Review
Molecular Methods: Blessing or Curse?

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Abstract Conservation genetic studies make use of molecular methods to obtain valuable information which help optimizing management strategies especially for threatened species. This chapter presents an overview of different molecular markers (microsatellites, AFLPs, RFLPs, RAPDs, mtDNA, allozymes) and their applications in conservation and genetic studies. Microsatellites have shown to be, though expensive, currently the most popular genetic marker as the high degree of polymorphism is ideal to study small geographical scales of species. RFLPs, RAPDs and allozymes still represent useful markers for studies of both, small and larger geographical scales. Low degree of polymorphism, no detection of alleles and low reproducibility characterize some drawbacks. To examine phylogeography MtDNA seems to be the best choice.

1 Introduction

Conservation studies rely increasingly on molecular methods to evaluate species "status quo," historical distributions (biogeographical traits), and to develop management strategies for the restoration of populations. Since the invention of the polymerase chain reaction in the late 1980s (Mullis et al. 1996), further possibilities have opened up to apply genetic tools for diverse biological methods (see Fig. 1). Zhang and Hewitt (2003) revealed that, among the 1,758 primary papers and primer notes published between 1994 and 2003 in the journal of *Molecular Ecology*, 29.8 and 42.5% were indexed with mitochondrial and microsatellite DNA markers, respectively. Nevertheless, many more genetic methods exist to answer different ecological and genetic questions. Scientists, unfamiliar with the most commonly used genetic methods in the "conservation genetics field," tend to get lost in the different molecular techniques as each has its own advantages and disadvantages,

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and can be applied in diverse forms. However, all these molecular techniques aim to detect genetic variation and differences within species, populations, or even individuals. This is generally achieved by comparing special DNA sections "marked" by the individual genetic method.

This chapter aims to present an overview of the most commonly used molecular methods during the last two decades (microsatellites, AFLPs, RFLPs, RAPDs, mtDNA, and allozymes) and the variety of ecological questions which can be answered with each method.

2 Molecular Markers and Their Application

2.1 Microsatellites

Microsatellites consist of short, tandemly repeated sequences of 1–6 base pairs within the nucleus of the cell (Palo et al. 1995; Ashley 1999). They have an elevated rate of mutation due to "slipped-strand mispairing" (Levinson and Gutman 1987; Palo et al. 1995; Eisen 1999), resulting in a high proportion of polymorphism even between closely related lines (Semagn et al. 2006 and references therein). Resulting variations (alleles) are scored through differing banding patterns. This marker is neutral to selection and is inherited co-dominantly as a standard Mendelian trait (Meglécz and Solignac 1998; Ashley 1999; Luikart and England 1999).

Microsatellites are the most commonly applied molecular marker in ecological research (Fig. 1). Their use in ecology and conservation studies is very broad and ranges from the identification of population genetic differentiation, demographic

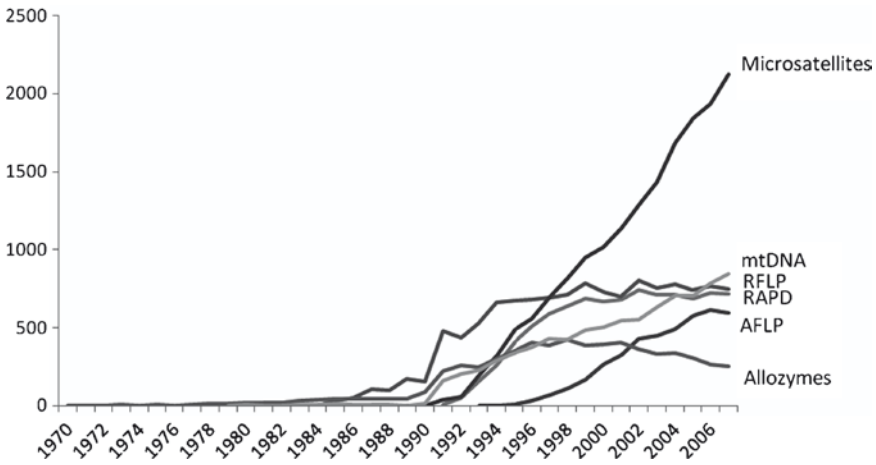

Fig. 1 Number of publications (selected biological subject) between 1970 and 2007 employing mtDNA, Allozymes, Microsatellites, RFLPs, RAPDs and AFLPs found via ISI web of knowledge

changes (e.g., bottlenecks, changes in effective population sizes (Ne), genetic drift), to parentage analysis and the definition of management units (e.g., Jones and Ardren 2003; Wan et al. 2004; Leberg 2005; Olivieri et al. 2008; Orsini et al. 2008). Minimal preserved or dry samples are suitable for the microsatellite technique, allowing the analysis of ancient population patterns and genetic changes over time (Bruford and Wayne 1993; Meglécz and Solignac 1998). However, microsatellites may have limited phylogenetic value due to homoplasy (Goldstein and Pollock 1997; Doyle et al. 1998; Selkoe and Toonen 2006). An informative review about microsatellites and their drawbacks can be found in the study by Selkoe and Toonen (2006).

2.2 Mitochondrial DNA

Mitochondrial DNA (mtDNA) is an extra-chromosomal genome in the cell mitochondria that resides outside of the nucleus, and is inherited from mother with no paternal contribution (Avise 1991). The obtained PCR products are sequenced and banding patterns analyzed.

The theory of relatively constant mutation rates (molecular clock) (Lushai et al. 2003) is used to estimate time scales in which populations are split up, allowing the detection of, e.g., species dispersals and dispersal centers (Riddle and Honeycutt 1990; Rocha et al. 2008). Thus, genetic variation can be investigated on a broader geographical scale to unravel the historic (historical or recent) barriers to gene flow (Avise 2000) and genetic basis of speciation and evolution of species and genera. Due to higher evolutionary rates of mtDNA relative to the nuclear genome (Wan et al. 2004 and references therein), this marker is preferred in constructing phylogenies and inferring evolutionary history, and is therefore ideal for within- and between-species comparisons (DeYoung and Honeycutt 2005; Behura 2006). Furthermore, mtDNA is believed to be the best tool for resolving taxonomic problems (Wan et al. 2004), identifying regions of endemism (Proudfoot et al. 2006), and Evolutionary Significant Units (ESUs) (Wan et al. 2004). Drawbacks of mtDNA analyses include hybridization, introgression, and incomplete lineage sorting. Moreover, mtDNA is of little use in investigating the recent loss of genetic variation and any individual-level events such as identity, individual dispersal, and mating systems (Wan et al. 2004).

2.3 Allozyme

Allozymes are enzyme variants due to allelic differences and can be visualized through protein electrophoresis. This technique was developed to quantify the genetic and geographic variation in wildlife populations, and it remains a cost-effective and straightforward method (Avise 1994). Genetic variations caused by mutations are expressed as amino acid replacements due to changes in protein compositions, and are resolved as bands (alleles) on electrophoretic gels (DeYoung and Honeycutt 2005).

Allozymes have been important in plant biosystematics (see van der Bank et al. 2001) and are suitable for the detection of genetic variation within and between populations (Steiner and Joslyn 1979; Bartlett 1981; Loxdale et al. 1985). Due to a relatively low average heterozygosity, the application of this marker is suitable for geographically broader scales of extant species (van der Bank et al. 2001; Schmitt 2007). Even the analysis of parentage as single-locus polymorphism is sometimes possible with this marker (Chakraborty and Hedrick 1983; Meagher and Thompson 1986). Similarly, levels of hybridization, introgression, gene flow, and polyploidy can be studied. On the contrary, allozymes have limited phylogenetic value (e.g., Lowe et al. 2004 and references therein), represent phenotypic traits, and might be subject to selection. Another significant drawback is that only fresh material can be used to extract the proteins.

2.4 Multilocus DNA Marker Systems

Three of the commonly used multilocus DNA marker systems in evolutionary, taxonomic, ecological, phylogenetic, and genetic studies are RFLPs, RAPDs, and AFLPs (DeYoung and Honeycutt 2005; Behura 2006; Agarwal et al. 2008). All these markers generate banding patterns that are scored for variation. In all three markers, the detected variation is caused by either point mutation within recognition sequences as well as insertions and/or deletions between the recognition sites, which may lead to an underestimation of genetic variation (DeYoung and Honeycutt 2005). Since none of the described multilocus markers is specific to a certain target organism DNA, there is a risk of false variation generated by contaminations (Sunnucks 2000). Furthermore, dominance of some of the markers (RAPD and AFLP) does not allow for a detection of alleles.

Depending on the sampling strategy, these markers can cover a wide spatial range, allowing for a detailed fine scale analysis of population structure between individuals, especially with AFLPs (Meudt and Clarke 2007), up to taxonomically and spatially coarse studies (e.g., Despres et al. 2003). While RFLP, RAPD, or AFLP are unsuitable to estimate mutation rates or alike, and are thus inappropriate for temporal studies (i.e., evolutionary), they provide a detailed image of the present species or population genetic state.

2.4.1 RFLP: Restriction Fragment Length Polymorphism

Restriction fragment length polymorphisms (Botstein et al. 1980) are highly polymorphic, co-dominantly inherited markers based on the use of restriction enzymes which can be applied as single and multilocus probes with the banding patterns resulting from multilocus probes.

The technique generates highly reproducible banding patterns and is characterized by a high heritability (Lowe et al. 2004; Semagn et al. 2006; Agarwal et al.

2008). It is used in areas such as population and conservation genetics, diversity (e.g., Apostolidis et al. 2008), phylogenetics (e.g., Hu et al. 2008), linkage mapping (e.g., Tanksley et al. 1989), or cultivar identification (e.g., Busti et al. 2004), though their main application is within human genetics (Weising et al. 2005). As RFLPs require relatively large amounts of DNA, they have recently been replaced by PCR-RFLPs or AFLP analyses in most ecological studies.

2.4.2 RAPD: Random Amplified Polymorphic DNA

The dominant marker system RAPD, introduced by Williams et al. (1990), is based on arbitrarily amplifying DNA sequences during PCR, without prior knowledge of the organism sequence, using 10 nucleotide primers (Lowe et al. 2004, Weising et al. 2005). One of the main problems associated with RAPDs is their susceptibility to changes in reaction conditions leading to reproducibility problems (Jones et al. 1997; Agarwal et al. 2008; Assmann et al. 2007). Due to these problems, some peer-reviewed journals (e.g., Molecular Ecology) have recently changed their policy and publish RAPD data only in exceptional cases.

RAPDs have been used in many fields, among them are studies on population and conservation genetics (e.g., Kim et al. 2008), phylogenetics (e.g., Simmons et al. 2007), and linkage mapping (e.g., Sun et al. 2008).

2.4.3 AFLP: Amplified Fragment Length Polymorphism

AFLPs are dominant markers based on a combination of the RFLP and PCR techniques and were developed by Vos et al. (1995). Also, they do not require any previous sequence information and are based on the digestion of DNA by restriction enzymes and adapter ligation (resulting in universal primer binding sites), with two subsequent PCRs using specific primers. AFLPs are highly reproducible (Meudt and Clarke 2007; Agarwal et al. 2008) and primers can be combined to yield a large set of combinations, though this may also lead to a clustering of markers with certain restriction enzymes (Keim et al. 1997; Young et al. 1999; Saal and Wricke 2002).

AFLPs find wide application in studies on population genetics, diversity, and differentiation (e.g., Abbott et al. 2008; Tang et al. 2008), phylogenetics and taxonomy (e.g., Brouat et al. 2004; Schenk et al. 2008), hybridization (e.g., Volkova et al. 2008), linkage, gene, and genome mapping (e.g., Olmstead et al. 2008; Radoev et al. 2008), assignments (e.g., Yang et al. 2008) and kinship (e.g., Hardy et al. 2006). Although they are dominant markers, the large number of loci gives them a high statistical power (Meudt and Clarke 2007). Therefore, they are well suited for intraspecific studies (distinguishing between closely related individuals), where many loci are necessary (i.e., high genomic heterogeneity, low genetic variability), in polyploids, and in systems with hybridization occurring (Meudt and Clarke 2007).

Table 1 Advantages, disadvantages and different features of microsatellites, Allozymes, mtDNA, RFLPs, RAPDs, and AFLPs

	Advantages	Disadvantages	Genomic abundance	DNA amount required	Polymorphism	Inheritance	Single loci
Micro-satellites	Medium costs (1)No sequence information needed (4) High reproducibility & reliability (4)	High start-up costs (1, 4) Species-specific primer pairs (4) PCR-based problems (5)	Medium (1, 2) – high (4)	Low (1, 2)	Medium (2) – high (1,4)	Co-dominant (1, 3)	Yes (3)
Allozymes	Easy & safe protocol (4)Low costs (4, 8) Many enzyme systems (4) Suited for polyploids (4)	Underestimation of genetic variation (4) Not sure whether neutral or not (10) Limited to extant populations (10) Only forzen or fresh samples (10)	–	–	Low (4)	Co-dominat (3)	Yes (3)
mtDNA	Constant mutation rates (11) Available primers Neutral and selective regions (6) Inherited maternally (7)	Little practical value for population genetic studies (8) Expensive	–	–	High (6)	Co-dominant (3)	Yes (3, 8)
RFLP	Detection of nucleotide DNA and organelle DNA polymorphisms (4) Phylogenetic analysis (4) High repeatability (1, 2, 4)	Labor intensive use (1, 4) High start-up costs (1, 4) Comparability between studies difficult (4)	High (1, 2)	High (1, 2, 4)	Medium (1, 2, 4)	Co-dominant (1, 3)	Yes (3)

RAPD	Low costs (1, 4) Easy use (1, 4) No sequence information needed (4)	Low-intermediate reproducibility (1,2) Extensive criticism, see (4)	Very high (1, 2)	Low (1, 2)	Medium (2) – high (1)	Dominant (1, 3)	No (3)
AFLP	Medium costs (1)No sequence information needed (4) High reproducibility & reliability (4)	Initially difficult to set up (1)Technically demanding (4) Relatively high amounts of DNA needed (4)	Very high (1, 2)	Medium (1, 2) – high (4)	Medium (2) – very high (1,4)	Dominant (1, 3)	No (3)

Abbreviations: 1= Semagn et al. 2006; 2= Agarwal et al. 2008; 3= Sunnucks 2000; 4= Lowe et al. 2004; 5= Selkoe and Toonen 2006; 6= Wan et al. 2004; 7= Behura 2006; 8= Zhang and Hewitt 2003; 9= DeYoung and Honeycutt 2005; 10= van der Bank et al. 2001; 11= Lushai et al. 2003

3 Conclusions

The molecular methods presented in this chapter have question-related advantages and disadvantages (see Table 1). Before choosing a marker, it is essential to evaluate (1) which ecological question ought to be answered, (2) the spatial and temporal scales which ought to be explored, and (3) how exhaustive populations can be sampled (sample design; for detailed sample strategies, see Lowe et al. (2004), and (4) the available financial resources. Furthermore, the popularity of a specific marker might be important for the acceptance in a high ranking peer-reviewed journal, even though many markers could be suitable to answer the same question (Assmann et al. 2007).

In conclusion, we can say that microsatellites are currently one of the most popular genetic markers in ecological studies (see Fig. 1). Especially, the elevated rate of polymorphism is ideal to study small geographical scales of extant species (e.g., Finger et al. 2009). The use of allozyme markers is decreasing since several years (Fig. 1), although the low costs allow a high throughput for studying large geographical scales of extant species (Schmitt 2007). Similarly, AFLPs, RAPDs, and RFLPs can be used to study small to large geographical scales. These markers have their own drawbacks (AFLPs: no detection of alleles, RAPDs: low reproduc-

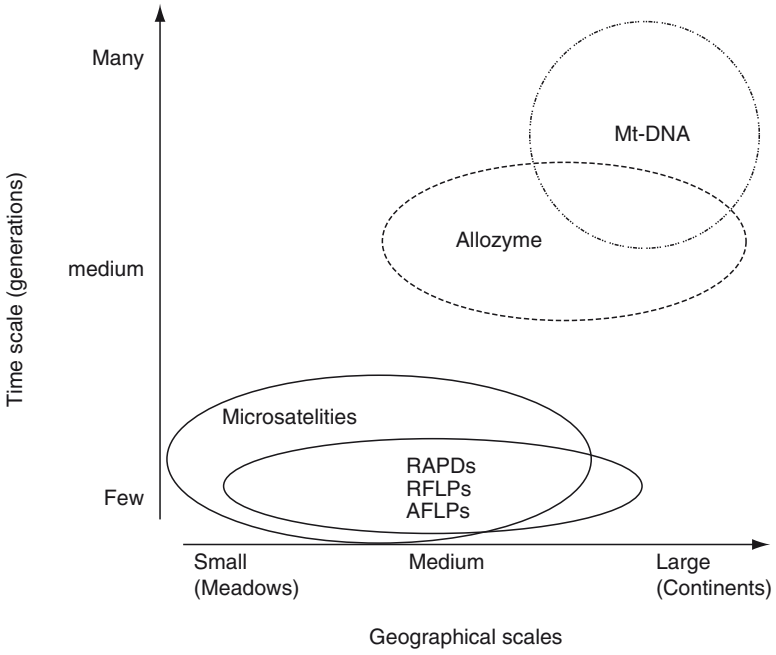

Fig. 2 Different time and geographical scales applicable using mtDNA, Allozymes, Microsatellites, RFLPs, RAPDs, and AFLPs

ibility, RFLPs: high amount of DNA required, see Table 1). Finally, mtDNA is the best choice to study species history and phylogeography on broad geographical scales or deeper timescales (see Fig. 2).

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