Bulgarian Journal of Agricultural Science, 20 (No 4) 2014, 949-956
Agricultural Academy

MAJOR FEATURES, MUTATION MECHANISM AND DEVELOPMENT OF MICROSATELLITES AS GENETIC MARKERS

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Abstract

TENEVA, A., N. TOMLEKOVA and G. GOUJGOULOVA, 2014. Major features, mutation mechanism and development of microsatellites as genetic markers. *Bulg. J. Agric. Sci.,* 20: 949-956

Recent advances in molecular technologies provide various approaches for DNA polymorphism studies. There are several marker systems used in animal genetic studies, such as SSRs (microsatellites), AFLPs, VNTRs, RAPD, RFLP, mtDNA and Y-chromosome specific markers. Until recently, the microsatellite DNA marker has been the most widely used, due to its easy use by simple PCR and to the high degree of polymorphism and informativeness. Furthermore, microsatellite loci have gained widespread use in genome mapping, phylogenetic and conservation genetics due to their abundance in eukaryotic organisms. Microsatellites have much more information than allozymes and mtDNA. This paper reviews the major features, mutation mechanism, development, advantages and limitations of microsatellites as genetic markers.

Key words: molecular markers, DNA polymorphism, microsatellites, mutation, slippage, transcriptome, indels

Introduction

The history of molecular genetics stems to early 1950 when F. Crick, J. Watson and M. Wilkins established the double helix model of DNA structure. Since then details of structure and function of DNA and genes have been clarified and started to use in determining the genomes. Methods for DNA cloning, sequencing and hybridization developed in the 1970s and DNA amplification and automated sequencing during 1980s led to the development of various classes of DNA markers.

The genome is complete genetic material of an organism, including the nuclear and mitochondrial genomes of animals and plants, and chloroplast genomes in plants. Inasmuch as mitochondrial and chloroplast genomes are small and contain a limited number of genes, the focus of genome investigations is on the nuclear genome (Reece, 2004; Berrot, 2004; Kahl, 2001; Dalle and Schantz, 2008; Primrose, 2008; Primrose et al., 2009).

Each organism`s genome is subject to mutation as an effect of the cellular metabolism and the environment, which interaction results in genetic variation (polymorphism). In connection with the selection and genetic drift, genetic variation occurs within and between individuals, species, and higher order taxonomic groups (Liu and Cordes, 2004; Poptsova, 2014).

There are three general classes of polymorphic genetic markers that are routinely used in population genetic and phylogenetic studies: allozymes, mitochondrial DNA, and nuclear DNA. They have been subject to a number of recent reviews (Avise, 1994; Sunnucks, 2000; Thuy et al., 2006; Oliveira et al., 2006; Ollivier, 2009).

The identification methods, such as typing of blood groups and the biochemical polymorphisms based on allozyme electrophoresis have proved their usefulness in the livestock species to understand the mutations of animal genome and for many years have been the standard tool in livestock genetic studies (Manwell and Baker, 1970; Widar et al., 1975; Tanaka et al.,1983; Oshi et al., 1990; Xuebin and Jianlin, 2000; Shixin and Jianlin, 2000; Rebedea et al., 2005; Graml et al., 2009; Yakubu and Aya, 2012). The classical molecular technique for studying genetic variation at codominant Mendelian in-

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herited loci - allozyme electrophoresis was developed in the 1960s and was dominating until the early 1990s. In the early 1980s the first population genetic studies based on analysis of mitochondrial DNA came into being (Avise, 1994). Moreover, the discriminating power of these techniques represents significant limitations (Van Zeveren et al., 1990a, b; Schlotterer, 2000; Isfan et al., 2012; Galal et al., 2013).

Recent advances in molecular technologies have brought the revolutionary changes in the field of genetics by providing new approaches for the genome analysis. Nowadays it is possible to uncover a large number of genetic polymorphisms at the DNA sequence level and to use them as molecular markers for the evaluation of genetic basis for the observed phenotypic variability in farm animals. At DNA level, types of genetic variation include: base substitutions, commonly referred to as single nucleotide polymorphisms (SNPs), insertions or deletions of nucleotide sequences (indels) within a locus, inversion of a segment of DNA within a locus, and rearrangement of DNA segments around a locus of interest. DNA marker technology can be applied to reveal these mutations (Liu and Cordes, 2004).

The generality of molecular markers used currently in animal genetic studies for typing of polymorphic loci are: microsatellite markers (simple tandem repeat, STR, SSRs), amplified fragment length polymorphisms (AFLPs), variable number of tandem repeats (VNTRs), random amplified polymorphic DNA (RAPD), single strand conformation polymorphisms (SSCPs), restriction fragment length polymorphisms (RFLPs),single nucleotide polymorphism (SNP), specific mtDNA and Y chromosome markers (O'Brien, 1991; Liu and Cordes, 2004).

Until recently, the microsatellite DNA markers have been the most widely used, due to their easy generation by simple PCR, and to the high degree of informativeness, huge abundance in eukaryotic organisms.

This paper reviews the occurrence, molecular base of SSR markers, polymorphism, mutation mechanism, development, advantages and disadvantages of SSRs.

Major Features of SSR Markers

A striking feature of the genomic organization in the eukaryotes is that the coding sequences constitute only a minor portion, about 5 to 10 per cent in the mammals, of the total genome (Hochgeschwender and Brennan, 1991).

The non-functional DNA is either the single copy DNA or the repetitive DNA. The repetitive elements may be interspersed in the genome or may occur as tandem repeats (Schmid and Jelinek, 1982). In mammals, two major groups of the interspersed repetitive elements can be recognised: the short interspersed elements (SINEs) and the long interspersed elements (LINEs) (Schmid and Jelinek, 1982; Singer et al., 1987) (Figure 1). Both types of the elements are proposed to have originated by the reverse flow of information by retro position (Rogers, 1985).

The repetitive elements arranged in the tandem are as common as the interspersed repeats. Tandem repeats are intrinsically highly variable sequences since repeat units are often lost or gained during replication or following unequal recombination events. The tandem repeats are broadly referred to as the satellite type of DNA. Owing to their low complexity and weakness, these repeats are often considered to be futile "junk" DNA (Jansen et al., 2012). Current findings show that tandem repeats are frequently found within promoters of stress-induced genes and within the coding regions of genes encoding cell-surface and regulatory proteins. Multiple changes in these repeats usually determine the phenotypic variability. This suggests that instead of being useless junk DNA, some variable tandem repeats are useful functional elements that facilitate the evolution and rapid adaptation to changing environments. Because changes in repeats are frequent and reversible, repeats provide a unique type of mutation that bridges the gap between rare genetic mutations, such as single nucleotide polymorphisms, and highly unstable but reversible epigenetic inheritance (Jansen et al., 2012). The satellite types are further classified according to the size or location of the repeat as satellite, telomeric, minisatellite and microsatellite DNA (Brutlag, 1980; Prosser et al., 1986; Jansen et al., 2012). The satellite DNA is characterized by huge arrays of the short or long repeats, spanning several millions of nucleotides and is the typical centromere sequences in many mammals (Singer, 1982). The telomeric DNA is characteristic of the telomeric regions of the DNA. The telomeric repeat

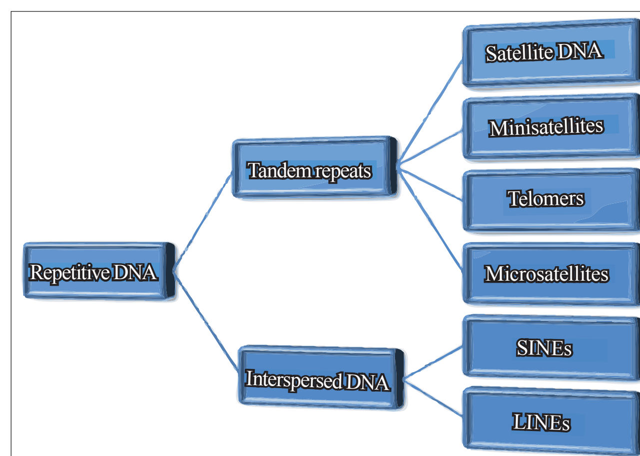

Fig. 1. Repetitive DNA of mammalian genome

region comprises about 10-15 kb and is site of the telomerase activity (Biessmann and Mason, 1992).

The third class of tandem repeats comprises the minisatellite DNA (Jeffreys et al., 1985). They consist of chromosomal regions containing tandem repeat units of a 10-50 base motif, flanked by conserved DNA restriction sites. A minisatellite profile consisting of many bands, usually within a 4-20 kb size range, is generated by using common multilocus probes that are able to hybridize to minisatellite sequences in different species. Minisatellite loci are also often referred to as Variable Number of Tandem Repeats (VNTR) loci. The term DNA fingerprinting was introduced for minisatellites, though DNA fingerprinting is now used in a more general way to refer to a DNA-based assay to uniquely identify individuals. The main advantages of minisatellites are their high level of polymorphism and high reproducibility. Disadvantages of minisatellites are similar to RFLPs due to the high similarity in methodological procedures. In addition, they detect repetitive sequences that primarily reside in heterochromatic regions near telomeres and centromeres of chromosomes, and thus they do not uniformly mark the genome. They are dominant markers, which reduce the potential information for genotyping (Dodgson et al., 1997).

The fourth class of the tandem repeats is referred to as the microsatellite (Goldstein and Pollock, 1997). They are more informative than allozyme polymorphism and mtDNA, and less informative than sequence mutation (Jarneand Lagoda, 1996).

Microsatellites are simple sequence tandem repeats (SSTRs) that are repeated several times at various points in the organism's DNA and are distributed in the genome on all chromosomes and all regions of the chromosome (Goldstein and Schlotterer, 1999; Zane et al., 2002). They belong to a class of highly mutable genomic sequences known as variable number of tandem repeat (VNTR) elements (Chambers and MacAvoy, 2000; Buschiazzo and Gemmel, 2006). In the microsatellite the repeat motifs of 1 to 6 base pairs are repeated up to a maximum of about 100 times. They appear to be abundant and are evenly distributed throughout the genome occurring once in about every 6 kb of the genome. Some regions like the centromeres, telomeres, nuclear organization regions and the interstitial heterochromatin have lower densities (Starling et al., 1990; Wintero et al., 1992).

Such repeats are highly variable enabling that location (polymorphic locus) to be tagged or used as a marker. The repeat units are generally di-, tri- tetra- or pentanucleotides (Tautz, 1989; Litt and Luty, 1989). The number of repeats is variable in populations of DNA and within the alleles of an individual.

They are present in both coding and noncoding regions and are usually characterized by a high degree of length polymorphism (Toth et al., 2000; Li et al., 2004). The origin of such polymorphism due to slippage events during DNA replication (Schlotterer and Tautz, 1992). In spite of their wide distribution in genes, microsatellites are predominantly located in noncoding regions (Metzgar et al., 2000). Only about 10–15% of microsatellites reside within coding regions (Moran, 1993). This distribution should be explained by negative selection against frame shift mutations in the translated sequences (Metzgar et al., 2000). Because the majority of microsatellites exist in the form of dinucleotide repeats, any mutation by expansion or construction would cause frame shift of the protein encoding open frames if they reside within the coding region. Most microsatellite loci are relatively small, ranging from a few to a few hundred repeats. The relatively small size of microsatellite loci is important for PCR-facilitated genotyping.

Based on the repeat composition, microsatellites have been classified depending upon their size, type of repeat unit and its location in the genome. Depending upon the number of nucleotides per repeat unit, SSR's have been classified as mono-, di-, tri-, tetra-, penta- or hexanucleotides (Figure 2). In comparison to other repeat types, tri- (and hexa-) nucleotide repeats are overrepresented in coding sequences which could be explained by selection against slippage mutations in these regions (Schlotterer and Harr, 2001).

Depending upon the arrangement of nucleotides in the repeat motifs, Weber (1990) used the terms perfect, imperfect and compound microsatellites for classification while Wang et al. (2009a) classified microsatellites as simple perfect, simple imperfect, compound perfect or compound imperfect. Perfect repeats are tandem arrays of a single repeat motif, while in imperfect repeats; perfect repeats are interrupted by non-repeat motifs at some locations. In compound microsatellites, two basic repeat motifs are present together in various configurations (Jarne et al., 1998). Jarne and Coworkers (1998) coined the terms pure and interrupted for perfect or imperfect repeats. As Oliveira et al. (2006) state the microsatellites are classified according to the type of repeat sequence as perfect, imperfect, interrupted or composite.

Most of the genomic SSRs are nuclear SSRs, however, microsatellites are also distributed in mitochondria and chloroplasts. Based on their location in the genome, microsatellites can be classified as nuclear (nuSSR), mitochondrial (mtSSR) or chloroplastic SSRs (cpSSR) (Figure 2).

The location of SSR in the genome determines its functional role. These have the potential to affect all aspects of genetic functions including gene regulation, development and evolution. A microsatellite located in a coding region can affect the activation of a gene and therefore, the expression of a protein. If located in a no coding or genic region, the microsatellite may impact gene regulation or gene transcription (Nidup et al., 2010). According to available large

scale database in wide range of organisms, UTRs (5`-UTR, 3`-UTR and introns) have more SSRs than the coding regions (Beuzen et al., 2000). Majority of intronic SSRs are monomeric and/or dimers in different taxonomic groups (Li et al., 2004). Due to the high microsatellite mutation rate it is expected that coding regions have a low microsatellite density because possibly lossing of functionality. Comparative studies (Tóthet al., 2000) in both coding and non-coding regions of different species have confirmed this hypothesis.

This means that selection against mutations that change the reading frame of a gene restrict the presence of coding regions, while microsatellites with repeats in multiples of three develop evenly in both regions (Metzgaret al., 2000). This is related to the fact that RNA bases are read as triplets.

The most common microsatellites in the mammals are the (A)n, (CA)n, (AAAT), and (AG)n (Beckmann and Weber, 1992). For the most motifs, the short stretches of the repeat units are generally more common than the longer stretches (Moran, 1993). Plants are rich in TA repeats, whereas in animals CA repeat is the most common. This is the general feature to differentiate plant and animal genomes (Powell et al., 1996).

Microsatellites are thought to occur approximately once every 10 kb. They are one of a class of highly variable, non-coding and considered to be selectively neutral, and thus more useful for genome mapping studies.

Microsatellite polymorphism

Microsatellite polymorphism is based on size differences due to varying numbers of repeat units contained by alleles at a given locus.

As compared to the most other types of the DNA sequences, the microsatellites are the highly polymorphic which makes them attractive as genetic markers (Goldstein and Shlotterer, 1999; Zane et al., 2000). For the naturally evolving DNA sequences, the amount of polymorphism is expected to be directly proportional to the mutation rate (Kimura, 1983; Goldstein and Pollock, 1997; Goldstein and Schlotterer, 1999).

The mutation rate of the microsatellites is several orders of the higher magnitude, often quoted in the range of 10^{-3} to 10^{-4} per locus per generation. The degree of polymorphism, at least for the mammalian (CA)n repeats is positively correlated with the average number of the repeat units. As a rule, the mammalian microsatellite repeats with less than 10 repeat units is likely to be the monomorphic. On the other hand, the repeats with an average number of iterated units exceeding 20 may possess the polymorphism information content values of 0.6 or more (Goldstein and Schlotterer, 1999).

Mutation mechanism

Hypermutability of microsatelitesis explained by two mechanisms suggested by Jakupciak and Wells (2000). The first involves polymeraseslippage during DNA replication, resulting in different number of tandem repeat (Tautz et al., 1989). Transient dissociation of the replicating DNA strands followed by subsequent reassociation would lead to slippage of the two strands, allowing the change of repeat numbers in the newly replicated DNA (Ellegren, 2004).

The second mechanism involves nonreciprocal recombination within the SSRs, leading to production of significant-

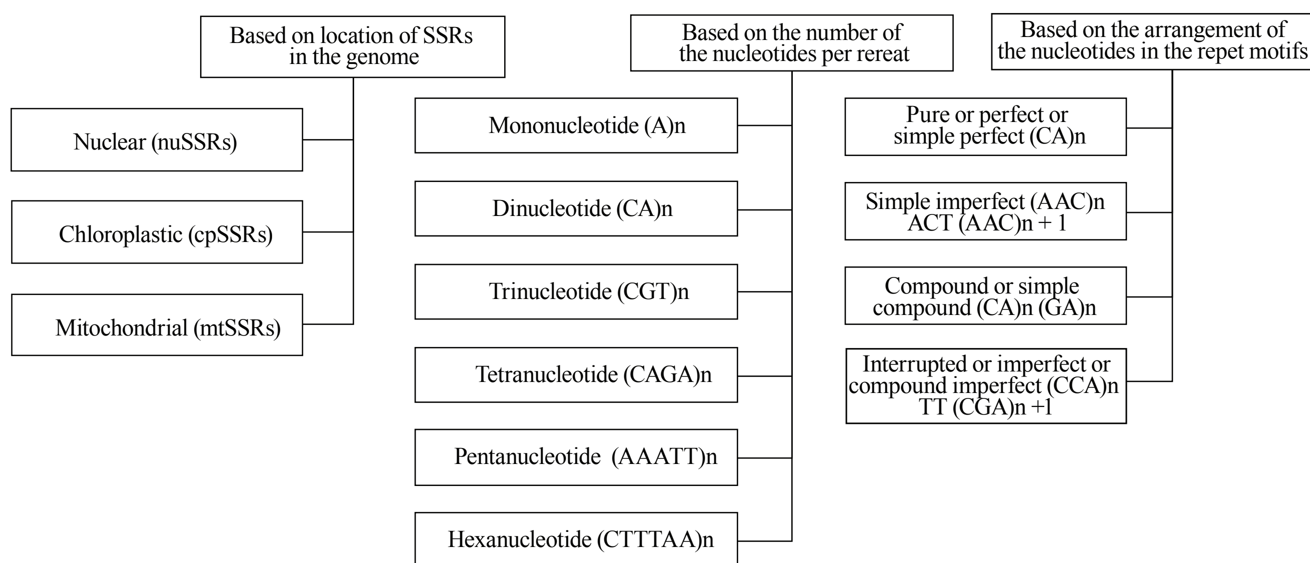

Fig. 2. Classification of the microsatellites

ly larger and smaller alleles (Goldstein and Schlotterer, 1999; Jakupciak and Wells, 2000; Schlotterer, 2000).

Length changes in microsatellite DNA are arised from replicationslippage - that is, transient dissociation of the replicating DNA strands followed by mispairing re-association (Ellegren, 2004). When the new synthesized strand realigns out of register, renewed replication will lead to the insertion or deletion of repeat units relative to the template strand. Most of these primary mutations are corrected by the mismatch repair system, and only the small fraction that was not repaired ends up as microsatellite mutation events.

In vitro experiments that use purified eukaryotic or prokaryotic enzymes confirm that DNA polymerase is the only enzymatic activity needed for slippage. Slippage involves DNA polymerase pausing, during which the polymerase dissociates from the DNA. On dissociation, only the terminal portion of the newly synthesized strand separates from the template and subsequently anneals to another repeat unit. Replication slippage also occurs during PCR amplification of microsatellite sequences *in vitro.* A characteristic feature of such amplifications is the presence of "stutter bands" that is, minor products that differ in size from the main product by multiples of the length of the repeat unit (Schlotterer and Tautz, 1992). Quantitative experiments show that the *Taq* polymerase slippage rate increases with the number of repeat units and is inversely correlated with repeat unit length (Jakupciak and Wells, 2000).

Recombination processes (involving unequal crossover or gene conversion) also generate mutations in the larger minisatellite sequences (Goldstein and Schlotterer, 1999).

Their high polymorphism, and PCR based analysis has made them one of the most popular genetic markers (Wright, 1994; Crawford and Cuthbertson, 1996; Li et al., 2004; Guimaraes et al., 2007; Kantartzi, 2013). With current molecular methods it is feasible to score microsatellite length polymorphisms for genetic analyses within and between populations.

There are two main techniques for microsatellite analysis. The first one requires probing complete digests of nDNA with simple sequence repeats (di-, tri-, or tetra- nucleotide repeats).

Alternatively they are genotyped using the PCR using primers targeted to the unique sequences flanking the microsatellite motif. PCR can easily be semi automated. The resulting PCR products are separated according to size by gel electrophoresis using either agarose gels or more commonly with higher resolution denaturing polyacrylamide gels (Boguski, et al., 1993; Katti et al., 2001; Ellis and Burke, 2007).

Development of microsatellites markers

Microsatellites are developed in two ways (Mburu and Hannote, 2005) - use and screening of DNA repositories sequences (e.g. Genbank, EMBL) and through cloning. The following step are: genomic DNA extraction from tissue, digestion of DNA with restriction enzyme, insertion of the fragments into often a plasmid DNA vector, transfer of plasmid clones into membranes, probing with labeled desirable repeats tandemly repeat oligonucloties probes, pick and bacterial culture of positive clones, extraction of plasmid DNA, and cutting of inserts with restriction enzyme, confirmation of the micorsatellite nature of the insert by Southern blotting, sequencing of the positive clones, analyze of the sequence to check for "good" primer sites and useful repeat length (generally at least 8 repeats), design of primer use software packages (OLIGOANALYZER 3.0 and PRIMERQUEST), screening of the loci. The quality of a genetic marker is typically measured by its heterozygosity in a population and the population information content (PIC).

Advantages of microsatellites as genetic markers

Microsatellite markers have a number of advantages over other molecular markers and have insensibly replaced allozymes and mtDNA.

Microsatellites are inherited in a Mendelian fashion as codominant markers (heterozygotes can be distinguished from homozygotes, in contrast to RAPDs and AFLPs which are "binary, 0/1"). This is one of the strengths of microsatellite markers in addition to their abundance, even genomic distribution, small locus size, and high polymorphism. They are locus-specific (in contrast to multi-locus markers such as minisatellites or RAPDs). However, use of microsatellite markers involves a large amount of up-front investment and effort. Each microsatellite locus has to be identified and its flanking region sequenced for the design of PCR primers. Due to polymerase slippage during replication, small size differences between alleles of a given microsatellite locus (as little as 2 bp in a locus comprised of dinucleotide repeats) are possible (Chambers and MacAvoy, 2000).

Microsatellite loci are typically short, this makes it easy to amplify the loci using PCR, and the amplified products can subsequently be analyzed on either "manual" sequencing gels or automated sequencing.

Microsatellites are relatively easy to isolate compared with minisatellites. The much higher variability at microsatellites results in increased power for a number of applications (Luikart and England, 1999; Weir et al., 2006). Only small amounts of tissue are required for typing microsatellites and these markers can be assayed using non-lethal fin clips and archived scale samples, facilitating retrospective analyses and the study of depleted populations. Moreover, there is potential for significant increases in the number of samples that can be genotyped in a day using automated fluorescent sequencers. For applications where a large number of loci

are required, such as genome mapping or identification of Quantitative Trait Loci (QTL), microsatellites offer a powerful alternative to other marker systems (Weir et al., 2006).

Limitations of microsatellites as genetic markers

Despite the advantages of microsatellite markers they have some constraints.

The major drawback of microsatellites is that they need to be isolated *de novo* from most species being examined for the first time (Zane et al., 2002). This is due to the fact that microsatellites are usually found in noncoding regions where the nucleotide substitution rate is higher than in coding regions. The strategy of designing universal primers matching conserved sequences, which is very effective for mitochondrial DNA, is more problematic for microsatellites (Kocher et al., 1989).

The other one of the main problems is the presence of so-called "null alleles". These are alleles that do not amplify in PCR reactions (Jarne et al., 1998).Null alleles occur when mutations take place in the primer binding regions of the microsatellite locus, i.e. not in the microsatellite DNA itself. The presence of null alleles at a locus causes severe problems, in particular in individual based analyses such as relatedness estimation and assignment tests, and usually it prefers to discard loci exhibiting null alleles (Quelleret al., 1993; Kantartzi, 2013).

The primers developed to amplify markers in one species may amplify the homologous markers in related species as well. Another important disadvantage of microsatellite alleles is that amplification of an allele via PCR often generates a ladder of bands (1 or 2 bp apart) when resolved on the standard denaturing polyacrylamide gels. These accessory bands (also known as stutter or shadow bands) are thought to be due to slipped-strands impairing during PCR or incomplete denaturation of amplification products. The practical outcome of PCR stutter is that it may cause problems scoring alleles (Tautz, 1989).

The disadvantage of microsatellites as markers include the requirement for existing molecular genetic information, the large amount of up front work for microsatellite development, and the tedious and labor intensive nature of microsatellite primer design, testing, and optimization of PCR conditions (Zhanjiang, 2007). Each microsatellite locus has to be identified and its flanking region sequenced for the design of PCR primers. Due topolymerase slippage during replication, small size differences between alleles of a given microsatellite locus (as little as 2 bp in a locus comprised of dinucleotide repeats) are possible.

Microsatellite DNA is rarely useful for higher-level systematic, because the mutation rate is too high (Kantartzi, 2013).

Conclusion

Since their detection, microsatellites markers are constantly being isolated and characterized in a great deal of livestock breeds. Microsatellite markers have a number of advantages over other molecular markers and have insensibly replaced allozymes and mtDNA.

Microsatellites are inherited in a Mendelian fashion as codominant markers; heterozygotes can be distinguished from homozygotes and are locus-specific (in contrast to multi-locus markers such as minisatellites or RAPDs), abundant genomic distribution, small locus size, and high polymorphism. The highest heterozygosity among all marker types causes of their high number of alleles. Since the SSRs are subjected to DNA replication slippage, microsatellites form a huge reservoir for polymorphic genetic markers

The features described have made microsatellites the unique marker system for identification of individuals such as parentage analysis, the construction of genetic linkage and QTL maps. DNA microsatellite markers have already found wide application in the control of genetic disorders in livestock animals as well as for comparative genome analysis because of their biological, technical and analytical advantages. The ultimate use of STR markers is for mapping quantitative trait loci (QTL) and in marker assisted selection (MAS) in order to practice genotypic selection. Consequently, microsatellites are markers of the choice for the animal genome analysis studies.

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Received April, 2, 2013; accepted for printing May, 22, 2014