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Molecular Markers: an Introduction and Applications

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Abstract

The dramatic development of molecular genetics has laid the groundwork for genomics. It has introduced new generations of molecular markers for use in the genetic improvement of farm animals. These markers provide more accurate genetic information and better understanding of the animal genetic resources. Scientists, unfamiliar with the different molecular techniques tend to get lost as each has its own advantages and disadvantages. This review represents a trail to shade alight on the different types of molecular markers by introducing a brief summary on the development of genetic markers including both the classical genetic markers and more advanced DNA-based molecular markers. This review could be helpful to better understand the characteristics of different genetic markers and the genetic diversity of animal genetic resources.

Keywords: SNP, microsatellite, molecular marker, genome, polymorphism.

Introduction

A genetic marker is a gene or DNA sequence with a known location on a chromosome and associated with a particular gene or trait. It can be described as a variation, which may arise due to mutation or alteration in the genomic loci that can be observed. A genetic marker may be a short DNA sequence, such as a sequence surrounding a single base-pair change (single nucleotide polymorphism, SNP), or a long one, like mini & micro satellites.

Recent years have witnessed a great interest towards molecular markers, revealing polymorphism at the DNA level, as they play an important role in animal genetics studies. Sometimes the term “Smart Breeding” is used to describe marker supported breeding strategies.

The main aim of the breeder is to select animal with superior genetic potential as parents for the next generation. The first attempt to improve animals used the phenotype of an animal for a specific trait as a tool for selection. This application uses external animal characteristics as a marker that called morphological markers (i.e. udder shape, coat color, body shape, skin structure, and anatomical characteristics) [1, 2]. These markers depend on visual observation and measurement to identify, classify, and characterize the genetic evolution of different species or populations. The conclusions reached through applying morphological markers are often not completely accurate when they used for the evaluation of farm animal genetics, because these markers based on subjective judgments and descriptions. Another type of markers represents by using of cytological markers that were included several criteria such as chromosome karyotypes, bandings, repeats, translocations, deletions, and inversions to investigate the genetic resources of

animals [3]. The chromosome mutations lead to genetic variation [4]. These mutations were used as markers to identify a certain location of the gene on a specific chromosome. In the domestic animals, cytological markers allow to investigate their genetic diversity by comparing the chromosome number and the structure between domesticated animals and their wild ancestors [5]. Cytological markers are still widely used in elucidating the origin and classification of species [6] because of their good properties, rapid economic and straightforward technique.

The third type of markers is biochemical markers, such as the blood type and isozymes. These markers represent biochemical traits that could be analyzed by protein electrophoresis. The differences in the amino acid composition of isozymes and soluble proteins were used to investigate the genetic variation within species and phylogenetic relationships between species [7]. The application of these markers was limited because the proteins and isozymes are not genetic materials. They are products of gene expression, so they could be affected by environmental factors [8]. Thus, the direction attention of researchers is converted to the molecular markers. The molecular markers are based on the nucleotide sequence mutations within the individual's genome; they are the most reliable markers available [3].

Marker assisted selection (MAS)

Selection is one of the most important tools to improve the performance of animals. It can be accomplished based on two types of data – pedigrees and phenotypes to estimate Best Linear Unbiased Prediction (BLUP) that combines these to generate estimated breeding values (EBVs). A third type of data is based on DNA markers to get a new approach named Marker assisted selection (MAS). The MAS can be based on DNA in linkage equilibrium with a quantitative trait locus (QTL) (LE-MAS)–LE refers to genotype frequencies at one locus are independent of genotype frequencies at the second locus, - molecular markers in linkage disequilibrium with a QTL (LD-MAS)–LD refers to the non-random association of alleles between two loci-, or based on selection of the actual mutation causing the QTL effect (Gene-MAS). All three types of MAS are being used in the livestock industries [9].

Molecular and quantitative genetics

The most economically important traits in livestock are quantitative, that they show continuous distributions. Two models have been proposed to explain the genetic variation among such traits, the infinitesimal model (the basis of quantitative genetics) and the finite loci model (the basis of molecular genetics). The infinitesimal model assumes that traits are determined by an infinite number of unlinked and additive loci, each with an infinitesimally small effect [10]. This model has been exceptionally valuable for animal breeding, and forms the basis for the breeding value estimation theory [11]. The finite loci model assumes the existence of a finite amount of genetically inherited material (the genome). There are a total of around 20000 genes or loci in the genome [12]. Many evidences confirmed that the distribution of the effect of these loci on quantitative traits could be classified to a few genes with large effect and a many of small effect [13, 14]. The search for these loci, particularly those of moderate to large effect, and the use of this information to increase the accuracy of selecting genetically superior animals, has been subjected to intensive research studies in the last two decades.

The first approach of applying molecular markers has been used is the candidate gene. It is assume a gene involved in a certain trait could show a mutation causing variation in that trait, and any variations in the DNA sequences, that are found, are tested for association with variation in the phenotypic trait [15]. Although this approach has achieved some success – for example a mutation that discovered in the estrogen receptor locus (ESR) which results in the increased litter size in pigs [16], but two problems have faced this approach. Firstly, candidate genes affecting a trait usually have a large number, so many genes must be sequenced and a large sample of animals is needed. Thus, the likelihood that the mutation may occur in non-coding DNA further increases the amount of sequencing required. Secondly, the mutation that associated with the phenotypic variation in a certain trait could occur in another gene that considered a non-candidate gene.

Up to now, many types of molecular markers have been utilized to detect the variation among individual and population. These markers can be classified into three groups; protein variants (allozymes), DNA sequencepolymorphism, DNA repeat variation. So it is very necessary to conduct a review on most important molecular markers.

Allozyme markers

Allozymes are enzyme variants due to allelic differences and can be visualized through protein electrophoresis. This technique was developed to quantify the genetic and geographic variation in wildlife populations, and it remains a cost-effective and straightforward method [17]. Genetic variations caused by mutations are expressed as amino acid replacements due to changes in protein compositions, and are resolved as bands (alleles) on electrophoretic gels [18].

These markers provided a valuable tool for population genetic studies in natural populations of woody plants [19]. They usually exhibit simple Mendelian inheritance and codominant expression, making genetic interpretations easy. In addition, allozyme analysis is relatively fast, inexpensive, and an extensive literature exists about it [20, 21]. However, allozymes have limitations, such as highly biased genomic sampling (only genes encoding well-documented, soluble proteins are detectable); a low number of markers, insufficient for examining major portions of the genome; occasional differences between tissues or ontogenetic stages; and difficulty in the standardization of experimental methods from laboratory to laboratory.

Mitochondrial DNA (mtDNA)

mtDNA is an extra-chromosomal genome in the cell mitochondria that resides outside of the nucleus, and is inherited from mother with no paternal contribution [17]. Due to higher evolutionary rates of mtDNA relative to the nuclear genome [22], this marker is preferred in constructing phylogenies and inferring evolutionary history, and is therefore, ideal for within- and between-species comparisons [18]. The drawbacks of mtDNA analyses include hybridization, introgression, and incomplete lineage sorting. Moreover, mtDNA is of little use in investigating the recent loss of genetic variation and any individual-level events such as identity, individual dispersal, and mating systems [22].

Restriction Fragment Length Polymorphism (RFLP)

The RFLP is a technique that is not widely used now, but it was one of the first techniques used for DNA analysis in forensic science and several other fields. The RFLP is defined by the existence of alternative alleles associated with restriction fragments that differ in size from each other.

The molecular basis of the RFLP is that nucleotide base substitutions, insertions, deletions, duplications, and inversions within the whole genome can remove or create new restriction sites [3].

Despite the fact that it is less widely used now, there have been numerous benefits to RFLP analysis. It plays an important role in allowing scientists to map the human genome as well as provide information on genetic diseases [23]. RFLP analysis is useful to find out where a specific gene for a disease lies on a chromosome. This is performed by looking at the DNA from a set of family members who suffer from a certain disease and then searching for RFLP alleles that share the same type of inheritance pattern for the condition. Using RFLP analysis, enable scientists to determine others who might be at risk for the disease or a carrier of the mutated gene.

The RFLP was also one of the first methods used for genetic typing - also known as genetic fingerprinting, profiling or testing. Despite that the RFLP have many benefits but it is still a slow and more tedious process compared to some of the newer DNA analysis techniques. Ii is also requires substantially larger sample sizes than other forms of analysis.

Random Amplification of Polymorphic DNA (RAPD):

In the last decade, the RAPD technique based on the polymerase chain reaction (PCR) has been one of the most commonly used molecular techniques to develop DNA markers [24].

The RAPD technology provides a quick and efficient screen for DNA sequence based polymorphism at a very large number of loci. The major advantage of RAPD includes that, it does not require pre-sequencing of DNA [25].

The RAPD analysis has been extensively used for various purposes which include identification and classification of accessions [26], identification of breeds [27] and genetic diversity analysis [28].

The principle of RAPD is that, a single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that

the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome [25]. Since the advantages of RAPDs are the technical simplicity and the independence of any prior DNA sequence information, [29, 30] it is viewed as having several advantages compared to RFLP and fingerprint [31].

A disadvantage of RAPD markers is the fact that the polymorphisms are detected only as the presence or absence of a band of a certain molecular weight, with no information on heterozygosity besides being dominantly inherited, and also show some problems with reproducibility of data [32].

Amplified Fragment Length Polymorphism (AFLP)

AFLP markers have found the widest application in analyses of genetic variation below the species level, particularly in investigations of population structure and differentiation [33].

AFLP methods rapidly generate hundreds of highly replicable markers from DNA; thus, they allow high-resolution genotyping of fingerprinting quality. The time and cost efficiency, reproducibility and resolution of AFLPs are superior or equal to those of other markers (RAPD, RFLP and microsatellites) [32]. AFLP markers have emerged as a major new type of genetic marker with broad application in systematic, pathotyping, population genetics, DNA fingerprinting and quantitative trait loci (QTL) mapping [34].

However, AFLPs are dominant bi-allelic markers, [35] and are unable to distinguish dominant homozygous from dominant heterozygous individuals [36]. The AFLP method is an ideal molecular approach for population genetics and genome typing, it is consequently widely applied to detect genetic polymorphisms, evaluate, and characterize animal genetic resources [37, 38, 39, 40].

Microsatellites

Microsatellites or simple sequence repeated (SSR) loci, which have been referred to in the literature as variable number of tandem repeats (VNTRs) and simple sequence length polymorphisms (SSLPs), are found throughout the nuclear genomes of most eukaryotes and to a lesser extent in prokaryotes [41, 42].

Microsatellites range from one to six nucleotides in length [43] and are classified as mono-, di-, tri-, tetra-, penta- and hexanucleotide repeats. The sequences of di-, tri- and tetranucleotide repeats are the most common choices for molecular genetic studies [44]. They are tandemly repeated (usually 5-20 times) in the genome with a minimum repeat length of 12 base-pairs [45, 46, 47].

The number of repeats is variable in populations of DNA and within the alleles of an individual. The sequence below has a 20 dinucleotide repeat (40bp) stretch of CA that is shown in **bold**.

CGTTCAATAAGCAAAAATCCATAGTTTTAGGAATGTGGGCT
GCTTGGTGTGATGTAGAAGGCGCCAATGCATCTCGACGTAT
GCGTATACGGGTTACCCCCTTTGCAATCAGTG**CACACACAC**
ACACACACACACACACACACACACACACACACAGTGCCAAGCA
AAAATAACGCCAAGCAGAACGAAGACGTTCTCGAGAACACC
AGAAGTTCGTGCTGTCGGGGCATGCGGCGAGTAAAGGGGAT

When a microsatellite flanked with fluorescent PCR primers then the amplification will give a pair of fluorescent allelic products which will vary in size according to their repeat length. A population might possess 5 alleles which vary in size as illustrated in Fig 1.

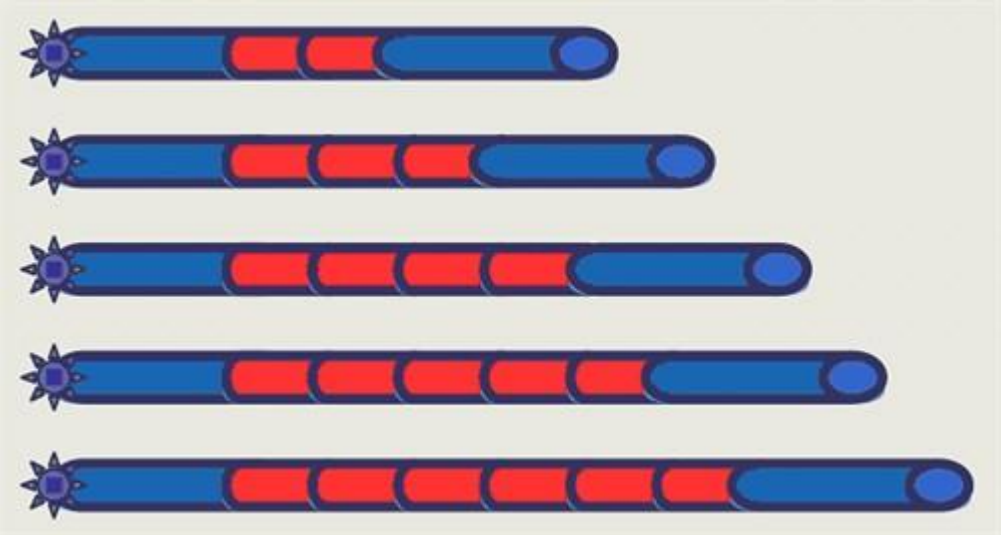

Figure 1: Five Alleles with Different Repeat Length

Microsatellites can be used as markers in genetic studies of linkage in families and linkage disequilibrium studies of populations. In linkage studies one can examine large number of families and see when the alleles of specific markers are inherited together with a phenotype in more cases than not. Microsatellite repeat are amplified with fluorescently labeled primers and then the alleles from each individual in a family are separated by size and the marker tested for linkage with another as shown in Figure 2.

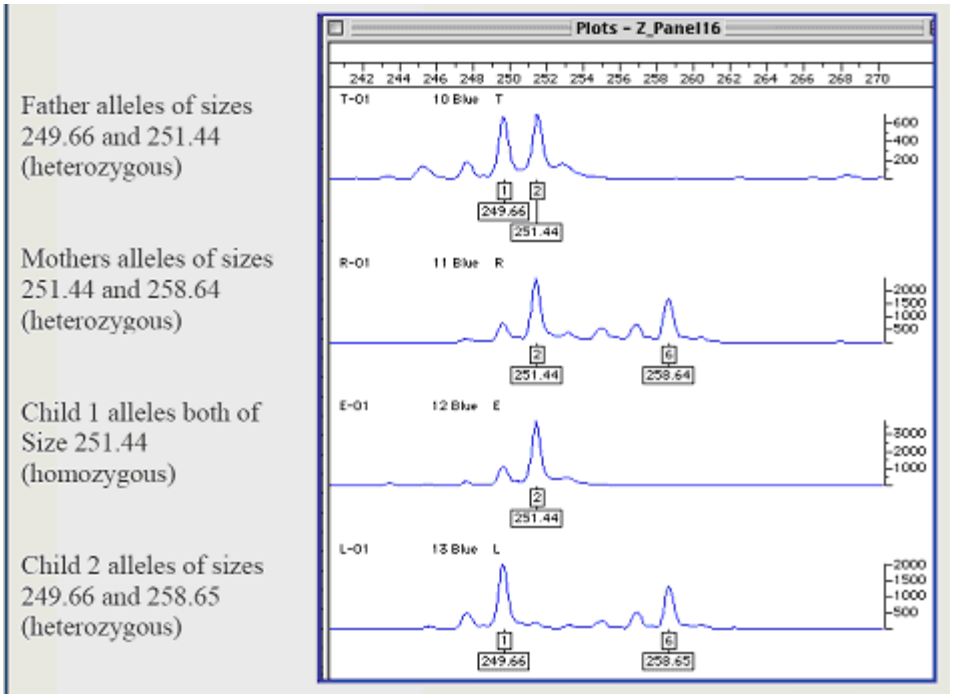

Figure 2: Raw of Genotyping Data

This approach assumes that a certain quantitative trait was affected by many unknown genes. So, this approach is looking for associations between the variation of allele and quantitative traits at the neutral DNA markers. The DNA marker is located on a chromosome and its inheritance can be monitored [48].

Microsatellites are the most commonly applied molecular marker in ecological research (Fig. 3).

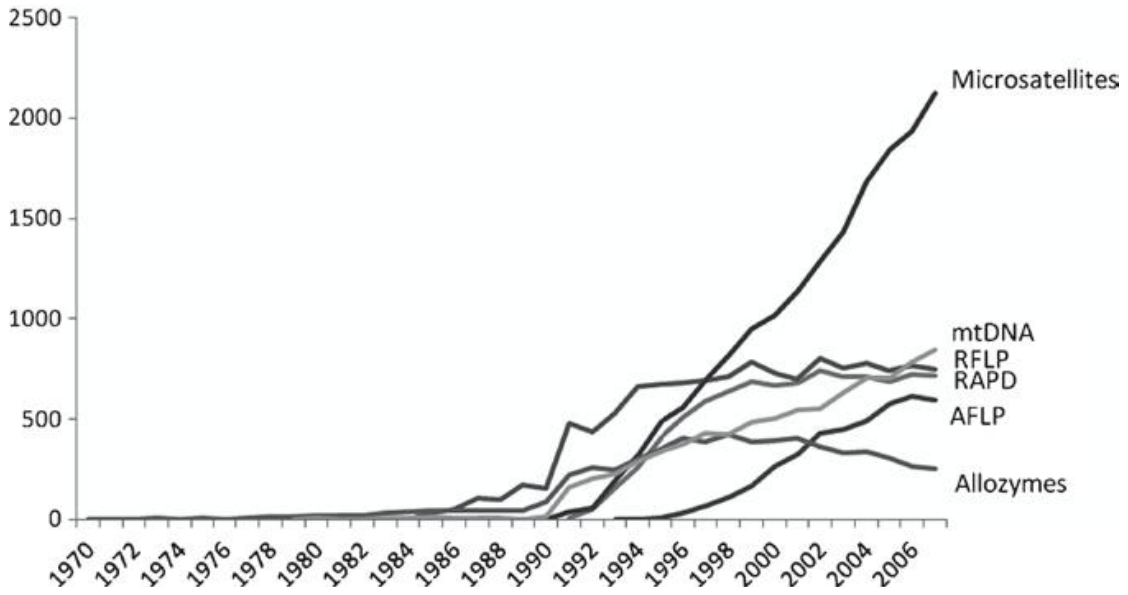

Figure 3: Number of publications (selected biological subject) between 1970 and 2007 employing mtDNA, Allozymes, Microsatellites, RFLPs, RAPDs and AFLPs found via ISI web of knowledge

Types of microsatellites

The types of SSRs could be classified based on:

1- Occurrence and source for development:

a. Genomic or nuclear microsatellites (gSSRs) - microsatellites isolated from the nuclear genome (genomic DNA of an organism with or without the construction of genomic DNA library).

b. EST or genic microsatellites (EST-SSRs) - microsatellites developed by data-mining or exploiting EST sequences deposited in public databases.

c. Organellar microsatellites [chloroplast SSRs (cpSSRs) and mitochondrial SSRs (mtSSRs)] - microsatellites developed from the chloroplast or mitochondrial genome of an organism.

2- The type of repeat sequence [49]:

a. Simple perfect - the repeat sequence is continuous and is not interrupted by any base not belonging to the motif [e.g. AGAGAGAGAGAG or (AG)6].

b. Simple imperfect - the arrays consist of one or more repeat units of different lengths [e.g. AGAGAGAGAGCTAGAGAG or (AG)5CT(AG)3].

c. Compound-perfect - the arrays are composed of two or more different repeat motifs of the same length [e.g. AGAGAGAGCGTGAGAGAGAG or (AG)4CGTG(AG)4].

d. Compound imperfect - motifs are interrupted by one or more repeats of different length [e.g. AGAGAGAGAGTCTCTCTC or (AG)5(TC)4].

3 - The length of repeat motif [50]:

a. Class I microsatellites- perfect SSRs of ≥20 nucleotides in length.

b. Class II microsatellites- perfect SSRs of ≥12 nucleotides and ≤20 nucleotides in length.

Microsatellite markers have several advantages as they are considered to be robust [51] and more variable and informative than RFLP, RAPD [52] and AFLPs [53]. Using the technique of PCR-based require only low quantities of template DNA [54, 55]. Moreover, these markers are considered to be the best marker system for the detection of intervarietal polymorphisms [56]. They are also useful for parentage analysis and for estimating the degree of relatedness of individuals or groups [51]. On the other hand these markers have several disadvantages: expensive, laborious and time-consuming [57, 58, 59]. The low frequency of SSRs in plants also hinders the large scale isolation of SSRs [60].

Homoplasy is another problem when applying microsatellites as a reliable tool for phylogenetic analysis because alleles considered to be identical in state are not necessarily identical by descent [61].

Single-nucleotide polymorphism (SNP)

In 1996, Lander proposed a new molecular marker technology named SNP. When a single nucleotide (A, T, C, or G) in the genome sequence is altered this will represent the SNP (Figure 3). In other words, it refers to a sequence polymorphism caused by a single nucleotide mutation at a specific locus in the DNA sequence [3].

This sort of polymorphism includes single base transitions, transversions, insertions and deletions [41], and the least frequent allele should have a frequency of 1% or greater [62]. Transitions are the most common (approx. 2/3) among all the SNP mutation types [63], as shown in Figure 4. Currently, SNP markers are one of the popular approach, despite they can be considered as a step backwards (simple bi-allelic co-dominant markers) when compared to the highly informative multi-allelic microsatellites. This popularity of the SNPs based on some preferred properties; they are abundant in the genome, genetically stable, and amenable to high throughput automated analysis [64]. The more recent SNP concept has basically arisen from the recent need for very high densities of genetic markers for the studies of multifactorial diseases [64].

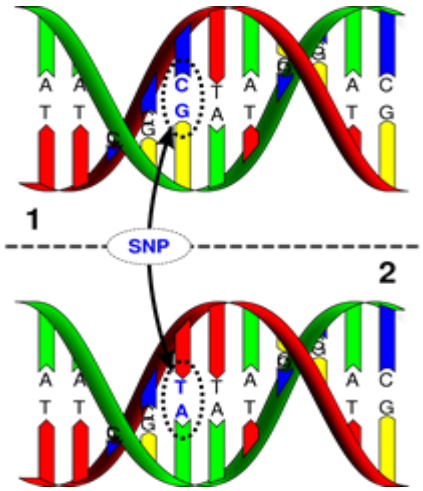

Figure 4: SNPs in DNA

The fundamental principle of SNPs is to hybridize detected DNA fragments with high-density DNA probe arrays (also called SNP chips); the SNP allele is then named according to the hybridization results [3].

SNPs are third generation molecular marker technology coming after RFLPs and SSRs [65]; it was successfully performed to investigate genetic variation among different species and breeds [66-68].

The role of SNPs in farm animals was very important concerning the population structure, genetic differentiation, origin, and evolution research [3]. On the other hand, the most important disadvantage of SNPs is the low level information obtained as compared with that of a highly polymorphic microsatellite but this can be solved by using a higher numbers of markers (SNP chips) and whole-genome sequencing [69-70].

Variations in the DNA sequences of humans can affect how humans develop diseases; respond to pathogens, chemicals, drugs, etc. However, their greatest importance in biomedical research is for comparing regions of the genome between cohorts (such as with matched cohorts with and without disease. The study of single nucleotide polymorphisms is also important in crop and livestock breeding programs.

DNA barcoding markers

A DNA barcode is a short DNA sequence from a standardized region of the genome used for identifying species. The essential aim of DNA barcoding is to use a large-scale screening of one or more reference genes in order to assign unknown individuals to species, and to enhance discovery of new species [71-72].

Biological taxonomists apply this principle to species classification. The first application of using the DNA sequences in systematical biological taxonomy (also called DNA taxonomy) was conducted by Tautz et al. [73] and then, Hebert et al. [71] 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.

The DNA barcoding has a high accuracy of 97.9% [59], and provides a new, quick, and convenient identification strategy for animal genetic diversity [74]. This approach like previous mentioned markers have some disadvantages represents by 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. Moreover, COI is an mtDNA sequence of maternal origin, which could bias species diversity [75, 76].

Conclusion

The accurate genetic evaluation of animals is the primary target for their conservation and utilization. Different methods have been developed and tested at the DNA sequence level. These methods provide a large number of markers and opening up new opportunities for evaluating diversity in farm animal genetic resources. Among all these methods, microsatellites remained the marker of choice for the past 15 years [77-80], due to their highly polymorphic and hence informative nature [81].

However, due to their complex and varied mutational patterns, as well as high genotyping error rates and relatively low density throughout the genome, they have recently received much scrutiny [82, 83]. More specifically, their application in estimating genetic diversity within and between populations was recently challenged by Väli et al. [82] after demonstrating that multilocus heterozygosity does not reflect genome-wide diversity, reinforcing a similar perspective initially highlighted over 30 years ago [84, 85]. The authors suggested instead that SNPs to get a more accurate means of assessing overall genomic diversity in natural populations.

Currently, SSR and SNP markers subjected to many researches to compare their validation for map built.

In a simulations prediction study [77], results showed that SNPs are at least two to six times more necessary to achieve the same resolution as microsatellites when used for individual identification and the study of parentage assessment and relatedness.

The SNP markers have promising advantages over microsatellite markers, due to high-throughput automated analysis, lower mutation rates and lower genotyping costs [77, 86].

In a study conducted by Ball et al. [87] a comparison among linkage maps built with microsatellites, SNPs and a combination of both markers was done to determine the ability of each method to produce accurate linkage maps. Results revealed that, although microsatellites are informative, they can provide misleading results because they have greater error rates than SNPs. The potential inflation in map and incorrect marker orders associated with the microsatellite genotyping led the Ball et al., [87] to formulate a conclusion as "we are not suggesting that microsatellites should be abandoned in mapping studies, but we do urge that appropriate error checking precautions are employed".

Defaveri et al. [88] stated that; for microsatellites, there is a standard procedure for genotyping involving the PCR and size determination of the amplified fragment by gel electrophoresis. Whereas, no standard method for analysis must be used in SNP genotyping and many techniques are available [64, 86].

Their results also confirmed that the genomic location of markers had a strong impact on the results generated from microsatellites, but not on those from SNPs. Results also reinforce the perspective that microsatellite variability does not reflect genome-wide diversity [82], and that this genomic heterogeneity can affect the estimates of demographic and selective forces acting in wild populations. However, some reports on humans [89] and plants [90], showed that the SNP markers can only be transferred to different mapping populations within the same species, but not across species. In view of these results, Wang et al. [91] claimed this will limit the applications of SNP markers on related minor species. In contrast, due to multiple alleles, cost-effectiveness, and transferability, SSR markers will continue to play an important role in different genetic studies in many minor plant and insect species in the future.

The continuous development of molecular markers along with innovation of new statistical methods and the available of software could be end the debate about this subject by identification which of them is the best.

This certainly will lead to more progress in application of molecular markers in animal breeding.

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