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MOLECULAR MARKERS IN FARM ANIMALS: ADVANCED TOOLS FOR EVALUATION OF GENETIC DIVERSITY

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Abstract

Biodiversity and genetic diversity research is required to meet current production needs in various environments and to allow sustained genetic improvement as well as to facilitate rapid adaptation to changing breeding objectives. Different levels of genetic diversity have important applications in conservation, restoration and agriculture which can improve our understanding of the interplay between evolution and ecology. The increasing availability of molecular markers in farm animals allows determining of genetic variations and biodiversity with high levels of accuracy and reproducibility.

Key Words: *Genetic diversity – molecular markers – farm animals*

INTRODUCTION

The most unique feature of Earth is the existence of life and the most extraordinary feature of life is its diversity. Biodiversity is the variety of life including variation among genes, species and functional traits. Three levels of biodiversity are commonly discussed;

1. Genetic diversity: is all the different genes contained in all the living species, including individual plants, animals, fungi, and microorganisms.
2. Species diversity: is all the different species, as well as the differences within and between different species.
3. Ecosystem diversity: is all the different habitats, biological communities and ecological processes ‘as well as variation within individual ecosystems (Cardinale *et al*., 2012).

The development of every species under its particular natural ecosystem, environmental, and socio-economic conditions has led to each having its own specific genetic characteristics which constitute the Earth’s species diversity. Mankind can learn and make use of these special genetic resources to develop animal production for human food needs (Yang *et al*., 2013).

Livestock biodiversity is important because the genes and gene combinations they carry may be useful in the future for traits that are presently unknown or difficult to define, e.g. disease and stress resistance, quality and composition of products, adaptation to different environments or farming systems (Boyazoglu, 1999).

In this context, the investigation of genetic relationships among species populations in order to study their origin, domestication and genetic structure, will provide a useful tool in supporting conservation decisions and designing of breeding schemes (Moioli *et al*. 2001). Moreover, farm animal genetic diversity is required to meet current production needs in various environments, to allow sustained genetic improvement, and to facilitate rapid adaptation to changing breeding objectives (Notter, 1999).

Diversity among organisms is a result of variations in DNA sequences and of environmental effects. Genetic variation is substantial and each individual of a species possesses a unique DNA sequence. DNA variations are mutations resulting from substitution of single nucleotides (single nucleotide polymorphisms – SNPs), insertion or deletion of DNA fragments of various lengths (from a single to several thousand nucleotides), or duplication or inversion of DNA fragments. DNA variations are classified as “neutral” when they cause no change in metabolic or phenotypic traits, and hence are not subjected to positive, negative, or balancing selection; otherwise, they are referred to as “functional”. Mutations in key nucleotides of a coding sequence may change the amino acid composition of a protein, and lead to new functional variants. Such variants may have an increased or decreased metabolic efficiency compared to the original “wild type”, may lose their functionality completely, or even gain a novel function. Mutations in regulatory regions may affect levels and patterns of gene expression; for example,

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turning genes on/off or under/overexpressing proteins in specific tissues at different development or physiological stages (Hughes *et al*. 2008).

Genetic Diversity In Farm Animals

Genetic diversity is defined as any measure that quantifies the magnitude of genetic variability within a population and it is a fundamental source of biodiversity (Hughes *et al*. 2008 and Sangwan, 2012). The knowledge of the genetic variability and process that underlie its origins and maintenance is vital to provide critical insights into the structure and dynamics of populations. Genetic variability also provides the opportunity for tracing the history of populations, species, and their ancestors through DNA based markers. Detailed knowledge of genetic variation within and among different breeds is very important for understanding and improving traits of economic importance. Hence, the future improvement is dependent on genetic variation present within breeds (Sangwan, 2012).

Intrapopulation genetic diversity is important for conservation of populations for two primary reasons: (1) genetically determined phenotypic variation is equated with adaptive potential of populations (Bell and Collins, 2008 and Hoffmann and Sgro, 2011), and (2) neutral genetic variation of natural populations reflects inbreeding and genetic drift, which reduce the viability of populations (Frankham, 2005 and O'Grady *et al*., 2006). Consequently, reduced intrapopulation genetic diversity, be it neutral or adaptive, is related to increased extinction risk in natural populations (Kahilainen *et al*., 2014). Sufficient genetic markers for evaluating the population structure and other aspects of available animal genetic resources are necessary to assess genetic diversity (Yang *et al*., 2013). Genetic markers provide information about allelic variation at a given locus. The increasing availability of molecular markers in farm animals allows the detailed analyses and evaluation of genetic diversity and furthermore the detection of genes influencing economically important traits. They are also the prerequisite for the identification of positional and functional candidate genes responsible for quantitative traits (Erhardt and Weimann, 2007).

Advances in molecular biotechnology have introduced new generations of molecular markers for use in the genetic improvement of farm animals. Consequently, more accurate genetic information can be obtained to better understand existing animal genetic resources (Yang *et al*., 2013). In earlier studies, morphological markers and ecogeographical factors were used to represent diversity, and after that, chromosomal karyotyping was developed. With the rapid development of modern biotechnology, biochemical markers, such as proteins and isozymes, were utilized. By the 1980s, many different types of DNA molecular markers including mitochondrial DNA (mtDNA) and nuclear DNA markers had been explored and played significant roles in the evaluation of genetic diversity in farm animals. In addition, with biotechnological and computer innovations, novel strategies such as wholegenome SNP chips and DNA Barcoding have emerged. DNA molecular marker techniques are widely applied in the fields of germplasm identification, phylogenetics, and genetic structural analysis. They overcome the limitations of morphological, cytological, and biochemical markers, namely the small numbers of such markers and the fact they can be environmentally influenced. The expansion in DNA information will facilitate study of genome-wide diversity; such information is much more precise for the assessment of genetic diversity than previous markers. This chapter will deal with a brief summary on the principles and advancements of primary genetic markers involved in assessments of animal genetic resources (Arif and Khan, 2009 and Yang *et al*., 2013).

Dna Based Markers

DNA markers are useful in both basic (phylogenetic analysis and search for useful genes) and applied research (marker assisted selection, paternity testing and food traceability). With the development of molecular biotechnology, molecular markers have made rapid progress. A molecular marker is based on the nucleotide sequence mutations within the individual's genome and they are the most reliable markers available. Molecular markers can be used for investigating genetic variations at the DNA level between different populations and individuals. Its advantage is being able to find genetic variations rapidly and directly. Molecular markers have developed quickly and they are becoming more and more informative. Up to now, various types of molecular markers have been utilized to evaluate DNA polymorphisms.

According to technical principles; there are three classes of DNA molecular markers: (i) Nucleic acid hybridization based on complementary bases, e.g., restriction fragment length polymorphisms (RFLPs), (ii) Polymerase Chain Reaction (PCR) based on DNA amplification, e.g., random amplification of polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLP), microsatellites or simple sequence repeats (SSRs) and (iii) Single nucleotide polymorphisms (SNPs). The first technique, RFLP, has

been decreasingly used due to the difficulties involved in manipulating high throughput sampling and the third technique, SNPs, represents high costs related to large-scale genotyping. However, the cost-effective PCR-based techniques have been largely used (Semagn *et al*. 2006).

Restriction Fragment Length Polymorphisms (RFLPs)

Hybridisation – based marker technologies use cDNA, cloned DNA elements, or synthetic oligonucleotides as probes, which are labeled with radioisotope or with conjugated enzymes that catalyze a colored reaction, to hybridize DNA. The DNA is either cleaved with restriction enzymes or amplified by PCR. RFLP is representative of this type of technology and was first developed in 1980 (Botstein *et al*., 1980 and Schimenti, 1998) to visualize the differences of the DNA structure based on the use of bacterial restriction enzymes that cut the DNA at sites with specific nucleotide sequences (Mburu and Hanotte, 2005). RFLP analysis is based on the ability of restriction enzymes (also called restriction endonucleases) to cleave DNA at specific target nucleotide sequences consisting usually of a four or six nucleotide pair target. For example, one of the more commonly used enzymes, Eco RI, cleaves DNA at the target sequence GAATTC. Each time the enzyme encounters this sequence, it cleaves the DNA molecule at that point (Fairbanks and Andersen, 1995).

Differences are evident when the length of fragments are different, implying that the restriction enzyme cut the DNA at unrelated locations. Restriction polymorphism occurs when mutations remove and existing restriction site or create a new restriction site. The alterations are detected by using a hybridization probe. The choice of the DNA probe is crucial in RFLP analysis. The identification of RFLPs requires the use of gel electrophoresis to separates the DNA fragments of differing sizes followed by transfer of the fragments to a nylon membrane (Southern blot) and visualization of specific DNA sequences using radioactive or chemiluminescent probes exposed to an X-ray film (Drinkwater and Hetzel, 1991; Smith and Smith, 1993; Albert *et al*., 1994; Lewin, 1994; Bishop *et al*., 1995 and Stein *et al*., 1996).

RFLP analysis is now a standard, well-tested procedure for estimation of genetic diversity. It has been used in thousands of studies in a variety of species (Fairbanks and Andersen, 1995). Because hybridization patterns obtained by RFLP analyses are much simpler than ordinary restriction-fragment patterns, it is possible to compare mtDNAs of distantly related species (Terachi and Tsunewaki, 1992). Each time an RFLP program is initiated in a new species, a new set of probes often needs to be developed. In a few cases, probes from a related species may be used if they are available. Probe generation by cloning and testing can be laborious and expensive. If probes are not readily available, RFLP programs may require a substantial initial investment of time and money before reliable data are available. Nonetheless, RFLP analysis remains a reliable and much-used technique for analysis of genetic diversity (Fairbanks and Andersen, 1995).

According to (Teneva, 2009), the main advantages of RFLP markers are: Produces co-dominant (also known as semi-dominant) markers - this allows discrimination of homo- and heterozygotic states in diploid organisms , Stable and reproducible - gives constant results over time, and location and Selective neutrality.

The disadvantages are the following (Teneva, 2009): Long methodology, Labour intensive, Requires high quality and large quantities of DNA, RFLPs limited the identification of the whole genome variation in animals and The reduced variability observed in domestic animals by inbreeding makes many RFLPs sites non-informative.

These disadvantages replace RFLP markers with new type of markers – PCR based microsatellite markers (Drinkwater and Hazel, 1991 and Vignal *et al*., 2002). RFLP markers are most widely applied in genome mapping, marker – aided breeding, systematic and evolution studies (Teneva, 2009).

Random Amplification of Polymorphic DNAs (RAPD)

Random Amplified Polymorphic DNA is also known as arbitrarily primed PCR (AP-PCR), or as a DNA amplification fingerprinting technique (DAF) and was described in 1990 (Williams *et al*., 1990). Random amplified polymorphic DNA (RAPD) markers are analyzed by using PCR to amplify the segments of nuclear DNA. The use of a single primer (usually 8–10 bp long) that attaches to both strands of DNA and low annealing temperatures increase the likelihood of amplifying multiple regions representing a particular locus (multi–locus). Although RAPD is a simple and inexpensive technique its major limitation is the inability to differentiate between homozygote and heterozygote; this marker is therefore regarded as a dominant type (Arif and Khan, 2009).

According Vignal *et al*. (2002) and Mburu and Hanotte (2005) the advantages of RAPD markers are following: cost effective; simple and quick, large number of bands are produced, no prior sequence knowledge is necessary, the required samples are very small because DNA will be amplified by PCR technique. There are also some disadvantages with

respect to other methods (Dodgson *et al.*, 1997 and Atienzar *et al.*, 2000):

Detection of polymorphism is limited, reproducibility of results maybe be inconsistent e.g. low annealing temperature may cause; Some unspecific non reproducible binding of primers; Dominant markers (homozygote cannot be discerned from heterozygote so allele frequencies cannot be estimated) and The RAPD primers are very sensitive to PCR conditions and this may lead to poor reproducibility.

It had also applications for the conservation of endangered species in avian species (Nusser *et al.*, 1996 and Tansley and Brown, 2000).

Amplified Fragment Length Polymorphisms Markers (AFLPs)

Amplified fragment length polymorphism (AFLP) is a multi–locus technique that involves restriction digestion and PCR amplification (Arif and Khan, 2009). This DNA fingerprinting technique detects DNA restriction fragments by means of PCR amplification. Genomic DNA is first digested by appropriate restriction enzymes. A subset of resultant fragments representing many loci is than ligated to synthetic adaptors and amplified with specified primers which are complementary to a selective sequence on the adaptors. Subsequent separation of the resultant fragments is performed on a highly resolving sequencing and visualized using autoradiography. Where radiolabeled nucleotides are not used in the PCR step, fluorescence or silver staining technique can be used to visualize the application products. AFLP is similar to RAPD assay in that no prior knowledge of the sequence is required. AFLP detect a greater number of loci than RAPD does (Teneva, 2009).

AFLPs are dominant biallelic markers (Vos *et al.*, 1995). Variations at many loci can be arrayed simultaneously to detect single nucleotide variations of unknown genomic regions, in which a given mutation may be frequently present in undetermined functional genes (Young *et al.*, 1999). AFLP provides an effective, rapid and economical tool for detecting a large number of polymorphic genetic markers that are highly reliable and reproducible, and are able to be genotyped automatically. AFLP is considered as the "gold standard" for molecular epidemiological studies of pathogenic microorganisms and it is also widely used in forensic science (Negrini *et al.*, 2007).

The AFLP technique has been used extensively to detect genetic polymorphisms, evaluate and characterize breed resources, assess the relationship between breeds, construct genetic maps and identify genes in the main livestock species - cattle, sheep, pigs (Marsan *et al.*, 2007). AFLP is the ideal molecular approach for population genetics and genome typing except microsatellites (Luikart *et al.*, 2003). Mburu and Hanotte (2005) have shown the most useful advantages and disadvantages of AFLPs: high sensitive method, large number of polymorphisms is generated, selective neutrality, highly reproducible, no prior sequence information or probe generation is needed, expensive technique, dominant markers, technically demanding.

Microsatellites or Simple Sequence Repeats (SSRs)

Microsatellite DNA also known as simple sequences repeats (SSRs) or short tandem repeats (STRs) : are DNA sequences of mono-, di-, tri-, tetra- and pentanucleotide units repeated in tandem, which are widely distributed in the genome (Powell *et al.*, 1996). Markers are classified according to the number of bases, i.e., short repeats (10- 30 bases) are microsatellites and longer repeats are minisatellites (between 10-100 bases). Microsatellites have been also been classified according to the type of repeated sequence presented: (i) perfect, when showing only perfect repetitions, e.g., (AT) 20, (ii) imperfect repeats, when the repeated sequence is interrupted by different nucleotides that are not repeated, e.g., (AT)12GC(AT)8, and (iii) composite, when there are two or more different motifs in tandem, e.g., (AT)7(GC)6. The composite repeats can be perfect or imperfect. The sequences of di-, tri- and tetranucleotide repeats are the most common choices for molecular genetic studies (Selkoe and Toonen, 2006).

With the advent of PCR technology in the mid-1980s (Mullis and Faloona, 1987 and Saiki *et al.*, 1985), new perspectives have evolved for molecular biology fields that have largely impacted several applied purposes. Microsatellites were detected in eukaryote genomes almost thirty years ago. These tandemly repeated motifs of variable lengths are distributed throughout the eukaryotic nuclear genome in both coding and noncoding regions (Jarne and Lagoda, 1996). They also appear in prokaryotic and eukaryotic organellar genomes, e.g., chloroplast (Powell *et al.*, 1996) and mitochondria (Soranzo *et al.*, 1999).

SSRs have the same advantages as RFLPs, and avoid the utilization of radioisotopes essential for RFLPs; it has higher repeatability and stability than RAPDs; compared to AFLP markers, SSRs are co-dominant markers and able to distinguish homozygotes from heterozygotes. Until recently, microsatellites were the markers most widely used for genetic diversity, mapping quantitative trait loci for production, and functional traits in farm animals (Hiendleder *et al.*,

2003 and Fang *et al*., 2005) . They have also been used for marker assisted selection practices (Montaldo *et al*., 1998).

The advantages and disadvantages of SSR markers have been reported by (Bishop *et al*., 1994). Its advantages are as follows: low quantities of template DNA required (10–100 ng), high polymorphism, co-dominant markers, high accuracy, high reproducibility, different microsatellites can be multiplexed in PCR, and they are amenable to automation. Its disadvantages include: time-consuming and expensive to develop, heterozygotes which may be misclassified as homozygotes when null-alleles occur because of mutations in the primer annealing sites, stutter bands may complicate accurate scoring of polymorphisms, underlying mutation model largely unknown, and microsatellite markers do help to identify neutral biodiversity but do not provide information on functional trait biodiversity. Despite these disadvantages, microsatellite markers are still popular nuclear DNA markers for the investigation of genetic variation among and within species (Yang *et al*., 2013).

Single Nucleotide Polymorphisms (SNPs)

Among the wide range of molecular markers developed, single nucleotides polymorphisms (SNPs) are the most abundant, are widely dispersed throughout genomes, and have variable distribution among species (Vignal *et al*., 2002). The availability of high-throughput SNP genotyping platforms makes it feasible to undertake high-density scans by using large numbers of SNP markers and are either distributed across the whole genome or focused in specific regions. The SNPs are useful in studying livestock genetic diversity and population structure (McKay *et al*., 2008 and Lin *et al*., 2010). A SNP is found where different nucleotides occur at the same position in the DNA sequence. These markers are abundant in the genome, genetically stable and amenable to high-throughput automated analysis. They are found in both coding and non-coding regions of the genome and are present at one SNP in every 1000 bp (Stoneking, 2001 and Vignal *et al*., 2002).

They are bi-allelic markers, indicating a specific polymorphism in two alleles only of a population. SNP in coding regions can be directly associated with the protein function and as the inheritance pattern is more stable, they are more suitable markers for selection over time (Beuzen *et al*., 2000). Most SNPs, actually about two of every three SNPs, involve the replacement of cytosine (C) with thymine (T). Many SNPs have no effect on cell function, but it is believed that they could predispose an organism to disease or influence their response to a drug.

SNPs are becoming especially important as markers because they are very stable, i.e. have very low mutation rates and can be amplified with PCR for testing. One disadvantage of these markers is the lower informational content compared with that of a highly polymorphic microsatellite, but it can be compensated by the use of a higher number of markers (Werner *et al*. 2002).

New Approaches For Animal Genetic Resourses

In addition to the classical markers discussed above, with the development of modern molecular techniques and the completion of the Human Genome Project, some new markers have emerged and are being used in the evaluation of farm animal genetic resources; these include high-density SNP arrays, whole-genome sequencing, and DNA barcoding.

SNP Markers and Whole-Genome Sequencing

Due to its abundance and uniform distribution throughout a genome, single nucleotide polymorphisms (SNPs) are considered to be the most desirable molecular markers and have been demonstrated to be efficient markers for developing high-density genome scan (Wang *et al*. 1998 and Gupta *et al*. 2008). Wholegenome sequencing and oligonucleotide microarray are the two main strategies used to create SNP markers. The most important factor limited the application of SNP array is its comparative high cost to researchers. Meanwhile, the researchers also focus on how many SNPs are positioned on the array and when low-, medium- or high-density SNP arrays are used. High-throughput and low-cost next generation sequencing is a powerful approach to identify SNPs. Sequencing a SNP detection panel of few genotypes identifies the potential SNPs and then a SNP validation panel of several genotypes is used to filter informative SNPs for SNP array development .The assay of SNP array commonly includes the three major steps: chip hybridization, chip scanning and raw data analysis. In the process, a set of expensive facility is needed. It is believed that the cost of SNP array assay will be gradually decreased. The reasonable prices and quick high throughput genotyping of SNP array are making the use of SNPs even more attractive and efficient. SNP array will accelerate genetics studies and bridges the gap between genomics and breeding in cereals (Xing, 2014).

DNA Barcoding

Barcoding is an automatic scanning and identification technology, which has emerged from practical computer technologies. Biological taxonomists apply

this principle to species classification, referring to a DNA barcode. A DNA barcode is a Short DNA sequence from a standardized region of the genome used for identifying species. The intent of DNA barcoding is to use large-scale screening of one or more reference genes in order to (i) assign unknown individuals to species, and (ii) enhance discovery of new species (Hebert *et al*. 2003). Tautz et al. 2002 were the first researchers to use the DNA sequences in systematical biological taxonomy (also called DNA taxonomy). Subsequently, (Hebert *et al*. 2003) proposed the concept of DNA Barcoding and suggested its use for a single mtDNA gene, mitochondrial cytochrome c oxidase I (COI), as a common sequence in animal DNA barcoding studies. Researchers can compile a public library of DNA barcodes linked to named specimens, which can provide a new master key for identifying species diversity (Hebert *et al*. 2004). Compared with time-consuming and inefficient traditional morphological classification (Huang *et al*. 2007), DNA Barcoding has a high accuracy of 97.9%, and provides us a new, quick, and convenient identification strategy for animal genetic diversity (Hajibabaei *et al*. 2006).

As with the other markers mentioned the DNA barcoding technique also has some disadvantages: 1) the genome fragments are very difficult to obtain and are relatively conservative and have no enough variations. Some organisms cannot be identified with COI because of the low evolution rates of COI sequences in some species. 2) COI is an mtDNA sequence of maternal origin, which could bias species diversity (Meyer *et al*. 2005; Meier et *al*. 2006). The above disadvantages can be compensated for by using one or more nuclear gene barcodes together to make a standardized analysis of animal genetic resources (Yang *et al*., 2013).

CONCLUSION

It is our responsibility to conserve, preserve as well as maintain the animal genetic diversity. The accurate evaluation of animal genetic resources is the basis for their conservation and utilization. With the advent of science and technology; several valuable methods for molecular marker analysis are currently available for estimation of genetic diversity. From the first demonstration of RFLPs to the current wholegenome sequencing, many methods have been developed and tested at the DNA sequence level, providing a large number of markers and opening new opportunities for evaluating diversity in farm animal genetic resources. The development of molecular markers will continue during the near future and will provide better understanding of animal genetic resources.

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