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Microsatellite Markers In The Sustainable Management Of Forest Genetic Resources

Renan Marcelo Portela[1], Lorhan Matheus De Souza Medalha[2], João Francisco Dos Santos Quadros[3], João Henrique De Lara[4], Quemuel Alves Feitosa[5], Jerffersoney Garcia Costa[6], João Gabriel Zanon Paludeto[7], Caio Augusto Fidelix Carneiro Gomes[8]

[1,2,3,4]*forest Engineering Department, Universidade Estadual Do Centro-Oeste, Brazil*
[5,6,7]*klabin S.A.*
[8]*forest Resources And Environmental Conservation Department, Virginia Polytechnic Institute And State University, Usa*

Abstract:
Forest species represent an incalculable source of current and potential genetic resources for humanity. Therefore, understanding and characterizing these resources are crucial to clarify significant aspects of species' biology for management, conservation, and genetic improvement purposes. In this context, the use of molecular techniques is vital, as they provide adequate and precise information, in contrast to phenotypic information or the use of morphological markers. Molecular analysis of plants using microsatellite markers has various applications in genetic conservation and plant breeding. With this in mind, the aim of this study was to review the key aspects related to microsatellite markers, their advantages and disadvantages compared to other markers, their main applications, and analyses performed in genetic conservation and forest improvement. This work was carried out through a bibliographic review. The consulted bibliography indicated that methods and methodologies in molecular biology are constantly being updated and evolving, and due to advancements in the molecular field and computational statistics, numerous applications and analyses for microsatellites have become possible, making molecular methods an indispensable tool for conservation, management, and forest improvement. These methods provide essential information to guide the best strategies and assist in decision-making.

Palavra-chave*: Microsatellites, Forest biotechnology, Diversity.*

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I. Introduction

Forestry species are a vast reservoir of genetic resources that are both extant and prospective for humankind. A significant portion of these resources, however, faces irreversible destruction before their comprehensive understanding and utilization, necessitating immediate conservation actions (Kageyama, 1987). Thus, identifying and characterizing these resources is critical to shed light on important biological aspects for their management, preservation, and genetic enhancement.

Employing molecular techniques is essential in this regard, as they yield accurate and reliable information, surpassing phenotypic data or morphological markers in effectiveness. This is due to their ability to detect genetic differences unaffected by environmental factors, and at an early age, without requiring the organism's full development (Kordrostami & Rahimi, 2015; Santos et al., 2016). Molecular plant analysis serves various roles in genetic conservation and plant improvement, including genetic diversity studies, inferences about genetic structure, gene flow, reproductive systems, effective population size, kinship analysis, pedigree reconstruction, resolving taxonomic issues, phylogenetic studies, genetic mapping, gene tagging, marker-assisted selection for pest and disease resistance, germplasm evaluation and characterization, gene introgression, wide genomic selection, and association with quantitative traits, as well as studies on genotype-environment interaction, and registering and protecting developed genetic materials (Oliveira, 2006; Borém & Caixeta, 2009; Kordrostami & Rahimi, 2015; Turchetto-Zolet et al., 2017).

Currently, a plethora of markers are available, each differing in principles, methodologies, and potential applications, as well as in technical, financial, and laboratory resources required. Among the available markers, microsatellites are presently the most popular (Turchetto-Zolet et al., 2017). Microsatellite markers are tandem repeat sequences consisting of 1 to 6 nucleotides. These markers are extensively used in genetic analyses across a wide range of organisms, mainly due to their high degree of polymorphism and cross-species transferability (Borém & Caixeta, 2009).

In forestry species, these markers are primarily used in studies related to kinship testing, reproductive systems, genetic diversity, and evolutionary studies. This is because they evolve rapidly, allowing for the detection of a high number of alleles per SSR locus even in germplasms with a narrow genetic base (Selkoe & Toonen, 2006). Advantages of these markers include their codominance, multiallelic nature, PCR-based methodology, widespread distribution in the genome, and the requirement of only a small DNA sample from the individuals being analyzed (Buso, 2003).

Therefore, the objective of this study is to review the main aspects concerning microsatellite markers, their pros and cons compared to other markers, and their principal applications and analyses in genetic conservation and forestry improvement.

II. Material and methods

The work was conducted through a literature review, using articles from databases such as the CAPES Portal, SciELO, ScienceDirect, and PubMed, as well as reference books, dissertations, and theses. Keywords used in the bibliographic search included molecular markers, molecular biology, microsatellite, analysis, applications, methodologies, genetics, plants, breeding, marker-assisted selection, diversity, among others. These terms were also used in different languages for a comprehensive search.

This summary provides an overview of the research methodology used in the study, emphasizing the extensive and multilingual literature review process.

III. Results and Discussion

Genetic Markers: Morphological, Biochemical, and Molecular

Morphological markers are phenotypic characteristics, generally easy to visualize and with Mendelian inheritance mechanisms. These markers, which can be used to assess genetic differences between two or more individuals, were the first type of genetic marker used, forming the basis of conventional genetic improvement in discrete traits. Thus, desirable phenotypic characteristics are selected in the parents.

There are several applications for this type of marker, including the identification of genotypes, characterization of variability, differentiation between accessions, and grouping (SOUZA and SORRELLS, 1991; ZHONG-HU, 1991). However, they are less efficient in genetic linkage maps for various plant species (HARTL and CLARK, 2010), among other applications. These studies are based on the presumption that morphological similarity reflects genetic similarity.

However, several limitations are associated with morphological markers in plant breeding (PATERSON et al., 1991; BORÉM and MIRANDA, 2017). These include a limited number of characteristics to use; significant effects of determinant genes affecting genetic analysis for important silvicultural traits; a limited number of traits that can be studied simultaneously due to gene interaction effects; and the result of gene-environment interactions altering phenotype expression, affecting the analysis (HARTL and CLARK, 2010).

Due to technological advances around the 1950s, another group of genetic markers, biochemical markers, emerged, including terpenes, proteins (isozymes, and alloenzymes) (TURCHETTO-ZOLET et al., 2017).

The first molecules used as biochemical genetic markers were secondary metabolites, such as anthocyanins and phenolic compounds, used to differentiate varieties of plant species (GROVER and SHARMA, 2016). Soon after, enzymatic markers, which were very important despite detecting a low degree of polymorphism, were used in various studies such as phylogenetic analyses, assessing genetic variability, identification of accessions, and more limitedly in associating traits of importance (BORÉM and MIRANDA, 2017), as well as underpinning evolutionary theories, notably the neutral theory of evolution, and the nearly neutral theory of evolution (KIMURA, 1968; KIMURA and OHTA, 1974; OHTA and GILLESPIE, 1996).

It is important to note that the basis of this type of marker comes from post-translation modifications of enzymes, potentially producing "conformational proteins," resulting in polymorphisms in response to environmental conditions, differences in enzymatic activities, and changes associated with different stages of development, besides having limited genome coverage (FERREIRA and GRATTAPAGLIA, 1998).

Biochemical markers lost popularity years later, due to the emergence of DNA molecular marker techniques, which can detect greater polymorphisms, allowing genotype analysis without the need for phenotypic expression, excluding the influence of the environment on polymorphism, with a broader sampling of the genome, as biochemical markers sample only regions active in gene expression.

In the 1980s, molecular marker technology was routinely used in a wide range of species, and since then, they have been refined and their methodologies have evolved in conjunction with advances in large-scale sequencing techniques. Generally, DNA markers can be classified into three categories: those based on hybridization, PCR-based, and sequencing-based.

Currently, there is a vast number of markers available for use, differing in their principles, methodologies, and potential applications, as well as the necessary technical, financial, and laboratory resources. Among the applications in genetics and forest improvement, one can cite the study of genetic diversity, inferences about

genetic structure, gene flow, reproductive system, effective size, analysis of kinship, pedigree reconstruction, resolution of taxonomic problems, phylogenetic studies, genetic mapping, gene tagging, and assisted selection for pest and disease resistance, germplasm evaluation and characterization, gene introgression, broad genomic selection, and association with quantitative traits, studies of genotype-environment interaction, registration, and protection of developed genetic materials, among others (FERREIRA and GRATTAPAGLIA, 1998; BORÉM and CAIXETA, 2009; RESENDE et al., 2012; GRATTAPAGLIA, 2014; BORÉM and MIRANDA, 2017).

Important Aspects in the Choice of Markers

As mentioned earlier, markers differ in their principles, methodologies, and potential applications, as well as the necessary technical, financial, and laboratory resources (Table 1). In this sense, choosing one or more of these markers is not always a simple task, depending on specific purposes.

Table 1: Important Characteristics in Relation to Other Types of Molecular Markers Commonly Used in Genetics and Forest Improvement.

Characteristics	RFLP	RAPD	AFLP	SSRs	SNPs
DNA needed (μg)	10	0.02	0.5–1.0	0.05	0.05
PCR-based	No	Yes	Yes	Yes	Yes
DNA Quality	High	High	Moderate	Moderate	High
Polymorphism Type	Single base change, insertion, deletion	Base change, insertion, deletion	Base change, insertion, deletion	Base change, repeat	Single base change, insertion, deletion
Dominance	Co-dominant	Dominant	Dominant/ Co-dominant	Co-dominant	Co-dominant
Reproducibility	High	Unstable	High	High	High
Analysis Cost	Low	Low	Moderate	High	Low
Development Cost	Low	Low	High	High	High
Sequence Data Needed	Yes	No	No	No	Very High
Accuracy	Very high	Very low	Medium	Medium	Very high
Genome Abundance	High	Much high	High	High	Medium
Polymorphism Level*	Low	Low to moderate	Low to moderate	High	High
Inheritance	Codominant	Dominant	Dominant	Codominant	Codominant
Use in Assisted Selection	Moderate	Low to moderate	Low to moderate	High	High

* The level of polymorphism (average heterozygosity) is a measure of the probability that two randomly chosen alleles can be distinguished; RFLP=Restriction Fragment Length Polymorphism; RAPD=Random Amplified Polymorphic DNA; AFLP=Amplified Fragment Length Polymorphism; SSR=Simple Sequence Repeats; SNPs=Single Nucleotide Polymorphism.

The ideal genetic marker should be highly polymorphic, exhibit codominant inheritance, and be evenly distributed throughout the genome. It should be easily accessible; analyses should be low-cost, high-yield, reproducible across laboratories, and transferable between populations and/or species. Unfortunately, no single type of marker meets all these criteria. However, according to the type of study, it is still possible to choose among different molecular markers to find the one that best suits the needs, considering:

- Availability of markers;
- Complexity of the technique and time investment;
- Estimated levels of polymorphisms in the studied population;
- Quantity and quality of available DNA;
- Transferability between laboratories, populations, pedigrees, and species;
- The size and structure of the population to be studied;
- Availability of technique and laboratory resources;
- Cost per unit of information and available budget;
- Type of marker inheritance (dominant/co-dominant) and genetic information required in the population.

SSR Markers, General Characteristics

Microsatellite markers are repeated sequences consisting of 1 to 6 nucleotides that are repeated side by side in a tandem arrangement. This type of marker is widely used in genetic analyses, in a vast range of living beings, especially due to its high degree of informativeness and transferability between species (BORÉM and CAIXETA, 2009).

These markers are used in forest species in studies related to parentage tests, reproductive systems, genetic diversity, and evolutionary studies, mainly because they are sequences with a high evolutionary rate. Even

when comparing germplasms with a narrow genetic base, it is usually possible to detect a high number of alleles at an SSR locus (SELKOE and TOONEN, 2006).

The polymorphisms at a locus are due to differences in the number of times the nucleotides are repeated at a certain locus, which can be mono-, di-, tri-, tetra-, penta-, and hexa-nucleotides. Microsatellites can also be classified according to the repeated sequences, which can be perfect (when the sequences are not interrupted by bases that are not part of the motif), imperfect (when there is the presence of some foreign base to the motif), interrupted (a small foreign sequence alternates the motif) and compound (two adjacent repeated sequences occur) (BUSO, 2003).

These variations consequently generate differences in the lengths of the segments, which can be detected by the polymerase chain reaction (PCR), and specifically visualized during the fragment separation stage on electrophoresis gel.

The advantages of these markers include being codominant, multiallelic, PCR-based, widely distributed throughout the genome, and requiring a small amount of DNA collected from the individuals to be analyzed (BUSO, 2003).

Genetic Structure in Plants

Genetic structure refers to how genetic variability is distributed among and within hierarchical levels, over time and space (YOUNG, 2001). In this regard, SSR markers, compared to other classes of markers, are highly polymorphic and widely used to answer various questions related to the genetics of plant populations.

Understanding the distribution of genetic variability among and within natural plant populations is essential to adopt competent strategies for ex situ and in situ germplasm conservation, and microsatellites are extremely useful for estimating population genetic parameters such as population structure, kinship and paternity analysis, and gene flow.

The way genetic variability is distributed among and within forest populations over time and space (Figure 1) is affected by evolutionary forces (mutation, selection, genetic drift and gene flow) and other important factors that contribute to structuring, such as species attributes, including the mode of reproduction, reproductive system, sexual system, pollination, and seed dispersal, phenology, successional stage; population attributes, including size, dynamics, density, and spatial pattern; landscape attributes, such as distances, environmental heterogeneity, and anthropogenic disturbances (LOVELESS and HAMRICK, 1984; 1987; HAMRICK and GODT, 1990; KEVIN et al., 2004; MARQUARDT and EPPERSON, 2004).

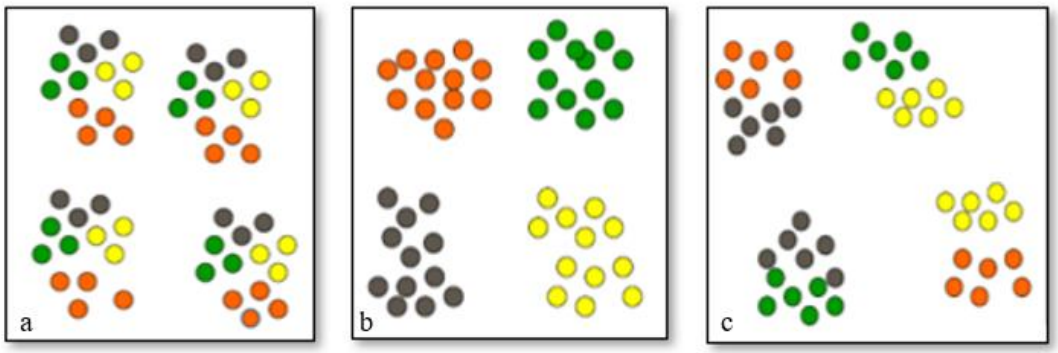
Knowing and identifying the patterns in which variability is distributed in natural populations enables the establishment of efficient conservation practices (FRANKEL et al., 1996), with this understanding being the basis for management techniques in natural forests and genetic conservation.

Different biometric measures have been proposed and used to study genetic diversity, aiming to elucidate the effects of genetic drift, estimate and predict the level of existing genetic variation, and verify its distribution.

An efficient metric widely used to assess population genetic structure is based on Wright's F-statistics (1951, 1978), which described a structure to measure variation in gene frequency among subpopulations, proposing three fixation indices: F_{IS}, F_{IT}, and F_{ST}, where the subscript letters indicate hierarchical population levels, as in the case 'I' is individual, 'S' is subpopulation, and 'T' is the total of subpopulations.

Considering that SSR markers may have many alleles at a locus, a common question relates to what is more efficient in genetic evaluation: a greater number of loci or alleles. Although there are no commonly accepted criteria for the selection of these primers, some authors discuss the subject.

Figure 1: Scheme of the arrangement of genetic structure among and within populations, where there is the same degree of genetic variability, but structured in different ways: 1.a: Genetic variability is not structured among populations; 1.b: Genetic variability organized among populations; 1.c: Genetic variability organized among and within populations.



Kalinowski (2002), using simulated data, showed that highly polymorphic loci provided better estimates of genetic distance compared to less polymorphic ones, and that an increase in the number of alleles was associated with a decrease in the coefficient of variation for each of the four genetic distances studied. These results indicate that there is no requirement to examine highly polymorphic loci or a large number of loci; the only requirement is that a sufficient number of alleles be examined.

Merritt et al. (2015) analyzed 6782 microsatellite markers published between 1997 and 2012, studying the correlations between observed and expected heterozygosity (*Ho* and *He*) and the number of alleles (*A*), according to the characteristics of the repeat type, motif length, motif region, repeat frequency, and size, where dinucleotide motifs exhibited significantly higher (*A*), (*Ho*), and (*He*) than most other motifs. Repeat frequency and motif region correlated positively with (*A*), (*Ho*), and (*He*), however, correlations with microsatellite size were minimal. Thus, lengths of dinucleotide motifs with high repeat frequencies may be the best when the researcher's goal is to target high genetic variation.

Petit et al. (2005) suggest that microsatellite loci with more repetitions generally show higher mutation rates. This is likely because DNA slippage increases proportionally with the number of repetitions. However, the high mutation rate of microsatellites can also invalidate some assumptions used in some conventional population structure analyses, as populations may share homoplastic alleles (ESTOUP et al., 2002). When this is ignored, it can lead to biased estimates (BALLOUX et al., 2000).

In this sense, an analogous statistic to F_{ST}, called R_{ST} (SLATKIN, 1995), was developed to consider the effects of mutation. Although R_{ST} performs better than Fst under some circumstances, it can also be sensitive to the details of the mutation process (BALLOUX AND GOUDET, 2002).

As the mutation rate varies widely between loci within species (DI RIENZO et al. 1998), an advantage of loci with high mutation rates is that genetic differentiation reaches equilibrium faster, offering the possibility of obtaining estimates from larger and more spaced populations.

When using microsatellite markers, and migration rates are low, *F*-statistics become sensitive to the mutation rate. However, considering a stepwise mutation model, under these conditions, Rst is independent of the mutation rate, and due to its high associated variance, it may be less accurate in reflecting population differentiation compared to Fst (BALLOUX and LUGON-MOULIN, 2002). Moreover, R_{ST} will be deflated when the pattern of mutations involves more than one repetition and the number of possible allelic states is finite (SLATKIN, 1995). The estimation and comparison of both statistics are relevant for critical comparison and careful interpretation of the data and can provide valuable information about the genetic structure of a population.

Collevatti et al. (2001) used microsatellite loci to investigate the genetic structure of the Caryocar brasiliense population and found that Fst was significantly lower (0.07) than R_{ST} (0.29) across all loci.

This occurs because of the high and variable mutation rates of microsatellites, which generally cause high levels of heterozygosity within the population. The fact that Fst is based on the infinite allele model, assuming that alleles are identical by descent, tends to underestimate population differentiation and produce lower values than their corresponding Rst values (SLATKIN, 1995).

In many cases, however, no significant differences are found between F_{ST} and R_{ST} values (NOVICK et al., 2002; CARVALHO, 2015).

Gene Flow

Gene flow is a generic term that refers to the migration of genes between and within populations. It can also be understood as the evolutionary change in allele frequency, resulting from the movement of gametes between populations (SLATKIN, 1985; 1989).

It is one of the most important population genetic parameters for purposes of genetic conservation and management because it can restrict evolutionary processes by being capable of preventing adaptation to local conditions and promoting evolution by increasing variability, spreading new genes and gene combinations into populations (SLATKIN, 1989). Without gene flow, populations may accumulate genetic differences over time, potentially becoming distinct species (SLATKIN, 1985).

The study of gene flow can be done in two basic ways: direct methods (using direct observations or estimates of dispersal distance and reproductive success) and indirect methods (using genetic data from samples of individuals in various subpopulations to infer migration rates) (SLATKIN, 1985; 1987; BEERLI and PALCZEWSKI, 2010). There are several indirect methods that can be estimated based on microsatellite markers, including estimators based on allele frequencies and analogs to the FST statistic (MICHALAKIS; EXCOFFIER, 1996); on the number of private alleles; on spatial autocorrelation; maximum likelihood estimators based on allele frequencies (RANNALA and HARTIGAN 1996; TUFTO et al. 1996); based on the genealogies of the sampled individuals, according to coalescence theory (KINGMAN, 1982) with migration rates estimated using procedures from (WAKELEY, 1998), (BAHLO; GRIFFITHS, 1998), and (BEERLI; FELSENSTEIN, 1999).

Effective Population Size

The concept of effective population size (*Ne*) refers to the number of individuals that reproduce and leave offspring, thereby transmitting genes to the next generation. This concept is fundamental for conservation, genetics, and breeding purposes. It is one of the main determinants of the loss of variability in populations threatened with extinction (SOLÉ-CAVA, 2001). The smaller the effective size of a population, the greater the effects of genetic drift, such as increased variance between populations, decreased diversity within populations, contributing to the fixation or loss of alleles (RIDLEY, 2006).

The effective size is conceptually equivalent to that of an idealized population in relation to the population model of Fisher (1930) and Wright (1931), considering that it reduces genetic variability at the same rate as the study population (SEBBENN, et al., 2005). Thus, the theoretical effective size may differ from the census size of a population, usually being smaller (WRIGHT, 1938).

In the literature, different types of estimators for the effective size are described, which can be based on microsatellite molecular markers, being used as a measure of genetic representativeness from a given sample (VENCOVSKY and CROSSA, 2003).

Kinship and Paternity Analysis

The use of molecular markers in plant paternity analysis and gene flow studies is common because allozymes usually do not have enough variability to determine kinship by exclusion (CHAKRABORTY et al.,1988), whereas each microsatellite locus has many relatively rare alleles, and in most cases, an individual can be excluded from paternity using just a few loci (DOW and ASHLEY, 1996; DOW et al., 1995).

Kinship can be estimated based on genetic data, which make use of molecular markers to estimate kinship based on a quantitative measure of relatedness or by genealogical methods that employ pedigree data based on relationships, such as full siblings, half-siblings, parent and child, etc.

Microsatellites, being codominant, allow in heterozygous diploids, once two alleles at a specific locus are known, the calculation of their complete allelic and genotypic composition, indicating that microsatellite markers are the most informative markers for calculating relationship coefficients (OLIVEIRA et al., 2006).

Currently, there are various methods for estimating kinship coefficients using molecular markers, which are well discussed in the literature (QUELLER and GOODNIGHT, 1989; LI et al., 1993; LYNCH and RITLAND, 1999; WANG, 2002) and, in general, can be categorized into two groups: those based on the method of moments and those based on likelihood methods. Estimators founded on the method of moments seek to estimate the relationship between individuals in terms of probabilities of identity by descent (IBD). Those based on likelihood methods estimate the probability of individuals fitting a given kinship (TAYLOR, 2015).

Characterization and Protection of Cultivars

For improved genotypes and species with a narrow genetic base, aiming to prove that a particular cultivar is unique, molecular information is more suitable and precise compared to the phenotypic information from morphological markers. The use of morphological descriptors in the characterization of cultivars is recommended; however, it presents limitations, being influenced by the environment, not stable, and many descriptors can only be evaluated in the adult phase of the plants, which requires time and physical space for the evaluations (VIEIRA, 2000).

Furthermore, in the case of characterizing cultivars with a narrow genetic base, the use of such descriptors may not be efficient, considering the morphological similarities. Therefore, it is necessary to obtain more stable markers, which, together with morphological descriptors, are efficient in characterization.

Marker-Assisted Selection and Association with Traits of Silvicultural Interest

The process of marker-assisted selection (MAS) in plant breeding programs is achieved by associating certain alleles with desirable traits to identify superior genotypes in segregating populations. This method offers potential benefits for traits with low heritability, which are difficult and/or costly to measure or are measured at a mature age.

In marker-assisted selection, success depends on the degree of association between the marker used and the trait of interest, such that the greater the association, the less chance of recombination between the marker and the gene controlling the trait, and the greater the efficiency of selection.

The goal is to concentrate in a single genotype different traits of interest such as pest and disease resistance; association with quantitative traits of silvicultural importance; or when phenotypic evaluation is costly, or in specific environments, such as in cases of saline stress, water deficit, photosynthetic efficiency, or when the trait manifests only in advanced stages of the tree cycle, so that MAS can be performed in the early stages, significantly reducing the time required for a selection cycle (FERREIRA and GRATTAPAGLIA, 1998).

For efficient implementation of MAS to occur, the gene/QTL associated with the desired trait must have been previously identified or mapped to monitor the presence of favorable alleles.

It is important to emphasize that with SSR markers, most of the time, what occurs is an association between alleles and traits, not meaning that it controls the traits (COLLARD, 2008).

It is also necessary to consider whether the trait is quantitative or qualitative, the mode of genetic action in relation to additivity, dominance or recessivity, the effect of the marker on phenotypic expression, and the efficiency with which the marker discriminates the trait of interest.

IV. Conclusion

Methods and methodologies in molecular biology are constantly being updated and evolved, being an indispensable tool for conservation, management, handling, and improvement, providing essential information in order to guide the best strategies and assist in decision-making. With advances in molecular and computational statistical methods, various applications for microsatellites in genetics and forest breeding have been made possible, mainly referring to:

- Verification and measurement of genetic diversity and effective population size;
- Planning the effects and consequences of the management actions and management of genetic resources;
- Identification of valuable populations for species conservation;
- Assessing gene flow in species or populations;
- Inferring whether populations are demographically connected or isolated;
- Elucidating and estimating the likely population origin of individuals and the taxonomic groups they belong to;
- Defining which populations or individuals to use for area recovery, conservation, maintenance of diversity, and controlled crossings;
- Understanding the importance of genetic diversity in terms of species perpetuation and resistance to ecosystem disturbances.
- Marker-assisted selection and association with traits of silvicultural interests;
- Analysis of kinship and paternity;
- Characterization and protection of cultivars.

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