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REVIEW

DNA marker technology for wildlife conservation

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Abstract Use of molecular markers for identification of protected species offers a greater promise in the field of conservation biology. The information on genetic diversity of wildlife is necessary to ascertain the genetically deteriorated populations so that better management plans can be established for their conservation. Accurate classification of these threatened species allows understanding of the species biology and identification of distinct populations that should be managed with utmost care. Molecular markers are versatile tools for identification of populations with genetic crisis by comparing genetic diversities that in turn helps to resolve taxonomic uncertainties and to establish management units within species. The genetic marker analysis also provides sensitive and useful tools for prevention of illegal hunting and poaching and for more effective implementation of the laws for protection of the endangered species. This review summarizes various tools of DNA markers technology for application in molecular diversity analysis with special emphasis on wildlife conservation.

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Contents

1. Mitochondrial DNA markers for wildlife conservation

Many of the conservation genetics studies have utilized the sequence information of mitochondrial DNA (mtDNA). The mitochondrial genome comprises a circular chromosome of DNA. Animal mtDNA ordinarily contains 36 or 37 genes; two for rRNAs, 22 for tRNAs and 12 or 13 for subunits of multimeric proteins of the inner mitochondrial membrane. In addition, there is a noncoding sequence termed the control region (CR) due to its role in replication and transcription of mtDNA molecules. Exons in the mtDNA circle are tightly packed with no spacing introns. Mitochondrial DNA is histone-free, has limited repair ability, and therefore has a relatively high mutation fixation rate (5–10 times that of nuclear DNA). Although mtDNA has evolved faster than the nuclear genome, the rate of evolution is different for different regions of mtDNA and has been used to examine various phylogenetic relationships. Several conserved primers have been developed that allow amplification of a number of regions of the mtDNA molecule in a wide range of species. Moreover, because most cells contain multiple copies of the mtDNA molecule, mtDNA sequences can often be obtained from very small amounts of tissue containing degraded DNA. The main uses of mtDNA sequences in conservation genetics include population structuring, resolving taxonomies, establishing interspecific hybridization and the detection of illegal hunting and poaching of endangered animals. However, the selection of appropriate bioinformatics tool plays an important role for reliable phylogenic inference using mitochondrial markers (Khan et al., 2008b). Table 1 provides quick information on numbers of samples and populations, names of taxa and types of mitochondrial markers used in some relevant studies.

1.1. Ribosomal DNA (12S and 16S rDNA)

Mitochondrial 12s rDNA is highly conserved and has been applied to illustrate phylogeny of higher categorical levels such as in phyla or subphyla. Whereas the 16s rDNA is usually applied for phylogenetic studies at mid-categorical levels such as in families or genera. It has been postulated that these sequences are useful for inferring moderate to long divergence times (Janczewski et al., 1995). Several investigators have used 12S rDNA sequences for wildlife forensic biology (Prakash et al., 2000; Gurdeep et al., 2004). Molecular phylogeny of elopomorph fishes using 12S rRNA sequences clearly separated monophyletic Elopomorpha from Clupeomorpha (Wang et al., 2003). Shukla et al. (2001) amplified the 12S rRNA gene using universal primers and then cloned and sequenced the 450 bp fragment for phylogenetic analysis of endangered species, Indian muntjac (*Muntiacus muntjak*). The 12S rRNA gene has been used to examine genetic variation in the endangered spur-thighed tortoise (*Testudo graeca*), an endangered species from broad distribution range (Alvarez et al., 2000; van der Kuyl et al., 2005). The 12S fragment of *Testudo graeca* was found to be somewhat less variable than the D-loop fragment, a finding compatible with the situation in mammals, where rRNA genes evolve slower than synonymous sites and the variable parts of the D-loop (Pesole et al., 1999). Recently, Pandey et al. (2007) have utilized 12S rDNA for molecular identification of Indian leopard, which is an endangered species except in Central Africa and India.

Lei et al. (2003) have examined the mitochondrial rRNA genes of Chinese antelopes and observed that average sequence divergence values for 16S and 12S rRNA genes are 9.9% and 6.3% respectively. Their phylogenetic analysis has revealed that Przewalski's gazelle is more closely related to Mongolian gazelle than Tibetan gazelle suggesting that the critically endangered Przewalski's gazelle should be treated as a species, not a subspecies of the Tibetan gazelle, which clearly warrants more attention from conservationists (Lei et al., 2003). A single base in the 16S rDNA sequences from the endangered species *Pinna nobilis* has been found to be different in all analyzed individuals from a single population sample (Chios island) differentiating it from the others (Katsares et al., 2008). Mitochondrial 16S rRNA has been used to elucidate the pattern of relationships and systematic status of 4 genera, including 9 species of skates living in the Mediterranean and Black Seas (Turan, 2008). A heminested PCR assay based on species-specific polymorphism at the mitochondrial 16S rRNA gene has been designed for the identification of seven pecora species including Blackbuck, Goral, Nilgai, Hog deer, Chital, Sambar and Thamin deer (Guha and Kashyap, 2005). Molecular studies on endangered Pecoran have shown lower sequence diversity in 16S rRNA gene as compared to cytochrome b gene, both between and within species however the 16S rRNA gene harbored a larger number of species-specific mutation sites than cytochrome b gene, suggesting that it could be more useful for species identification (Guha et al., 2006). NaNakorn et al. (2006) have assessed the level of genetic diversity of critically endangered Mekong giant catfish species using sequences of 16S rRNA and detected 4 haplotypes among 16 samples from natural populations. These findings may have important

Table 1 Application of mtDNA markers for conservation of wild animals.

No. of samples	No. of populations	Taxa	Study	Marker	Reference
21	1 Captive	Sun bear	Evolutionary significant unit	CR	Onuma et al. (2006)
47	3 Populations	Black muntjac	Population structure	CR	Wu et al. (2006)
40	1 Population	Chinese water deer	Genetic diversity	CR	Hu et al. (2006)
5	–	Roe, deer, horse, cow	Illegal hunting	Cyt b	An et al. (2007)
73	1 Population	Houbara bustard	Genetic diversity	CR	Idaghdour et al. (2004)
95	17 Locations	African sable	Genetic structure	CR, Cyt b	Pitra et al. (2002)
18 + 54	2 Locations	Rock-wallaby	Population structure	CR	Eldridge et al. (2001)
46	10 Sources	Tibetan gazelle	Genetic diversity	CR, Cyt b	Zhang and Jiang (2006)
17 + 42	Captive + field	Indian leopard	Species identification	12S rRNA	Pandey et al. (2007)
182	14 Zoos diff. countries	Oryx dammah	Genetic diversity	CR	Iyengar et al. (2007)
19 + 16 + 4 + 3 + 18	5 Locations	Oryx beisa	Genetic diversity	CR, Cyt b	Masembe et al. (2006)
21 + 3	2 Locations	Oryx leucoryx	Genetic diversity	CR	Khan et al. (in press)

implications for conservation of the Mekong giant catfish, especially in designing and implementing artificial breeding program for restocking purposes (NaNakorn et al., 2006). Recently, Khan et al. (2008a) have suggested the utility of 16S rRNA segment for molecular phylogeny of oryx at the genus and possibly species levels.

1.2. Mitochondrial protein coding genes

Compared to 12S and 16S rDNAs, the mitochondrial protein-coding genes evolve much faster and therefore regarded as powerful markers for inferring evolution history in lower categorical levels such as families, genera, and species. This feature of mtDNA in phylogeny is suitable for resolving taxonomic uncertainties in conservation genetics. Mitochondrial cytochrome b sequences have been used to understand the genetic diversity of Tibetan gazelle for better conservation planning (Zhang and Jiang, 2006). Partial mitochondrial cytochrome b genes of five mammalian specimens and Chromo-Helicase-DNA-binding (CHD) genes of five pheasants have been used to determine whether the specimens were from illegally hunted animals (An et al., 2007). Mitochondrial cytochrome b sequence analysis of cooked meat, remnants of the bird and the DNA obtained from the wooden chopping block revealed that the cooked meat and remnants of the bird were of a chicken, but the wooden chopping block was used to chop the meat of an endangered bird (Gupta et al., 2005). Partial cytochrome b based molecular phylogenetic trees and genetic distances have indicated that there is considerable genetic divergence between the Korean goral and the Chinese goral, but virtually none between Korean and Russian gorals suggesting the importance of molecular data for conservation of the goral populations of these regions (Min et al., 2004). Johnson and O'Brien (1997) have used NADH dehydrogenase subunit 5 together with 16S rRNA gene for phylogenetic analysis of multiple individuals of 35 species from Felidae family and recognized eight significant clusters or species clades that likely reflect separate monophyletic evolutionary radiations in the history of this family.

1.3. Non-coding or control region sequences

The control region (CR) is composed of three domains including ETAS (extended termination associated sequences) domain, central domain and CSB (conserved sequence block) domain. Each of the three domains presents a distinct pattern of variation; both ETAS and CSB domains evolve rapidly whereas the central domain remains taxonomically conservative. Iyengar et al. (2006) have compared the CR sequences from several captive animals with the sequences for oryx species and the subsequent phylogenetic analysis of sequence variations revealed a close grouping of *Oryx leucoryx* with *Oryx gazelle* rather than *Oryx dammah*. Recently, Khan et al. (in press) have observed typical sequence variation in the CR gene of 23 captive-bred and reintroduced *Oryx leucoryx* samples in the form of 7 haplotypes; one of these haplotypes has been reported earlier while the remaining 6 haplotypes are novel and represent different lineages from the founders. The CR sequences have been used to investigate the genetic status and evolutionary history of the Tibetan gazelle (Zhang and Jiang, 2006). Onuma et al. (2006) have sequenced the CR of the sun bear (*Helarctos malayanus*) using 21 DNA samples collected from confiscated animals to identify conservation units including evolutionarily significant units and management units. Wu et al. (2006) have used the partial CR (424 bp) sequences from 47 samples to assess the population structure and gene flow among the populations of black muntjac (*Muntiacus crinifrons*), a rare species endemic to China. The genetic diversity and population structure of the Chinese water deer have been investigated by analyzing the 403 bp fragment of the mitochondrial DNA CR revealing 18 different haplotypes in 40 samples (Hu et al., 2006). Idaghdour et al. (2004) have sequenced 854 bp of CR from 73 birds to describe their population genetic structure of *Chlamydotis undulata*, a declining cryptic desert bird whose range extends from North Africa to Central Asia. A single CR haplotype was identified in New Zealand population, while 17 haplotypes were found in Australian populations of brush-tailed rock-wallabies (*Petrogale penicillata*), which were introduced to New Zealand from Australia in the early 1870s and have experienced widespread population declines and extinctions (Eldridge et al., 2001).

2. Nuclear DNA markers for wildlife conservation

The most commonly used nuclear markers for DNA fingerprinting are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and micorsatellites or short sequence repeats (SSR). Both RAPD and AFLP markers do not require prior molecular information whereas SSR markers require sequence information to design primers. All these markers are widely used for analysis of

genetic diversity because of large number of loci, which can be screened simultaneously. The differentiating power of these markers is in the following order: SSR > AFLP > RAPD. Both RAPD and AFLP have two alleles per locus and therefore considered as dominant markers (presence or absence of a band). In contrast, SSRs are able to recognize both the alleles (co-dominant marker) and can easily differentiate between homozygotes and heterozygotes. The application of nuclear DNA markers in wildlife fingerprinting is summarized in Table 2.

2.1. Random amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) markers can be amplified relatively easily using standard protocol. There is no requirement for any prior genetic information about the species in question and the method is relatively simple and inexpensive. A more serious drawback of RAPDs is that the low stringency PCR required in the procedure results in a high genotyping error rate and in lower genotyping reproducibility compared to single locus markers. Chelomina et al. (1999) have used RAPD to examine genetic polymorphism in the Far-Eastern leopard subspecies and differented wild-living animals from those kept in captivity; the genetic diversity was found to be lower in animals from the zoo (D = 0.058) compared to natural population (D = 0.137). Neveu et al. (1998) have compared the genetic diversity in four captive groups of Microcebus murinus with wild mouse lemurs using 5 primers to amplify 98 different loci. They showed that the captive groups had lost genetic information with respect to the wild sample; the loss of genetic diversity in captive animal groups varied according to their number of founders and/or the management of their captive reproduction (Neveu et al., 1998). The RAPD analysis using 5 decamer primers to test 52 polymorphic loci of fifteen closed broodstock lines of the Pacific white shrimp revealed that the genetic variation loss probably related to bottleneck effects and inbreeding (Freitas et al., 2007). Rodrigues et al. (2007) have used RAPD markers to analyze population divergence and genetic variation within and between two populations of endangered Pampas deer. They displayed substantial genetic variation with all animals possessing unique RAPD phenotypes over 105 polymorphic bands produced by 15 primers suggesting the relevance of these findings for developing management guidelines for its conservation (Rodrigues et al., 2007). Maciuszonek et al. (2005) have demonstrated the application of population-specific RADP3 markers by resolving genetic group specific bands for four indigenous Polish goose breeds, suggesting endangered geese to be kept as separate flocks for their preservation. The estimation of genetic diversity in an Iberian imperial eagle population, one of the most threatened bird species in the world, has been carried out using 45 arbitrarily primers that amplified 614 loci (59.7% being polymorphic) in 25 individual eagles (Padilla et al., 2000). This RAPD method has revealed a high level of heterozygosity in this species while the genetic distances estimated could serve to establish more adequate mating to preserve genetic variability (Padilla et al., 2000). RAPD analysis has been used to determine the genetic diversity and the population structure of the endangered Blanca Cacerena bovine breed on the basis of 1048 loci produced by 71 primers (Parejo et al., 2002). These findings could be useful to plan more adequate mating in order to maintain the genetic diversity and to improve the efficiency of conservation for this breed (Parejo et al., 2002).

2.2. Amplified fragment length polymorphism

Amplified fragment length polymorphism (AFLP) markers are becoming one of the most popular tools for genetic analysis in the fields of evolutionary genetics and conservation of genetic resources. These markers are restriction fragments that are amplified by PCR following the addition of adapters to the fragments. Both RAPD and AFLP are multi-locus systems making use of primers that simultaneously bind to many different parts of a genome resulting in the amplification of many loci at the same time. However, the high stringency PCR used

Table 2 Application of nuclear DNA markers for conservation of wild animals.

No. of Samples	No. of populations	Taxa	Study	Marker	Reference
90	5 Locations	Australian tiger snake	Marker isolation	SSR	Scott et al. (2001)
13	–	Protected deer and other taxa	Illegal hunting	SSR	Fang and Wan (2002)
16	–	Japanese quail	Genetic variation	SSR	Ye et al. (1998)
66	Captive	Ostrich	Genetic structure	SSR	Kawka et al. (2007)
24 + 25 + 21 + 19	4 Locations	Rattlesnake	Genetic diversity	SSR, RAPD	Lougheed et al. (2000)
14 + 18	2 Populations	Pea fowl	Genetic diversity	RAPD	Chang et al. (2002)
70	4 Captive groups	Wild mouse	Genetic diversity	RAPD	Neveu et al. (1998)
134	2 Breeds	Cattle	Genetic diversity	RAPD	Yu et al. (2004)
72	4 Protected areas	Plains zebra	Genetic diversity	RAPD	Bowland et al. (2001)
25	–	Iberian imperial eagle	Genetic diversity	RAPD	Padilla et al. (2000)
38	Captive	Asiatic lions	Genetic diversity	RAPD	Shankaranarayanan et al. (1997)
24	–	Snake	Species tree	AFLP	Giannasi et al. (2001)
163	6 Locations	Topmouth cutler	Genetic diversity	AFLP	Wang et al. (2007)
32 + 32 + 32	3 Captive stocks	Asian arowana	Genetic diversity	AFLP, SSR	Yue et al. (2004)
21 + 3	Captive and reintroduced	Arabian oryx	Genetic diversity	SSR	Arif et al. (2010)

in the AFLP protocol amplifies markers that are more reliable than RAPD and minisatellites. Although per marker information in dominant and biallelic AFLP markers is low as compared to microsatellites, the generation of a large number of markers outweighs this limitation of AFLP. Zenger et al. (2006) have used AFLP to evaluate genetic diversity in the endangered sand tiger shark (*Carcharodon taurus*) and the great white shark (*Carcharodon carcharias*); both the species displayed relatively high levels of allelic diversity with a total of 59 and 78 polymorphic loci respectively. Giannasi et al. (2001) have investigated the use of AFLP to aid determination of the species tree for 24 specimens of a medically important snake and suggested that AFLP may prove a valuable aid in determining species trees at fine taxonomic levels to facilitate the incorporation of molecular data into such activities as antivenom production and conservation management. Inbreeding estimates based on both AFLP and microsatellite markers using empirical data from 179 wild and captive-bred old-field mice have been found to correlate strongly with pedigree-based inbreeding coefficients suggesting AFLP markers as a valuable tool for estimating inbreeding coefficient in natural populations and for examining correlations between heterozygosity and fitness (Dasmahapatra et al., 2008). Lucchini (2003) have compared the multi-locus AFLP results with single-locus markers (microsatellites) and haploid organellar marker (mtDNA) sequences and suggested that the AFLP technique could be very useful in a wide range of conservation studies.

2.3. Microsatellites or simple sequence repeats

Microsatellites or simple sequence repeats (SSRs) were the first PCR-based markers to become widely used for molecular fingerprinting. Microsatellites are short sections of DNA where a simple motif, generally 1-5 bp long is repeated up to about 100 times. Microsatellite markers are highly polymorphic, abundant and fairly evenly distributed throughout eukaryotic genomes. They are also co-dominant markers as heterozygotes can be discriminated from homozygotes because alleles at a particular microsatellite locus vary in the number of tandem repeats and can be differentiated on the basis of the resulting differences in sequence length. The popularity of these markers is due to their ease of amplification by PCR, their co-dominant nature and their typically high levels of allelic diversity at different loci. However, due to complex and tiring methodology, microsatellite loci have not been identified in the majority of species. Some cross-species amplification of microsatellites is possible between closely related species but high mutation rates in the sequences flanking the microsatellites severely hamper the designing of conserved primers for universal application. Therefore, new species-specific microsatellite markers have to be isolated whenever a species is studied for the first time, a process that is both time consuming and expensive. Recently, Arif et al. (2010) have demonstrated a fair degree of molecular diversity in captive bred and reintroduced population of Arabian oryx using an array of seven microsatellite loci. Ryberg et al. (2002) have used microsatellite markers to investigate patterns of genetic variation within and between populations of alligators distributed at coastal and inland localities in Texas. Lougheed et al. (2000) have compared the genetic differentiation among populations of the threatened massasauga rattlesnake using microsatellites and RAPD. Both types of markers have been found to be suitable for defining broad-scale genetic structures in snake populations and can provide important inputs into conservation initiatives of focal taxa however microsatellites are superior for detecting structure at limited spatial scales (Lougheed et al., 2000). Yue et al. (2004) have applied microsatellite and AFLP markers for monitoring of genetic diversity of three Asian arowana, a highly endangered fish species. Kawka et al. (2007) assessed the genetic variability within and among the 3 ostrich breeds and also evaluated the genetic distance between them using microsatellite markers. Chan et al. (2008) have developed 10 microsatellite markers using feathers as DNA source from kakerori suggesting the usefulness of isolated loci in studying population genetics of this endangered forest bird. Banhos et al. (2008) have characterized the microsatellite loci for the conservation and management of the Neotropical harpy eagle in view of providing an excellent set of molecular tools for the conservation and management of wild and captive harpy eagles. Li et al. (2007) have demonstrated the usefulness of the two multiplexed microsatellite systems for genetic diversity studies on two populations of black tiger shrimp. Multiplexing of microsatellite markers can be performed either using a single dye labeling (if amplicons are of different size) or different dye labeling (if amplicons are of same size).

3. Conclusion

Molecular markers play an important role in estimating the relatedness between the individuals by comparing the genotypes at a number of polymorphic loci. Several types of molecular markers are available but none of them can be regarded as optimal for all applications. Most of the systematic and phylogenetic studies have utilized approaches with mtDNA sequencing. However, mtDNA phylogeny represents only the geneology of a particular gene that is almost only maternally inherited. Therefore additional markers targeting nuclear DNA, such as RAPD, AFLP or microsatellites need to be used for more accurate interpretation of population genetics, biodiversity, phylogeny and forensics.

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