to the suppressive action of *Bd* on host immune function (Fites et al. 2013). Thus,

resistance to chytridiomycosis may be related to a species' ability to escape the immunosuppressive activity of the fungus.

In summary, genomic surveys suggest natural variation in both pathogen virulence and host immunity, but the interactions between these two components have not been adequately addressed to allow predictions of which species or communities have the potential to recover after exposure to *Bd*. Nonetheless, genomic approaches have begun to unravel the mechanisms underlying the evolution of pathogen virulence and host resistance not only in *Bd*, but now in the newly emerged *B. salamandrivorans* that is specific to salamanders (Box 2).

Box 2 Genomics of the Newly Emerged *Batrachochytrium salamandrivorans* (*Bsal*)

A major breakthrough in amphibian disease ecology was the discovery of a new chytrid species, *Batrachochytrium salamandrivorans* (*Bsal*) that infects salamanders. The new species is morphologically, genetically, and functionally distinct from *B. dendrobatidis* and was discovered as a pathogen of fire salamanders (*Salamandra salamandra*) in Europe (Martel et al. 2013) but was likely introduced from eastern Asia (Martel et al. 2014). The recent arrival of *B. salamandrivorans* in Europe was followed by rapid expansion of its geographical distribution and host range, confirming the unprecedented threat that this chytrid fungus poses to amphibians globally (Stegen et al. 2017). *Bd* and *Bsal* diverged an estimated 67 million years ago, and they have different host species ranges, with *Bsal* mostly infecting a single order, Caudata (salamanders; Martel et al. 2014), while *Bd* infects many species across all three orders of Amphibia (Fisher et al. 2009). Scientists predicted that this evolutionary jump to amphibian hosts was facilitated by the acquisition of common ancestral traits, whereas subsequent differentiation of infection strategies has been the result of lineage-specific adaptations (Farrer et al. 2017). To test this prediction, they sequenced the genomes of *Bd* and *Bsal* and compared them to those of two related saprobic chytrids. The results show that evolutionary adaptation to infect amphibians is correlated with the acquisition of genes encoding secreted proteins that are unique to the genus *Batrachochytrium. Bd* and *Bsal* share 542 gene clusters that are not found in the two saprobic chytrids and include specific functions related to cell wall modification and candidate secreted effectors (Farrer et al. 2017). Several of these lineage-specific protein families are highly expressed during in vivo infection of salamanders; these upregulated genes likely include key virulence factors. Among these are the M36 metalloproteases implicated in chytrid pathogenicity (Farrer et al. 2013; Martel et al. 2014) as well as at least two large families of secreted proteins with no recognizable functional domains, which are very highly expressed and may represent novel virulence factors unique to *Bsal*. Characterizing the genomic architecture of pathogens with different virulence, and the expression

(continued)

Box 2 (continued)
of pathogen and host genes during the infection process, will yield important information on how these pathogens have specialized and their potential impact on other salamander species.

Salamandra salamandra fastuosa from Guipúzcoa, Basque country, Spain (Photo credit: Guillermo Velo-Antón)

3.3 Captive Breeding

Captive breeding programs are a stopgap approach to conserving those populations that have declined to the point where their persistence in the wild is in doubt. With the catastrophic declines of many amphibian species and populations in the last few decades associated with *Bd* and other factors, ex situ captive breeding has become an important management strategy for amphibian conservation (Harding et al. 2016). Although a suite of complex technical and ethical issues surround captive breeding (Seigel and Dodd 2002; Trenham and Marsh 2002), it will likely remain an important conservation strategy for endangered amphibian species for the foreseeable future.

MPS and population genomics can maximize the potential for success of captive breeding programs by identifying mating pairs that are not too closely related – potentially resulting in inbreeding depression – or from divergent populations, which could result in outbreeding depression. When individuals are brought into captivity, their relatedness is typically unknown, meaning that closely related individuals

might be mated, resulting in inbred offspring with low fitness (Kardos et al. 2016). Genomic datasets consisting of genotypes at hundreds or thousands of SNP loci generated from RADseq allow precise estimation of relatedness (Weir et al. 2006), facilitating selective breeding to maximize success of ex situ breeding programs.

Genomics can also be used to more precisely estimate genetic divergence among populations to identify pairs of populations that are so divergent that crosses between individuals from these populations could result in outbreeding depression (Frankham et al. 2011). Several genomic approaches are available for characterizing the degree of adaptive divergence among populations (see Sect. 2.5); thus, genomics can play a key role in making sure that only individuals from adaptively similar populations are crossed as part of captive breeding programs.

4 Challenges and Solutions for Genomic Studies of Amphibians

4.1 Few Reference Genomes

Increasing numbers of mammal and bird species (Zhang et al. 2014) are becoming genome enabled, yet amphibians remain largely "genome disabled." Even for nonavian reptiles and fishes (Bernardi et al. 2012), the number of published reference genomes is increasing rapidly. Extant amphibians, or Lissamphibia, represent over 300 million years of evolution and include 7,934 species as of September 30, 2018 (AmphibiaWeb 2018). Additionally, amphibians arguably are the most endangered clade of vertebrates as well, with one third of species listed as endangered by the IUCN (Hoffmann et al. 2010). Despite this tremendous age, diversity, and threat level, only four reference genomes had been published by the end of 2017: the model organisms *Xenopus tropicalis* (diploid) and *X. laevis* (a tetraploid), the Tibetan frog, *Nanorana parkeri*, and finally, the American bullfrog, *Rana catesbeiana* (see Table 1 for citations). Fortunately, 2018 has seen this number double, with publication or prepublication of reference genomes announced for the African bullfrog, *Pyxicephalus adspersus* (Denton et al. 2018), cane toad, *Rhinella marina* (Edwards et al. 2018), strawberry dart-poison frog (Rogers et al. 2018), and two independent efforts at assembling the enormous axolotl genome (Nowoshilow et al. 2018; Smith et al. 2018). Reference genomes provide a deeper understanding of demographic history and are a key resource for studying functional variation that may be adaptive (Steiner et al. 2013). Knowing the genomic position of genes is essential for understanding the interaction between natural selection and recombination including gene conversion (Hoban et al. 2016). Genomic information can greatly enhance management efforts for captive and wild populations of threatened species by identifying loci involved in inbreeding depression and disease susceptibility (Johnson and Koepfli 2014).

In prioritizing amphibian species for whole genome sequencing (WGS), the Genome 10K consortium suggested three criteria (Haussler et al. 2009). First, species of special conservation concern may receive an immediate and applied benefit to becoming genome enabled. Ironically, the most endangered amphibian species may be so rare that access to genomics-quality samples becomes very difficult. Second, WGS projects should maximize phylogenetic diversity to provide genomic "outposts" across the phylogeny of Lissamphibia. Each WGS will facilitate genomic studies of a constellation of related species, and any comparative genomics questions will require data from as many distinctive lineages as possible. Thus, initial WGS projects should maximize phylogenetic diversity (Faith 1992), e.g., by sequencing at least one species per taxonomic family of amphibians (Table 2). Third, and arguably the most important, each WGS requires a community of curators and users attracted to a given species for scientific questions and dedicated to providing support for quality control and continued development of annotation and other resources. Reference genomes that are not curated and updated risk becoming inaccessible or obsolete. In fact, reference genomes are almost never "done," rather they progressively improve in terms of length, contiguity, accuracy, and annotations. So too, not all genomes need to be completed to chromosome-level, phased scaffolds. Genome 10K has recommended that each major taxonomic group (clades with a stem age of up to 50 million years, e.g., taxonomic families; Table 2) should be represented by a very high-quality reference referred to as a "platinum genome" (Koepfli et al. 2015). Under the aegis of Genome 10K, Phase I and Phase II of the Vertebrate Genomes Project (VGP) aim to produce at least one near-gapless, chromosome-level, phased genome assembly representing each vertebrate lineage, including amphibians, which predates the K–Pg mass extinction event (https://www.rockefeller.edu/research/vertebrate-genomes-project/vertebrate-genomes-project-plan/).

4.2 The Genome Size Problem

Amphibian genomes are undoubtedly among the largest of all organisms (Elliott and Gregory 2015). While genome size information is lacking for the majority of amphibian species, and most of these data come from microscopy rather than from more precise flow cytometry methods, preliminary trends can be inferred from the www.genomesize.com database (Gregory 2011) augmented by a recent study (Liedtke et al. 2018). Salamander genomes start at around 14 Gb with an interquartile range of 25–44 Gb ($n = 170$), with waterdog or mudpuppy (*Necturus* spp.) genome sizes ranging from 84 to an astounding 118 Gb, the equivalent of 37 human genomes! Frog genome sizes show an interquartile range of about 3–5 Gb but range from 1 to 11 Gb ($n = 272$: Fig. 1). Caecilian genome sizes show an interquartile range of about 5–8 Gb, but range from 3 to 12 Gb ($n = 22$; Fig. 1). While amphibian genomes can tolerate a surprising amount of polyploidy (Schmid et al. 2015; Session et al. 2016), gigantism in amphibian genomes appears to be more commonly caused by proliferation of transposable elements (TE) such as long

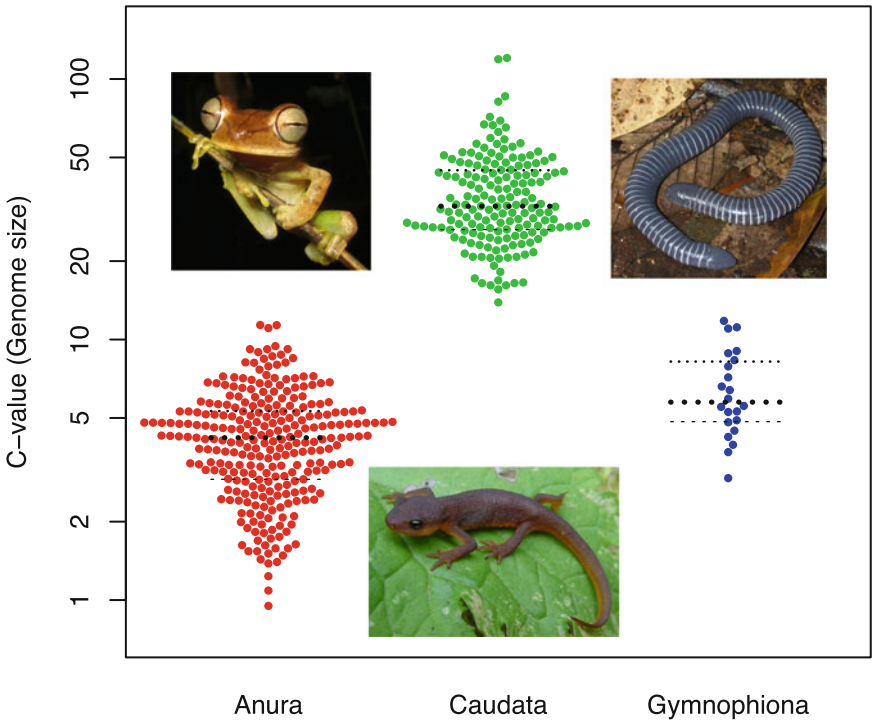

Fig. 1 Variation in genome size within and among taxonomic orders of amphibians. Data consist of 464 species in Gregory (2011) and Liedtke et al. (2018), combining data across multiple techniques for genome size estimation. When multiple estimates existed for a species, the mean of the estimated genome sizes was assumed. Heavy dashed lines are the median values; light dashed lines are the first and third quartiles. Y-axis is on a natural log scale and reports C-values in picograms (pg), where 1 pg = 978 megabases of DNA sequence. The largest values are approximately 120 pg (118 Gb) for waterdogs (salamanders of the diploid genus, *Necturus*), while the smallest genome at 0.95 pg (929 Mb) was reported for the ornate burrowing frog, *Platyplectrum* (*Limnodynastes*) *ornatum*, of Australia. These largest and smallest values were obtained by Feulgen densitometry on red blood cells. As a point of reference, a human genome is 3.3 pg (3.23 Gb). Frog, salamander, and caecilian photos are of *Boana* aff. *fasciata* from Município Cotriguaçu, Mato Grosso, Brazil (Photo credit: Kelly R. Zamudio), *Taricha granulosa* from Benton County, Oregon, USA (Photo credit: W. Chris Funk), and *Siphonops annulatus* from Panguana, Huánuco, Peru (Photo credit: Andrew J. Crawford), respectively

terminal repeat (LTR) retrotransposons (Sun et al. 2012). Some frog species have secondarily evolved smaller genomes, potentially driven by natural selection for faster development time in xeric habitats (Liedtke et al. 2018), as species with larger genomes have slower embryonic development times (Jockusch 1997).

An estimated 31% of frog species have a genome size smaller than that of a human (data from Liedtke et al. 2018), which might suggest that size is not the only stumbling block to producing reference genomes for amphibians. Indeed, sequencing all the DNA in a genome is simple and each year less expensive. Assembling a

genome, however, is vastly more challenging. The worst-case scenario for whole genome assembly would be a genome rife with recently proliferated (i.e., very similar) repetitive elements. This is what has been found in the strawberry dart-poison frog (*Oophaga pumilio*): 4.76 of its 6.76 Gb genome is made up of recently expanded TE families, including 1 Gb of just the *Gypsy* family, making assembly a colossal challenge (Rogers et al. 2018).

In addition to the size, ploidy, and abundant TEs in their genomes, amphibians face further challenges to WGS. Many MPS and scaffolding techniques, such as optical mapping, require large amounts of ultrahigh molecular weight (uHMW) DNA as input (Wong et al. 2012), yet some amphibians rank among the smallest vertebrates, making it difficult to obtain large quantities of DNA for scaffolding (e.g., Rittmeyer et al. 2012). Finally, amphibians are in sharp decline around the world, and some of the most fascinating frogs have not been seen in decades (e.g., the gastric-brooding frog, *Rheobatrachus silus* of Australia; Table 2). Access to abundant, fresh, uHMW DNA is already impossible for many of the species that could most benefit from genomic tools.

We predict that the next few years will witness a renaissance in WGS and related approaches to amphibian genomics. We suggest three strategies that will help drive global amphibian genomics. First, we need more data on genome size variation in amphibians, i.e., a "1,000 genome sizes" project. Closely related species may vary substantially in genome size; thus, within each clade of interest, WGS efforts can be focused on species with smaller genomes with lower ploidy. We recommend flow cytometry (Vinogradov 1998), but imaging (Hardie et al. 2002) and quantitative PCR can also be used (Wilhelm et al. 2003). Karyotyping is still important to evaluate ploidy, check for heteromorphic sex chromosomes, and inform studies of synteny (Bogart 1973). Blood samples for flow cytometry should be collected along with routine tissue sampling in the field. Karyotyping requires slightly more specialized preparation with live animals.

Second, for many questions in amphibian genomics, a catalog of expressed genes may be sufficient. Very large diploid amphibian genomes do not present any additional challenges for transcriptomic studies, as these genomes likely have a similar complement of protein-coding genes as other vertebrates (Sun et al. 2015). A single RNAseq experiment (Wang et al. 2009) costs only a few hundred US dollars including sequencing but can provide 1,000s of complete, de novo assembled protein-coding genes for use directly in phylogenomics, comparative genomics, and molecular evolution (Huang et al. 2016). These gene sequences can also be used for developing target capture probes for phylogenomics and population studies (McCartney-Melstad et al. 2016; Portik et al. 2016). Perhaps the major limitation to implementing RNAseq studies is access to fresh, well-preserved RNA samples, e.g., flash-frozen, stored in nucleic acid preservation (NAP) buffer (Camacho-Sanchez et al. 2013), or both. RNA sampling should be a routine part of field collecting, whenever possible. RNAseq data also greatly improves eukaryotic reference genome annotation and can improve scaffolding (Yandell and Ence 2012). Transcriptomic data collection should be a first step in the genetic characterization of every amphibian species of special scientific interest or conservation concern (Table 2).

Third, amphibians may be among the greatest beneficiaries of recent advances in genome scaffolding. While MPS platforms, such as PacBio and Oxford Nanopore, offer long-read technology in the 10's of Kb range, additional service providers, such as 10X Genomics and Dovetail's Chicago method, are focusing on using linked DNA to assemble the resulting contigs into scaffolds in the 100's of Kb range. Hi-C methods fix full chromosomes in situ with the goal of creating Mb-scale assemblies, and optical mapping can be used to create chromosome-scale assemblies. We should know soon whether these new approaches using medium- and long-range positional information will finally achieve the assembly of very large and highly repetitive genomes such as those of frogs or perhaps even of salamanders (Koepfli et al. 2015).

4.3 High Population Structure

The tendency of amphibians to have low dispersal, and therefore high population structure, can reduce power to detect loci under positive selection using genome scans. The premise of genome scans is that loci with "outlier behavior," such as F_{ST} values significantly higher than the genome-wide average site-wise F_{ST} value, might be under positive selection (Beaumont and Nichols 1996). If genome-wide average F_{ST} values are low due to high gene flow, then the power to identify high F_{ST} outliers should be high. But if all loci have high F_{ST} values because of restricted gene flow, then the power to identify loci under divergent selection may be reduced. Conversely, if most loci have high F_{ST} values, some may be incorrectly identified as high F_{ST} outliers, resulting in type I errors, a potentially more insidious problem.

Due to the potential for type I errors, multiple lines of independent evidence should be used to test whether outlier loci are truly under divergent selection and adaptive. First, if a reference genome is available for the study species or close relative, loci can be mapped to determine if they fall in or near exons or regulatory regions (Manel et al. 2016). Second, multiple independent environmental gradients of a given type of gradient (e.g., multiple, similar elevational gradients) should be sampled to test whether the same loci are identified as outliers consistently, providing additional evidence that the gradient of interest is the cause of directional selection. Third, genotype-by-environment association (GEA) tests can be used to determine whether allele frequencies at loci identified as outliers are correlated with the gradient (s) hypothesized to cause adaptive evolution (Joost et al. 2007; Coop et al. 2010; Frichot et al. 2013). Finally, experimental approaches should ideally be used to determine potential adaptive functions of the candidate loci. For example, reciprocal transplant experiments could be conducted in which tadpoles are moved to ponds in different environments to test how allele frequencies change at outliers in mismatched environments (Soria-Carrasco et al. 2014). The prediction is that allele frequency of immigrants at loci involved in local adaptation should become more similar to the frequency of residents. Alternatively, it may soon be feasible to use CRISPR/Cas gene editing to silence genes or replace specific alleles to test their effects on fitness in different environments. Thus, there are many different follow-up analyses that can be conducted to increase confidence that loci truly are involved in local adaptation.

5 Recommendations

5.1 Maximizing the Potential of Genomics to Transform Amphibian Research

The possibilities for harnessing genomics to advance understanding of amphibian evolution, ecology, behavior, and conservation are essentially limitless. Ultimately, the general idea of MPS is not complex: MPS provides huge quantities of sequence data from wherever a researcher wants to look in the genome. It is up to us as scientists to select the best species and approaches to address long-standing questions in novel and exciting ways.

In Sects. 2 and 3 above, we pointed out different ways in which genomics will advance understanding of amphibian ecology, evolution, and behavior and improve conservation management of this imperiled taxon. For example, genomics provides tremendous opportunities to test for and understand the genetic basis of local adaptation. Many amphibian species occur across heterogeneous environments, suggesting the capacity to adapt to diverse habitat and climatic conditions, which can now be dissected using MPS. Genomics also provides unprecedented power to infer the history of diversification and speciation, providing the opportunity to understand how the diverse traits of amphibians (reproductive modes, behavior, habitat, size, morphology, etc.) influence speciation mechanisms. Amphibians have been the unfortunate victims of one of the worst disease epidemics ever known: chytridiomycosis caused by *Batrachochytrium dendrobatidis* and *B. salamandrivorans*. Some populations, however, have recovered after epidemics caused by these diseases, suggesting the evolution of resistance. Genomics can help identify individuals and populations that are resistant, for example, using genome-wide association tests (GWAS), and that could therefore serve as sources for supplementation of populations without resistance. Our newfound ability to peer deeply and broadly into the genomes of amphibians thanks to the emergence of MPS will greatly improve our understanding of their biology and our capacity to make informed decisions to conserve them.

To maximize the potential of genomics to transform our understanding of amphibian biology, we argue that herpetologists and genomicists should strive for three things: taxonomic diversity, multiple scales, and integration. By "taxonomic diversity," we mean that for strong inferences about evolutionary and ecological processes across all Amphibia, we need to investigate these processes in as many groups of amphibians as possible. Too often, a handful of species serve as models on which conclusions for an entire class of organisms are based. Yet amphibians are extremely diverse in terms of their life histories, reproductive modes, habitats, genome sizes and structure, and interactions with other species (Wells 2007; Zamudio et al. 2016b). The best way to understand ecological and evolutionary processes for all Lissamphibia is to study these processes in multiple taxonomic families.

"Multiple scales" refer to investigating questions at different levels of biological organization, from species to populations to individuals to tissues, which we argue provides the deepest understanding. For example, the strongest studies of adaptation examine (1) how species' traits are related to environmental variation using comparative phylogenetics at the species level, (2) patterns and function of phenotypic and genetic divergence among populations in relation to environmental heterogeneity, (3) the genetic basis of variation among individuals in traits, and (4) organismal physiology.

Finally, as we have highlighted throughout our chapter, integration of genomics with other established methods in ecology and evolution will provide the strongest inferences. These include field observations, field and laboratory experiments, and modeling. As scientists, we are attracted to the power of new technologies and have a tendency to dismiss "old" scientific approaches, but we must remember that in evolution, ecology, behavior, and conservation, no technological breakthrough can replace astute and careful observation, controlled experiments, and clear quantitative thinking formalized with models.

5.2 Getting Started

We conclude with recommendations for those interested in applying MPS and genomics to a question about their favorite amphibian species, but who do not yet have experience in this area. Our first recommendation is to simply recognize that developing a genomics component to a research program will take time, patience, and determination. Learning how to prep MPS libraries and especially learning bioinformatics takes significant investments of time. Many things can go wrong with library preparations and computational analyses. Expect problems and allow time for troubleshooting. Second, team up with other researchers who are experts in genomics and bioinformatics. Although anyone who is determined and persistent can learn these approaches, the learning curve will be less steep with the guidance of an experienced collaborator. Third, get formal training in genomics and bioinformatics in courses or workshops. Not only will this rapidly increase one's knowledge of the field, it will also provide the opportunity to meet experts in the field, providing you with a network of colleagues whom you can call on to answer questions. The fourth and most important recommendation is to make sure one understands the fundamental principles of population genetics (Allendorf 2017). Ultimately, population genomics is nothing more than population genetics "writ large" and is based on the same powerful population genetics theory developed by early pioneers in the field (Fisher 1930; Haldane 1930; Wright 1931) and by more recent theoreticians (Kingman 1982). Piles of DNA sequence data are useless without an understanding of theory to come up with well-grounded, interesting hypotheses to test by rigorously analyzing data and correctly interpreting the results.

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