Modern Molecular Methods for Amphibian Conservation

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Amphibians are declining globally at unprecedented rates. To direct conservation efforts, global amphibian assessments are being conducted to characterize biodiversity and evolutionary relationships among species, as well as amphibian population and species' health. Modern molecular methods are facilitating such characterization, and we highlight techniques for rapidly increasing the availability of data for making taxonomic distinctions. When diversity is characterized, and populations and the species most vulnerable to declines or extinctions are identified, it is then critical to understand factors causing declines to develop mitigation strategies. We discuss molecular approaches and their applications for addressing some of the leading hypotheses for amphibian declines, including habitat loss, emerging infectious diseases, chemical contaminants, and global climate change.

Keywords: amphibian declines, landscape genetics, next-generation sequencing, genomics, conservation

The loss of global biodiversity is a grand challenge for the 21st century, as recognized by the National Research Council (2001). Amphibians are a marquee example of biodiversity loss, with extinction rates exceeding the background rate by at least 200 times over the past few decades (Stuart et al. 2004, Roelants et al. 2007). Amphibians are thought to be among the most vulnerable vertebrates, with nearly 50% of all populations declining and 32% of species threatened with extinction (Stuart et al. 2004). Because of their permeable skin and life cycle, which most often includes stages in water and on land, amphibians are particularly sensitive to environmental degradation in different environments (Blaustein 1994).

Amphibian population health is affected by two classes of factors that can act additively or synergistically. Class I factors are considered straightforward or historical in nature; they include habitat alteration and degradation, overexploitation for use in the pet trade or for food, and invasive or nonnative species that either outcompete or prey upon native amphibians (Collins and Storfer 2003). Class II factors are considered to be enigmatic; these include emerging infectious diseases, global climate change, and environmental contaminants (Collins and Storfer 2003). The mechanisms by which these enigmatic factors act are not well understood because of the lack of predictability concerning, for example, the extent of climate change and subsequent organismal responses. Yet leading hypotheses cite such enigmatic factors as major contributors to many of the world's amphibian declines (Stuart et al. 2004).

The extent of amphibian declines and recently documented rapid population and species' extinctions necessitate urgent conservation measures (Lips et al. 2006, Mendelson et al. 2006). Recent technological advances allow genetic assessments of amphibian population and species status (e.g., by estimating effective population size and genetic diversity) in relatively short periods of time, even months (Beebee 2005). By contrast, demographic studies may take many years to resolve population trends accurately because amphibian population census sizes fluctuate widely from year to year (Pechmann et al. 1991). In addition, large census counts may reflect the genetic contributions of only a few individuals because many amphibian species can lay large numbers of eggs per season. Thus, estimating the genetically effective population size (see the glossary in box 1) is generally more informative than demographic studies for understanding the overall health of a population (Hedrick 2001).

Molecular methods are thus extremely suitable for assessing the status of amphibian populations and species. The purpose of this review is to highlight current and future applications of modern molecular techniques in two main sections. First, we discuss methods for cataloging amphibian population genetic and species-level diversity. Next we summarize approaches for addressing several of the major hypotheses for amphibian declines, including (a) landscape genetics to elucidate the effects of habitat loss; (b) quantitative polymerase chain reaction (PCR [box 1]) to address problems with emerging infectious diseases; and (c) genomic approaches to assess variation in population and species-

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level susceptibility to contaminants, diseases, and declines. This review is not meant to be exhaustive, as other insightful reviews of amphibian conservation genetics exist (e.g., Jehle and Arntzen 2002, Beebee 2005). A glossary of terms related to molecular methods is found in box 1.

Current methods for characterizing amphibian biodiversity

Scientists first exchanged anecdotal stories of declining amphibian populations in the late 1980s and early 1990s (Blaustein 1994), and despite early concerns with distinguishing apparent declines from wide demographic fluctuations typical of amphibian populations (Pechmann et al. 1991), a consensus was later reached that declines were real (Collins and Storfer 2003). A global amphibian assessment was conducted shortly after the turn of the century to assess the extent of the problem (Stuart et al. 2004). It is now estimated that there are more than 6300 amphibian species worldwide, with 2469 (43%) species having declining populations, approximately 0.5% increasing, and 27% considered stable (Stuart et al. 2004; *www.amphibiaweb.org*). However, variation in the ways in which population trends are approximated has resulted in debates as to whether declines are over- or underestimated (e.g., in Brazil; see Eterovick et al. 2005, Pimenta et al. 2005).

Overestimates of the number of species in decline can lead to finite conservation resources being expended diffusely across too many taxa, not all of which are truly in decline. Current assessments of amphibian species' status may overestimate declines as a result of characteristically low detection probabilities (MacKenzie et al. 2002). In contrast, underestimates of decline may miss some of the most threatened taxa. Indeed, field surveys may underestimate declines because in recent years "cryptic species" (Bickford et al. 2007)—those that are morphologically indistinguishable but genetically distinct from described taxa—have been increasingly discovered. These reasons, in addition to the rapid rate of many amphibian declines (Mendelson et al. 2006) and the fact that proper abundance data are deficient for nearly 30% of amphibian taxa (Stuart et al. 2004), make it critical to assess global amphibian diversity as quickly as possible.

Although new amphibian species have traditionally been discovered through a combination of fieldwork and morphological methods, genetic techniques are increasingly being used for assessments and clarifications of amphibian diversity (e.g., Frost et al. 2005, Roelants et al. 2007, Wiens 2008). Indeed, genetic techniques have been used to reveal cryptic species in a number of amphibian genera, including *Aneides* (Rissler and Apodaca 2007), *Batrachoseps* (Jockusch et al. 2001), *Plethodon* (Highton 1995), and *Rana* (Stuart et al. 2005). Genetic studies are also more frequently used for resolving phylogenetic relationships (box 1) among amphibian species, which can help focus conservation efforts. For example, high extinction proneness appears attributable to phylogenetic relatedness in some groups, a likely result of shared traits within some lineages of hyloid anurans (Corey and Waite 2007), plethodontid salamanders, and ranid frogs (Lockwood et al. 2002).

Nonetheless, amphibian phylogenetic reconstructions (Frost et al. 2005, Roelants et al. 2007, Wiens 2008) are still debated, and more data are needed to better support species distinctions and evolutionary relationships among taxonomic groups. Below, we discuss recent advances in DNA sequencing for aiding species identification and phylogenetic reconstructions, as well as single-nucleotide polymorphism (SNP [box 1]) analyses for species-level and population-level characterization of genetic diversity.

Applications of DNA sequencing

Two main types of DNA sequencing are used today: traditional dye-terminator sequencing and, less frequently, next-generation sequencing (box 2). The principal difference between traditional DNA sequencing and next-generation sequencing (e.g., pyrosequencing [box 1]; 454 sequencing, by Roche; sequencing-by-synthesis, by Illumina; SOLiD, by Applied Biosystems) is the number of fragments of DNA that can be sequenced simultaneously (box 2; Mardis 2008). Typically, traditional sequencing is performed on capillary machines, whereby 48 or 96 DNA sequence fragments are generated per run. In contrast, next-generation sequencing simultaneously generates hundreds of thousands of fragments in parallel. Next-generation sequencing can provide approximately 150 million base pairs of data in a single instrument run, whereas capillary sequencing would require hundreds to thousands of runs to produce the same quantity of data. Next-generation sequencing is thus considerably more time- and cost-effective than capillary sequencing for large-scale projects such as genome assembly (Mardis 2008). Although next-generation sequencing is used primarily for genomic studies because of its high cost (see Mardis 2008), in the future it could be used to dramatically increase the quantity of genetic data for classifying diversity at the population, species, and phylogenetic levels.

The sequencing method now most often used to describe amphibian diversity is dye-terminator sequencing. Once DNA sequences are generated, they are aligned with populations or other species of interest. Then, computer software is used to reconstruct phylogenetic trees, wherein taxa with more similar DNA sequences appear closer together on the tree than do taxa with more dissimilar sequences. It is assumed that taxa that have more similar sequences are more closely related evolutionarily, sharing more recent common ancestors than more genetically differentiated taxa. In this way, DNA sequences from newly sampled or unknown taxa can be placed within their appropriate evolutionary groups.

Before the turn of the century, genetic-based taxonomic decisions most often relied on trees developed from a single genetic locus, which are not necessarily expected to be congruent with "true" species trees (Liu et al. 2008). That is, data from a single locus might not reflect genomewide evolutionary rates, yielding incorrect taxonomic assignments (Liu et al. 2008). Sequence data from multiple loci often allow more

robust estimations of genetic distinctiveness, genetic diversity, and phylogenetic relationships because different loci can evolve at different rates (Maddison and Knowles 2006). For example, Weisrock and colleagues (2006) found consistent and strong support across multiple nuclear loci for genetic distinctiveness of the endangered Mexican Lake Patzcuaro salamander (*Ambystoma dumerilii*) and the Puerto Hondo stream salamander (*Ambystoma ordinarium*), whereas mitochondrial DNA (mtDNA [box 1]) analyses alone failed to support the genetic integrity of the two species. Similarly, a multilocus mtDNA study recently revealed an underestimation of species richness of *Hoplobatrachus* and *Euphlyctis*

Box 1. A glossary of key terms.

Amplified fragment length polymorphisms (AFLPs): Genetic markers for population genetic and genomic studies, isolated using restriction enzymes and random polymerase chain reaction (PCR) primers. AFLPs are dominant loci (band is present or not) and many AFLP loci—up to several hundred—can be generated at one time.

cDNA library: A collection of clones representing complementary DNA (cDNA) from many different genes throughout an organism's genome. cDNA is created by extracting mRNA and using reverse transcriptase PCR to produce the cDNA sequence, which can then be inserted into a plasmid clone and sequenced.

Ecotilling: A technique for detecting single-nucleotide polymorphisms in natural populations based on detecting nucleotide mismatches among samples.

Expressed sequence tags (ESTs): Short sequences of gene transcripts that represent only expressed portions of genes. A library of several hundred to thousands of ESTs is created by sequencing mRNA, converting mRNA to cDNA using reverse transcriptase PCR, and finally inserting cDNA in a plasmid clone to create a cDNA library from which ESTs can be sequenced.

Functional genomics: An emerging field that applies a variety of molecular techniques (through the use of completely sequenced genomes, microarrays, EST libraries, etc.) to identify and understand the function of specific genes of interest and their possible interactions with other genes or environmental influences.

Genetically effective population size: The number of breeding individuals that contribute genes to future generations, which is nearly always lower than the census population size.

Linkage disequilibrium: Nonrandom association of alleles at two or more loci. These alleles may or may not be on the same chromosome.

Linkage map: A representation of linked genetic loci within and among chromosomes, reflecting rates of recombination between loci.

Microarray: A chip that contains a large number of microscopic cDNA (probes) that can be used to detect levels of gene expression through cDNA hybridization and fluorescence.

Microsatellites: Short sequences of noncoding DNA characterized by tandem repeats of several nucleotides (e.g., GATA). Owing to rapid rates of mutation, microsatellites are highly appropriate for fine-scale population genetic studies.

Mitochondrial DNA (mtDNA): DNA contained within the mitochondria, which is inherited matrilineally, and often used for phylogenetic and population genetic studies.

Orthologous: Refers to genes found in different species that have highly similar DNA and amino acid sequence, suggesting similar function.

Phylogenetic: Related to reconstructing the evolutionary relationships both within and between individual taxa as well as groups of taxa.

Polymerase chain reaction (PCR): Method used to amplify fragments of DNA at an exponential rate. Double-stranded DNA is denatured, a primer molecule is annealed at each end of the target fragment, and *Taq* DNA polymerase is used for nucleotide extension to create a new DNA fragment molecule.

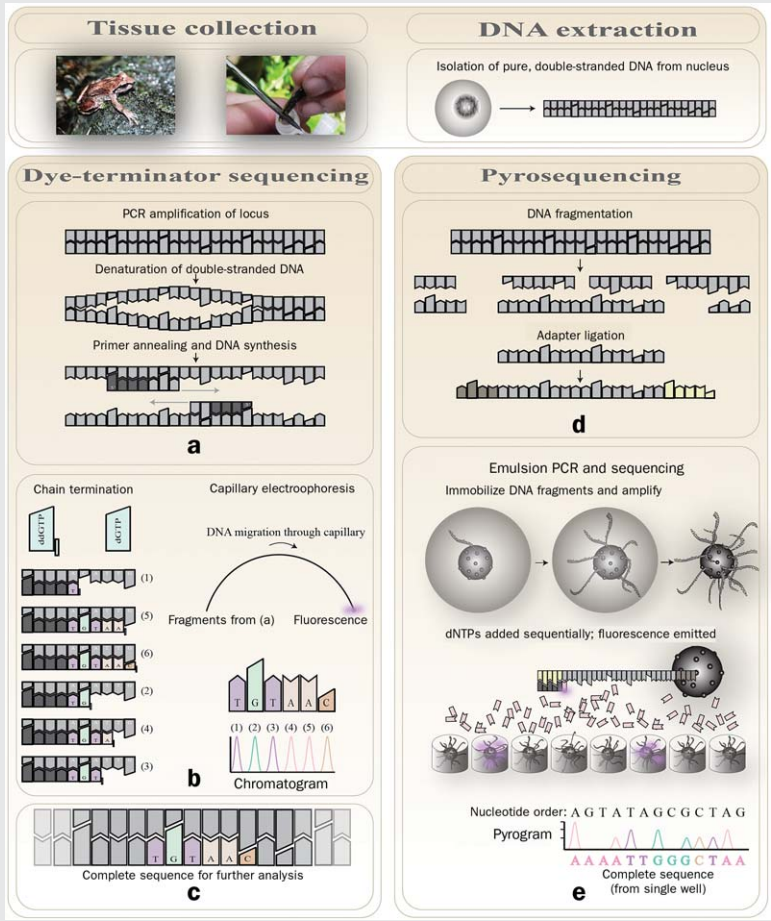
Pyrosequencing: A sequencing technique in which nucleotides are added one at a time to a template strand of DNA and the specific base identified at each step (based on emitted light). Pyrosequencing currently allows for more than 100,000 template DNA fragments to be sequenced simultaneously.

Quantitative trait loci (QTL): Segments of DNA that most likely contain a gene in close physical linkage (on a chromosome) to a phenotypic trait of interest.

Selective sweeps: The process in which an allele under selection increases or decreases in frequency within a population and, as a result, other alleles in linkage disequilibrium with that allele also increase or decrease in frequency.

Single-nucleotide polymorphisms (SNPs): Genetic markers that represent variation at a single DNA nucleotide site. SNPs occur throughout the genome, in both protein-encoding and noncoding loci, and are useful for a variety of population genetic and genomic applications.

Box 2. DNA sequencing techniques.



First, tissue is extracted from a study organism and DNA is extracted (top, right and left). Then DNA is sequenced using one of two major techniques: dye-terminator sequencing (left) or next-generation sequencing (right). In dye-terminator sequencing. the polymerase chain reaction (PCR) is used to replicate specific segments of DNA for sequencing by using primers with unique DNA base-pair sequences that complement targeted areas of the genome of the species being researched. Double-stranded DNA (dsDNA) is then heated to denaturation, becoming separated into single strands (ssDNA). Primers bind to the single strands, and a bioengineered DNA polymerase adds complementary nucleotides to each strand, resulting in two daughter strands (a). These steps are repeated, usually for 30 to 40 cycles, resulting in billions of copies of the targeted region of DNA from a single original copy. (b) Having used PCR to generate an abundance of copies for the target region, these fragments are used as a template in a sequencing reaction. A single primer is used to begin the synthesis of the complementary strand. A mixture of deoxynucleotide triphosphates (dNTPs) and dideoxynucleotide triphosphates (ddNTPs) are included. Each ddNTP has a different fluorescent dye incorporated into the molecule. If a ddNTP is incorporated into the newly synthesized strand, the stand can no longer be extended. In a probabilistic fashion, ddNTPs terminate chains at every nucleotide site. Fragments are size-fractionated through an electrophoretic gel matrix within the capillary: smaller fragments move more rapidly through the gel matrix, and movement of longer fragments is retarded. The fluorescent dye attached to each ddNTP will fluoresce when stimulated with a laser at the end of the capillary array. The fluorescence from the first fragment to emerge from the capillary corresponds to the first position (site) in the target DNA sequence. Fluorescence (wavelengths differ for each of the four nucleotides) is recorded to resolve the sequence of the target region. (c) Once the original target DNA sequence is fully compiled, a number of applications are possible for comparisons with other sequences within and among species.

In the panel on the right is an example of next-generation sequencing, namely, a pyrosequencing workflow implemented by 454 Life Sciences on the Genome Sequencer FLX (Roche Biosciences, Inc). (d) Genomic DNA is fractionated into small single-stranded fragments (250 nucleotides on average). Small molecules (adaptors) are attached to each ssDNA fragment. (e) Fragmented ssDNA is immobilized on beads coated with complementary RNA fragments of random sequence. As the DNA sequence of the sample is unknown, adaptors (of known sequence) allow for primer recognition in subsequent steps. In micelles, emulsion PCR is used to generate numerous copies of a particular fragment, each attached to a single bead. Roughly 400,000 PCR reactions occur simultaneously. Once fragments are PCR amplified, the DNA-bead complex is released from micelles and complexes are forced into wells (approximately 44 micrometers in diameter).

In subsequent sequencing reactions that occur in each well, the same nucleotides (e.g., all dATPs) are flowed across the entire set of wells in a sequential manner (e.g., first dATP, next dGTP, etc.), the order of which is predetermined. Each nucleotide has a pyrophosphate attached; if the particular nucleotide is complementary to the first free position in the fragment being sequenced, pyrophosphate is released from the nucleotide and reacts with sulfurylase, which causes chemiluminescent emission of light. The presence or absence of emitted light is recorded for each flowed nucleotide and for each well. The intensity of chemiluminescence within a well from a particular flowed nucleotide is proportional to the number of adjacent sites that have the same nucleotide in the sequenced fragment (e.g., a string of three adjacent A's will emit more light than a string of two A's). With the known order of flowed nucleotides and chemiluminescence intensity produced by each nucleotide in each well, the original sequence of the target genome can be identified once the generated data from pyrosequencing is assembled.

frogs in Asia (Shafiqul Alam et al. 2008). In contrast, a recent two-locus study suggested an overestimation of the number of species in the frog genus *Huia* (Stuart 2008). These studies illustrate the utility of using multiple loci to avoid under- or overestimation of taxonomic diversity, respectively.

DNA barcoding. In an attempt to standardize the segment of the genome analyzed, DNA barcoding uses "universal" PCR primers to generate short DNA sequences (approximately 800 nucleotides) that vary among species (Hajibabaei et al. 2007). The universality of the primers most commonly used to sequence mtDNA loci, such as cytochrome c oxidase subunit I (i.e., COI or *cox1*), facilitates coverage over wide taxonomic diversity. However, because mtDNA is inherited only maternally, overestimations of population distinctiveness can result from patterns of male-biased dispersal and genetic exchange. Thus, mtDNA barcoding seems more useful for rapid identification of specimens to species, given a sufficiently large reference database, than for within-species assessments of genetic diversity (Hajibabaei et al. 2007). However, taxonomic decisions made on the basis of DNA barcodes should be viewed with some caution because they suffer from the potential inaccuracies associated with the use of single loci.

Prior phylogenetic and barcoding studies have resulted in the availability of thousands of amphibian DNA sequences on electronic databases for assessments of diversity and taxonomic comparisons (see *www.barcodinglife.org* and Genbank, *www.ncbi.nlm.nih.gov*). However, the current availability of large amounts of DNA sequence data does not mean that all taxonomic problems will be resolved (reviewed in Wiens 2008). A leading challenge for future phylogenetics studies is the choice of segments of DNA to analyze and issues associated with combining data from different loci (see Wiens 2008 and references therein).

Single-nucleotide polymorphisms. Single base-pair variations at a certain site in an organism's genome—single-nucleotide polymorphisms, or SNPs—represent a ubiquitous and versatile class of genetic marker (Brumfield et al. 2003). Single-nucleotide polymorphisms are biparentally inherited and expressed, commonly have two alleles (e.g., an adenine, or A, nucleotide versus a guanine, or G, nucleotide), can occur in protein coding and noncoding regions of the genome, and are easily visualized using automated capillary sequencing methods (box 2) or with real-time PCR (box 3). With newly developed techniques for discovering single base-pair variations (e.g., ecotilling [box 1]; Gilchrist et al. 2006) and next-generation sequencing of parallel genomes (box 2), SNP discovery is becoming rapid and relatively inexpensive. By using DNA sequence data available online (e.g., Genbank), new SNP loci can often be characterized even without having to generate new sequence data (Brumfield et al. 2003).

Single-nucleotide polymorphism analyses are powerful for a variety of applications, including assessing genetic distinctiveness among populations (Palsbøll et al. 2007), detecting cryptic species (Shaffer and Thomson 2007), and confirming species membership (Brumfield et al. 2003). These analyses can also be used to track threatened species that are overexploited in commercial trade. For example, Apostolidis and colleagues (2007) developed a set of diagnostic SNP loci for rapid identification of the area of origin of specimens of the brown trout (*Salmo trutta*). In addition, SNP studies can be used to identify patterns of introgression, a biodiversity-threatening process resulting from the introduction of nonnative species that breed with and thereby dilute the genetic uniqueness of native species. Fitzpatrick and Shaffer (2007) used SNPs to assess the extent of genetic introgression of invasive barred tiger salamanders (*Ambystoma tigrinum mavortium*) in native populations of the threatened California tiger salamander (*Ambystoma californiense*). Higher fitness of hybrids relative to purebred *A. californiense* has recently been documented, potentially compromising the persistence of genetically pure populations of *A. californiense* (Fitzpatrick and Shaffer 2007).

Landscape genetics approaches to amphibian conservation

An important cause of amphibian declines and extinctions is habitat alteration and destruction (Stuart et al. 2004). Land-use change that causes habitat loss and fragmentation can restrict dispersal and, consequently, the exchange of genes (i.e., gene flow) that maintains genetic variation among populations (Slatkin 1987, Hedrick 2001). Restricted gene flow can thus result in reduced genetic diversity and higher susceptibility to the impacts of inbreeding, which can in turn further exacerbate demographic problems associated with small population size (Hedrick 2001). In addition, small, isolated populations may lack the genetic variability to adapt to future environmental change (see references in Hedrick 2001).

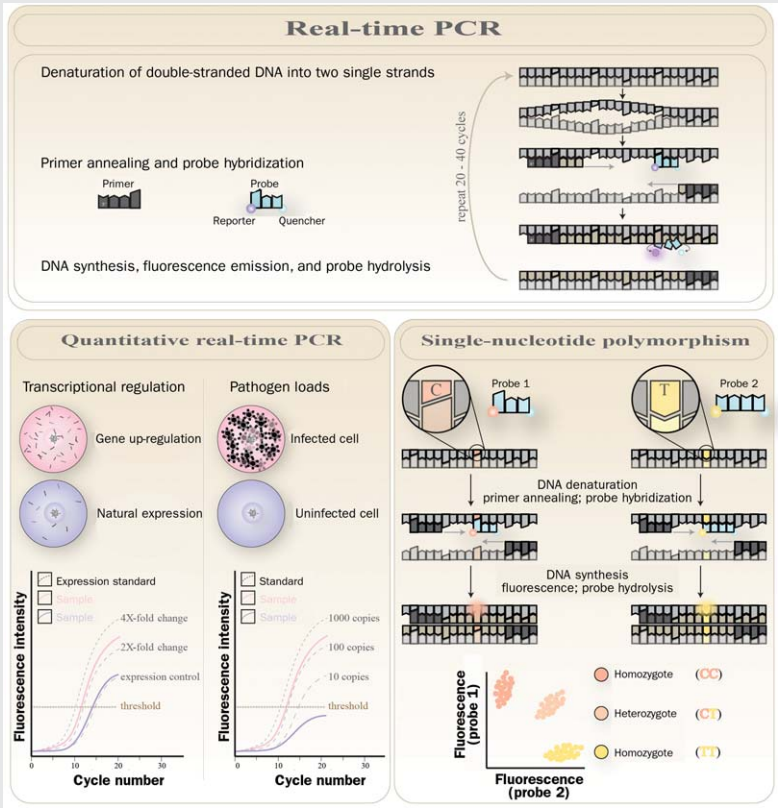
A new and exciting area of research, termed "landscape genetics" (Manel et al. 2003), holds great promise for assessing the effects of land-use change on amphibian population connectivity. While traditional population genetics studies often assume genetic connectivity along direct (Euclidean) routes between sites, landscape genetics studies explicitly quantify the effects of landscape composition, configuration, and matrix quality on genetic connectivity and spatial genetic variation (figure 1; Storfer et al. 2007). Most commonly, hypervariable genetic markers, such as microsatellites (box 1; reviewed in Jehle and Arntzen 2002) and SNPs, have been used in landscape genetics studies because they provide high statistical power to distinguish genetic diversity at the individual, group, or population level (Storfer et al. 2007).

Landscape genetics studies are particularly pertinent for amphibian research because amphibians generally have limited dispersal ability and high breeding-site fidelity (Beebee 2005). Amphibian movement is often limited by landscape features such as the distribution of moisture, shade, and topographic relief (Spear et al. 2005, Giordano et al. 2007). Landscape genetics studies can elucidate the specific

habitat features that affect amphibian genetic connectivity and the influence of land-use change on these features.

Studies that compare genetic diversity in developed versus undeveloped sites can be informative in this regard. For example, Noel and colleagues (2007) studied populations of the eastern red-backed salamander (*Plethodon cinereus*) in urban and undeveloped mountainous areas. They found that the urbanized mountain population showed lower rates of

Box 3. Real-time PCR.



As depicted, the TaqMan method (Applied Biosystems, Inc.) simultaneously generates new fragments of targeted regions of DNA or RNA and quantifies the number of fragments that are present in each cycle of the polymerase chain reaction (PCR). The process is much like standard PCR: double-stranded DNA is denatured into two single-stranded DNA (ssDNA) fragments. Primers anneal to appropriate sites in the sample DNA, one primer on each ssDNA fragment. Within the target region, a probe exhibiting a unique sequence hybridizes with complementary nucleotides of a single strand within the target region. In this implementation of real-time PCR, probes have two different attached fluorophores, a reporter and a quencher. As complementary nucleotides are added to each template strand, the nucleotides of the probe are hydrolyzed. This cleaves both fluorophores, and fluorescence is emitted. Each newly synthesized strand of DNA acts as template for each subsequent cycle of real-time PCR.

Quantitative real-time PCR. Quantitative real-time PCR is a sensitive method of detection offering both relative and absolute means of DNA or RNA quantification. If a researcher is interested in quantifying gene-expression differences among samples (often informed by microarray or expressed sequence tag studies), messenger RNA (mRNA) transcripts must first be converted into DNA. For this purpose, reverse-transcriptase PCR (not depicted) is used; in this technique, a fragment of DNA, complementary to the mRNA transcript of interest, is synthesized by reverse transcriptase. Once mRNA is converted into DNA, the PCR process proceeds as described above.

Standardized accumulation curves (gray) allow for relative or absolute quantification of DNA (or RNA) or original mRNA in samples using known quantities of DNA or standardized levels of gene expression. With additional fragments from the target region, free probe molecules will readily hybridize, and new DNA synthesis in each PCR cycle will result in greater fluorescence (the reporter and quencher fluorophores are hydrolyzed).

In the case of transcriptional regulation, the plot suggests that transcription of the pink sample was up-regulated (perhaps as a stress response to an environmental contaminant); in contrast, transcription for the gene in the purple sample is not distinguishable from transcriptional activity of the chosen "housekeeping" gene (i.e., the expression control) and would be considered to be neither up-regulated nor down-regulated. Similarly, the pink sample is infected with a high pathogen load, whereas the purple sample is not distinguishable from uninfected tissue. The more DNA copies there are, the more fluorescent probe copies will bond to the single-stranded DNA after denaturation; when complementary nucleotides are added to the strand with the probe attached, the more copies of the probe will be released and fluoresce brighter at an earlier cycle.

Single-nucleotide polymorphism. The nucleotide sequence in the probes is designed to exploit the single nucleotide differences among samples. Only individuals with a C at the single-nucleotide polymorphism (SNP) locus will allow hybridization of probe 1; only DNA expressing a T allele at this particular locus will hybridize with probe 2. Thus, individuals with the C allele will have a high ratio of probe 1 fluorescence to that of probe 2 fluorescence, and vice versa. Diploid individuals may express both alleles, and will bind probe 1 as often as probe 2. Visually, these differences can be detected by fluorescence difference, and individuals are scored as being heterozygous or homozygous for one or the other allele. Heterozygotes could be either individuals of the same population or hybrids from species with distinct SNP alleles fixed at this particular SNP locus.

gene flow and genetic diversity than did the forested mountain population (Noel et al. 2007). Thus, red-backed salamander populations may require habitat corridors among patches or translocations across unsuitable habitat to maintain genetic diversity in urban areas.

Landscape genetics studies can also reveal counterintuitive features that facilitate gene flow. For example, Spear and colleagues (2005) showed that tiger salamander gene flow was positively correlated with open shrub habitat in northern Yellowstone National Park. Although contrary to the initial hypothesis of dispersal through forested corridors, salamanders were very likely dispersing though post-1988 fire-regenerated shrub habitat, which provided shade and moisture lower to the ground than trees could. Without this study, then, management efforts for the tiger salamander could fail to appreciate the importance of maintaining shrub habitat in dispersal corridors.

Landscape variables can also act as barriers to gene flow. For example, recent studies have shown that mountains or areas of high topographic relief are barriers to both frog (Funk et al. 2005) and salamander (Giordano et al. 2007) dispersal and gene flow. Landscape limitations on gene flow may lead to divergent life histories or adaptation to different local conditions (Slatkin 1987, Giordano et al. 2007), so genetically differentiated populations may require distinct management practices (Storfer 1999).

Finally, landscape genetic studies may be used to predict responses to proposed development actions or other future habitat change. This may be accomplished, for example, through the use of least-cost modeling (figure 1) to test the influence of alternative paths through a particular landscape to reveal characteristics most positively correlated with gene flow. When considering a range of forest types and elevations, Cushman and colleagues (2006) determined that continuous forest at intermediate elevations best explained connectivity among populations of black bear (*Ursus americanus*). Other studies have attempted to produce maps of future landscape configuration using techniques such as landowner surveys of predicted land use (Pocewicz et al. 2008), simulations of future forest environments posited on alternative harvest rotations (Gustafson et al. 2001), or simulations of habitat alteration under varying climate change scenarios. These anticipated changes can be modeled onto the present landscape to predict future modification of landscape variables critical for facilitating or impeding gene flow. Although landscape genetics studies have yet to incorporate such simulations, this approach is promising for guiding future development actions (Storfer et al. 2007).

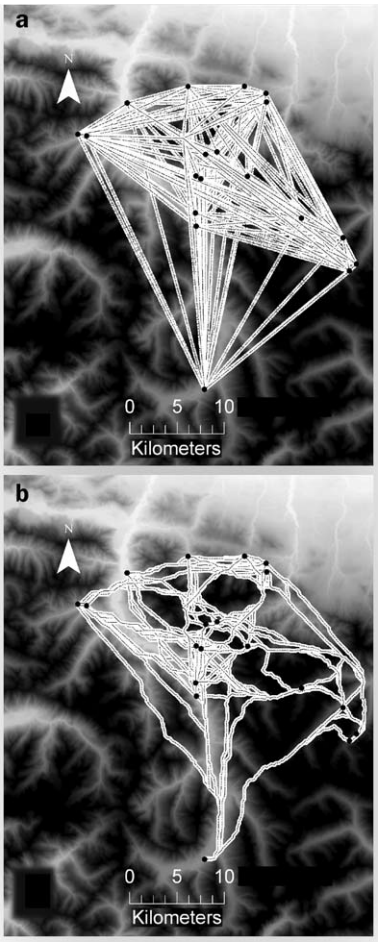


Figure 1. Landscape genetics uses information from geographic information systems to assess the influence of landscape features on gene flow among populations. This figure illustrates the example using a digital elevation model map of a portion of the Olympic Peninsula in Washington, with darker areas representing high elevation and lighter areas representing low elevation. Dots represent sampling localities for the tailed frog (Ascaphus truei). (a) Lines indicate straight-line Euclidean distances connecting sampling sites, as often used to calculate isolation-by-distance metrics. (b) Lines indicate least-cost paths that minimize areas of nonforest and high solar radiation along routes of connectivity among sampling sites. Note that the least-cost paths tend to avoid high-elevation regions, which are correlated with alpine meadows (nonforest) in this study area.

Real-time PCR approaches to amphibian conservation

Real-time PCR can have several important uses for amphibian conservation, including visualization of SNPs for characterizing population- and species-level diversity and identifying emerging infectious diseases implicated in some of the more enigmatic cases of amphibian declines (box 3). Emerging infectious diseases—and in particular two types of pathogens, ranaviruses and the chytrid fungus *Batrachochytrium dendrobatidis (Bd)*—are causes of widespread amphibian epizootics and declines (Daszak et al. 2003, Stuart et al. 2004). In some areas, pathogens such as *Bd* can cause catastrophic declines of many species, as has been shown in Central America (Lips et al. 2006). Other parasites also affect amphibian populations. For instance, the fungus *Saprolegnia ferax* (Daszak et al. 2003) and a recently discov-

ered *Perkinsus*-like protist (Davis et al. 2007) are implicated in localized declines, whereas parasitic worms in the trematode genus *Ribeiroia* are implicated in widespread deformities (Johnson et al. 2007).

Given the diversity of pathogens emerging in amphibian populations and the precipitous nature of many resulting epizootics, a crucial step in responding to pathogen-mediated declines is to rapidly and correctly identify the responsible infectious agent. Much like amphibian species identification, parasite species identification is often achieved with molecular methods such as DNA sequencing and SNP analysis. Then, to predict pathogen effects on host populations, it is crucial to know pathogen prevalence (percentage of individuals infected) in natural populations for predictive population dynamics modeling (see references in De Castro and Bolker 2005).

Knowledge of pathogen prevalence in host populations aided accurate predictions of *Bd* spread through populations of mountain yellow-legged frogs (*Rana muscosa*) in the California Sierra Nevada using theoretical modeling (Rachowicz and Briggs 2007). In fact, one of the most important determinants of *Bd* spread in a population of yellow-legged frogs is pathogen load on individual hosts, which is detected using real-time PCR (Rachowicz and Briggs 2007).

Batrachochytrium dendrobatidis has also been implicated in population-level extinctions for a wide variety of taxa in both tropical and temperate zones (Daszak et al. 2003, Lips et al. 2006, Rachowicz and Briggs 2007). In theory, however, pathogens should not cause host extinctions, because when host density drops below a critical threshold, pathogen transmission becomes exceedingly rare, driving the pathogen locally extinct (De Castro and Bolker 2005). Pathogens can persist in sparse host populations by residing in less-susceptible reservoir species or persisting in the abiotic environment (De Castro and Bolker 2005), both of which are apparently the case for *Bd* (Lips et al. 2006, Mitchell et al. 2008). Indeed, persistence of *Bd* in the abiotic environment appears important for explaining host extinction dynamics (Mitchell et al. 2008).

Quantitative real-time PCR (box 3) is a recent advance that is highly sensitive for detecting low quantities of DNA or protein products. Real-time PCR is up to 1000-fold more sensitive than traditional PCR (box 3) and can be used for assessing pathogen presence and infection levels on biotic hosts or reservoir species. Researchers developed a real-time PCR assay that is sensitive enough to detect a single *Bd* zoospore equivalent (the microscopic, free-swimming stage of the fungus) (Boyle et al. 2004), which is useful for sampling both animals and abiotic environmental samples (Walker et al. 2007). Widespread surveys have shown *Bd* to be present on several continents (Daszak et al. 2003, Stuart et al. 2004, Lips et al. 2006, Weldon and Du Preez 2006).

Quantitative PCR methods have also been developed for environmental detection of *Ambystoma tigrinum* virus (Brunner et al. 2004), a ranavirus infecting tiger salamanders and implicated in the declines of the endangered Sonoran tiger salamander (Jancovich et al. 1997). These methods can also be used following laboratory experiments to determine species' susceptibility, such as that of the endangered California tiger salamander (Picco et al. 2007), or pathogen loads in experimentally infected individuals (e.g., Forson and Storfer 2006). Based on the major capsid protein DNA sequence, a highly conserved structural gene among ranavirus strains (Chinchar 2002), real-time PCR protocols should easily be developed for ranaviruses isolated from other species. Similar implementations of real-time PCR could be developed for detecting other pathogen species such as *Saprolegnia* and *Ribeiroia.*

Genomic approaches to amphibian conservation

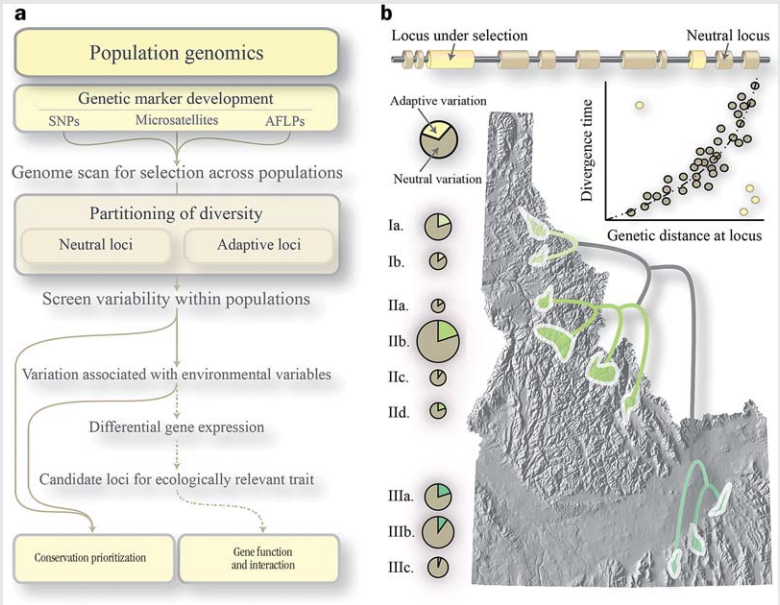
Perhaps one of the biggest questions in amphibian conservation is, Why are some populations and species susceptible to decline and extinction, while other populations and species are resistant? Genomic approaches hold great promise for addressing this question mechanistically by allowing assessment of the genes underlying the variation in species' susceptibility to such stressors as pathogens and chemical contaminants. Knowledge of genes associated with resistance or plasticity in response to environmental stressors can lead to focused conservation efforts on particular populations or species to maintain such adaptive variation. In addition, understanding spatial genetic variation in such functional genes can guide possible translocations of individuals with particular adaptive variants into populations lacking them. Such approaches are generally not feasible at present because of the lack of genomic resources for non–model species, but the techniques discussed below may be used in the future as they become more accessible and cost effective.

Several recent approaches have been used to discover candidate genes associated with adaptive genetic variation. First, we discuss the development of large numbers of variable loci for "population genomics" (Luikart et al. 2003), or linkage disequilibrium mapping (box 1; Stinchcombe and Hoekstra 2008). Next, we highlight methods for identifying genes that underlie adaptive genetic variation using microarray analyses developed from expressed sequence tag (EST [box 1]) libraries (Bouck and Vision 2007), cDNA libraries (box 1), or large amounts of DNA sequence data (e.g., from next-generation sequencing). Finally, we discuss methods to understand the function of genes associated with adaptive variation (i.e., "functional genomics"), and, in turn, to screen natural populations for variation in genes that may be correlated with susceptibility to declines or extinction, one goal of the emerging field of ecological genomics.

Population genomics. Population genomic analyses that entail development of large numbers of highly variable genetic markers such as microsatellites, SNPs, or amplified fragment length polymorphisms (AFLPs [box 1]) are perhaps most feasible for genomic amphibian population assessments. Whereas population genetics studies typically assess presumed selectively neutral genetic variation to estimate levels

Box 4. Population genomics.

With an abundance of anonymous loci (microsatellites, amplified fragment length polymorphisms, single-nucleotide polymorphisms, etc.), a genomewide scan for loci under selection is performed (a). As shown, under neutral evolution, higher levels of genetic divergence (among populations) are expected to be associated with longer periods of evolutionary independence. Loci that differ significantly from this null expectation are deemed outliers and are presumed to be under selection (or at least are genetically linked to fitness-determining loci). Loci that are more diverged among populations than expected are under divergent selection; loci that are less divergent than expected may be under stabilizing selection. If outlier loci are isolated, functional genomic techniques can be used to hypothesize and confirm gene function for ecologically relevant traits (e.g., timing of metamorphosis, adaptation to environmental variables). (b) The size of the pie charts indicates overall variability (e.g., average allelic diversity within each population); relative proportions of adaptive variation (proportion of nonneutral loci for which allele frequencies in the population differ significantly from those of other populations) and neutral variation (proportion of neutral loci for which allele frequencies in the population differ significantly from those of other populations) are indicated by colored and gray slices, respectively. Patterns of partitioned adaptive and neutral variation among populations may prove useful for conservation prioritization. For instance, on the basis of the population adaptive index, population IIb might receive highest conservation priority.



of inbreeding or genetic structuring among populations, population genomics focuses on large numbers of loci spread throughout the genome to identify individual markers that are most likely under selection (box 4; Luikart et al. 2003, Stinchcombe and Hoekstra 2008). Loci that are statistical outliers and show patterns distinct from those exhibited by the majority of loci studied across populations in different environments are assumed to be under selection or linked to those under selection (box 4; Luikart et al. 2003, Stinchcombe and Hoekstra 2008). For example, an assessment of 392 AFLPs in the common frog (*Rana temporaria*) among populations at varying altitudes in the Alps revealed 8 candidate loci potentially involved in adaptation to altitude (Bonin et al. 2006). In the same manner, linkage disequilibrium mapping tests (box 1) for statistical associations between particular molecular markers and phenotypic variation (Mitchell-Olds and Schmitt 2006, Stinchcombe and Hoekstra 2008). Identification of outlier loci can also indicate the occurrence of selective sweeps (box 1; Schlötterer 2002), in which particular populations exposed to a stressor, such as a highly virulent disease, exhibit significantly higher frequencies of certain alleles than do other populations. Such alleles may thus be interpreted as candidate loci that confer disease resistance.

One approach for prioritizing conservation of adaptive variation is to estimate the population adaptive index (PAI) among populations (Bonin et al. 2007); the PAI measures the percentage of loci in a population that significantly diverged from other populations. Populations with a higher PAI are assumed to have more loci under selection and thus harbor more adaptive genetic variation than those with a lower PAI. Nonetheless, population genetics studies most commonly measure neutral genetic diversity, which may not accurately reflect the level of adaptive diversity (Hedrick 2001). As an example, the two populations of the common frog harboring the most adaptive genetic diversity as estimated by the PAI were different from the two populations with the highest diversity as estimated by neutral markers (Bonin et al. 2007). Because estimates of adaptive variation may predict responses to future environmental change better than neutral variation estimates, measures such as the PAI may prove useful for targeting conservation efforts.

Expressed sequence tag libraries and microarrays. Expressed sequence tag libraries are generated by isolating transcribed messenger RNA from tissues of interest (e.g., liver tissue) from individuals exposed to different environments or those representing different genotypes (see figure 1 in Bouck and Vision 2007). Messenger RNA is then converted to cDNA using reverse-transcriptase PCR. An EST library is created by cloning cDNA fragments into plasmid vectors, and the DNA of the clones is then sequenced, revealing only protein-encoding DNA sequences (Bouck and Vision 2007).

The EST libraries can be used for a variety of applications to guide large-scale sequencing projects, to identify candidate genes, or to assess variation in gene expression using microarrays (box 1). A large EST library has been generated for the Mexican axolotl (*Ambystoma mexicanum*) and the tiger salamander as a resource for other genomic studies (*www.ambystoma.org*; Smith et al. 2005). This library was also used to create a linkage map (box 1) presumed to correspond with the 14 chromosomes in the haploid genomes of *A. tigrinum tigrinum* and *A. mexicanum* (Smith et al. 2005). The linkage map was used to determine chromosomal regions that influence phenotypic variation (also called quantitative trait loci, or QTLs [box 1]), such as metamorphic timing (Smith et al. 2005). Interestingly, tiger salamanders are phenotypically plastic in metamorphic timing, whereas axolotls fail to metamorphose. An understanding of the genetic basis of such differences may yield important insights into potential responses to environmental change. For example, climatic warming may increase the ephemerality of amphibian aquatic habitats, and therefore require metamorphosis in some areas earlier than is necessary at present. Researchers have also investigated two hybridizing species of fire-bellied toads, *Bombina bombina,* which breeds in permanent habitats, and *Bombima variegata,* which breeds in ephemeral habitats (Nürnberger et al. 2003). Hybrids from the contact zone allowed development of a genome linkage map of the two species to identify loci implicated in adaptation to habitat ephemerality or other environmental features (Nürnberger et al. 2003).

Expressed sequence tags can also be used to construct microarrays, a powerful tool for assessing variation in gene expression under different environmental conditions (Bouck and Vision 2007). A microarray is a chip that contains thousands of cDNA probes (developed from EST libraries or whole genome libraries) that will hybridize to host cDNA labeled with fluorescent dyes. Messenger RNA is extracted from animals under different environmental conditions, reverse-transcribed into cDNAs, and allowed to hybridize to the chip. The more a particular gene is expressed, the more it will hybridize to a particular cDNA probe on the chip, resulting in higher fluorescence.

Significant challenges that affect the utility of microarrays for amphibians include (a) their limited availability for non–model species and the high costs of new development; (b) concern that their utility for field-based studies is limited because of the high sensitivity of gene expression to slight variation in environmental conditions (Travers et al. 2007); and (c) the fact that many gene expression arrays are not complete genome arrays, and thus potentially miss some functionally important genes. Nonetheless, custom array development for new species, achieved by contracting with commercial outfits (such as NimbleGen) or by designing them in labs equipped for this purpose, is becoming increasingly affordable. Whereas microarrays have been developed for a few model species such as *A. mexicanum* and *A. tigrinum* and for two species of African clawed frog, *Xenopus laevis* and *Xenopus tropicalis,* properly designed cross-species amplification may work in closely related species (Travers et al. 2007).

Despite these challenges, microarray analyses are beginning to yield insights that may aid amphibian conservation efforts. Using a microarray developed for *A. mexicanum,* Page and colleagues (2007) showed that transcript abundances of several keratin genes dramatically increased in this species in response to exposure to thyroid hormone, which is critical in amphibian development and metamorphosis. Inasmuch as environmental chemicals often mimic natural chemicals such as thyroid hormone and can act as endocrine disruptors, such microarray analyses may be useful for assessing amphibian responses to chemical contaminants. Studies could also be performed to elucidate gene expression differences between infected or disease-free individuals within a species or among species that vary in disease susceptibility. For example, ranaviral infections of axolotls result in extremely high rates of mortality (Cotter et al. 2008), whereas African clawed frogs tend to survive and clear infections (Morales and Robert 2007). A microarray study revealed that axolotls infected with a ranavirus apparently fail to mount an effective T-cell mediated immune response (Cotter et al. 2008), whereas *Xenopus* does mount such a response (Morales and Robert 2007), potentially explaining differences in susceptibility between the two species.

Functional and ecological genomics. The biological interpretation of results from candidate gene or gene expression assays is often difficult, and determining gene function remains a challenge in genomics, particularly for non–model organisms (Mitchell-Olds and Schmitt 2006, Travers et al. 2007). Inasmuch as complex traits are influenced by a suite of interacting gene products and by genotype-by-environment interactions, functional genomics provides a framework for understanding the mechanistic processes underlying phenotypic variation (Mitchell-Olds and Schmitt 2006, Bouck and Vision 2007). Once a pool of candidate genes is identified, gene function can be verified through targeted mutations in the gene locus and posttranscriptional gene silencing by interference RNA (reviewed in Novina and Sharp 2004), but these are laborious techniques. When candidate genes are altered or silenced, the function of the gene can be verified if the phenotypic consequences match expectations (e.g., the loss of the putative protein product associated with that gene). Candidate genes could also be cloned into a vector, with their function confirmed if the same protein product is produced (Novina and Sharp 2004). At present, techniques such as gene silencing are feasible only for model species, but indirect methods can be used to infer gene function for non–model species.

Indirect methods for inferring functional properties of genes include cluster analysis (e.g., expression data from microarrays or EST libraries) and gene annotation by comparing observed DNA or cDNA sequences with existing databases. Signatures of transcriptional expression patterns are

often conserved among orthologous genes (box 1), even in widely divergent taxa (Lamason et al. 2005). Thus, although functional gene sequences may be lacking for a particular amphibian species under study, they may be present for related taxa, and thus may contribute to inferences about the studied species. Moreover, the characterization of complete genomic sequences and gene functions of many model organisms (*Caenorhabditis elegans, Drosophila melanogaster, Homo sapiens*) are used to hypothesize the function of orthologous genes in non–model organisms (Kohn et al. 2006). The Joint Genome Institute (*genome.jgi-psf.org/Xentr4/Xentr4.home.html*) is now assembling the *X. tropicalis* genome. The mapping of this genome will be an important step for understanding the relationship between genome assembly and gene function, and will assist with functional genomic annotations in other amphibian species.

Ecological genomics, a field that investigates variation in genomes and genes across natural environments and can be used to apply functional genomic techniques to conservation biology, has been addressed in a number of recent reviews (e.g., Kohn et al. 2006, Landry and Aubin-Horth 2007). Once we understand the genes underlying the traits that are particularly important for variation in susceptibility to declines, we can begin to understand how these genes vary among natural populations and species. Related population genomic approaches may be quite useful for guiding decisions regarding population-level conservation, particularly if the goal is to focus on conservation of adaptive, rather than neutral, genetic variation (Kohn et al. 2006).

Conclusions

Given the urgency of global amphibian declines, challenges lie ahead for conservation and management. In this article we have described molecular approaches to continue the important global assessment of amphibian biodiversity, as well as to address some of the key hypotheses associated with amphibian declines. First, we highlighted recent advances in automated DNA sequencing methods that will help to rapidly classify newly discovered diversity, as well as to refine the evolutionary relationships of existing diversity. Accurate classification of existing as well as incipient diversity is critical for focusing conservation efforts. We then discussed applications of some modern molecular approaches to address leading hypotheses for amphibian declines. Landscape genetics is a relatively new approach that may help assess effects of current and future habitat alteration on amphibian population connectivity. Understanding the particular landscape features important for the maintenance of gene flow may help guide future development actions so as to minimize their impacts. Real-time PCR can be useful for identifying and characterizing the prevalence of emerging infectious diseases implicated in global amphibian epizootics and declines. Genomic techniques, although in their infancy for application to natural populations, hold promise for understanding variation in adaptive genetic diversity and genes underlying variation in resistance and susceptibility to environmental stressors and diseases. We hope that such techniques will become more readily available for the study of non–model organisms to guide effective management and conservation.

Overall, amphibian declines are often influenced by a complex interaction of multiple factors. We have focused on molecular approaches for studying several of these, including habitat loss, emerging infectious diseases, climate change, and environmental contaminants. Our list is not exhaustive, nor do we extensively consider methods to investigate the interactions of factors. Nonetheless, we hope that the guidance we provide for some existing conservation projects will prove helpful and provoke ideas for application of new methods for future studies.

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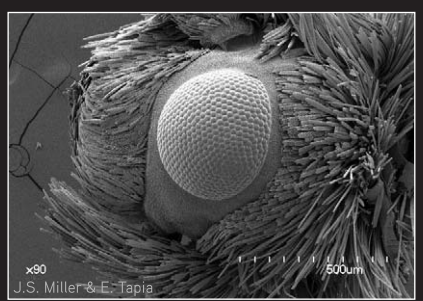

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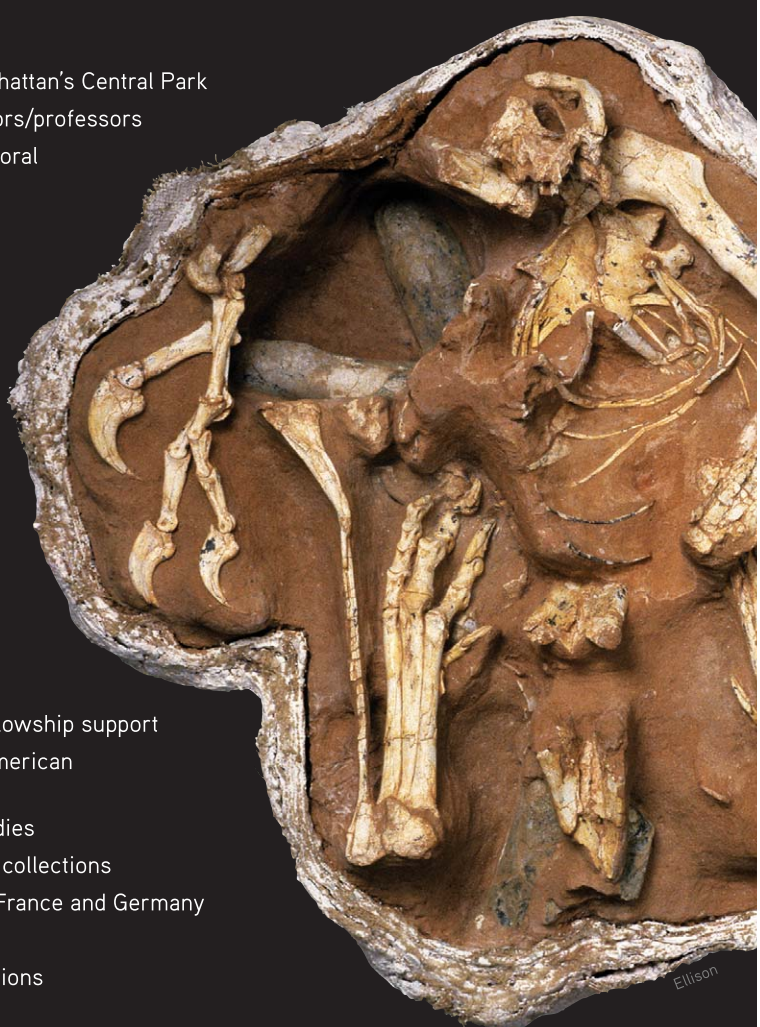

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