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A STUDY ON POPULATION GENETICS AND MOLECULAR MARKERS WITH ITS APPLICATIONS IN CONSERVATION OF WILDLIFE

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ABSTRACT

Genetic diversity and its measurement have vital importance in analysis, appreciative and management of population and individuals. Allozymes electrophoresis has ability to observe genetic variation and it is also considered as a standard tool in population genetic studies of conservation. But in the recent year, it has been increasingly replaced by DNA markers. Various molecular markers, protein or DNA (mitochondrial or nuclear DNA) such as minisatellite, microsatellites, RAPDs, are now being used in conservation. In this review, we have provided basics of population genetics (DNA) and overview of commonly used molecular markers in the conservation of wild life.

KEYWORDS: **Allozymes, Molecular markers, Mitochondrial DNA, Nuclear DNA Microsatellite, Minisatellite**

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INTRODUCTION

Genetic variation is distributed among species, and its population can be detailed by population genetics, it explains how evolutionary impact of mutation, migrations and their interaction with selection in large population affect the distribution of genetic variability. Genetic diversity described by quantitative variation which explains the variations within and among population, comparison of inbred without bred population, resemblance among relatives. Mostly large population of animals and plants contain extensive genetic diversity. However, levels of genetic diversity are often reduced island population and endangered species. Genetic variation is reduced by the slow process of mutation which follows generation to generations (Allendrof & Leary 1986). Early markers at the time of Mendlians include compound of flower and fruit colour. Protein electrophoresis proved a new source of marker and allow individuals to be identified themselves as homozygotes and heterzygotes at given locus. Early stage of 1960's, study of molecular genetics is limited with protein. Sudden change of protein to isozymes with molecular forms of enzymes and isozymes (Markert & Moller 1959) was dominant way during 1980 and proved as important method to evolutionary biology (Markert & Moller 1959). Variable genetic sites or single polypeptide species produce isozymes. Separation of all of these proteins is done, according to their mobility and molecular weight in an electric field. Isozymes variation is an indirect indicator of genetic change, at the molecular (DNA) level. Polymerase chain reaction (PCR) based method of genetic analysis of wild population, such as RAPD (Randomly amplified polymorphism DNA), microsatellites and AFLP (amplified fragment length polymorphism) are used to analyse genetic variation among the population. In addition, maternal inheritance of mitochondrial marker has often proved to be useful genetic marker (Queller & Goodnight 1989), (Park & Moran 1994). The choice of appropriate marker for a particular task, to be based on the genetic difference and similarities among the individuals of population. Normally rapidly evolving DNA (mini and microsatellite DNA) generates more genetic variation among the individuals and the population that provides information about individual's identity, paternity determination and intraspecific genetic isolation among populations. On the other hand genetic marker with moderate evolutionary rates (allozymes, mtDNA) control region resolves genetic distance at intermediate taxonomic levels. Resolution of deep branching patterns or divergence among distantly related taxa require highly conserved region (e.g. ribosomal RNA genes) within which evolutionary changes accumulate slowly (Moritz 1994), (Avise 1995). Two major goals of conservation genetics are to minimize the loss of genetic variation in wild population and to define the taxonomic units that is critically endangered rare population of species and sub- species in relation to (Mac & Lande 1991) this endangered means, a high probability of extinction in a short time has been predicted (Hedrick 1986). Suggested that low genetic variation in a species might be indicative of recent population bottleneck, and such a bottleneck potentially indicative of recent population bottleneck, and such bottleneck potentially vulnerability to extinction. In small populations genetic drift tends to reduce genetic variation, leading eventually to homozygosity and loss of evolutionary adaptability to the environmental changes. Here we have attempted to provide an overview of currently available molecular markers and the application of these molecular techniques in conservation biology. The aims of the current review are (i) to review basics of commonly used molecular markers (ii) and evaluate the potential and limitation of molecular markers.

Molecular marker in conservation

According to Hedrick & Miller 1992 genetics contribute not only to the conservation of species, but also to the conservation of whole population. (Gaston 2000) Suggested that biodiversity include the three levels of study that is genetics, species and ecosystem. Most of studies consider biodiversity data at single level whereas multiple level of diversity

provides the data over a wide spectrum. Genetic diversity is essential to coordinate the population and species with the evolutionary and ecological time periods. (Frankham 'et al.' 2012) it is described genetic variation by interaction of many genes known as quantitative genetic variation. Molecular genetic marker works as analysis of either proteins (isozymes or allozymes) or specific DNA sequence. DNA molecules show high polymorphism and stability as compared to proteins, it plays a major role as molecular markers for rapid identification of wildlife. Hence, recognition of genetic variability in natural or animals reared in captivity. Today, many molecular methods are available for studying the population for conservation but they are basically categorized under two types of marker, namely protein and DNA. There are three general ways to study population genetics and phylogenetic studies: (1) allozymes or isozymes (2) Mitochondrial DNA (3) Nuclear DNA (Avese 'et al.'1995)

1) Alozymes or Isozymes –

Isozymes have been found to be an ideal gene product in studies of conservation genetics. The term of isozymes refers to a functionally similar form of an enzymes, including all polymers of subunits produced by different gene loci or referring to different biochemical forms of an enzymes identified by electrophoresis. The term allozymes refers to the subset of isozymes demonstrating different allelic forms of the some gene locus or different allelic forms of nuclear encoded enzymes. Both isozymes and allozymes data have been used for analysis of genetic variability in natural species. there are two main ways in using enzymes data for analysis banding pattern, (i) that changes in the mobility of enzymes in electric field (Shaw 'et al.' 1970) (ii) the enzymes expression may be co-dominant, i.e., all alleles at a locus are expressed (Buth 1984). In avian biology, enzymes data have been used to obtain quantitative estimation of genetic variation in natural populations. To study the patterns of gene activation of allozymes and isozymes data has been in systematic studies of birds (Driesel 'et al.' 2004) several methods are present for the separation of native form of enzymes by electrophoresis method of separation, which differ by its supporting medium which is generally starch gel which includes both horizontal and vertical, polyacrylamide gel, agarose gel and cellulose acetate gel. (Murphy & Matson 1986), (Murphy & Crabtree 1985) Stability of enzymes basically depends on the following conditions that are (a) Temperature (b) Properties of the separation matrix (c) pH value of the running buffer. Polyacrylamid has greater lead over other types of standard, as it takes shorter time for electrophoresis separation of proteins. It Produce high resolution and sharpness of banding pattern, handling of gel is easy and can be stored for longer time after fixation and drying (Buth & Murphy 1999). The basic principle of enzymes visualization in situ is to present an enzyme with a solution containing an enzymes specific substrate reaction product that indicates the migration of enzymes molecules (Shaw 'et al.' 1970). The most basic hypothesis that evolutionary biologist use in enzymes data is, the changes in mobility of enzymes in the electric field reflecting changes in the encoding DNA sequences. Thus, if the banding pattern of any two individuals differ, it may be assumed that these difference are genetically based and heritable (Matson 1984) and second hypothesis is the co-dominant expression of enzymes for analysis i.e., all the alleles at a locus are expresses (Buth & Murphy 1999) the most important comparison of enzymes, or their activities in particular tissue and organelle. For example, liver may have different enzymes than RBC or muscles (Murphy & Matson 1986). Although protein based markers isozyme and allozymes, have achieved a wide spread applications in population characterization, whereas the technique has certain limitation too (Smith 1990). Allozymes degrade rapidly after death, at a high temperature, so use of allozymes at a molecular level require fresh tissue or blood, long term storage is one of the most important problem with allozyme electrophoresis, because some of enzymes are not stable for long time. For screening of large numbers of loci require multiple tissues such as liver, kidney, heart, brain and blood corpuscles (RBC and WBC), However, in case of

endangered species protected under law and are not permitted for killing or where population size drastically reduced or when there was a loss of individuals from natural habitat, so experimental tissue material is limited (Utter 'et al.' 1987), (Grant 1984) it limits the application of isozymes is deviation from co dominant expression of allozymes. Null alleles (those with reduced or no expression of protein product) were detected by reduced staining intensity of the same single isozymes on the same gel, complete absence of enzymes activity may indicate null genetic variability (Utter 'et al.' 1987). Electrophoresis revealed considerable variation among species at some protein – coding loci, but the level of differentiation among population of the same species was often found low. One consequence of this is that individual's identification or paternity designation is not usually possible with allozymes. Further improvements in the resolution of genetic difference clearly require direct examination of DNA sequence.

(2) Mitochondrial DNA

The eukaryotic cell contain two structurally independent genomes compartmentalized in the nucleus and mitochondria or chloroplast, each genome is unique in the organization and genetics of the cell and they are partially dependent to each other. Coordinating certain vital function essential for cell survival. The DNA markers are derived from these genomes. Mitochondria are cytoplasmic organelle in eukaryote cells where respiration takes place within the mitochondria which has its own genetic material or DNA. The mitochondrial DNA is circular molecule, 15-20 kilo base in length and contains about 37 genes, including two gene for rRNAs (12 and 16S) 22 mt tRNA and 13 for protein coding gene, which are vital for cellular metabolism. Mitochondrial DNA is haploid, which is each mitochondrion contains only one type DNA (Brentzen 'et al.' 1988), (Birky 'et al.' 1983). Mitochondrial DNA markers are more popular because they provide greater variability and sensitivity to drift. Mitochondrial DNA possesses conserved gene order. Devoid of introns, lacks recombination and small in size, which makes analysis of sequence data relatively easy, as compared to more complex nuclear genome. (Engstrom 'et al.' 2002) Mitochondrial DNA-RFLP data have been widely used for studying evolutionary history, status and management of endangered species population of African Cheetah Florida Panther (Wayne 'et al.' 1986) (Gilbert 'et al.'1991), (Brien 'et al.' 1990). DNA based identification of species are now under extensive applications using the potential tool of polymerase chain reaction (PCR). Most DNA methods reported involves sequence analysis of mitochondrial DNA (Parson 'et al.' 2000), (Guha & Kashyap 2005). The mitochondrial genome of vertebrate has been extensively used over nuclear DNA based marker for resolving phylogenetic relationship at different evolutionary depth due to its unique properties, including presence of strictly orthologous (Gilbert 'et al.' 1991) genes, lack of translation, unique rate of nucleotide substitution and easy isolation (Avise 1996), (Grechko 2002).These unique features along with high copy number of mitochondrial DNA per cell compared to nuclear DNA makes it more powerful tool than nuclear DNA for identification of unknown biological material (Wilson 'et al.'1995). Species identification problems are very much important in conservation and management of endangered species population. Mitochondrial DNA serve as genetic marker in forensics analysis and used as important tool in the conservation and rehabilitation programmes and also a number of applications in fisheries biology management and aquaculture (Billington 2003).

Multiple Arbitrary Primer Markers

Assays that target a segment of DNA of unknown function are included under this category. A general class of PCR –based technique used to detect such anonymous or arbitrary amplicon profiling. Randomly amplified polymorphic DNA (RAPD) and Amplified fragment length polymorphism (AFLP).

Randomly amplified polymorphic DNA (RAPD)

For routine analysis of molecular genetic variations in wild life, RAPD markers are

generally useful for the analysis of genetic variation. RAPD markers consist of relatively short DNA fragment about 200- 2000 base pairs long, amplified via PCR by small arbitrary primers with >50% GC content RAPDs are PCR–based molecular marker that may substantially reduce time, labour, and cost required for molecular analysis RAPDs involve the use of single DNA primer to direct amplification under PCR based amplification of random sequences (Grosberg 'et al.' 1996). Typically, single primer is used and must anneal to priming sites plus the intervening sequences of nucleotides was termed as a RAPD locus and the amplified products are known as RAPD marker, the amplified product can be resolved on agarose gel or PAGE and visualized generally by ethidium bromide or silver staining (Grosberg 'et al.' 1996). RAPD polymorphisms presumably are based on mismatches in primer binding sites. Depending on the number of inverted complementary priming sites in an individual's genome and the length of intervening DNA sequence within a locus, a given primer may amplify none to several amplicons and therefore, usually result in the presence or absence of an amplified product from single locus (Wilson 'et al.' 1990),(Welsh 'et al.'1990),(Xiao-Bing 'et al.' 2002). Some of the most common application of RAPD markers in conservation biology include species/subspecies identification and detection of genetic difference within and between populations in a variety of species (Hardrys 'et al.' 1992), (Hardrys 'et al.' 1993), (Neveu 'et al.' 1998), (Paterson and Snyder 1999),(Cooper 2000), (Vucetich 'et al.' 2001). RAPDs has played a very important role in the selection process for desired genotypic characteristics, leading to the production of species specific, genome specific or chromosome specific markers.

RAPD bands are expected to be unique to every species (Dinesh 'et al.'1993) hence can be used as species or subspecies-specific markers in the study of intra specific genetic variations. The degree of variability observed for many primers suggests that the RAPD technique is useful for the variety of question, including individual's identification, paternity analysis, strain identification, diversity analysis, phylogenetics or systematic, genome mapping and analysis of gene expression (Cheng 1997), (Parker 'et al.' 1998). A high resolution molecular genetic marker is useful only if it shows reproducibility in result without artifact and is easily scorable. Susceptibility to such amplification artefact is potentially a very serious problem with PCR-RAPD. Different PCR condition can yield differential product. RAPD-PCR involves the multiple parameters such as primer size, concentration of Taq polymerase, dNTP and magnesium chloride and PCR condition. Primer size will determine the degree of the specificity in genome scanning. It is most likely that shorter primer, having a shorter target site, would anneal more frequently and direct the amplification of unreasonably large number of RAPD bands than a primer twice as long, As the length of the primer increase, the chance of finding perfect or near perfect homologies between target site decreases, consequently increasing the probability of random non reproducible amplification patterns (Reidy 'et al.' 1992), (Hadrys 'et al.' 1992). RAPD bands are usually scored as discrete present/ absent characters, in which some bands consistently amplify more strongly than other and given band in different individuals may quantitatively vary in intensity from visible to invisible. Furthermore, bright bands tend to amplify more reliably than faint ones. A locus could be scored as null/null homozygote. Some variation in the band intensity appears to be both repeatable and heritable (Hunt & Page 1992), (Levitan & Grosberg 1993). It is again difficult to differentiate the homozygotes and heterozygotes in population.

Amplified Fragment Length polymorphism (AFLP)

AFLP provide a large number of polymorphism in a single polyacrylamide gel. This is PCR based technique, which requires no probe or previous sequence as needed by RFLP. It is more efficient marker than RFLP and RAPD and also more useful than microsatellite loci among individuals in population (Campbell 'et al.' 2003). The limit the application of AFLP is that, to get a large number of unrelated fragments that are visible along with the polymorphic fragments.

(3) Nuclear DNA Markers

In wildlife study nuclear DNA markers have several advantages over protein (isozymes and allozymes) mitochondrial or cytogenetic markers due to high level of variation detectable directly in a variety of organisms. It is a recombined form of both the parents, so various population parameters simultaneously resolve the individual identification. A small quantity of sample is required from any kind of source such as bone, blood, surgically remove tissue epithelial cells in fecal pellets or scat, dried and preserved animal or even fossilized materials without killing the animal, since in wildlife forensic or conservation study, the source or number of animal availability is limited and even killing of single individuals is unethical and prohibited by law. In addition, unlike mitochondrial DNA, nuclear protein–coding genes and introns tend to evolve more slowly (Prychitko 'et al,' 1997), (Prychitko 'et al.' 2000), (Prychitko & Moore, 2000), (Vidal & Hedge, 2005).

Minisatellite

Nuclear DNA contains both, unique single copy gene and non unique duplicated or repetitive regions; the latter include microsatellite and minisatellites, being variable markers identified in most of the eukaryotes. The repeated units are linked together as consecutive tandem repeats and these repetitive sites may be found at one locus or at many regions scattered throughout the genome. Variation in the number of repeat units is common in eukaryotes. These variable numbers of tandem repeats (VNTR) are also known as satellite DNA, large repetitive units of satellite DNA are often associated with heterochromatin near the centromere. The smaller units are made up of repeated units consisting of highly conserved core areas of short oligonucleotide sequence (10-65 base pair) rich in GC nucleotide and highly polymorphic which are generally termed as minisatellite that are strong together in a long arrays. The array sizes vary considerably within and between the genome due to difference in the number of tandem repeat units across 10-25 loci (Jeffreys 'et al.' 1985a,b), Minisatellite DNAs are inherited in mendelian fashion. RFLP analysis of minisatellite DNAs reveal unique inherited banding pattern for each individual, ideally suited for individual genetic profile parentage and forensic analysis, (Buke 1989),(Burke & Bruford, 1987), (Woodruff 1990). Burke & Bruford 1987 published the first study of DNA fingerprinting in a wild bird, the house sparrow (*Passer domesticus*). (Nybom 'et al.' 1990) applied the technique to paternity analysis *in Malus*, *Prunus* and *Rubus*. These results proved ground breaking and stimulated the application of DNA fingerprinting in many subsequent ecological studies. Further, multilocus DNA fingerprinting probes, other than Jeffreys, (Jeffreys 'et al.'1985) were added to the repertoire available to ecologists. A recent modification of this method is multilocus microsatellite fingerprinting, in which a synthetic oligonucleotide consisting of a tandemly repeated simple sequence (e. g. $(CCA)_5$ is used as probe.

The minisatellite DNA fingerprintings are of two types, multilocus and single locus, multilocus DNA fingerprinting involves complex banding pattern that makes it less suitable for population genetic degree of relatedness, (Lynch 1988), (Burke 1989), (Chan 'et al.' 2007), (Jordan 'et al.' 2002) In contrast single locus fingerprinting resolves polymorphism associated with different loci sequentially and therefore, more suitable for population studies with less technical statically and theoretical difficulties generally associated with multilocus DNA fingerprinting. This procedure involves genomic DNA library construction, screening and sequencing for probe development followed by southern hybridization under very inflexible conditions. A single-locus minisatellite probe is able to detect variation only at specific hyper variable minisatellite locus, consequently providing allelic diversity, mean number of fragments per locus and allele frequencies within and among population. However, the development of single-locus probe for a particular species is more expensive, labour intensive and time consuming than multilocus probes and providing the best option for quick screening of population for estimation of heterozygosity (Hanotte 'et al.'1992) relatedness pedigree analysis and translocation between populations (Fleischer 'et al.' 1995).

Microsatellite

Microsatellites are tandem repeats of mono, di-tri or tetra nucleotides, generally less than 300bp in total length but can be much larger (Tautz 1989) (Weber & May 1989) (Hughes & Queller 1993) Microsatellite markers are widely distributed every 20-30 kb, on an average, throughout each eukaryotic genome these marker are also called simple sequence repeats (SSRs) or variable numbers of tandem repeats (VNTRs). These marker are multilocus, exhibit a higher degree of polymorphism than mt. DNA markers and are bi parentally inherited and co-dominant rather than completely dominant like RAPD. (Parker 'et al.'1998). The simplest microsatellite are mononucleotide SSRs of A (e.g.AAA = $(A)_3$). The most commonly used (and most abundant in human and probably most other mammals) microsatellite is AC (which = CA = GT=TG). There is some disagreement on the exact size range for microsatellites. In general way repeat that is composed of 1-4 or 1-6 bp repeat units is considered a microsatellites. Generally, there need to be more tandem repeat units for a microsatellite to be hyper variable, (Weber 1990), (Tautz 1989), (Weber & May 1989), (Tautz 'et al.' 1986) Microsatellite tends to be distributed in the euchromatin. Microsatellites are useful for four important reasons. First the total size of microsatellites tends to be small. The repeats tend to be less than 100 bp in the total length. Second, the DNA flanking the microsatellite tends to be single copy nuclear DNA. Therefore PCR primer may be designed from the DNA flanking a particular repeat to amplify a specific repeat faithfully. Third, microsatellite mutate at a high rate (on the order of 10^{-4} to 10^{-5} per locus per generation for di-nucleotide) finally, they tend to mutate by gaining or loosing repeat units. For example one allele of a locus (i.e. a specific chunk of DNA on a chromosome) may be ACACACACACACACACACAC = $(AC)_{10}$. Another allele for the same place the same locus may be screened for size variation (something relatively cheap and easy compared to sequencing) to detect alleles and there should be many allele in most population. Thus PCR product of a given microsatellite locus may be screened for size variation to detect alleles and there should be many alleles in most populations given the mutation rate. Basically all eukaryotes have microsatellites (Hamada 'et al.' 1982), (Hamada 'et al.' 1982) however, distribution and frequency of certain types of microsatellite seems to vary greatly across major taxomomic group (e.g. Birds and mammals). Mammals and social insect seems to have an abundance of microsatellite. The human genome is estimated to have about 30,000 AC microsatellite loci (cite). Birds have approximately 10% as many microsatellites as typical mammals. Alligator have more microsatellites than birds but significantly less than most mammals.

The disadvantage of microsatellite is that it needs construction of genomic library and identification of microsatellite clones from this genomic library for a new species which can be time consuming and costly. In most of the studies known primer don't amplify the same locus across the taxa unless microsatellite region is flanked by highly conserved sequence where priming sites are located (Ellegren 1992) (Fitzsimmons 1998), (Fitzsimmons 1995).Therefore microsatellite based DNA fingerprinting has been less recommended for quick genetic assessment of endangered or threatened species (Moorea 'et al,' 1991). In the recent year only, the availability of the DNA based marker in population genetics (John 'et al.' 1994).

Choice of marker-

(Park & Moran, 1994) described the increasing availability of molecular marker provide a universally applicable and objective approach for comparative analysis of species, population and individual identity. Choice of genetic marker depends on mostly two factors these are, rate and mode of evolution of the genetic marker, Mode of inheritance (maternal, biparental) and expression (dominant, co-dominant) (table 1). The genetic divergence among populations results in virtually no shared alleles. To date no single molecular technique has proven to be optimal for resolving major genetic concerns in conservation biology. Each technique has strengths and weakness generally based upon the equilibrium between repeatability, cost and

development time and detection of genetic polymorphism (table 1). Choice of marker depends on the following factors these are; availability of samples, sensitivity of marker, availability of marker, rapid development and screening, multilocus or single-locus, relative cost, organelle and nuclear DNA. The most suitable and relevant genetic markers for genetic databases are allozymes, microsatellites, DNA-sequence (both nuclear and mtDNA), RFLP (PCR-amplified segments) and SNPs.

CONCLUSION

The main purpose of this review was to provide an overview of molecular markers widely used in conservation genetics. These closely developed markers can identify closely related species, and its population. So it is important to evaluate genetic data. It is apparent that no one molecular marker is superior for all common application and it is not possible to say that no marker could exist, on the basis of evidence from one or more markers. Either nuclear or mitochondrial genome is considered, this conclusion cannot be reached; increasing the number of alleles per locus does not always increase the probability of detecting significant difference. Thus, it is recommended that at least two markers, (mitochondrial and nuclear) should be utilized for each case. Availability of potential technique and time and costs of materials decide which marker should be use. Major attribution of molecular markers commonly used in conservation of wildlife genetics (Parker & Moran, 1994), (Parker 'et al.' 1998) are tabulated as below (table 1).

Marker	General ease	PCR	Expression	Loci	Genome	Overall variation	No. of loci available	Data for analysis
Allozymes	Electrophoresis (Native PAGE, SDS)	No	Co-dom.	Single	Nuclear	Low	Moderate	Direct
mtDNA RFLPs	Extraction, digestion, electrophoresis	No	Varies/ Co-dom	single	Organelle	variable	Single	Direct
mtDNA sequence	Extraction,PCR, electrophoresis analysis	Yes	Dom.	single	Organelle	variable	Single	Direct
RAPD/AFLP	Extraction,PCR analysis	Yes	Dom.	Multilocus	Nuclear	High	Many	Limited
Multilocus mini/micro satellite fingerprints Nuclear RFLPs	Extraction, digestion, electrophoresis, gelblot hybridization, analysis.	No	Co-dom.	Multilocus	Nuclear	High/ variable	Many	Limited
VNTRs: Mini satellites VNTRs:Microsatel lites	Primer development may be long, extraction, PCR electrophoresis	Few/ yes	Co-dom.	Single	Nuclear	High	Many	Indirect

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