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REVIEW:

MICROSATELLITE MARKERS IN AMPHIBIAN CONSERVATION GENETICS

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Recent technical advances allow straightforward access to genetic information directly drawn from DNA. The present article highlights the suitability of high variation molecular genetic markers, such as microsatellites, for studies relevant to amphibian conservation. Molecular markers appear particularly useful for i) measuring local gene flow and migration, ii) assigning individuals to their most likely population of origin, iii) measuring effective population size through the between-generation comparison of allele frequencies, and iv) detecting past demographic bottlenecks through allele frequency distortions. We demonstrate the use of some newly developed analytical tools on newt (*Triturus* sp.) microsatellite data, discuss practical aspects of using microsatellites for amphibians, and outline potential future research directions.

Key words: amphibians, conservation, microsatellites, *Triturus cristatus*

INTRODUCTION

The introduction of enzyme electrophoretic techniques in the 1970s enabled direct access to genetic information from wild populations (Lewontin, 1991). In the European amphibian fauna, protein variants facilitated the description of several previously unrecognized species (e.g. Busack, 1986; Beerli *et al.*, 1994; Arntzen & García-París, 1995; Lanza *et al.*, 1995 - reviewed in Veith, 1996 and García-París & Jockush, 1999). However, owing to the relatively low level of genetic variation documented by protein variants, their application for evolutionary and ecological inferences was often limited to large-scale analyses, typically at the level of species and subspecies (e.g. Rafinski & Babik, 2000). Only in the last one or two decades have laboratory advancements such as the advent of routine sequencing and PCR (Polymerase Chain Reaction) technology permitted access to genetic information from across geographical ranges (Alexandrino *et al.*, 2000, 2001; Riberon *et al.*, 2001; Zeisset & Beebee, 2001). Such information can, for example, be used for the definition of 'Evolutionary Significant Units', an operational level of organization for assessing biodiversity independent from taxonomic hierarchy (Moritz, 1994; Crandall *et al.*, 2000).

One class of newly developed DNA-based markers – microsatellite loci (Goldstein & Schlötterer, 1999) – is currently receiving particular attention. Microsatellites occur in high numbers in every eukaryote genome, and consist of tandem repetitive units of DNA typically less than five basepairs in length, with a high variability due to different repeat numbers (e.g. [CA]n); for more information on specific properties of amphibian microsatellites see, for example, Neff & Gross (2001). Microsatellites are amplified with specific PCR primers and the different alleles separated along an electrophoretic gradient in routine laboratory procedures. However, the development of statistical tools for the analysis of the data has lagged behind, and some new methods, such as computer-aided Maximum Likelihood, Bayesian statistics and Markov Chain Monte Carlo procedures (for overviews see Beaumont & Bruford, 1999; Luikart & England, 1999; Sunnucks, 2000) are not yet included in the standard toolbox of many population ecologists.

Most amphibians depend on both aquatic and terrestrial habitats, and for protection and management plans their local population dynamics as well as the degree of population connectivity must be considered (Semlitsch, 2000). Furthermore, amphibians have relatively low dispersal abilities and are often philopatric, leading to distinct populations that can represent unique genetic entities despite geographic proximity (Kimberling *et al.*, 1996; Waldmann & Tocher, 1997; Driscoll, 1999; Scribner *et al.*, 2001). Amphibians therefore appear highly suitable for addressing population and conservation genetic issues, but are as yet under-represented in this research area. For example, the otherwise prominently debated global amphibian population decline is only little studied from a genetic point of view (but see Shaffer *et al.*, 2000), although there is evidence that the amount of genetic variation could have an impact on fitness-related traits (Rowe *et al.*, 1999). The aim of the present article is to demonstrate the power and utility of highly variable DNA-based markers and some recently developed analytical methods for population studies on amphibians, and the value of such studies for conservation issues.

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METAPOPULATION PROCESSES

Amphibians are well suited to address questions at the level of metapopulations (e.g. Hanski, 1998), and have in recent years become a focus for studies on the effect of landscapes and landscape alterations to wildlife (e.g. Halley *et al.*, 1996; Vos *et al.*, 2001*a*). Population size fluctuations can be addressed in a straightforward way with standard field methods, but to assess the exchange of individuals between populations at the landscape scale is difficult with fieldwork alone. We anticipate that the most ground-breaking applications of fine-tuned, DNA-based markers will be at the regional, metapopulation scale. Questions on how processes like source-sink dynamics, population connectivity and extinction-recolonization frequencies affect the maintenance of within-species genetic diversity have so far only been addressed through modelling (Whitlock & Barton, 1997; Pannell & Charlesworth, 2000; Higgins & Lynch, 2001). We now have the molecular tools at hand for obtaining empirical data on such issues, and amphibians are particularly promising study organisms in this respect.

Allozymes have already revealed some influences of human-induced landscape fragmentation on the genetic structure of European anurans of the genera *Bufo* and *Rana* (Reh & Seitz, 1990; Hitchings & Beebee, 1997; 1998), and lower genetic differentiation in natural situations relative to human-altered regions (Seppä & Laurila, 1999). Using microsatellites, Garner *et al.* (submitted) demonstrated for *R. latastei* that populations at the periphery of the distribution range are genetically depleted and should be considered as particular conservation units. Vos *et al.* (2001*b*) showed that linear barriers to dispersal such as roads had a significant impact on genetic distance measures of European moor frogs (*R. arvalis*), whereas only little fine-scale genetic differentiation was found for North American frogs (*R. sylvatica*) in a highly dynamic wetland landscape (Newman & Squire, 2001). Moreover, Rowe *et al.* (2000*a*) demonstrated the fit of a mixed 'island/island-mainland' metapopulation model to three sets of neighbouring breeding sites of remnant natterjack toad (*B. calamita*) populations in Britain. However, all four microsatellite-based studies have not yet fully explored the new analytical tools now available (e.g. Beerli & Felsenstein, 2001), and further insights into fine-scale metapopulation processes are to be expected in future studies.

Because not only the connectivity of populations but also the availability of summer habitats shapes the amphibian metapopulation structure (Pope *et al.*, 2000), detailed quantitative assessment of all utilized aquatic and terrestrial habitats is required to predict accurately the determinants of metapopulation processes. Among the most promising approaches to studies addressing this issue is perhaps the combination of data derived from microsatellites with fine-scaled landscape data – such as those obtained using GIS techniques – to identify colonization routes along environmental gradients (Arntzen *et al.*, in preparation).

INDIVIDUAL IDENTIFICATION AND ASSIGNMENT METHODS

Estimates of population structure and local migration have traditionally been obtained through the identification of individuals from phenotypic features, or some sort of tag. Alternatively, the number of alleles segregating over a panel of microsatellite loci enables the genetic recognition of individuals without physical marking ("genetic tagging", Palsböll, 1999; Taberlet & Luikart, 1999). Genetic tagging may be particularly recommendable for amphibians, as it circumvents traditional physical markings frequently claimed to be harmful, such as the removal of several toes.

Among the latest developments in the toolbox of the population geneticist are methods to classify individuals according to the most likely population of origin, based on their genotype (Rannala & Mountain, 1997; Waser & Strobeck, 1998; Dawson & Belkhir, 2001; Hansen *et al.*, 2001). Although such "assignment tests" are not new (Jamieson, 1965), they became widely applicable only with the availability of large amounts of genotypic data in combination with high computational speed and appropriate software (GENECLASS: Cornuet *et al.*, 1999; WHICHRUN: Banks & Eichert, 2000; STRUCTURE: Prichard *et al.*, 2000). Using a sufficient number of variable loci in combination with an adequate sample size the approach is surprisingly powerful, even when the reference populations are genetically rather similar (Bernatchez & Duchesne, 2000).

Assignment methods are currently far from fully explored in amphibian conservation, despite several potential applications. Illegal cases of collecting and trade could be revealed by genetically tracing the source population of involved individuals (as done for fish, Primmer *et al.*, 2000). Similarly, the source of local introductions could be inferred, an important issue when determining whether a species belongs to a regional fauna or not (Szymura, 1998; Arntzen, 2001; Zeisset & Beebee, 2001). The most promising application of assignment methods for conservation-related research on amphibians, however, lies not in tracing "alien" individuals, but in measuring between-population connectivity at a scale equal to or smaller than the migratory range of the species under study.

To provide an example of the power of assignment methods, we applied the likelihood Bayesian approach provided by GENECLASS to two French *Triturus cristatus* populations ca. 10 km apart (data from Jehle *et al.*, 2001). Thirty-five and 168 adults (48% and 29% of the estimated population census sizes, respectively) and some larval offspring (40 and 87 individuals, respectively) were assayed for eight microsatellite loci with between two and nine alleles each. The populations were differentiated at the level of Fst=0.045. We tested the null hypothesis that individual larvae would be as-

signed to their true parent population. From 74-88% of the larvae were correctly classified ($P<0.001$ for both populations, G-test for goodness of fit, $G=25.3$ and 20.1, respectively, df=1). In another analysis, without having any knowledge about potential source populations, we also estimated the probability for every adult individual that its genotype belongs to the population where it was captured. Eight adult newts (6%) did not fit the genetic profile of the population in which they were caught ($P<0.01$). Such individuals could be immigrants or their offspring, although a rigorous assessment of migration patterns would require genetic data from all regional populations, in order to assign such putative migrants to their most likely origin. It also has to be kept in mind that the results of assignment tests are inferential and, in contrast to physical capture-mark-recapture, are not based on actually recorded movements.

Measuring Effective Population Size

The size of a population is generally taken to be the total number of individuals at a certain locality, but from an evolutionary point of view, only those individuals which are successful in reproduction are important. Therefore, the census size of a population is distinguished from the "effective population size" (N_e, Wright, 1931). Current efforts for protecting and sustaining endangered and rare species often focus on the maintenance of genetic diversity (Sherwin & Moritz, 2000; Hedrick & Kalinowski, 2000; Hedrick, 2001), and it is the effective population size that determines the amount of genetic variation maintained over time. Intuitively one might expect the effective population size to be close to the adult population census size, but parameters such as reproductive failures, skewed sex ratios and substantial reproductive skews caused by specific mating systems can bias N_e up to several orders of magnitude below census size (Frankham, 1995). Because N_e is not easy to measure, comprehensive data are not yet available for many taxa, rendering the routine use of N_e in practical conservation controversial (Mace & Lande, 1991).

Measures of N_e can be obtained through demographic methods that incorporate life-history data into analytical equations (Nunney & Elam, 1994; Basset *et al.*, 2000). Unfortunately, obtaining precise life-table parameters – and particularly their variances – can be difficult. Genetic methods, however, enable calculation of N_e from one or more genetic samples without detailed life-history knowledge (Schwartz *et al.*, 1998). The "temporal method", which is based on two samples taken from one population, is particularly straightforward (Waples, 1989; Williamson & Slatkin, 1999). As N_e increases, genetic drift – and therefore the temporal change in allele frequencies – decreases. Assuming that selection, mutation, migration and population subdivision is negligible, one parameter can be estimated from the other (Fig. 1), with highly polymorphic loci such as microsatellites providing particularly precise estimates (Turner *et al.*, 2001). The main practical disadvantage of the temporal method is that at least two samples are required, ideally with several generations between sampling dates. For discussions on possible violations of assumptions of the temporal method when using microsatellites, see Jehle *et al.* (2001, and references therein).

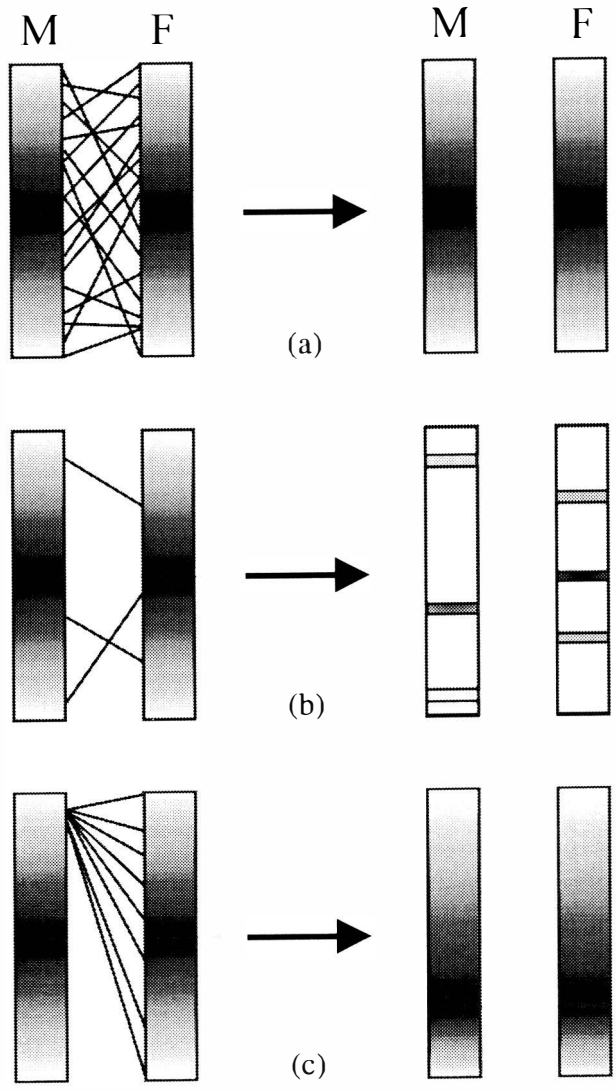

FIG. 1. Schematic and simplified representation on the effect of effective population size on between-generation changes in the spectrum of allele frequencies; (a) large numbers of breeders: the amount of genetic diversity does not change markedly between generations; (b) low number of breeders: the maintained amount of genetic diversity decreases; (c) skewed operational sex ratio: the allele frequency distribution becomes distorted. In both (b) and (c), the between-generation variance in allele frequencies is high, and the effective population size is low. M, males; F, females.

In amphibians, the methodological basis of measuring N_e ranges from counting the number of egg clutches to various demographic and genetic estimates (Merrell, 1968; Gill, 1978; Easteal, 1985; Berven & Grudzien, 1990; Driscoll, 1999; Seppä & Laurila 1999). Estimates of N_e with the temporal method were made for common toads (*Bufo bufo*, Scribner *et al.*, 1997, based on minisatellites), north American salamanders (*Ambystoma*

macrodactylum, Funk *et al.*, 1999, based on allozymes) and European newts (*Triturus cristatus* and *T. marmoratus*, Jehle *et al.*, 2001, based on microsatellites). The toad study revealed that just 1% of the adult population successfully reproduced in a particular year. In newts, N_e was 10-20% of the adult population census size. The difference in N_e between toads and newts was in accordance with knowledge of the species' reproductive modes, characterized by large and small variances in reproductive success, respectively. A potentially very accurate method for calculating the effective number of breeders in a population would be to reconstruct paternal genotypes for offspring with known mothers (Pearse *et al.*, 2001), but such data are difficult to obtain in natural amphibian populations.

Notwithstanding some methodological differences, effective population size in amphibians has generally been estimated as under a hundred individuals, whereas the minimum effective population size required to maintain genetic variation sufficient for demographically viable populations is thought to be between 500 and 5000 individuals (Franklin & Frankham, 1998; Lynch & Lande, 1998). Given that many European amphibian species are subject to increasing population isolation, these findings suggest that the long-term survival of many populations is in danger, more so than field ecological studies would reveal. Future studies could also model spatial genetic structure by combining N_e measures with an assessment of population connectivity. This would enable the assessment of the effective size of a whole metapopulation (Whitlock & Barton, 1997), a measure that could determine the rate of within-species genetic erosion on a large scale.

Detecting Past Population Bottlenecks

Genetic bottlenecks occur when populations experience severe, temporary reductions in their effective size, and can dramatically reduce the genetic diversity of populations. For example, a high degree of inbreeding depression is often interpreted as the result of a past population bottleneck (Hedrick & Kalinowski, 2000). Traditional measures of genetic diversity, such as heterozygosity and allelic diversity, can be used to infer a past bottleneck, but require a reference sample either from before the event or from another, non-bottlenecked population (Spencer *et al.*, 2000; for a simulation program on the effects of bottlenecks on genetic diversity see e.g. England & Osler, 2001). Statistical methods have recently been developed to infer the demographic history of a population from a single genetic sample. One method is based on the premise that bottlenecking gives rise to an excess of heterozygotes compared to the level of heterozygosity expected at mutation-drift equilibrium, because under bottlenecks rare alleles have a higher risk of going extinct than common alleles (Cornuet & Luikart, 1996; Luikart *et al.*, 1998; Garza & Williamson, 2001, see Fig. 2). Depending on the sample size and marker variability, this method can detect such a heterozygote excess for up to about ten generations after its occurrence (Luikart *et al.*, 1998*a*), although false bottlenecks signals can appear in the data when the populations are not fully isolated (Pope *et al.*, 2000).

The ability to trace back population bottlenecks opens the door for addressing a variety of questions. The detection of past bottlenecks could, for example, indicate the decimation of a population due to disease, or the colonization of a newly formed habitat. Bottlenecks are also expected to occur during extinctions-recolonization processes in metapopulations, which are of vital importance for the maintenance of overall genetic variation (Whitlock & Barton, 1997). Such events could now be reconstructed from genetic data, without historical knowledge of population demography. Furthermore, for studies which aim to trace population reductions over longer time scales, additional maximum likelihood and coalescent-based methods are available in the literature (Beaumont, 1999; Goldstein *et al.*, 1999).

For amphibians, the distribution of microsatellite allele frequencies has been proven to be very powerful by successfully identifying known bottlenecks in British *B.*

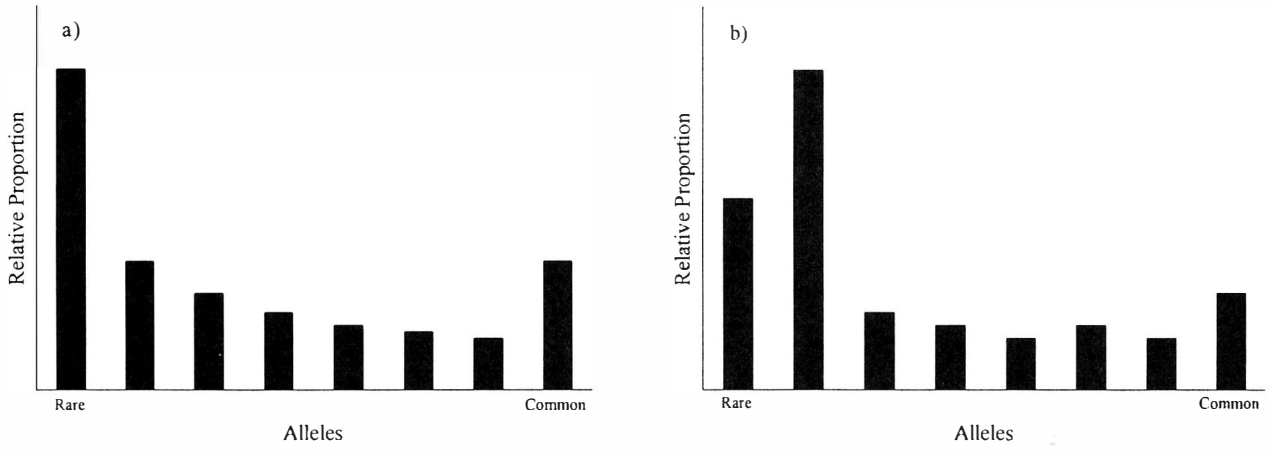

FIG. 2. Schematic representation of the consequences of a population bottleneck on gene diversity; (a) unbottlenecked population, (b) bottlenecked population. Rare alleles, under mutation-drift equilibrium more abundant than high-frequency alleles, have a higher probability of becoming extinct under bottlenecks, leading to a distortion of allelic frequencies which is detectable with hypervariable markers several generations after a bottleneck occurred. After Luikart *et al.* (1998).

calamita populations (Beebee & Rowe, 2001), but, based on allozyme data, has failed to detect significant bottlenecks associated with an introduction of the newt *T. carnifex* (Arntzen, 2001). In another example, we found evidence for one *T. cristatus* populations having experienced a genetic bottleneck, in line with the documented recent colonization of the study area (Jehle *et al.*, 2001). However, we noted that support for the case crucially depended on the microsatellite mutation mechanism assumed to operate. Microsatellites mostly mutate through the addition or deletion of one repeat unit, following a stepwise mutation model (SMM) or, alternatively, by a certain number of repeats simultaneously, following the infinite allele model (IAM, Goldstein & Schlötterer, 1999). We estimated the contributions of SMM and IAM over all loci, using a Markov chain method implemented in the software MISAT (Nielsen, 1997), as approximately 95% and 5%, respectively. Under these mixed model conditions significant bottlenecks were not found, similar to assuming the SMM alone. However, it should be mentioned that, unless the bottleneck is very severe, the statistical power of one-sample tests is lower than when an additional sample before the bottleneck occurred is available (Luikart *et al.*, 1998b). This could, for example, be achieved by extracting DNA from museum specimens (as in Beebee *et al.*, 1998).

PRACTICAL CONSIDERATIONS

Apart from their high variability, DNA-based markers offer several practical advantages over allozyme methods. Samples can be stored in concentrated ethanol, rendering the immediate freezing of samples, as usual for enzyme electrophoresis, unnecessary. Furthermore, DNA can be extracted from "old" (dried, alcohol – or even formalin – preserved) material, such as that kept in museum collections (Beebee *et al.*, 1998). For any PCR based assay, minute amounts of tissue are required, allowing non-destructive sampling (Taberlet & Luikart, 1999). Amphibian DNA samples can be obtained by removing a toe (Gonser & Collura, 1996), a procedure which at the same time can serve as a physical mark (e.g. for population size estimated with capture-mark-recapture). In urodeles, samples can be obtained by taking the tail tip (Arntzen *et al.*, 1999); larval amphibians can be non-destructively sampled by clipping the tail-fin (Rowe *et al.*, 1999), or by removing an external gill (Jehle *et al.*, 2000).

User-friendly software for performing the Maximum Likelihood, Bayesian and Monte Carlo methods is now freely available from the internet (for an overview of sources see, for example, Luikart & England, 1999), complementing the packages designed for "traditional" population genetic analyses (e.g. GENEPOP, Raymond & Rousset, 1995). However, when using microsatellite-based data it should be borne in mind that estimating population genetic parameters which depend on mutational processes can be ambiguous, as precise mutation mechanisms of microsatellites are still subject to debate and can also vary from locus to locus.

Co-dominant markers such as microsatellites are highly informative, but their biggest disadvantage is probably that they cannot be applied "off the bench". In fact, the development of the required PCR primers can be costly and time-consuming, a potentially prohibitive fact when planning a study with limited duration and finance. Within amphibians, it has been shown for ranid frogs that cross-species amplification success rates are significantly lower than for birds and mammals (Primmer & Merilä, in press). The often very large genome of urodele amphibians makes the development of successfully amplifying primers particularly difficult for them (Garner, 2002), rendering enrichment procedures highly recommended for obtaining a sufficient number of amplifiable and polymorphic loci (e. g. following Gibbs *et al.*, 1997). For European amphibians, published microsatellite PCR primers are so far available for *Bufo bufo* (Scribner *et al.*, 1994; Brede *et al.*, 2001), *B. calamita* (Rowe *et al.*, 1997; Rowe *et al.*, 2000*b*), *Hyla arborea* (Arens *et al.*, 2000), *Rana arvalis* (Vos *et al.*, 2001*b*), *R. latastei* (Garner & Tomio, 2001), *R. lessonae/R. ridibunda* (Garner *et al.*, 2000; Zeisset *et al.*, 2000), *R. temporaria* (Berlin *et al.*, 2000; Rowe & Beebee, 2001), and *Triturus cristatus/T. marmoratus* (Krupa *et al.*, 2002). Once developed, the primer systems enable the efficient derivation of genotypes, currently at a cost of about 1 Euro per datum point.

FUTURE DIRECTIONS

High-throughput microsatellite genotyping is currently facilitated by, for example, using PRC primers labelled with fluorescent dyes, in combination with semi-automated sequencing machines and associated technology such as pipetting robots. Microsatellites are in the vast majority of cases situated in non-coding regions, and their neutral character is a basic assumption underlying the above-described analytical methods. However, this also implies that they only reflect indirectly the genetic variation relevant for fitness and adaptation. The great majority of ecological and demographic characteristics relevant for population viability are based on quantitative genetic traits, which are typically determined by multiple loci with various and additive effects (Falconer & Mackay, 1996). Assuming that a large number of loci is known (in the order of >100 distributed across the whole genome), such QTLs ("Quantitative Trait Loci") could be mapped on the basis of microsatellites, an approach currently used for better-known model species. Microsatellites also play a significant role in the upcoming field of "population genomics", where numerous loci are sampled across the whole genome, with locus-specific effects of population genetic parameters being distinguishable from the general sample distribution across all loci (e. g. Black *et al.*, 2001).

Beyond microsatellites, another genetic marker widely applied for other vertebrates and directly related

to fitness, the MHC ("Major Histocompatibility Complex", e.g. Edwards & Hedrick, 1998), has, to our knowledge, not yet been applied in amphibian conservation studies. Technical advances might in the future lead to a shift from marker-based data to information directly at the sequence level, such as information derived from single-basepair substitutions ("Single Nucleotide Polymorphisms", SNPs). However, despite being promising for phylogeographic studies, due to their low mutation rate their usefulness for fine-scale population inferences might be limited. Recent technical advances also include microarrays or "DNA chips", which are microscopic plates on which short DNA strands can be bound and visualized. For example, in combination with ESTs ("Expressed Sequence Tags"), which mark those regions in the genome which are expressed under certain circumstances, microarrays now would enable to relate the activity of specific genes to environmental conditions such as ecological stress. However, these methods are as yet beyond the financial scope of most amphibian ecologists.

Genetic considerations are probably most useful when incorporated early in a species' conservation plan, when the existence of some robust populations across a species' geographical range offers the possibility of a variety of creative solutions to conservation problems (Hedrick, 2001). Many European amphibian species suffer serious declines but are not yet exposed to the imminent risk of extinction, rendering molecular studies particularly timely. However, an increased knowledge of the population genetic structure alone is not sufficient to guarantee conservation, and new scientific findings based on high variation genetic markers will only help amphibian conservation when integrated into current and future action plans.

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