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Heterologous microsatellite markers/SSR used in buffaloes species

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Abstract

Assessment of genetic variability present in a breed is vital and it should be a basic component for working out conservation strategies and for designing genetic improvement programs for a particular breed. Molecular markers have been comprehensively exploited, throughout the world, to access this variability as they contribute information on every region of the genome, regardless of the level of gene expression. Microsatellites markers/ Simple sequence repeats are highly polymorphic and are presently the most favored molecular markers, essentially owing to the option of blending their analysis with use of the polymerase chain reaction (PCR). They are simple tandem repeats motifs of 2-6 bases in length. The SSR are popularly known as microsatellites. The most thoroughly studied type to date is of dC-dA type repeats. Since the number of tandem repeats at a locus can vary greatly, SSR markers tend to be amongst the most polymorphic genetic marker types. For example, one allele might have 10 copies of the AC tandem repeat (CA)₁₀, whereas another allele would have 11 copies (CA)₁₁, another 12 copies (CA)₁₂, and so forth. The frequency of occurrence of one SSR per 6-10 Kb. The microsatellite markers used in characterization of a buffalo population has recommended by FAO (2007) out of which 25 usable markers have been identified and are mostly used in diversity analysis at ICAR National Bureau of Animal Genetic Resources (NBAGR).

Keywords: microsatellite markers, SSR, IAM, SMM

1. Introduction

India has vast animal genetic resources reflected by 13 breeds of buffaloes, 41 breeds of cattle, 42 breeds of sheep, 28 of goats, 9 of camel, 7 for pigs, 7 for horse & ponies, 1 for donkey, 1 for duck, 8 of poultry, 1 of yak and 1 for geese and few type mithun, quail and others distributed in various agro climatic zones of the country. In the recent past, there is perception amongst the farmers and breeders in the breeding tract about the deterioration in the form, size, quality, growth, reproduction and production potentialities of cattle breeds due to changes in the utility, cropping pattern, breeding objectives and agro-biodiversity of the breeding tract [1]. The first step for the sustainable use of domestic animal genetic resources is the gathering of information about the genetic variability through characterization of breeds, it also helps in prioritization of breeds for conservation. Also, there is an urgent need to document the diversity of our livestock genetic resources and to design strategies for their sustainable conservation.

Over the last few decades, the use of molecular markers has played an increasing role in animal breeding and genetics. Molecular markers are essential for mapping genes of interest, marker-assisted breeding, and cloning genes using mapping-based cloning strategies. Other uses of molecular markers include gene introgression through backcrossing, germplasm characterization and phylogenetic analysis [2]. Of the various classes of existing markers, microsatellites have emerged as the markers of choice for animal breeding applications. Restriction fragment length polymorphism (RFLP) analyses are not easily scalable to high-throughput methods, and random amplification of polymorphic DNA (RAPD) assays are often not reproducible or transferable between laboratories. Although both microsatellites and amplified fragment length polymorphisms (AFLPs) can both be used to efficiently identify polymorphisms, microsatellite-based methods are more readily automated.

Recent developments in molecular biology and statistics have opened the possibility of identifying and using genomic variation for the characterization of livestock. There are two main categories of genomic information that can be used for this purpose. They are the genes with known effects on the expression of certain protein and genes with effects detected on the characteristic in statistical terms.

The first category, which also known as candidate gene approach used extensively for livestock improvement but has limited use in characterization because of low level of polymorphism. The second group of markers is based on polymorphic sequences of the DNA, which corresponds to genes with detectable variation by means of RFLP, microsatellites or other similar molecular systems [3]. Microsatellites have been effectively exploited to understand bovine domestication and migration pattern and to evaluate genetic diversity and relationships among populations [4, 5, 6, 7, 8, 9]. The main objectives of this review are the following: (i) to review the basic principles and characteristics of commonly used microsatellite markers (ii) to outline the advantages and limitations of these markers and (iii) to provide examples of how microsatellites have been used in molecular breeding programs.

2. Microsatellites: the marker of choice

The term microsatellite was first coined by Litt M, and J A Luty, 1989 [10]. Microsatellites are simple repeated motifs consisting of 2 to 6 base pairs, and they can be found in both coding and non-coding regions. The mutation rate of this type of genetic marker has been estimated to be between 10^{-2} and 10^{-4} per generation. The primary advantage of microsatellites as genetic markers is that they are inherited in a Mendelian fashion as codominant markers. Furthermore, high polymorphism rates, high abundance and a broad distribution throughout the genome have made microsatellites one of the most popular genetic markers for use in animal breeding programs. However, significant drawbacks do exist with respect to using microsatellite-based methods, including relatively high development costs and technical challenges during the construction of enriched libraries and species-specific primers.

3. Advantages of microsatellites as genetic markers

- Locus-specific (in contrast to multi-locus markers such as

minisatellites or RAPDs)

- Codominant (heterozygotes can be distinguished from homozygotes, in contrast to RAPDs and AFLPs which are "binary, 0/1")
- PCR-based (means we need only tiny amounts of tissue; works on highly degraded or "ancient" DNA)
- Highly polymorphic ("hypervariable") - provides considerable pattern
- Useful at a range of scales from individual ID to fine-scale phylogenies

4. Microsatellite markers panel for Buffalo species

The FAO and the ISAG/FAO Advisory Group on Animal Genetic Diversity have proposed panels of 25 Microsatellite markers for major livestock species. The microsatellite markers recommended by FAO for the genetic distancing studies in buffaloes are CSRM060 CSSM019 CSSM033 CSSM045 CSSM047 CSSM057 ETH003 ILSTS058 ILSTS026 HEL013 ILSTS030 ILSTS033 ILSTS019 ILSTS056 ILSTS089 CSSM066 ILSTS036 ILSTS095 ILSTS029 ILSTS028 ILSTS025 ILSTS052 ILSTS060 BM1818 and ILSTS061. Ideally, all 25 markers should be used for characterization of populations. Working group recommended the following criteria to select appropriate microsatellites

- Free access to microsatellite markers.
- Microsatellite loci should be present on different chromosomes.
- Markers should follow Mendelian inheritance.
- Each locus should exhibit at least four alleles.

4.1 Fluorescent labelled Primers for microsatellite marker

The following labelled gene primers forward (P1) and unlabelled reverse (P2) as suggested by FAO for genetic diversity analysis were used for 25 simple sequence repeat / microsatellite markers.

Table 1: Microsatellite marker used for diversity analysis

Marker	Sequence	Allele size	Chromo. Location	Dye
CSRM060	For-5'-AAGATGTGATCCAAGAGAGAGGCA-3' Rev-5'-AGGACCAGATCGTGAAAGGCATAG-3'	92-136	10	VIC
CSSM019	For-5'-TTGTCAGCAACTTCTTGTATCTTT-3' Rev-5'-TGTTTTAAGCCACCCAATTATTTG-3'	131-161	01	NED
CSSM033	For-5'-CACTGTGAATGCATGTGTGTGAGC-3' Rev-5'-CCCATGATAAGAGTGCAGATGACT-3'	149-175	17	PET
CSSM045	For-5'-TAGAGGCACAAGCAAACCTAACAC-3' Rev-5'-TTGGAAAGATGCAGTAGAACTCAT-3'	102-122	02	FAM
CSSM047	For-5'-TCTCTGTCTCTATCACTATATTGC-3' Rev-5'-CTGGGCACCTGAAACTATCATCAT-3'	127-162	03	NED
CSSM057	For-5'-GTCGCTGGATAAAACAATTTAAAGT-3' Rev-5'-TGTGGTGTTTAACCTTGTAATCT-3'	102-130	09	FAM
ETH003	For-5'-GAACCTGCCTCTCCTGCATTGG-3' Rev-5'-ACTCTGCCTGTGGCCAAGTAGG-3'	96-192	03	NED
ILSTS058	For-5'-GCCTTACTACCTTTCCAGC-3' Rev-5'-CATCCTGACTTTGGCTGTGG-3'	118-182	17	NED
ILSTS026	For-5'-CTGAATTGGCTCCAAAGGCC-3' Rev-5'-AAACAGAAGTCCAGGGCTGC-3'	131-153	02	NED
HEL013	For-5'-TAAGGACTTGAGATAAGGAG-3' Rev-5'-CCATCTACCTCCATCTTAAC-3'	158-198	11	VIC
ILSTS030	For-5'-CTGCAGTTCTGCATATGTGG-3' Rev-5'-CTTAGACAACAGGGTTTGG-3'	146-158	02	PET
ILSTS033	For-5'-TATTAGAGTGGCTCAGTGCC-3' Rev-5'-ATGCAGACAGTTTATAGAGG-3'	126-138	12	FAM
ILSTS019	For-5'-AAGGGACCTCATGTAGAAGC-3'	169-185	29	VIC

	Rev-5'-ACTTTTGGACCCTGTAGTGC-3'			
ILSTS056	For-5'-GCTACTGAGTGATGGTAGGG-3' Rev-5'-AATATAGCCCTGGAGGATGG-3'	132-178	17	PET
ILSTS089	For-5'-AATCCGTGGACTGAGGAGC-3' Rev-5'-AAGGAACCTTCAACCTGAGG-3'	106-144	12	FAM
CSSM066	For-5'-ACACAAATCCTTTCTGCCAGCTGA-3' Rev-5'-AATTTAATGCACCTGAGGAGCTTGG-3'	142-210	29	VIC
ILSTS036	For-5'-GAGTATTATGCTTGGGAGGC-3' Rev-5'-AGACAGGATGGGAAGTCACC-3'	122-172	14	NED
ILSTS095	For-5'-GAAAGATGTTGCTAGTGGGG-3' Rev-5'-ATTCTCCTGTGAACCTCTCC-3'	187-219	11	VIC
ILSTS029	For-5'-TGTTTTGATGGAACACAGCC-3' Rev-5'-TGGATTTAGACCAGGGTTGG-3'	140-180	21	PET
ILSTS028	For-5'-TCCAGATTTTGTACCAGACC-3' Rev-5'-GTCATGTCATACCTTTGAGC-3'	143-173	03	PET
ILSTS025	For-5'-GTTACCTTTATATAAGACTCCC-3' Rev-5'-AATTTCTGGCTGACTTGGACC-3'	110-144	11	FAM
ILSTS052	For-5'-CTGTCCTTTAAGAACAAACC-3' Rev-5'-TGCAACTTAGGCTATTGACG-3'	135-179	02	PET
ILSTS060	For-5'-TAGGCAAAGTCGGCAGC-3' Rev-5'-TTAAGGGGACACCAGCCC-3'	150-204	19	VIC
BM1818	For-5'-AGCTGGGAATATAACCAAAGG-3' Rev-5'-AGTGCTTCAAGGTCCATGC-3'	229-279	28	FAM
ILSTS061	For-5'-AAATTATAGGGGCCATACGG-3' Rev-5'-TGGCCTACCCTACCATTTC-3'	109-165	23	FAM

5. Population genetics analysis

5.1 Within-breed analysis

Expected heterozygosity or allelic richness within breeds indicates the influence of drift on breed diversity, where decreased heterozygosity is associated with increased drift. Differences between expected and observed heterozygosity as well as departure from Hardy-Weinberg equilibrium indicate nonrandom mating or the existence of population substructures [11]. The presence of inbreeding can be tested by F statistics in particular by testing if the F_{IS} parameter is significantly greater than zero [12].

5.2 Among breeds analysis

Total diversity can be partitioned in a within-breed and among-breeds component of variation. These components and others can be quantified by analysis of molecular variance (AMOVA) analysis and reflect history and breeding practices [13]. Normally, 50 to 90 per cent of the total diversity corresponds to the within-breed component which dependent upon the breeds sampled. The calculation of genetic distances among breeds is based on gene frequency data followed by visualization of relationships in trees, and networks [14]. Nei's standard genetic distance D_s has been used most commonly in studies of natural populations in evolutionary genetics and has

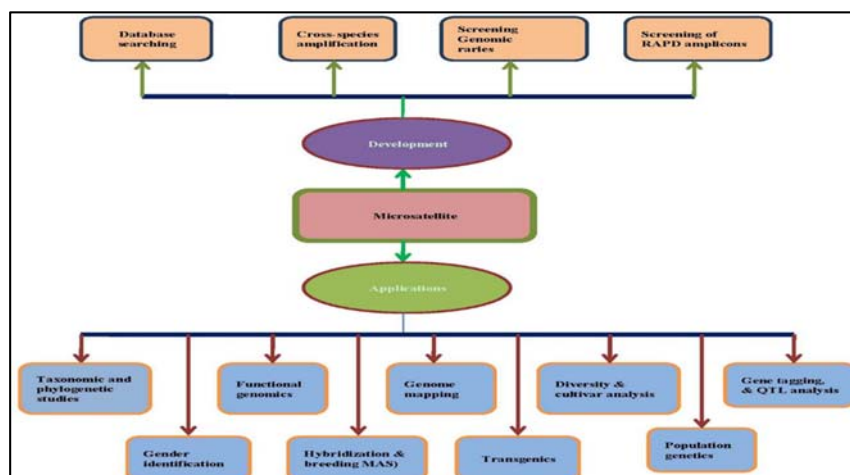
the advantage that it is linear in time [15]. Distance measures based on Wright's F_{ST} statistic, which may be estimated via the DR distance may be more appropriate for short-term evolution such as the divergence between livestock breeds [16].

6. Evolution of microsatellite markers

Mechanisms of mutation are believed to be unequal crossover during recombination, polymerase slippage and especially slipped-strand mispairing during replication resulting in the addition or loss of one or a small number of repeats. There is several mutation models considered for microsatellites.

6.1. The infinite allele model (IAM): Assumes that all new alleles are unique or microsatellite mutations may create an infinite number of repeated units and allelic states not present in the population. Each mutation creates a novel allele at a given rate, u . Consequently, this model does not allow for homoplasy. Identical alleles share the same ancestry and are identical-by-descent (IBD), unlike in other models [17].

Development and applications of microsatellite markers at a glance [18]



6.2 Stepwise mutation model (SMM): Involve addition or deletion of one repeat. Under this scenario, each mutation creates a novel allele either by adding or deleting a single repeated unit of the microsatellite, with an equal probability $u/2$ in both directions. Consequently, alleles of very different sizes will be more distantly related than alleles of similar sizes. Therefore, unlike the other models, the SMM has a memory of allele size [19].

6.3 Generalised Stepwise Model or Two Phase Model: Mutations are also described as or as a combination of single and multiple steps by the two-phase mutation model (TPM). The main difference in the models is that once a mutation has occurred it has a probability p of being a onestep mutation, and a probability $1-p$ of being a multi-step mutation [20].

6.4 In the K-allele model (KAM), the number of possible alleles is K . The probability for any allele to mutate to any other $(K - 1)$ allelic state is identical. Hence, a given allele will mutate to any of the remaining alleles at a rate $u/(K - 1)$. This model allows for homoplasmy, that is, alleles that are identical-in-state (IIS), but not identical by descent (IBD). Note that the IAM is a special case of the KAM, with $K = \infty$ (hence lacking homoplasmy).

Based on these models, it has been suggested that microsatellites that have a more IAM-like evolution (i.e. composite repeats) should be those best suited to study population questions such as population subdivision and genetic relationships since they will contain the lowest levels of homoplasmy.

Here is an example of a microsatellite sequence which contains a repeat unit and forward and reverse primer-sites. GCCAAGCTTGCATGCCTGCAGGTCGACTCTAGAGGA TCCCAAGTGTATGTGCATACACGTG CACACACACA CACACACACACAGAGGGTGTGCACATGTGCATGCAC ACTCCAAGAGACAGTG CCTAGTAAAGTGTCTCAGCA CCATCTGCAGCAAACAGGTTCTGCAAAAACCAATCC CAACTGA TGTTCCACAGTGACACTGT

From beginning of forward primer to end of reverse primer, the above is 131 bp Repeat is CA11. The repeat unit is highlighted in red, while the forward and reverse primers are highlighted in blue and green.

7. How do we develop microsatellite primers?

- Extract DNA from tissue (wide variety of possible methods depending upon tissue type).
- Fragment the genome. Cut our genomic DNA into suitable size fragments with restriction enzymes. Generally, restriction enzymes that produce mean fragment sizes in the range of 300-600 bp are the desired goal.
- Insert the fragments into plasmids. This step allows cloning of the fragments -- producing many copies of the 300-600 bp pieces we have inserted in the plasmids.
- Plate the plasmids on a nylon membrane.
- Probe the membrane with labeled oligonucleotides of desirable repeats (e.g., AC10).
- Culture the positive clones (the plasmid-fragments that bonded with the oligo probes).
- Cut the insert out of the plasmids with restriction enzymes and run them out on an agarose gel.
- Use Southern transfers to probe the digest again with labeled oligos. This serves: a) to verify the presence of the repeat and b) to allow us to estimate the size of the inser.

- Sequence the positive clones that make it through all the above selection steps.
- Select, analyze the sequence to check for "good" primer sites and useful repeat length (generally at least 8 repeats and it is often best to have more -- depending upon our intended application we may want long pure repeats or we may be interested in shorter interrupted repeats, which may have lower mutation rates). Several software packages are available that can help in primer selection (Oligo, Primer, Mac Vector).
- Order the locus-specific primers (Generally these will be 20-30 bp sections of the flanking regions not immediately adjacent to the repeat unit).

8. Conclusion

When combined with previously described conserved loci, the microsatellite markers will help in the study of wide range of genetic studies, including parentage and population analyses, but will also now enable comparisons of genetic diversity among different species (and populations) at the same set of loci, with no or reduced bias. Finally, the approach used here can be applied to other taxa in which appropriate genome sequences are available.

Microsatellites have been found to be highly polymorphic, genome-specific, and abundant co-dominant, and they have become important genetic markers in animal breeding programs especially for improving disease resistance.

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