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MICROSATELLITE MARKERS: A BREAKTHROUGH IN EVOLUTIONARY BIOLOGY

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Abstract: Repetitive sequences of DNA have explored a large amount of genetic data within the living organism. By the help of these repetitive sequences of DNA it is easy to study the genetic diversity within the organisms. Molecular Markers are the powerful tools for analysis of genetic biodiversity. Microsatellites are Simple Sequence Repeats of 1-6 nucleotides. Microsatellites are abundant throughout the genome and show high level of polymorphism. They are found to have a great potential to provide genetic information within an organism, diagnosis of disease of genetic origin and in evolutionary studies. In this review we tried to summarize the every aspect of microsatellites including features, application and drawbacks.

Keywords: Biodiversity, Genome, Polymorphism

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INTRODUCTION

Characterization and quantification of genetic diversity has long been a major goal in evolutionary biology. Knowledge on the genetic diversity within and among closely related crop varieties is essential for a rational use of genetic resources. Repetitive sequences of genomic DNA has opened a great means to estimate the genetic information associated with a particular organism. It becomes easy to study the genetic diversity by the help of these repetitive sequences. Molecular markers offer numerous advantages over conventional phenotype based alternatives as they are stable and detectable in all tissues despite of growth, differentiation, discrimination, defence or development status of the cell are not confounded by the environment, pleiotropic and epistatic effects.

The concept of genetic markers is not a new one; in nineteenth century phenotype-based genetic markers were used by Gregor Mendel in his experiment. Subsequently, phenotypebased genetic markers for Drosophila led to the establishment of the theory of genetic linkage. The restrictions of phenotype based genetic markers led to the development of more general and useful direct DNA based markers that became known as molecular markers. Molecular markers offer numerous advantages over conventional phenotype based alternatives as they are stable and detectable in all tissues despite of growth, differentiation, discrimination, defence or development status of the cell are not confounded by the environment, pleiotropic and epistatic effects (Agarwal et al*.,* 2008).

Molecular markers should not be considered as regular genes, because they do not possess any biological effect and can be considered as invariable landmarks within the genome. They are identifiable DNA sequences, found at precise locations of the genome, and their transmission is conducted by the universal laws of inheritance from one generation to the next (Semagn et al*.,* 2006).

Frequently used molecular markers are RFLP (Becker et al., 1995, Paran and Michelmore, 1993), RAPD (Tingey and Deltufo, 1993, Williams et al., 1990), SSRs (Levinson and Gutman, 1987), ISSRs (Albani and Wilkinson, 1998, Blair et al., 1999), AFLP (Mackill et al., 1996, Thomas et al., 1995, Vos et al., 1995, Zhu et al*.,* 1998) and SNPs (Vieux, et al., 2002) are presently available to assess the variability and diversity at molecular level (Joshi et al., 2000).

MICROSATELLITE MARKERS

Microsatellites are 1-6 nucleotides long and are arranged in a simple internal repeat structure throughout the genome of an individual. These are unique sequences in species. Microsatellite or (Simple Sequence Repeats) SSRs have been found to have great potential to provide genetic

information within species. They have been used for variety of purposes which includes gene tagging, estimation-determination of genetic diversity, physical mapping and genome mapping. Microsatellite markers, developed from genomic libraries, can belong to either the transcribed region or the non transcribed region of the genome. The function of microsatellite is almost unknown. SSRs are especially suited to distinguish closely related genotypes, because of their high degree of variability, they are, therefore, favoured in population studies (Smith and Devey, 1994) and for the identification of closely related cultivars (Vosman et al*.,* 1992).

In plants, microsatellite frequency is negatively correlated with genome size. This has been attributed to the fact that microsatellites are underrepresented in the repetitive parts of the plant genome that are involved in expansion of genome like long terminal repeats of retrotransposons (Morgante et al*.,* 2002). SSRs constitute a rather large fraction of noncoding DNA and are relatively rare in regions of protein-coding. For example, all of the observed 101 mono-, di- and tetranucleotide SSRs were classified in noncoding regions across 54 plant species (Wang et al*.,* 1994). The strengths of microsatellites include the codominance of alleles, their high genomic presence in eukaryotes and their random distribution from beginning to the end of the genome, with preferential relationship in low-copy regions (Morgante et al*.,* 2002) low quantities of template DNA required (10-100 ng), high genomic abundance, random distribution from beginning to the end of the genome, band profiles, high level of polymorphism can be interpreted in terms of loci and alleles, codominant markers, allele sizes can be determined with utmost accuracy, comparison among different gels feasible using size standard, greater reproducibility, distinct microsatellites may be multiplexed in PCR, wide range of applications, amenable to automation. (Goldstein et al*.,* 1995, Jarne and Lagoda, 1996, Goldstein and Schlotterer, 1999). Vos et al*.,* 1995 are stating that the heterozygosity of SSRs is seven to ten times higher than that of RFLPs. Despite many advantages, microsatellite markers also have several challenges and pitfalls that at best complicate the assessment of data, and limit their utility at its worst and confound their analysis.

HISTORY

With the advent of PCR technology in the mid 1980s (Mullis & Faloona, 1987, Saiki et al*.,* 1985), new perspectives have evolved for molecular biology fields that have largely impacted several applied purposes such as diagnostics, plant breeding programs, animal breeding system, forensics and others. Microsatellites were detected in eukaryote genomes almost thirty years ago and they are the most promising PCR-based markers. Microsatellites are tandemly repeated motifs of variable lengths that are

distributed throughout the eukaryotic nuclear genome in both coding and noncoding regions (Jarne & Lagoda, 1996). They also appear in prokaryotic and eukaryotic organellar genomes, e.g., chloroplast (Powell et al*.,* 1995) and mitochondria (Soranzo et al*.,* 1999).

Microsatellites were first identified in humans in 1981 by sequence analysis of alleles at the β globin locus (Miesfeld et al*.,* 1981, Spritz 1981) and subsequently found to be naturally occurring and ubiquitous in prokaryotic and eukaryotic genomes (Tautz and Renz 1984, Jeffreys et al*.,* 1985, Tautz 1989, Thoren et al*.,*, 1995, Toth et al*.,*, 2000).

Litt & Luty first used the term "microsatellites" in 1989 when analyzing the abundance and dispersion of $(TG)_n$ in the cardiac actin gene. Identification of SSRs in gene sequences of plant species was carried out as early as 1993 by Morgante and Olivieri. Soranzo et al*.,* (1999) was the first to show length variation at a mitochondrial SSR locus in conifers.

FEATURES AND IDENTIFICATION

Microsatellites were originally designed to research degenerative and neurology diseases in humans but showed great applicability for other species. Microsatellites range from one to six nucleotides in length (van Oppen et al*.,* 2000) and are classified as mono-, di-, tri-, tetra-, penta- and hexanucleo- tide repeats. They are tandemly repeated (usually 5-20 times) in the genome with a minimum repeat length of 12 base-pairs (Goodfellow 1992, Vaughan and Lloyd 2003, Ellegren 2004) which are widely distributed in the genome.

Many authors classified the markers 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., $(AT)_{12}GC(AT)_8$, 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 & Toonen, 2006).

In addition to their co-dominant feature, i.e., the identification of all alleles of a given locus, microsatellites can also be amplified using polymerase chain reaction (PCR) in stringent conditions that usually only permit the amplification of single loci, thus facilitating data integration (Bravo et al*.,*2006). Microsatellites are widely distributed throughout the genome, highly polymorphic and transferable between species. These features provide the foundation for their successful application

in a wide range of fundamental and applicable fields (Chistiakov et al.,2006).

The presence of SSRs in eukaryotes was verified from diverse genome regions, including 3´- UTRs, 5´-UTRs, exons and introns (Rajendrakumar et al.,2007). Furthermore, their localization could potentially interfere with different aspects of DNA structure, DNA recombination, DNA replication and gene expression as illustrated by Chistiakov et al., (2006). The transposable elements might contain one or more sites that are predisposed to microsatellite formation and enables SSRs dispersion throughout the genome (Bhargava & Fuentes, 2010). Microsatellites are also commonly located in proximity of interspersed repetitive elements, such as short interspersed repeats (SINEs) and long interspersed repeats (LINEs). Kashi et al., (1997) reported that in promoter regions, the presence and length of SSRs could influence transcriptional activity.

The microsatellites can also be present in organellar genomes, such as chloroplast and mitochondria, and nuclear DNA. Powell et al., (1995) provided experimental evidence of length variation in the mononucleotide repeats of the chloroplast genome of angiosperms, and polymorphisms within these regions might be used to study both intraspecific and interspecific variability.

Knowledge of the complete genome sequence of many species in the public domain now permits the determination of SSR frequencies at the whole genome level, decreases the economic limitations and accelerates the process of SSR analysis. The accessibility and data analysis of microsatellite content in whole genome sequences would also facilitate comprehensive studies on the direct role of microsatellites in genome organization, recombination, gene regulation, quantitative genetic variation and the evolution of genes (Katti et al.,2001). The density analyses of SSRs in fully sequenced eukaryotic genomes showed a higher density in mammals and the initial analysis of the human genome sequence concluded that approximately 3% of all DNA is represented by SSRs. The human genome is estimated to contain on an average 10-fold more microsatellites than plant genomes (Powell et al.,1996). The analyses of microsatellite distribution in the genomes of many species revealed that compared with Drosophila, Arabidopsis, Caenorhabditis elegans and yeast, human chromosomes 21 and 22 are rich in mono- and tetranucleotide repeats. Drosophila chromosomes have higher frequencies of di- and trinucleotide repeats and, surprisingly, the C. elegans genome contains less SSRs per million base pairs of sequence than the yeast genome (Katti et al., 2001).

ISOLATION AND ANALYSIS

Isolation

The methods of SSR loci isolation have been improved much more than first time and

several protocols were published. There are published reviews concerning this topic (Weising et al., 2005, Zane et al., 2002), but with the recent development of technology and evolution of methodology, new methods and modifications have been proposed.

The published microsatellite isolation protocols can be grouped into three types:

(i) the standard method, where a library is screened for repeated sequences,

(ii) the automated method, where the SSR sequences are searched in sequence databases and

(iii) the sequencing method, where the whole genome or parts of the genome are sequenced using high-throughput technologies.

Each of these methods was modified and optimized to many species and conditions, generating a large number of protocols. Here, we will present an overview of the commonly used protocols.

Standard method

In Standard method creation of a library is an required step. There are various protocols to create and screen a genomic, cDNA or PCR fragment library [revised by Mittal & Dubey (2009) and Weising et al., (2005)], but the main steps can be summarized as follows:

1. The DNA is fragmented by sonication or enzymatic digestion.

2. The DNA fragments are ligated into a vector and transformed into Escherichia coli.

3. The clones are analyzed for the presence of SSR sequences by Southern blot. Then, the positive clones are sequenced.

The number of positive clones obtained by this method ranges from 0.04 to 12%, with the lowest yields occurring in birds (Zane et al., 2002). These protocols are efficient; however, the cost of developing a microsatellite marker is high because the use of a total genomic DNA library requires the evaluation of a large number of clones to find those containing repeated sequences. Ito et al. (1992) proposed the use of a biotinylated oligonucleotide to screen the plasmids of a restriction fragment library. The oligonucleotide and plasmid interact to form a triple helix, and the positive clones could be recovered using streptavidin coated magnetic beads. Subsequently, the microsatellite-enriched plasmids are purified and transformed into E. coli. This technique is limited to sequence motifs that are capable of triple helix formation (such as GA- and GAA-repeats).

Another technique to increase the number of positive clones or enrich the libraries relies on the extension of the library of single-stranded genomic DNA using repeat specific primers. For example, Paetkau (1999) amplified genomic libraries using biotinylated oligonucleotides that were complementary to the microsatellite sequence, as primers. The single-stranded

biotinylated sequences were recovered with streptavidin bound to magnetic particles, made double-stranded and transformed into E. coli. In this case, the enrichment efficiency was 100% for the dinucleotide $(CA)_{18}$. However, the enrichment efficiency depends on the size of the genomic library.

The most popular enrichment methods for SSR sequences are based on hybridization selection (Weising et al., 2005). Therefore, the following steps are done after DNA fragmentation:

1. The DNA fragments are ligated to adapters and amplified by PCR.

2. The PCR products are hybridized to microsatellite sequences that are attached to nylon membranes or biotin, and the hybrid sequences are eluted from the membrane or recovered via streptavidin-coated magnetic beads.

3. The selected PCR products are ligated into a vector and transformed into E. coli.

Researchers using hybridization selection have reported up to 80% of clones containing a microsatellite. Using two rounds of amplification and hybridization with biotin/streptavidin, Kandpal et al., (1994) generated a high enrichment efficiency of approximately 90% for CA repeats.

Yue et al., (2009) described another method to enrich microsatellite libraries. These authors applied a duplex-specific nuclease to normalize a pool of cDNA prior to cloning and generated 30 times more positive clones as compared with direct sequencing methods. Recently, Santana et al., (2009) and Malausa et al., (2011) applied pyrosequencing to enriched DNA libraries of many species and demonstrated that this methodology is more rapid, effective and economical than others.

Automated method

Microsatellite identification and development is also made possible through the use of public DNA databases to search for repeated sequences. Initially, database searches were performed using unspecific alignment tools, such as BLASTN (Altschul et al., 1990). Subsequently, several computer-based software programs were developed and the SSR search became easier. Mittal & Dubey (2009) reported a list of programs, their applications and references. As microsatellites located in expressed sequences are more conserved and gene related, many studies have described and applied EST-SSRs (Varshney et al., 2005).

This automated approach reduces the costs associated with microsatellite marker development but is limited to species with available sequences.

Sequencing method

The new high-throughput sequencing technologies have allowed whole or expressed genome sequencing (Abdelkrim et al., 2009, Mikheyev et al., 2010). These technologies do not require the creation of

libraries (total DNA or RNA can be sequenced), produce a huge amount of sequences quickly and because many steps have been skipped, have lower costs than other methods.

Following the isolation of microsatellite sequences, it is necessary to develop PCR primer pairs flanking these sequences to test new loci for robust amplification, genomic copy number and sufficient polymorphism. Arthofer et al. (2011) reviewed published research concerning microsatellite isolation and showed that approximately half of all loci were lost due to inconsistent PCR amplification, multicopy status in the genome or monomorphism, regardless of the isolation strategy used. Moreover, these authors demonstrated the applicability of high-resolution melting (HRM) analyses to screen candidate loci for marker development, reducing the costs of traditional tests.

Analyses

In microsatellite loci analyses, variations in the amplification product size are related to the number of repeated motifs and would indicate the polymorphism level of that specific locus in a population. There are many protocols to amplify and detect microsatellite loci variation. Weising et al. (2005) described the most frequently used methods.

The protocol choice depends on the availability of equipment and reagents and the desired accuracy of the polymorphism detection. Agarose gels stained with ethidium bromide are easy to handle and are one of the cheapest protocols but do not allow precise fragment size determination. However, one of the most accurate methods requires an automated sequencer and fluorescent-labeled primers. The combined use of multiplex reactions (with primers labeled with different fluorochromes) with capillary DNA sequencers allow high- precision genotyping and high-throughput.

Regardless of the electrophoretic technique chosen to determine the banding pattern of the amplified fragments, the next step is statistical analysis. Molecular markers with known band sizes are usually added to electrophoresis gels to estimate the fragment size.

There are several methods and computer programs that can be used in data analysis, depending on the final application. Excoffier & Heckel (2006), Labate (2000) and Weising et al. (2005) reviewed many of them and summarized their main applications. Several statistical analyses are based on genetic distances, and as a initial step, the pairwise similarity is quantified. Mostly, the similarity index is calculated from band sharing data and the complement to this index is the genetic distance between the samples (Weising et al.,2005). When large number of samples is involved, it is difficult to interpret genetic distances. In these instances, the use of ordination, clustering and dendrograms condenses the

differences into fewer characters and permits the visualization of these entries in a multidimensional space (Weising et al., 2005).

TRANSFERABILITY OF MICROSATELLITES

SSRs are transferable because their flanking regions are highly conserved across taxa, allowing cross-species amplification, i.e., primers developed in one species can be used in others of the same genus or family, especially for vertebrates, such as reptiles and mammals (Peakall et al.,1998, Rico et al.,1996). The transferability of SSRs derived from EST databases (EST-SSR) is greater than that of SSRs derived from enriched genomic DNA libraries. The EST-SSRs originate from expressed regions, and hence, they are more conserved across a number of related species than non-coding regions (Varshney et al.,2005).

Many researchers have studied the transferability of SSRs. Zhao et al., (2011) showed the high transferability (86%) of Brachypodium SSR markers to Miscanthus sinensis. Moreover, 18 (31%) of the transferable markers produced perfect polymorphic and easy-scoring bands, consequently, this study confirms the significance of Brachypodium as a model plant for Miscanthus. Faria et al., (2010) used Eucalyptus EST databases to develop, select and conduct a detailed characterization of a novel set of 20 microsatellite markers that are polymorphic and transferable across 6 of the major Eucalyptus plant species. The primers were developed from more conserved transcribed regions, therefore, the transferability and polymorphism of these microsatellites likely extended to the other 300 or more species within the same subgenus Symphyomyrtus, further highlighting their applied value for Eucalyptus genetics and breeding. Pépin et al. (1995) showed that an estimated 40 percent of the microsatellites isolated from cattle were useful to study the caprine genome and characterize economically important genetic loci in this species. Moreover, bovine microsatellites were shown to be useful tools for the study of the genetic diversity of Artiodactyla. Dawson et al. (2010) developed primer sets for 33 polymorphic loci that are highly useful in the study of passerine, shorebirds and other non-passerine birds and for genotyping in species belonging to the Passeridae and Fringillidae families.

ROLE OF MICROSATELLITE MARKERS WITHIN GENOME

The location of the microsatellite within the genome decides its functional role (Lawson and Zhang 2006). Hence, SSRs have the potential to affect all aspects of genetic function including gene regulation-development-evolution (Kashi and King 2006, Lawson and Zhang 2006) and microsatellites have been described as “mutator alleles” for this reason. A microsatellite situated in a coding region can affect the activation of a gene and therefore, the expression of a protein. If

located in a noncoding or genic region, e.g., the 5'- untranslated regions (UTRs) or introns, the microsatellite may impact gene regulation or gene transcription (Lawson and Zhang 2006). Comparative studies in insects, with some exceptions (Thoren et al., 1995, Toth et al., 2000) have suggested that microsatellite length and frequency correlate with genome size (Hancock 1996). In plants, the general frequency of microsatellites was not only shown to be inversely related to genome size, but the percentage of repetitive DNA appeared to remain constant in coding regions (Morgante et al., 2002) with dicots having more mononucleotide repeats and monocots having more trinucleotide repeats (Lawson and Zhang, 2006).

Microsatellite genesis is an evolutionarily dynamic process and has proven to be exceedingly complex (Ellegren, 2004, Pearson et al., 2005). Trying to understand the process and mechanism may help us to analyze the data and explain the results obtained from microsatellites. Possible explanations for microsatellite genesis include single-stranded DNA slippage, double-stranded DNA recombination, mismatch/double strand break repair, and retrotransposition. During DNA replication, slipping of DNA polymerase III on the DNA template strand at the repeat region can cause the newly created DNA strand to expand or contract in the repeat region if the mismatches are not repaired. DNA slippage has been confirmed in vitro by endonuclease digestion, mutation analysis, and synthesis of simple sequence repeat DNA without using polymerase chain reaction (PCR), and amplification and sequence of DNA containing SSRs using PCR (Streisinger and Owen, 1985, Schlotterer and Tautz, 1992, Murray et al., 1993). Recombination by unequal crossover or gene conversion in the region containing SSRs may also lead to expansion or contraction of the repeat length (Richard and Paques 2000). Microsatellite generation had been found to be accompanied by retroposition events by analysis of a portion-sequenced human and rice genome DNA (Nadir et al., 1996, Temnykh et al., 2001).

APPLICATIONS

Due to all of the previously discussed features, microsatellites have been a class of molecular markers chosen for diverse applications. In this review, the SSR applications will be summarized into four categories:

(i) Genetic diversity and individual identification,

(ii) Genome mapping and marker-assisted selection,

(iii) Population and phylogenetic relationships and

(iv) Bioinvasion and epidemiology.

Genetic diversity and individual identification

Genetic diversity refers to any variation in nucleotides, genes, chromosomes or whole genomes of organisms (Wang et al.,2009a). Genetic diversity can be assessed among different accessions/individuals within same species (intraspecific), among species (interspecific) and between genus and families (Mittal & Dubey, 2009).

Even crops with advanced studies in genomics (e.g., rice, corn, soybean and apple) have been recently evaluated by SSRs to access the genetic diversity. As mentioned previously, large-scale screening requires low-cost technologies. In a recent publication, Ali et al., (2011) evaluated the genetic and agro-morphological diversity of rice (Oryza sativa) among subpopulations and their geographic distribution. A selection of 409 Asian landraces and cultivars were chosen from 79 countries representing all of the major rice growing regions of the world. This rice diversity panel with the accompanying genetic and phenotypic information provides a valuable foundation for association mapping and understanding the basis of both genotypic and phenotypic differences within and between subpopulations.

Microsatellite markers have also been used for plants with poor genomic knowledge. For example, in an interspecific analysis, Hoshino et al. (2006) evaluated 76 accessions of 34 species from nine Arachis sections and showed that this germplasm bank possessed high variability, even when a species was represented by few accessions. This information was used to maintain Arachis genetic diversity during the storage and conservation process. Beatty & Provan (2011) published research utilizing intraspecific analysis through SSR markers. These authors assessed the genetic diversity of glacial and temperate plant species, respectively Orthilia secunda (one-sided wintergreen) and Monotropa hypopitys (yellow bird's nest). In this case, microsatellites were extremely useful to evaluate biogeographical distributions and the impact of changes in the species ranges on total intraspecific diversity. These authors concluded the following: "given that future species distribution modeling suggests northern range shifts and loss of suitable habitat in the southern parts of the species' current distribution, extinction of genetically diverse rear edge populations could have a significant effect in the range wide intraspecific diversity of both species, but particularly in M. hypopitys".

The great variability detected by microsatellites could be used to identify a person, a cultivar or a population. A set of SSR markers could be selected for each species/situation to distinguish one cultivar/genotype from all others. This practice is employed to protect the intellectual property rights of new varieties by commercial companies (Wang et al., 2009a). It is also used in paternity testing, when a progeny inherits one allele from the male parent and another allele from the

female parent (Chistiakov et al., 2006). The genotypic profile is highly discriminating, which suggests that a random individual would have a low probability of matching a given genotype and if only a few potential parents are being consider, paternity could be determined by exclusion (Weising et al., 2005).

"Assignment tests" (assignment of an individual to the population) can be used in forensics, conservation biology and molecular ecology. An interesting example is the study of Primmer et al. (2000), which used this approach to identify a case of fishing competition fraud. The assignment of the SSR genotype of the suspect fish to its most likely original population indicated a high level of improbability that the fish originated from Lake Saimaa (where the competition occurred). When this evidence was presented, the offender confessed purchasing the salmon at a local fish shop and criminal charges were laid.

Genome mapping and marker-assisted selection

Genome mapping includes genetic, comparative, physical and association mapping. Genetic mapping is one of the major research fields in which microsatellites have been applied because they are highly polymorphic and require a small amount of DNA for each test. Linkage maps are known as recombination maps and define the order and distance of loci along a chromosome on the basis of inheritance in families or mapping populations (Chistiakov et al., 2006). Association mapping links a locus to a phenotypic trait and comparative mapping aligns chromosome fragments of related species based on genetic mapping to trace the history of chromosome rearrangements during the evolution of a species (Wang et al., 2009a). However, in physical mapping, markers anchor large pieces of DNA fragments, such as bacterial artificial clones (BACs), and provide the actual physical distance between the markers (Wang et al.,2009a). Apotikar et al. (2011) constructed a SSR- based skeleton linkage map of two linkage groups of sorghum in a population of 135 recombinant inbred lines derived from a cross between IS18551 (resistant to shoot fly) and 296B (susceptible to shoot fly) varieties. The authors found 14 markers that were mapped to each linkage group and three quantitative trait loci (QTL) governing more than one trait (pleiotropic QTLs). The identification of genomic regions/QTLs that influence resistance can help breeders to introgress them into the breeding lines using the linked molecular markers. Baranski et al., (2010) analyzed the flesh color and growth related traits in salmonids with 128 informative microsatellite loci, distributed across all 29 linkage groups, in individuals from four F2 families. Chromosomes 26 and 4 presented the strongest evidence for significant QTLs that affect flesh color, while chromosomes 10, 5 and 4 presented the strongest evidence for significant QTLs that affect growth traits (length and weight). These potential QTLs

provide a starting point for further characterization of the genetic components underlying flesh color and growth in salmonids and are strong candidates for marker-assisted selection.

The use of the markers to indicate the presence of a gene (trait) is the basis for marker- assisted selection (MAS). Therefore, the construction of high-density and high-resolution genetic maps is necessary to select for markers that are tightly linked to the target locus (gene) (Chistiakov et al*.,* 2006). Once a linkage is established between a locus and the gene of interest, the inheritance of the gene can be traced, which could greatly enhance the efficiency of breeding programs (Wang et al*.,* 2009a).

Population and phylogenetic relationships

Microsatellite markers can be used to determine the population structure within and among populations (Wang et al*.,* 2009a). Evaluations of population differentiation permit the estimation of the migration rate between populations, assuming that these populations are in equilibrium (e.g., no selection, identical mutation rates and generation time) (Weising et al*.,* 2005). In plants, migration rates correspond with the gene flow through seeds and pollen (Weising et al*.,* 2005). Microsatellite markers are a powerful system for revealing inter or intraspecific phylogenetic relationships, even in closely related species (Wang et al*.,* 2009a). Phylogenetic relationships reflect the relatedness of a group of species based on a calculated genetic distance in their evolutionary history. Genomic SSRs, specifically EST-SSR markers, are the best choice for cross-species phylogenetics (Mittal & Dubey, 2009). However, the high incidence of homoplasy increases evolutionary distances and might undermine the confidence of the phylogenetic hypotheses, compromise the accuracy of the analysis and limit the depth of the phylogenetic inference (Jarne & Lagoda, 1996). Another problem with SSR-based phylogenetic inference is that primer transferability might not work well in all taxa and even when it is possible to amplify, the sequences might not be similar enough to permit a confident orthology assessment. Flanking regions of microsatellites have also been used in phylogenetic relationships between species and families because they evolve more slowly than repeated sequences (Chistiakov et al*.,* 2006).

Microsatellites have been used successfully in some phylogenetic cases. Using EST-SSR markers derived from Medicago, cowpea and soybean, the genetic diversity of the USDA Lespedeza germplasm collection was assessed and its phylogenetic relationship with the genus Kummerowia was clarified (Wang et al*.,*2009b), despite the fact that phylogenetic analysis with morphological reexamination provides a more complete approach to classify accessions in plant germplasm collection and conservation. Orsini et al*.,* (2004) used a set of 48 polymorphic microsatellites derived from

Drosophila virilis to infer phylogenetic relationships in the D. virilis clade and found results consistent with previous studies (D. virilis and D. lummei were the most basal group of the species). Furthermore, these authors detected differentiations between D. americana texana, D. americana americana and D. novamexicana that were previously supported by FST analyses and a model-based clustering method for multilocus genotype data. Rout et al., (2008) assessed the phylogenetic relationships of Indian goats using 17 microsatellite markers. Breeds were sampled from their natural habitats, covering different agroclimatic zones. Analyses showed that the results of the microsatellite analysis were consistent with mitochondrial DNA data, which classifies Indian goat populations into distinct genetic groups or breeds. The phylogenetic and principal component analysis showed the clustering of goats according to their geographical origin. The authors concluded that although the goat breeding tracts overlapped and spread countrywide, they still maintain genetic distinctions while in their natural habitats.

In the scope of biodiversity conservation and evolutionary genetics, microsatellites have been used to contribute accurate information on issues of population dynamics, demography and ecological/biological factors intrinsic to species and populations. Palstra et al. (2007) examined the population structure and connectivity of Atlantic salmon (Salmo salar) from Newfoundland and Labrador, which are regions where populations of this species are relatively pristine. Using the genetic variation of 13 microsatellite loci from samples (n=1346) collected from a total of 20 rivers, the connectivity at several regional and temporal scales was verified, and the hypothesis that the predominant direction of the gene flow is from large into small populations was tested. However, this hypothesis was rejected by evidence that the temporal scale in which gene flow is assessed affects the directionality of migration. Whereas large populations tend to function as sources of dispersal over contemporary timescales, such patterns are often changed and even reversed over evolutionary and coalescent-derived timescales. Furthermore, these patterns of population structure vary among different regions and are compatible with demographic and life- history attributes. No evidence for sex-biased dispersal underlying gene flow asymmetry was found. These results are inconsistent with generalizations concerning the directionality of the gene flow in Atlantic salmon and emphasize the necessity of detailed regional study, if such information is to be meaningfully applied in conservation and management of salmonids.

Becquet et al. (2007) used 310 microsatellite markers genotyped in 78 common chimpanzees and six bonobos, allowing a high-resolution genetic analysis of chimpanzee population structure. These chimpanzees have been traditionally

classified into three populations: western, central and eastern. While the morphological or behavioural differences are small, genetic studies of mitochondrial DNA and the Y chromosome have supported the geography-based designations. The findings showed that the populations seem to be discontinuous and provided weak evidence for gradients of variation reflecting hybridization among chimpanzee populations. In addition, the results demonstrated that central and eastern chimpanzees are more closely related to each other in time than to western chimpanzees.

Bioinvasions and epidemiology

The analysis of genetic diversity, population structure and demographic inferences using microsatellite has been useful to elucidate the processes of bioinvasion, understand the epidemiological patterns and aid in controlling and eradicating diseases.

The characterization of the genetic structure of invasive populations is important because genetically variable populations tend to be more successful as invaders than those that are relatively genetically homogeneous, and genetic data might provide an important tool to resource managers concerned with invasion risk assessments and predictions. To examine the invasion genetics of the Eurasian spiny water flea, Bythotrephes longimanus, which is a predacious zooplankter with increased range in Europe that is rapidly invading inland waterbodies throughout North America's Great Lakes region, Colautti et al. (2005) employed microsatellite markers. Three populations where B. longimanus has been historically present (Switzerland, Italy and Finland), a European-introduced population (the Netherlands) and three North American populations (Lakes Erie, Superior and Shebandowan) were sampled. Consistent with a bottleneck during colonization, the average heterozygosities of the four European populations were higher than the three North American populations. The pairwise FST estimated among North American populations was not significantly different from zero and was much lower than that among European populations. This result is consistent with a scenario of higher gene flow among North American populations. The assignment tests identified several migrant genotypes in all introduced populations (the Netherlands, Erie, Superior and Shebandowan), but rarely in native ones (Switzerland, Italy and Finland). A large number of genotypes from North America were assigned to Italian populations, suggesting a second invasion in the region of northern Italy that was previously unidentified. These results support a bottleneck in the invasion of North American populations that has been largely offset by the gene flow from multiple native sources and among introduced populations.

Microsatellites have also been chosen to evaluate the genetic variability and dynamics of the invasion of Ambrosia artemisiifolia, an aggressive North American

annual weed, found particularly in sunflower and cornfields. Besides its economic impact on crop yield, this plant represents a major health problem because of strongly allergenic pollen. The results of Genton et al. (2005) suggested that the French invasive populations include plants from a mixture of sources. The reduced diversity in populations distant from the original introduction area indicated that ragweed range expansion probably occurred through sequential bottlenecks from the original populations and not from subsequent new introductions.

Understanding the epidemiology of the disease is related to knowledge about the basic biology of the organisms involved. Population genetic studies can provide information about the taxonomic status of species, the spatial limits of populations and the nature of the gene flow among populations. Examples of the important results in this approach are Pérez de Rosas et al. (2007) and Fitzpatrick et al. (2008). Pérez de Rosas et al. (2007) examined the genetic structure in populations of the Chagas disease vector, Triatoma infestans. Levels of genetic variability (assessed by microsatellites) were compared in populations of T. infestans from areas with different periods after insecticide treatment and from areas that never received treatment. These authors found that genetic drift and limited gene flow appear to have generated a substantial degree of genetic differentiation among the populations of T. infestans and the microgeographical analysis supports the existence of subdivision in T. infestans populations. Levels of genetic diversity in the majority of T. infestans populations from insecticide-treated localities were similar or higher than those detected in populations from areas without treatment. This study supports the hypothesis of vector population recovery from survivors of the insecticide-treated areas, and therefore highlights the value of population genetic analyses in assessing the effectiveness of the Chagas disease vector control programs. Fitzpatrick et al. (2008) investigated the identity of silvatic Rhodnius (vector of Chagas' disease) using sequencing and microsatellites and whether silvatic populations of Rhodnius are isolated from domestic populations in Venezuela. Sequencing confirmed the presence of R. prolixus in palms and that silvatic bugs can colonize houses. The analyses of microsatellites revealed a lack of genetic structure between silvatic and domestic ecotopes (non-significant FST values), which is indicative of unrestricted gene flow. These results demonstrate that silvatic R. prolixus presents an unquestionable threat to the control of Chagas disease in Venezuela.

LIMITATIONS

One of the main drawbacks of microsatellites is that high development costs are involved if there is availability of adequate primer sequences for the species which are of interest, it would be easy to

apply them to unstudied group. Although microsatellites are in principle codominant markers, mutations in the primer annealing sites may result in the occurrence of null alleles (no amplification of the intended PCR product), which may lead to errors in genotype scoring. A very common observation in microsatellite analysis is the appearance of stutter bands that are artifacts in the technique that occur by DNA slippage during PCR amplification. Interpretation of band profiles could be complicated because size determination of the fragments is more difficult and heterozygotes may be confused with homozygotes (Kumar et al. 2009). Recombination could potentially change the SSR length by unequal crossing over or by gene conversion (Brohele and Ellegren, 1999, Hancock, 1999, Jakupciak and Wells, 2000, Richard and Paques, 2000).

Although microsatellites have many advantages over other molecular markers, all data sets might include some errors and genotyping errors remain a subject in population genetics because they might bias the final conclusions (Bonin et al. 2004). Microsatellite genotyping errors result from many variables (reagent quality, Taq polymerase error or contamination), as reviewed by Pompanon et al. (2005), and the primary consequence is the misinterpretation of allele banding patterns.

Microsatellite markers are mainly limited by:

1. Null alleles: locus deletion or mutations in the annealing primer site prevent locus amplification and heterozygous identification and lead to erroneous estimations of allele frequencies and segregation rates. Primer redesign might resolve this problem.

2. Homoplasy: alleles identical in state (length) but not by descent are homoplasic alleles (Jarne & Lagoda, 1996). They can be identical in length but not in sequence or identical in length and sequence but with different evolutionary history (Anmarkrud et al. 2008). Because homoplasy is disregarded, the actual divergence between populations is underestimated. Sequencing could be used to identify differences in sequences, but differences in evolutionary history can only be identified by mutations documented in known pedigrees.

3. Linkage disequilibrium: deviations from the random association of alleles in a population, which are primarily caused by population substructuring and high levels of inbreeding (Weising et al. 2005). It is especially problematic for population studies and paternal exclusion. Computer programs or an offspring analysis could detect the problems.

CONCLUSION

Numerous evidences have shown approach of molecular genetics by microsatellites. The utilization of microsatellites has been demonstrated by a large number of studies applying this marker and by the variety of

areas that apply microsatellites for several purposes. Microsatellites have proven to be an important tool in diagnosis and treatment of diseases and disorders of genetic origin, mapping and analysis of genome, tracing back and evolutionary history and in several phylogenetic studies. Furthermore, novel technologies have enabled the development of markers for previously neglected species through the generation of new sequences and a more refined search in databases. Nevertheless, there are some bottlenecks that need to be overcome as they hamper the best and widespread use of SSR data, e.g., an exchangeable data format to allow users to access different kinds of analyses and computer programs easily (Excoffier & Heckel, 2006) and the best understanding about microsatellite evolution and mutation mechanisms.

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