Research Journal of Recent Sciences _____ ISSN 2277-2502
Vol. **6(7),** 53-62, July **(2017)** *Res. J. Recent Sci.*

Review Paper

Molecular markers based characterization and conservation of wild animals

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Available online at: www.isca.in
Received 13th May 2017, revised 16th June 2017, accepted 28th June 2017

Abstract

Simultaneous presence of various animal genetic resources in a given area or country is known as animal biodiversity. Africa has rich wildlife resources serving as a major tourist attraction. Wildlife biodiversity (WLBD) is an important asset for developing countries in uplifting their economy. Hence, characterization, conservation and maintenance of WLBD should be given top priority. Global climate change has resulted in the depletion of wildlife habitat and is responsible for extinction of many species. Characterization helps us to distinguish variation within and between different organisms and guide us for proper conservation of populations, species and or strains. Characterization can be done based on phenotype, biochemical polymorphism and molecular based markers. Molecular studies based on mitochondrial DNA (mtDNA) and nuclear DNA are more popular as they save time, minimize long term investigation cost and are efficient in information generation. In this article, we reviewed the various molecular markers used in the characterization of wildlife, namely: Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellite and Single Nucleotide Polymorphisms (SNPs).

Keywords: Biodiversity, Molecular markers, DNA forensics, Wildlife, Africa.

Introduction

Biodiversity refers to the genetic variability of species, populations within species, within and between populations. Simultaneous, presence of various animal genetic resources in a given region or country is known as animal biodiversity. Genetic diversity is a guarantee to a given population to evolve and adapt to the sudden changes in environmental conditions[1]. Biodiversity offers multiple opportunities for development and improving human well-being. It is the basis for essential environmental services upon which life on Earth depends. Thus, its conservation and sustainable use are of critical importance.

Africa has underdeveloped tourism industry which attracts very less numbers of foreign tourists globally. The continent is rich in wild animal resources which is the major tourist attraction. Ethiopia's geographical location and physical feature has resulted in the diversification of wildlife[2]. Ethiopia possesses diverse agro ecology and is known to be home of several unique habitats. Out of the 277 mammalian species found in Ethiopia, 31 are endemic[3]. Rural Africans neither want to give up land to wildlife nor have wildlife nearby[4]. Like in many other African countries, many of Ethiopia's protected areas exist only on records, while others have declined in size or quality[5]. The development of tourism sector in Ethiopia is relatively less as compared to other African countries; however, it is serving as a major source of foreign exchange earnings in the country, claiming an average of 23.34% from 1995 to 2007[6]. Hence, considering Ethiopia's great potential for wildlife tourism industry into account, the sector should be given high priority. And proper conservation, characterization and maintenance of wildlife should be given maximum importance.

Nowadays, biodiversity loss is one of the greatest problems of the world. Knowing the genetic profile of species that are threatened with extinction is a prerequisite for management of ecological, the species and/or populations[7]. The biological diversity of earth is rapidly diminishing largely as a result of global climate change[8]. Molecular techniques are increasingly used in the conservation of biological diversity in response to the alarming extinction of different species[7]. Undoubtedly, many conservation geneticists study the genetic markers to make decision for conservation of endangered wild animals. Choosing effective profiling technique is rather very critical step, as inappropriate information on molecular data may result in incorrect conservation actions[9]. Application of both karyotypes and molecular genetic markers to conservation of wild animals has been put in practice[10].

Identification and characterization are important steps towards crafting sustainable conservation strategies of wild animals. Studying genetic variability within and between populations, estimation of effective population size and possible bottlenecks are some of the practices carried out in wild animal

characterization. Various methods have been developed over the years for the characterization of wild animals and these methods are largely categorized into phenotypic, biochemical and molecular markers. Phenotypic characterization of wild animals is based on the physical appearance of the animal such as the size, shape, color etc. This method of characterization can be considered as the first step towards describing the animal, but not sufficient in revealing the existing genetic variability (among or within populations) for developing sustainable conservation strategies. Characterization based on biochemical markers (blood proteins, isozymes and allozymes) involve the detection of polymorphism on electrophoresis separation of the proteins of interest according to their molecular weight and followed by specific staining for visualization[11,12]. Gel electrophoresis can also reveal genetic polymorphism at protein or at an enzyme level[13]. The major drawbacks associated with both phenotypic and biochemical marker is their inefficient ability to pick up the existing genetic polymorphism within population. More importantly, they could be influenced by age and environmental factors. Therefore, in this review various molecular markers viz. Restriction Fragment Length Polymorphism (RFLP), Random Amplification of Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellite (short tandem repeats), Single Nucleotide Polymorphisms (SNPs) that are more efficient in polymorphism detection are discussed.

Molecular characterization

The molecular characterization is based on studies of mitochondrial DNA (mtDNA) and nuclear DNA. Both are more popular as they save time; minimize long term investigation cost and efficient in information generation. Variations of appropriate genetic markers in addition to demographic and geographic parameters analysis enable to deduce about population and evolutionary processes[14]. Genetic markers are specific chromosomal regions varying in the DNA sequence that allows detection of the genetic differences of individuals in a population. Advancement in molecular biology technique such as Polymerase Chain Reaction (PCR) has made molecular markers studies easy, affordable and popular[15].

Molecular (DNA) markers are chosen over biochemical and phenotypic markers to characterize wild animals because they are relatively abundant, independent of growth and physiological state and provide a more powerful source of detecting genetic polymorphism[16-18]. Molecular markers can be grouped into two major classes; mtDNA markers (D-loop region, cytochrome b gene, 12S rDNA and 16S rDNA genes) and nuclear DNA markers (RFLP, RAPD, AFLP, Microsatellite and SNPs)[19].

Mitochondrial DNA (mtDNA) markers

It is a known fact that mitochondria are the only source of mammalian cytoplasmic genetic information. Mammalian mitochondrial DNA is a small circular DNA with size of 15-25 kb[20]. Each mammalian mitochondrion DNA contains 37 genes viz.13 protein-coding genes including cytochrome b, 2 ribosomal RNA (rRNA) genes and 22 transfer RNA (tRNA) genes[21]. Each mitochondrial DNA molecule contains a non-coding region called displacement loop (D loop) that regulates initiation of replication and transcription[22]. This region stretches around 1kb and it is amenable to PCR amplification before sequencing to find out molecular variability. The sequence of 12S rRNA is highly conserved and usually used in taxonomy for high category level (phyla and subphyla), whereas the 16S rRNA is used for mid category classification[19]. Among the molecular markers, mtDNA is very useful in population studies, phylogenetics, and maternal inheritance studies. It lacks DNA recombination and evolves at a faster rate[23]. Different parts of mtDNA evolve at different rate; hence, the rRNA genes evolve approximately 100-fold quicker than their nuclear counterparts and the D-loop evolves 5-times faster than the rest of the mtDNA. Various studies have indicated the importance of this technique in phylogenetics of wild animals[24-27].

Restriction fragment length polymorphism

RFLP was one of the first techniques to be widely used for detection of variation at molecular level. This technique reveals variation in the length of DNA fragments produced by specific restriction enzymes from genomic DNA of two or more individuals of a species[28]. The technique consists of isolation of DNA, digestion of the DNA with restriction enzyme, separation of the DNA fragments with gel electrophoresis according to their size followed by hybridization using fluorescently or radioactively labeled probes (Figure-1)[29]. The difference among individuals in the length of restriction fragments is largely because of the loss of a cleavage site or formation of a new one[30]. Insertion or deletion of certain DNA sequence can also alter the size of restriction fragments. RFLP can be used to study genetic distance, population variation, gene flow, effective population size, and analyses of parentage and relatedness. Some of the advantages of RFLP include: it gives reproducible results within and among laboratories and distinguish co-dominant markers. Polymorphic markers are described as co-dominant or dominant if they can distinguish between homozygotes and heterozygotes[31]. However, there are some drawbacks associated with RFLP analysis such as the requirement of relatively high quality and large quantity of DNA (2-10mg), labor intensive and the requirement of probes for detection. The development of PCR based RFLP analysis (PCR-RFLP) has made the technique to be less laborious with relatively small quantity of DNA and possibility of detection without use of probes. Among other DNA based markers, PCR-RFLP has been used in very few studies[26].

Randomly amplified polymorphic DNA

RAPD is a molecular technique[32] which uses short arbitrary primers (10-12 base pairs) that bind randomly to multiple places

in the genome and subsequently amplify these regions by PCR[28]. The PCR products are then separated using electrophoresis on either agarose or polyacrylamide gel. The presence or absence of bands is used for detecting polymorphism among individuals (Figure-2).

RAPD lacks reproducibility within and among laboratories, but it has been used for molecular characterization of wild animals because it is cost effective, simple and quick method as compared to other molecular techniques. Helen *et al.*[33] reported the existence of high genetic diversity and the occurrence of intense and constant gene flow in red fox (*Vulpes vulpes*) populations using RAPD markers. Padilla *et al.*[34] used 45 RAPD primers to study genetic diversity of Iberian imperial eagle (*Aquila Adalberti*) and observed high heterozygosity in this species. Freitas *et al.*[35] revealed the importance of RAPD marker for conservation of fish species. Gouin *et al.*[36] indicated the loss of genetic diversity among 21 Crayfish (*Austropotamobius pallipes*) based on four RAPD primers. Saudi Arabian Oryx (*Oryx leucoryx*) populations were characterized based on 20 RAPD primers and indicated sufficient variability within this populations[37].

Amplified fragment length polymorphism

AFLP is a molecular technique that combines restriction based RFLP marker and PCR based RAPD marker[28]. This technique is based on the amplification of subsets of genomic restriction fragments. DNA is cut with restriction enzymes and double-stranded adapters are ligated to the ends of the DNA-fragments to generate template DNA for amplification. The sequence of the adapters and the adjacent restriction site serve as primer binding site[38]. The amplified fragments are then separated by electrophoresis and the variations are scored based on presence or absence of bands (Figure-3). Even though, it is advantageous in its quick scan and detection of whole genome polymorphisms, high reproducibility, needs no prior sequence information & probe generation, but it is technically expensive and labor intensive. Some studies have revealed the importance of AFLP markers for identification and characterization of species and populations[39-41].

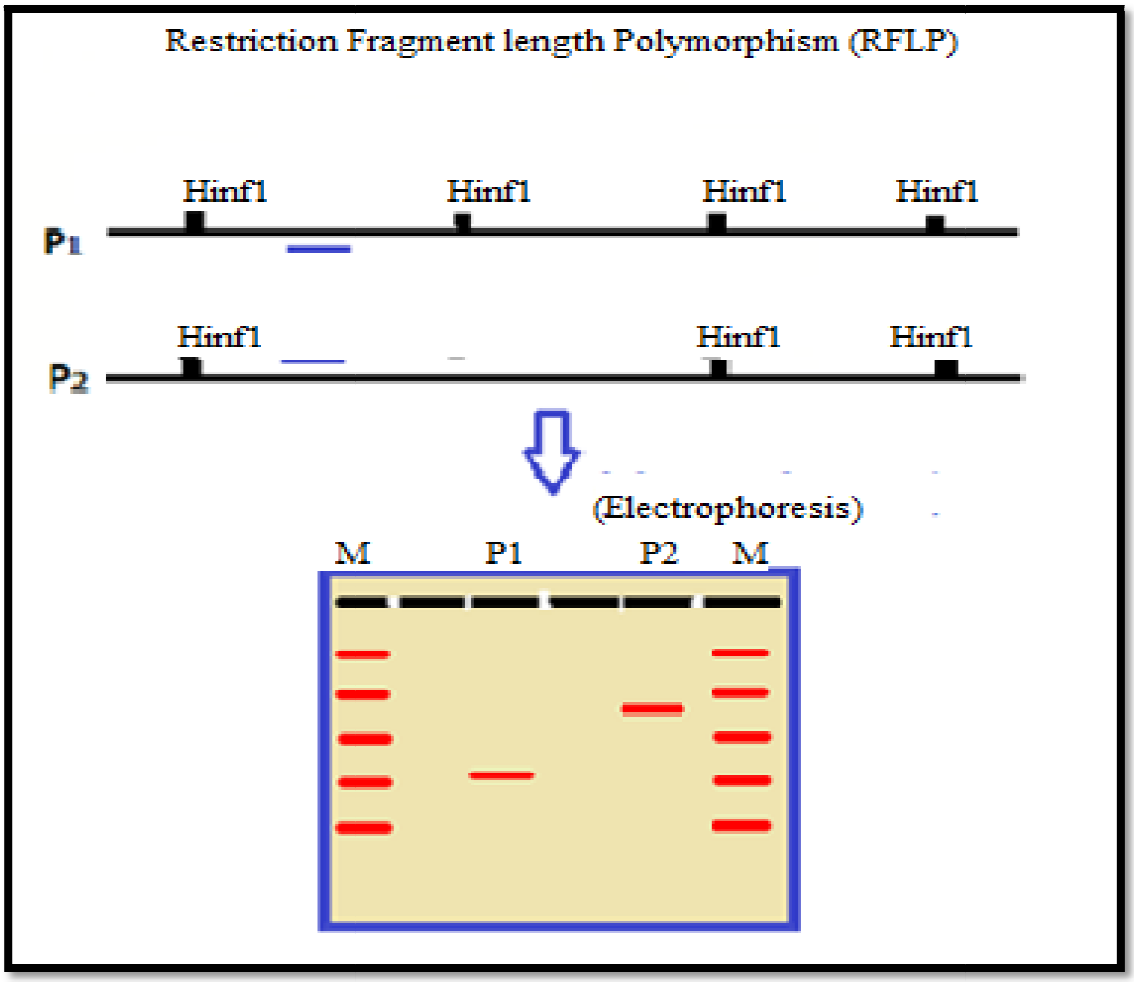

Figure-1: Enzyme digestion of DNA into fragments and their subsequent gel separation and the detection of allelic variation in two animals from population 1 and 2.

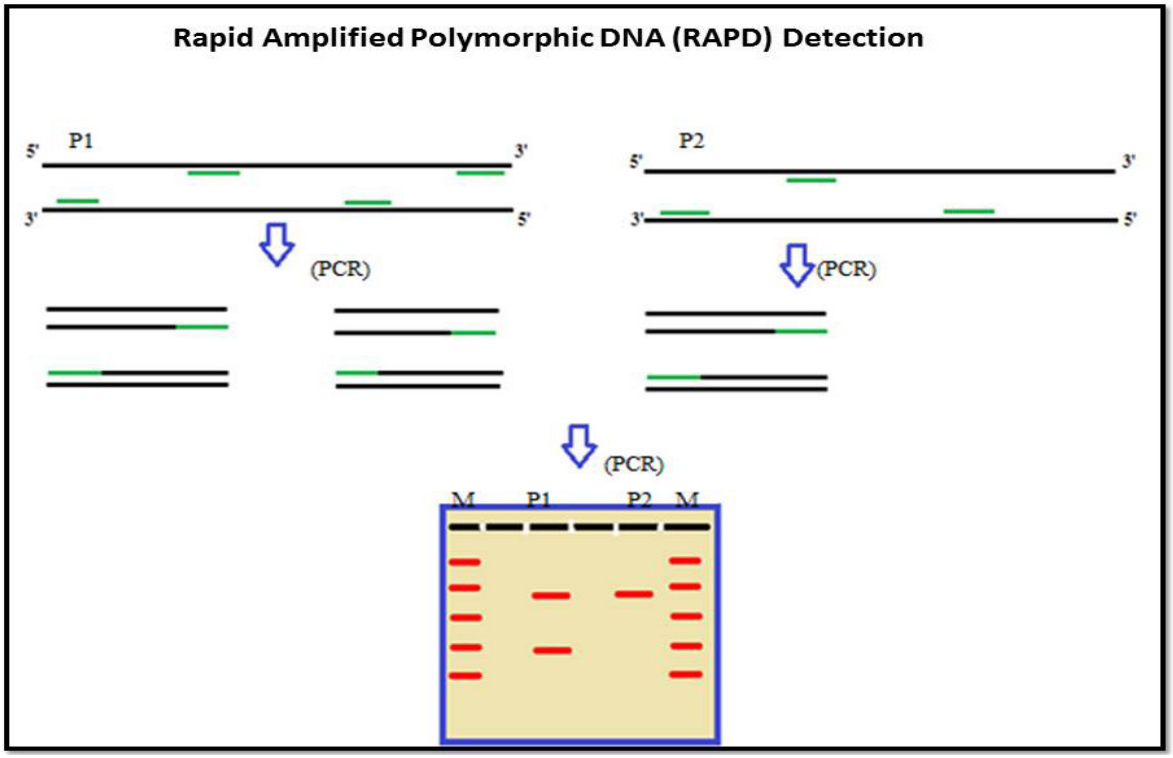

Figure-2: Variation detection between two animals from population 1 and 2 using RAPD.

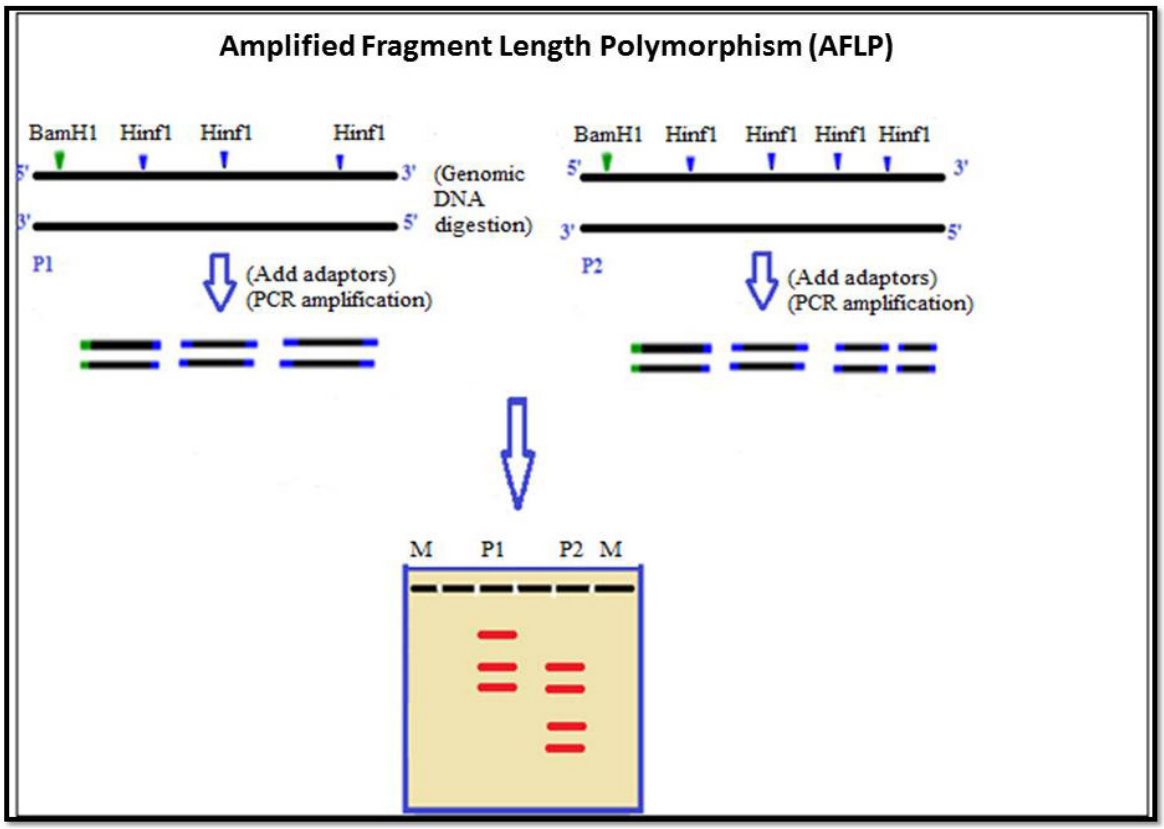

Figure-3: Schematic diagram depicting the variation of two individuals (P1 and P2) using Amplified fragment length Polymorphism.

Microsatellite markers

Microsatellite is a sequence of short tandem repeated segments of 1-6 base pairs at a unique physical location in the genome. It is a class of repetitive DNA elements[42,43] . The di-, tri- or tetra-nucleotide repeats are arranged in tandem arrays consisting of 5 – 50 copies, such as $(AT)_{29}$, $(CAC)_{16}$ or $(GACA)_{32}$. Polymorphism of individuals is due to variation on the number of repeats (Figure-4). The primary mutational mechanism leading to changes in microsatellite length is DNA polymerase template slippage caused by mismatches between DNA strands while being replicated[44]. DNA strands may dissociate and then free-associate incorrectly during replication. In this misaligned state leads to insertion or deletion of repeat units, thus altering allele length.

Several important characters make microsatellites as informative marker to study variation within and among populations. Microsatellites are variable and exhibit a high level of allelic variation, co-dominantly inherited and thus applied to study genetic variability, population structure and gene flow among populations. Currently, microsatellite markers are found to be suitable molecular markers to study wild animals' genetic resources. Various studies have also confirmed this fact.

Eblate *et al.*[45] reported polymorphism of six microsatellite markers in African Antelope species. Liukkonen *et al.*[46] indicated the usefulness of microsatellite markers to differentiate between released and wild partridges in Finland. The purple Swamphen (*Porphyrio porphyrio*) were characterized using 10 microsatellite markers[47].

Single nucleotide polymorphisms

SNP reveals variation in DNA sequence where a single nucleotide in the sequence has changed at least in one percent of the population[28,48]. SNPs are reported to be the most frequent variation in DNA[49]. They are primarily detected by DNA sequencing (Figure-5), but RFLP and primer extension with allele specific probes are also employed for SNPs analysis. Variations among individuals using SNPs can also be detected by single strand conformation polymorphism[50]. They are the most abundant molecular marker systems ever known in the genome with high geneticvariability[51]. They are highly reproducible and very informative. However, they are relatively expensive and require prior knowledge of sequence. A large number of SNPs have already been developed in human but the limited number of SNPs for many species creates a hurdle to their application in population genetic studies.

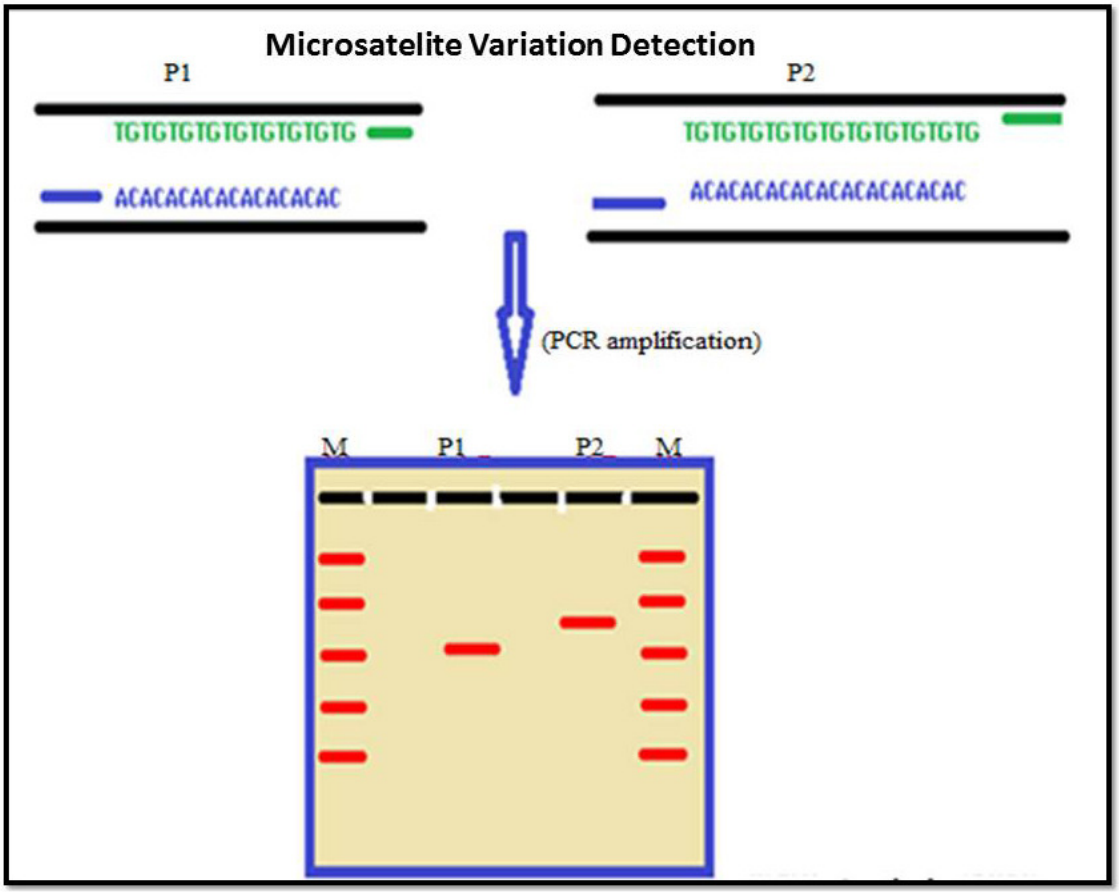

Figure-4: Schematic drawing showing how microsatellite variation (short P1 and long P2) can be detected using gel electrophoresis after PCR amplification: standard DNA marker, P: Population.

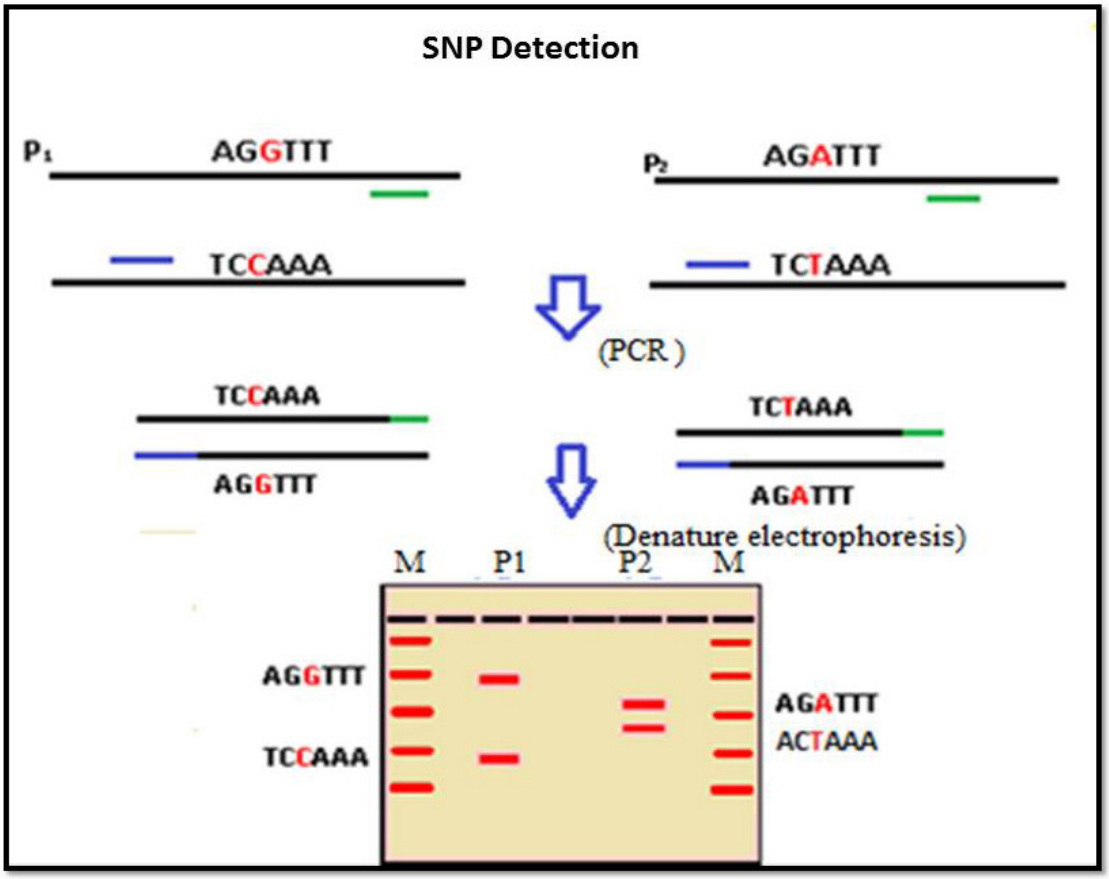

Figure-5: SNP detection by single standard conformation polymorphism in two animals from population 1 and 2.

Molecular techniques and wild animals conservation

Our planet is losing wildlife at a frightening pace and the number of strains, breeds and species being discovered each year is almost insignificant. Not only human made habitat destruction but illegal hunting and trade are also thought to contribute for the wildlife biological genetic resource shrinkage[52]. For instance, it was reported that illegal trade of wildlife parts and products annually worth more than 20 billion USA dollars in black market[53]. Consequences of climate change for wildlife include altered ecosystems, altered species composition, increased incidence of human-wildlife conflict, wild land fires, and spread of invasive species & infectious diseases[54]. Studies conducted in diffent parts of the world imply that climate change drives an increase in wildlife attacks. Hence, responding to global climate change and law enforcement are therefore, believed to play significant part in conservation of wild lives. Responding to climate change involves encouraging campigns for evergreen areas protection and natural resources conservation as these safeguard habitant of wild animals and their survival. DNA forensics is a promising tool to address questions relating to the identification of species, populations, geographic origin, family relatedness and individual identity. Thereby offering support to the crime investigation teams and law enforcement agencies[55]. A summary of literature for species and individual identification in animals using different animal parts is given in Table-1. By using various molecular markers it is possible to detect unique genetic variations in endangered population or species. Knowledge of genetic diversity may enable us to develop proper breeding programs to minimize inbreeding and safeguard the loss of genetic variation.

Conclusion

Molecular markers investigation is most accurate and more informative than phenotypic and biochemical methods of characterization. Molecular markers are very useful as they provide accurate information on genetic variability within and among animal populations thereby helping to develop appropriate conservation strategies. Choosing effective profiling technique is very critical step as selection of inappropriate study technique may result in incorrect conservation actions. Hence, characterization for conservation and maintenance of WLBD should be done wisely. Government and conservationists need to develop innovative ways and means for protecting natural habitants of wild animals. Awareness among local people should be done so that community based conservation of natural resources can be encouraged.

Table-1: Summarized literature to trace back sources of different wild animals.

Sl.	Samples details	Reference
01.	Bile crystals; Asiatic black bear	56
02.	Blood, horn; Rhinoceros	57
03.	Blood; Eurasian badger	58
04.	Blood; Tiger	59
05.	Caviar; Sturgeons and paddlefish	60
06.	Feathers; Scarlet macaw	61
07.	Hair, leather; Asiatic black bear	62
08.	Horn; Rhinoceros	63
09.	Ivory; Elephant	64
10.	Meat, carcass; Reedbuck	65
11.	Meat, hair; Roe deer	58
12.	Meat; Indian peafowl	66
13.	Meat; Whale species	67
14.	Skin; Crocodile	68
15.	Solid tissue; Mule deer	69
16.	Solid tissue; Wild boar	70
17.	Tail tissue; Sea horse	71
18.	Tissue; Cobra snake	72
19.	Tusk; African Elephant	73
20.	Wool; Tibetan Antelope	74

Adopted from Panday *et al.*[75] Licensee Bentham Open.

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