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Review

Development and Use of Simple Sequence Repeats (SSRs) Markers for Sugarcane Breeding and Genetic Studies

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Abstract: Recently-developed molecular markers are becoming powerful tools, with applications in crop genetics and improvement. Microsatellites, or simple sequence repeats (SSRs), are widely used in genetic fingerprinting, kinship analysis, and population genetics, because of the advantages of high variability from co-dominant and multi-allelic polymorphisms, and accurate and rapid detection. However, more recent evidence suggests they may play an important role in genome evolution and provide hotspots of recombination. This review describes the development of SSR markers through different techniques, and the detection of SSR markers and applications for sugarcane genetic research and breeding, such as cultivar identification, genetic diversity, genome mapping, quantitative trait loci (QTL) analysis, paternity analysis, cross-species transferability, segregation analysis, phylogenetic relationships, and identification of wild cross hybrids. We also discuss the advantages and disadvantages of SSR markers and highlight some future perspectives.

Keywords: simple sequence repeats (SSRs); molecular marker; sugarcane genetic research; sugarcane breeding

1. Introduction

Sugarcane (*Saccharum* spp.) is a major global crop, not only required for the production of biofuels such as ethanol, but it also produces 80% of total dietary sugar globally [1], and 92% of the sugar consumed in China [2]. Modern cultivars exhibit complex aneu-polyploid genomes with chromosome numbers ranging from 100 to 130, and are inter-specific hybrids derived from crosses among *S. barberi* ($2n = 111–120$), *S. officinarum* ($2n = 80$, $x = 10$), *S. robustum* ($2n = 60$ and 80, $x = 10$), *S. sinense* ($2n = 81–124$), and *S. spontaneum* ($2n = 40–128$, $x = 8$) [3,4], constituting approximately 80% of *S. officinarum*, 10–15% of *S. spontaneum*, and 5–10% recombinant chromosomes [4]. Currently, all commercial sugarcane breeding populations in the world share a narrow genetic base, due to their ancient origins from a small number of popular hybrids, such as POJ2878, Co419, and NCo310, which were developed in the early 1900s. These varieties were developed from complex interspecific hybridization through the Nobilization breeding process among wild clones of *S. spontaneum* and *S. officinarum*. Modern commercial cultivars represent the outcomes of 15–20 genotypes of the nobilized cultivars developed in India and Java from 100 years ago within the nobilized germplasm [3]. Thus, the genetic base of sugarcane commercial cultivars has narrowed, potentially explaining the slow progress currently being experienced by sugarcane breeding and improvement projects [3]. Although sugarcane is a major crop globally, relatively little effort has been expended on sugarcane genetics, likely due to life history

factors (e.g., heterozygosity and a longer breeding and selection cycle) that make conventional plant breeding programs time consuming and ineffective.

In sugarcane development, selection of parents for crossing needs careful characterization and assessment of the germplasm, as well as thorough knowledge and breeding techniques. Molecular DNA markers can be helpful tools for this process. Molecular marker technologies have been used in genetic diversity studies, molecular assisted selection (MAS), paternity analysis, quantitative trait loci (QTL) mapping, cultivar identification, phylogenetic relationships, and genetic mapping. Various molecular markers are being developed and used in sugarcane genetic studies, including simple sequence repeats (SSRs) [5], inter simple sequence repeat (ISSRs), restriction fragment length polymorphisms (RFLPs) [6], random amplification of polymorphic DNAs (RAPDs) [7], amplified fragment length polymorphisms (AFLPs) [8], and single nucleotide polymorphisms (SNPs) [9]. Among these, SSRs, also known as microsatellites, are very advantageous for a variety of applications in sugarcane genetics research and breeding, because they are multi-allele, co-dominant, highly informative, occur with high relative abundance and good coverage across the genome, and are experimentally reproducible [5,10,11]. SSRs have a few advantages over SNPs using modern genotyping platforms, though they are low throughput and might cost more per unit. SSR markers have been used in an extensive range of fundamental and applicable fields, including genetic analysis at the individual, population, cultivar, and species levels [12]. In this review, we describe the development of and applications for SSRs in sugarcane genetic research and breeding, which we believe could be useful for future crop improvement programs.

2. Development of Microsatellite (SSR) Markers

In recent years, a number of different molecular marker systems have been developed for use in sugarcane, and SSRs have been shown to be the most powerful [10,13]. SSRs are sequence blocks containing DNA motifs of 1 to 6 base pairs repeated between 5–50 times and flanked by sequences that are generally unique in the genome, but conserved in organisms [10,14]. SSRs are classified into mono-, di-, tri-, tetra-, penta-, or hexa-SSRs based on the number of repeated base pairs, and into perfect, imperfect, and compound SSRs, which display perfect repetitions, interruption with novel nucleotides, and two or more tandem motifs [15]. Wang et al. [16] further classified SSRs as simple perfect, simple imperfect, compound perfect, or compound imperfect.

SSR markers can be sorted by genomic or expressed sequence tag (EST) levels. SSRs can be classified as nuclear (nuSSR), mitochondrial (mtSSR) [17], or chloroplast SSRs (cpSSR) according to their location in the genome [18] (Table 1). Most genomic SSRs are nuclear SSRs. In sugarcane, ESTs led to significant advances in gene identification by providing biological proof of transcript isoforms, as well as predicted and newly discovered genes. ESTs are also useful in accessing the genetic information of species with complex genomes, including sugarcane [19]. Scientific reports have identified more than 5000 novel SSRs [20–22] using the SUCEST database (sugarcane ESTs; [19,23]). The conventional process of construction and screening of a genomic library is time-consuming, demanding, and costly, though after completion, the operating cost for these markers is practical [19]. Therefore, alternative approaches have been designed to reduce the time for SSR isolation and to substantially increase the number of SSR loci produced [24,25]. These methods include: (i) Next-generation sequencing or high throughput sequencing, (ii) EST libraries search, and (iii) enriched-genomic libraries search. Selected published studies on SSRs in sugarcane are listed in Table 2.

Table 1. Classification of microsatellites (Adapted from Kalia et al. [26]).

Classification	Motif
(A) Based on the number of nucleotides per repeats:	
Mono $(A)_{10}$	A
Di $(CT)_5$	CTCTCTCTCT
Tri $(CTG)_3$	CTGCTGCTG
Tetra $(CAGA)_4$	CAGACAGACAGACAGA
Penta $(AAATT)_5$	AAATTAAATTAAATT
Hexa $(CTTTAA)_6$	CTTTAACTTTAACTTTAA
(B) Based on the arrangement of nucleotides in the repeat motifs:	
Perfect repeat	CTCTCTCTCTCT
Compound repeat	CTCTCTCACACA
Imperfect repeat	CTCTCTACTCTCT
Region of cryptic simplicity	GTGTACACAGT
(C) Based on the location of SSRs in the genome:	
Nuclear (nuSSRs)	
Chloroplast (cpSSRs)	
Mitochondrial (mtSSRs)	

Table 2. Recent reports on the development of sugarcane SSRs through different techniques.

Method	Description	Reference
ESTs libraries	Development of 51 EST-SSRs from SUCEST database for the comparison of EST-SSRs with genomic SSR's to establish relationship among 18 sugarcane clones.	[21]
ESTs libraries	Development of 351 EST-SSRs from the 4085 non redundant EST sequences of two Indian sugarcane cultivars, out of which 227 were evaluated in sugarcane.	[27]
ESTs libraries	Oliveira designed 342 EST-SSR primer pairs, of which 224 amplified polymorphic bands in 18 sugarcane varieties.	[22]
ESTs libraries	A total of 2335 EST's functional microsatellite marker developed from the sugarcane sequence tag database (SUCEST) using bioinformatics tools.	[28]
ESTs libraries	Development of 722 EST-SSRs from the 8760 sucrose related EST sequences harvested from NCBI database and their application in sugarcane molecular breeding.	[29]
ESTs libraries	Development of 267 EST-SSRs markers through computational approach from 10,000 ESTs sequences for genetic diversity, cross-species transferability among *Poaceae* plants and bulk segregation analysis.	[30]
Enriched-genomic libraries	Development of 21 microsatellite markers from sugarcane (*Saccharum* spp.) ESTs and tested on 5 sugarcane clones and cross transferable to *Erianthus* and sorghum.	[24]
Enriched-genomic libraries	Development of informative genomic microsatellite markers from two hybrid sugarcane cultivars enriched with 18 different repeat-motifs for efficient genotyping applications in sugarcane.	[31]
Enriched-genomic libraries	Development of a total of 5675 microsatellite markers in autopolyploid sugarcane and comparative analysis of conserved microsatellites in sorghum and sugarcane	[32]
Enriched-genomic libraries	Development of 26 SSR's from a sugarcane interspecific hybrid of ISH 100 to study the population genetics structure across the species. SSR's primer pairs were assessed on 8 individuals sampled from one population.	[33]
Next-generation sequencing	A total of 1682 candidate loci were used to developed 174 primer pairs and validate on eight *Erianthus arundinaceus* accessions.	[34]
Next-generation sequencing	Illumina RNA-Seq platform were used on the de novo assembly of the sugarcane transcriptome, and a large number of molecular markers were found, including 5106 SSRs and 708,125 SNPs.	[35]
Next-generation sequencing	Development of 15 polymorphic SSR markers using 3730 XL Automated DNA Sequencer for 164 individuals of 18 populations of Chinese wild *E. arundinaceus.*	[36]

2.1. Next-Generation Sequencing or High Throughput Sequencing

Recent developments in plant science, particularly emerging genomic technologies, have the potential to increase the understanding of sugarcane genetics and breeding. Next-generation sequencing (NGS) technology contributed significantly to improving crop genetics and breeding [35]. In commercial programs, genotypes which are of interest to breeders, such as the parental genotypes of populations used for mapping, can be sequenced through NGS to generate genome or transcriptome data. This data can be further aligned with that of well-described model species, or of closely-related major crop species [37]. Sequencing large genomes is an expensive process, even with the use of NGS.

The primary resource for the study of sugarcane genetics is the considerable EST information available in public databases. Sugarcane transcriptome studies began in South Africa [38,39], and the largest EST collection to date (~238,000 ESTs) was developed through the Brazilian SUCEST project [19, 23]. This collection resulted in biotechnological improvements by vastly expanding the potential molecular markers and sequence information available for sugarcane breeding programs. Furthermore, Cardoso-Silva et al. [35] used the Illumina RNA-Seq platform for de novo assembly of the sugarcane transcriptome, and described a great number of novel markers, including 5106 SSRs and 708,125 SNPs. That study mentioned 5000 undescribed genes, representing more than half of the expected sugarcane genes that were absent from existing sugarcane databases. Zhang et al. [36] further developed 15 polymorphic SSR markers from the important germplasm resource Chinese wild sugarcane *Erianthus arundinaceus* using the 3730XL Automated DNA Sequencer.

2.2. Expressed Sequence Tag (EST) Libraries

Sugarcane libraries show a low level of enrichment, and the cost of developing plant SSR libraries is relatively high, making the importance of developing alternative methods readily apparent. Because EST-SSR databases (dbESTs) can identify markers directly associated with a particular trait, they are currently an important source of candidate genes [40]. The most complete dbEST for sugarcane is the sugarcane expressed sequence tag project (SUCEST), which contains 237,954 EST sequences in 43,141 clusters [23,41], which is 267 times more than currently available for *Saccharum* in the NCBI Expressed Sequence Tags database (http://ncbi.nlm.nih.gov/dbEST/). Starting from library construction, several disadvantages seem to be common, especially in species like sugarcane with large genomes [20]. Problems include the presence of one-side flanks in sequenced fragments, and the low efficacy and specificity of hybridization [31]. Pinto et al. [21] derived 51 EST–SSRs from SUCEST and compared these markers with 50 genomic SSRs (gSSR) based on their allele number and ability to establish genetic connection among 18 examined sugarcane clones. The majority of EST-SSR loci had four to six alleles and 15% had Polymorphism information content (PIC) values around 0.90, while gSSR loci had seven to nine alleles and 35% reached a PIC of 0.90. All known ESTs are collected in the sugarcane gene index (v 3.0), which contains 282,683 ESTs, 499 complete cDNA sequences, and 121,342 unique assembled sequences (uni-genes) [21].

Vicentini [42] found that there are still more than 10,000 sugarcane coding genes that have yet to be identified. This clearly indicates the extensive work required in order to develop the sugarcane transcriptome. Moreover, Silva [43] identified 420 SSRs using the SUCEST sugarcane database, and accomplished high levels of polymorphism with a set of 20 primers screened across multiple commercial cultivars and *Saccharum* species. Kushwah [29] used the publicly available non-enriched EST database and screened a new set of 722 EST-SSRs from the 8760 sucrose related EST sequences and designed primers.

2.3. Enriched Genomic Libraries

The latest developments in SSR techniques have increased the efficiency of SSR characterization for cultivars and species in which little to no previous sequence knowledge exists [33]. Such markers have been constructed through use of publicly available sugarcane ESTs and unigene sequences

corresponding to expressed genome components [20,31]. EST-derived SSR markers have demonstrated a low level of polymorphism and a high degree of cross-transferability for *Saccharum* [25]. SSR markers developed from genomic sequences show a higher level of polymorphism [21]. A few sugarcane genomic SSR markers (International Sugarcane Microsatellite Consortium, ISMC; http://www.scu.edu.au/research/cpcg) were established from the SSR enriched genomic library [24] and assessed for their utility in genomic analysis [20,44,45]. A set of SSR markers was generated from an enriched genomic DNA library constructed from Q124 (*Saccharum* hybrid) [24]. Sequencing of 798 genomic DNA clones from an enriched SSR library resulted in 457 inserts containing SSR repeat motifs in sugarcane. Parida et al. [31] developed sugarcane enriched genomic microsatellites (SEGMS), yielding 6318 clones from enriched genomic libraries of two sugarcane hybrid cultivars, which were further sequenced to produce 4.16 Mb high-quality sequences. SSRs identified in 1261 of the 5742 non-redundant clones accounted for 22% of the enrichment of the libraries. Parshant et al. [33] studied patterns of population genetic structure by developing SSR primers from the sugarcane interspecific hybrid ISH 100. Altogether, 26 SSR primers were selected, of which 13 (50%) exhibited polymorphism.

3. Detection Systems for SSRs

3.1. Capillary Electrophoresis

SSR-capillary electrophoresis/fluorescence detection (SSR-CE/FD) and fragment analysis by capillary electrophoresis are frequently used to overcome some of the aforementioned problems, and to identify higher numbers of polymorphic SSRs. These methods can develop a highly precise and effective SSR marker assay [5,13], even permitting a perfect and reproducible score for highly variable SSR loci. In contrast, other techniques, including lab gel electrophoresis techniques with silver or ethidium bromide staining, can cause allele miscalling due to stutters, primer dimers, and other PCR artifacts. The use of modern methods can significantly diminish the time and effort required. However, problems can occur during amplification and should therefore be identified before SSR-CE/FD data analysis (Figure 1).

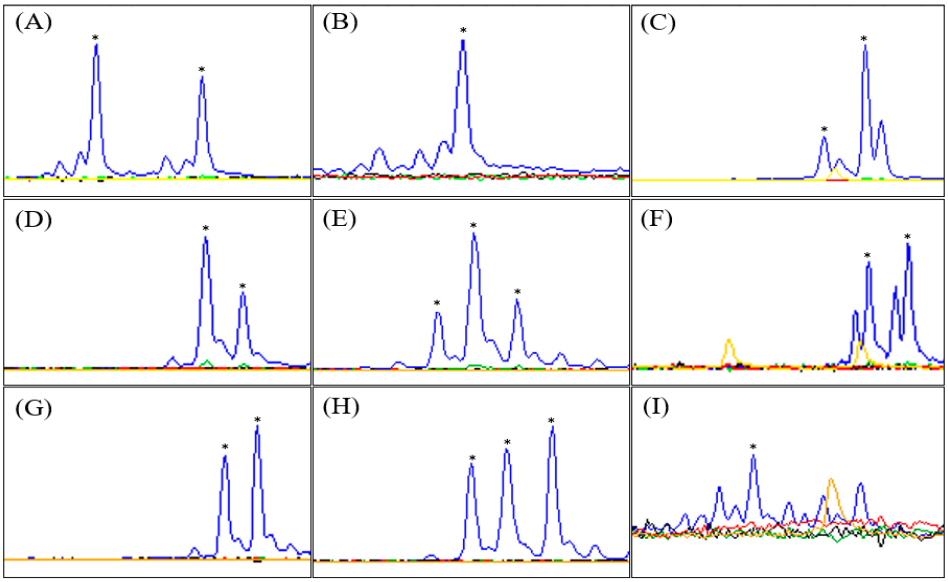

Figure 1. Figure 1. SSR profile generated through automatic capillary electrophoresis programming: Correct profile (**A**), excessive stuttering (**B**), weak alleles before (**C**), artefactual band (**D**), low heterozygosity peak (**E**), split peaks (**F**), null alleles (**G**), tri-allelic pattern (**H**), and noisy peaks (**I**). *, Correct alleles.

(1) Incorrect Alleles (Figure 1A): Both the amplification alleles should be considered correct for further data analysis.

(2) Excessive stuttering (Figure 1B): When PCR products that differ from the original template by one or more repeats are amplified, the interpretation of electrophoregrams is confounded. The resulting stutter bands are typically shorter than the original fragment due to a tendency toward contraction [46,47].

(3) Null alleles (Figure 1C,G): Non-amplifying alleles fail to appear when in the homozygote condition, which is typically interpreted as reaction failure, and they result in an apparent homozygote in the heterozygote condition [48]. Null alleles are formed by mutations in the flanking region at primer binding sites. Thus, when null alleles are present, the observed patterns of banding are not representative of the true range of genotypes.

(4) Primer-dimers, artefactual bands (Figure 1D), and tri-allelic patterns (Figure 1H): Each can be caused by the misplacing of primers [49,50]. Although these artifacts could be avoided during interpretation when they do not affect the calling of alleles, their presence may indicate a need for redesign to enable automatic interpretation of electrophoregrams.

(5) Low heterozygote peak height ratios (Figure 1E): These are indicated by very low amplification of the corresponding allele, and are the result of mutations in the flanking region at primer binding sites. Strategies for avoidance include tactics similar to those for null alleles.

(6) Split peaks (Figure 1F): These are characterized by the presence of an original fragment and an extra peak 1 bp longer corresponding to an adenylated fragment, and are triggered by addition of a single nucleotide (typically an adenine) to PCR fragments by Taq polymerase [51,52]. Incomplete adenylation results in double peaks, which compromises automatic peak recognition, especially for heterozygote genotypes with nearby alleles.

(7) Noisy peaks (Figure 1I): A collected signal can include high noise when an electrochemical detector is used for capillary electrophoresis (CE). Noisy peaks obscure analysis and negatively affect the accuracy of the results. Some chemo-metrics methods are used to eliminate the noise from the true peaks [53]. According to the description and complete protocol for capillary electrophoresis programming by Pan et al. (2003 and 2007) [14,44], an SSR allele was defined as a true, unique "Plus-adenine" DNA fragment that formed a measurable fluorescence peak during CE; while other DNA fragments that revealed measurable, but inconsistent, fluorescence peaks during CE as results of "dinosaur tails" or "Minus-adenine" are not scored (Figure 2).

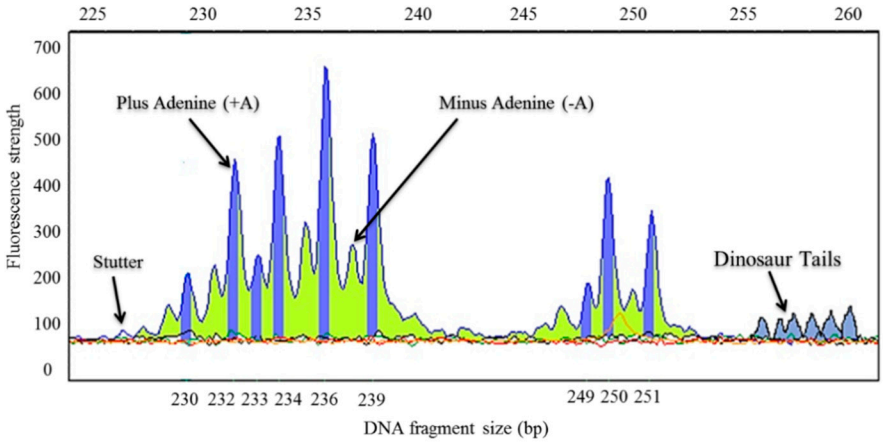

Figure 2. Several types of irregular and true peaks found in capillary electrophoregrams of sugarcane microsatellite DNA products: "Minus Adenine (−A)" low strength peaks (indicated by green colors) differ from "Plus-A" peaks by a single adenine nucleotide and can be omitted during scoring, followed by "Plus Adenine (+A)", the blue peaks, which represent true peaks and can be picked up. "Stutters" are peaks less than 1/3 of the height of the regular peaks that are larger in size by a single repeat unit; "Dinosaur tails" appear as one or several arrays of very low height peaks in a higher molecular weight zone. The Y-axis shows the fluorescence strength or yield of the amplified DNA fragment. The X-axis represents base pair sizes of DNA fragments.

3.2. Polyacrylamide Gel Electrophoresis

Capillary electrophoresis and fluorescent detection of SSR fragments (SSR-CE/FD) [54] is complex, time-consuming, and expensive. On the other hand, polyacrylamide gel electrophoresis with silver staining is an inexpensive alternative, originally defined for ultrasensitive detection of polypeptides. A detailed procedure and protocol of polyacrylamide gel electrophoresis (PAGE) and scoring of SSR-PAGE allele's data was described by Ahmad et al. [12]. Polyacrylamide gel shows high resolution as compared with agarose gel, but some non-specific alleles might also be detected in polyacrylamide gel. The presence of minor and non-specific products increases the difficulty of the identification of legitimate alleles; moreover, accurately calculating the size of an allele is difficult because of differences in migration between gel lanes, particularly when size markers are in the outer lanes. The sensitivity and accuracy quantified by limiting dilution are higher for CE as compared to PAGE [55].

4. Application of SSR markers

Although a variety of PCR-based markers are accessible for genetic analysis and plant breeding, SSRs are preferred for use in sugarcane [56], due to their co-dominance, high detection ability, abundance, and uniform distribution (Table 3). SSRs are highly conserved across closely related species of sugarcane, and this sequence conservation allows for successful amplification [45,57,58]. SSRs can give high levels of genetic information and are therefore useful in estimates of genetic variability and in genomic analysis across species. SSRs are more robust [12], variable, and informative than markers based on RFLPs, RAPDs, and AFLPs [6,8].

Records of pedigree and phenotypic traits from the sugarcane germplasm provide previous estimates of genetic diversity. More recent studies have shown that SSRs can be used for fingerprinting clones [44], mapping studies [59], and calculations of genetic diversity. Some recent published reports on the use of microsatellites in sugarcane breeding and genetics are listed in Table 4.

Table 3. Comparison of different types of molecular markers in plant applications.

	EST-SSRs	**Genic-SSRs**	**AFLPs**	**RFLPs**	**RAPDs/ISSRs**	**SNP**
Classification	PCR-Based	PCR-Based	PCR-Based	Hybridization based	PCR-Based	Sequence Based
Degree of polymorphism	Low	High	Low-Moderate	Low	Low-Moderate	High
Dominance	Co-dominant	Co-dominant	Dominant	Co-dominant	Dominant	Co-dominant
Interspecific transferability	High	Moderate	Low-Moderate	Moderate-High	Low-Moderate	High
Utility among commonly used markers	High	High	Low-Moderate	Moderate	Low-Moderate	Moderate-High
Production cost and labor involved	Low	High	Low-Moderate	High	Low-Moderate	Moderate
Application	Phylogeny, Gene mapping, Fingerprinting, Genetic diversity	Disease resistance, Genetic diversity, Starch contents, DNA fingerprinting	Genetic mapping and QTL linkage, QTL mapping, Paternity tests	Fingerprinting Genome mapping, Disease analysis, Genetic diversity,	Phylogeny, Genetic identity, Parentage, Clone strains identification, Gene mapping	Haplotype mapping, Linkage Disequilibrium, Disease and Trait association
Usage in plant species	Sugarcane, Rice, Maize, Tomato, Apple, Cereals	Rice, Sugarcane, Maize, Pineapple, Fruits crops	Palm, Sugarcane, Wheat, Rice, Fruits Crops	Wheat, Melon, Sugarcane, Barley, Potato	Broccoli, Mulberry, Cherry, Cucurbits, Sugarcane, Radish	Rice, Maize, Wheat, Sugarcane, Brassica rapa

Table 4. Application of SSR markers in sugarcane genetics and breeding.

Application	Description	Reference
Cultivar identification	A total of 84 clones were identified, including 58 commercial varieties, 17 clones in the final phase of the SASRI selection program for release and 9 imported varieties, using four SSR markers.	[60]
Cross-species transferability	Validation and polymorphism study of 227 EST-SSRs with 124 important Indian sugarcane cultivars for cross-species transferability and their utility in revealing population structure and genetic diversity.	[27]
Fingerprinting and genetic diversity	Establishment of the molecular identities (ID) of 91 nationally or provincially released Chinese sugarcane varieties and to evaluate the extent of genetic diversity among these varieties using SSR DNA markers and two fingerprinting systems.	[12]
Fingerprinting and genetic diversity	Use of 21 simple sequence repeat (SSR) markers for DNA fingerprinting and diversity analysis on 20 sugarcane (*Saccharum* spp) cultivars resistant and susceptible to red rot.	[61]
Genetic diversity	Fluorescence-labeled seven gSSR and eight EST-SSR markers were used for the genetic diversity among 181 sugarcane genotypes.	[62]
Genetic diversity	A total of 84 genotypes from the *Saccharum barberi*, *S. spontaneum*, *S. officinarum*, Indian and non-Indian commercial cultivars were evaluated for the genetic diversity using seven sugarcane cDNA derived SSRs, nine gSSRs and 16 uni-gene SSRs markers.	[63]
Genetic variability	Eighteen sugarcane genotypes including 13 active cultivars and five elite QT-series clones bred locally were screened for genetic variability with 21 SSR primer pairs.	[64]
Genome mapping	Genetic variability of 42 *E. arundinaceus* accessions native to Japan based on nuclear DNA content and 31 simple sequence repeat (SSR) markers	[34]
Genome mapping	Low cost single strand conformation polymorphism of 16 genomic and 12 EST-SSRs marker and its utility in genetic evaluation of 22 sugarcane genotypes.	[28]
Paternity analysis	Paternity analysis of 76 sugarcane progenies from four crosses was analyzed using three highly polymorphic SSR markers.	[65]
QTL mapping	Identification of quantitative trait loci controlling sucrose content based on an enriched genetic linkage map of sugarcane (*Saccharum* spp. hybrids) cultivar 'LCP 85–384 using 65 microsatellites markers.	[66]
QTL mapping	Identification of marker-trait associations for morphological descriptors and yield component traits in sugarcane using 174 SSRs in a panel of 92 sugarcane varieties from sub-tropical India.	[67]
Segregation analysis	Investigation of SSR marker segregation among 964 single pollens and 288 self-progenies (S_1) of sugarcane cultivar LCP 85–384.	[68]
Segregation analysis	Segregation of a multiallelic sugarcane SSR marker SMC336BS among single pollens of a sugarcane cultivar L99–233 as well as its F_1 progenies of a bi-parental cross between HoCP 00–950 (female) and L 99–233 (male).	[69]
SSR-assisted identification	Identification of the crosses of sugarcane (*Saccharum* spp.) × an intergeneric hybrid (*Erianthus arundinaceus* × *Saccharum spontaneum*) and their progenies by SSR and sequence-related amplified polymorphism (SRAP) molecular markers.	[70]
SSR-assisted identification	Identification of 57 progenies of sugarcane × *Narenga porphyrocoma* (Hance) Bor. by nine pairs of SSR primers.	[71]

4.1. Cultivars Identification

Because of sugarcane's genomic complexity, the development and identification of increased numbers of SSR markers is needed to distinguish between accessions, clones, and cultivars [22]. After a slow start, some high-throughput SSRs were developed and are now commonly used for these applications [25]. SSR markers are useful in screening of sugarcane cultivars and in pedigree analysis because they represent a single locus [72,73]. Because they represent multiple alleles, these markers are reliable across a wide range of genetic sources [74,75].

With the advent of capillary electrophoresis CE-based sugarcane SSR genotyping, breeders of sugarcane are able to accurately control the genetic characteristics of their sugarcane varieties and advanced breeding lines, and identify any sugarcane clone that may be mis-labeled [14,44]. Pan [5] constructed a local SSR identity database containing SSR-based molecular information for 1004 sugarcane clones. These included 237 clones from 2005, 238 from 2006, 339 from 2007, and 190 from 2008. Oliveira et al. [22] identified 224 EST-SSR primer pairs, which amplified polymorphic bands in 18 sugarcane varieties. Pan et al. [14] ensured the genetic identity of 116 Louisiana commercial sugarcane clones with 21 SSR markers using capillary electrophoresis programming. Ahmad et al. [12] established molecular identities (ID) of 91 Chinese sugarcane cultivars using SSR DNA markers and two fingerprinting systems, CE and PAGE, which detected 151 and 117 SSR alleles, respectively.

4.2. Genetic Diversity/Phylogenetic Relationship

SSR markers are commonly used in sugarcane to measure genetic variation at the molecular level [76]. Genetic diversity was previously identified based on pedigree records and phenotypic traits [59]. However, the ability of phenotypic traits to reveal the true level of diversity within the sugarcane germplasm is limited by the substantial effect of environmental factors [57]. The assessment of genetic diversity and the construction of phylogenetic relationships in sugarcane will give essential data for classification of plant germplasm accessions and breeding program selections [11,62]. Moreover, this information will be beneficial for classification of accessions in sugarcane plant germplasm collections and in studies of taxonomy [56]. You et al. [62] evaluated genetic diversity among 181 clones by using seven gSSR and eight EST-SSR primer pairs together with a capillary electrophoresis genotyping platform. Nayak et al. [73] screened out 36 SSR markers on 1002 genotypes of sugarcane and related grasses, and recorded 209 alleles for important agronomic traits. Sharma et al. [74] measured genetic diversity across 40 sugarcane genotypes and their parents by using 26 SSR primers. Tena et al. [75] used 22 SSR markers to amplify a total of 260 alleles in a study of introduced sugarcane accessions in Ethiopia to determine population relationships and differentiation. Fu et al. [64] employed 18 sugarcane genotypes, including 13 active cultivars and five elite QT-series clones, to determine genetic variability with 21 SSR primer pairs.

4.3. Genome Mapping

Modern sugarcane breeding primarily aims to increase sugar yield, biomass, and abiotic stress resistance [77]. Molecular markers allow the development of genome maps that facilitate understanding of genetic structure. They could also be used to identify genes and QTL associated with traits of agricultural importance in order to assist in MAS [78,79]. Linkage and partial genetic maps have been constructed for *S. spontaneum* [80–82], *S. officinarum* [83,84], and for some modern sugarcane cultivars [66,85–87]. These maps were created for species using full-sib (F_1) individuals (i.e., the pseudo-test cross strategy) [88–91]. Yet, existing studies are limited by the complex genome, and describe only the most observable, primarily quantitative agronomic traits, resulting in identification of typically small effect markers [84,92].

Gouy et al. [77] conducted genome wide association (GWA) mapping of 183 sugarcane accessions, genotyped with 3327 AFLP, DArT, and SSR markers and phenotypes for 13 traits related to morphology, yield, bagasse residue content, and resistance to disease. Siraree et al. [67] used 1546 marker loci produced by 174 SSR primers and phenotypic characterization for 32 traits to complete a panel of 92 sugarcane varieties from sub-tropical areas in India. Edmé et al. [58] developed a map of *S. spontaneum/S. officinarum* using 193 SSR loci, though genome coverage in these maps is until now incomplete. Few maps have yet been constructed using selfing-derived populations. Aitken et al. [45] constructed a map of an Australian cultivar Q165 from a segregating F_1 population, by using 72 SSR primers of double-dose and repulsion phase linkage, which resulted in 127 of the 136 LGs falling into eight homo(eo)logy groups (HG).

4.4. Quantitative Trait Loci (QTL) Analysis

QTL mapping typically employs populations resulting from a bi-parental cross [93]. In sugarcane, SSRs can be used to analyze QTLs that can contribute to the identification of candidate genes for traits of agronomic interest, including sugar yield, biotic and abiotic resistance, and overall quality [58,64,94]. Mapping and stability of QTLs is made difficult because many genetic and external factors influence the expression of the final phenotype, and studies therefore must include a high number of segregating genotypes to ensure precision [58,95].

Ming et al. [94] identified 36 QTLs that affected sucrose content in two interspecific *Saccharum* F_1 populations. In this study, 102 associations were found between six agronomic traits and DNA markers. Liu et al. [66] identified 24 putative QTL markers using an enriched genetic linkage map of Louisiana sugarcane cultivar LCP 85–384, two of which were simplex SSR markers (SMC545MS_147e and SEGM302_251). Hoarau et al. [95] detected 40 putative quantitative trait alleles (QTAs) in sugarcane that were associated with plant traits of interest. Previous QTL studies on sucrose content in sugarcane have used variable sources, including populations derived from crosses with *S. spontaneum* [89,94], commercial cultivars [87], and those from self-pollination of a clone [45,95]. Nunes et al. [96] used both DArT (Diversity arrays technology) and SSR markers on two sugarcane populations, including 81 genotypes from selfing (RB97327), resulting in 392 DArT and 57 SSR polymorphic markers, and 91 sugarcane genotypes from outcrossing (RB97327 × RB72454), which gave 632 DArT and 79 SSR polymorphic markers. Gutierrez et al. [97] assembled a genetic linkage map by selective genotyping of 89 pseudo F_2 progenies of a cross between resistant "LCP 85–384" and susceptible "L 99-226" using 1948 single dose (SD) markers produced from SSR, eSSR, and SNPs. Out of these, 1437 single dose markers were mapped onto 294 linkage groups. In addition, they covered 19,464 cM with 120 and 138 LGs consigned to the resistant and susceptible parent, respectively.

4.5. Paternity Analysis

Although plant breeders and researchers commonly use morphology to identify cultivars and hybrids, these traits can be heavily influenced by the environment and across developmental stages [72,98]. Genetic recombination in breeding and other studies has been accomplished using both bi-parental and poly-crosses [35,99]. In non-consolidated active germplasm banks, poly-crosses are commonly adopted due to the high numerical involvement of the parent population. These parents are tested for their potential identity in order to categorize males and females [100].

Compared to the bi-parental approach, poly-crosses are easier to obtain and are more cost effective. However, a major limitation is the loss of pedigree information of the obtained progenies [98], for which only the female parent is known. However, molecular markers permit the identification of the male parent, allowing pedigree reconstruction [5,77,101]. Xavier et al. [100] used these techniques to identify the male parent of 41 elite clones from poly-cross families. In this study, SSR primer pairs identified the most likely male parent by determination of markers present in the clone but absent in the female parent. Tew and Pan [98] used seven highly polymorphic SSR markers to identify 87 selfing progenies from poly-crosses which were genotyped along with the parents. This study produced 51 polymorphic SSR alleles, of which 15 were parent-specific. Similarly, Santos et al. [65] analyzed the paternity of seventy-six progenies using three highly polymorphic SSR markers from bi-parental crosses to identify progeny from pollen of unknown origin.

4.6. Cross-Species Transferability

EST-SSRs are considered to have increased inter-specific transferability over that of genomic-SSRs in sugarcane, because they arise from transcribed regions and are highly conserved in homologues [48,77]. They have thus proven useful for comparison studies, including genomics and mapping among species, as well as for studies of evolutionary patterns [27,102]. In sugarcane, these markers show

elevated levels of polymorphism, including in commercial cultivars, progenitors, and representatives of *Erianthus* and *Sorghum* [94,102].

Beyond their higher transferability, EST-SSR markers are preferred over gSSR markers for breeding and genetic programs because of their superiority in gene tagging [77]. Singh et al. [27] used 27 unigene-derived sugarcane microsatellite (UGSM) primers to estimate cross transferability among 19 accessions, including cultivars and hybrids of sugarcane, as well as related and divergent species and genera. Their study found a high level of polymorphism (96.3%) among these samples, and cross-transferability of 98.0% and 88.27% within the *Saccharum* complex and across cereals, respectively. Ul haq et al. [30] procured 10,000 ESTs across 20 plants, and used a computational method to evaluate 267 EST-SSRs and synthesize 63 EST-SSRs. Among those, expansion genetics allowed identification of 42 markers amplifying 519 alleles. Selvi et al. [103] used a set of 34 SSR primer pairs from maize (*Zea mays*) and found repeatable amplifications in 14 *Saccharum* clones, hybrids, and the related genus *Erianthus*, equaling 41.17% cross transferability. Singh et al. [27] established 351 EST-SSRs in two Indian sugarcane cultivars based on 4085 non-redundant EST sequences, and of these, 227 were evaluated in sugarcane, related species, and cereals. Cross transferability in these groups ranged from 87.0–93.4%, 80.0–87.0%, and 76.0–80.0%, respectively. Cordeiro et al. [25] used 21 SSR markers, of which 17 were polymorphic and proved cross-transferable to genera including *Erianthus* and *Sorghum*.

4.7. Segregation Analysis

Commercial sugarcane cultivars are heterozygous aneupolyploid or interspecific hybrids, which initially arose from the breeding of several *Saccharum* species with *S. officinarum* [104]. This narrow genetic base could restrict advancement in sugarcane breeding programs. A interspecific hybridization strategy used in the early 1900s introgessed genes and traits of choice from wild relatives into sugarcane cultivars to increase the genetic variability, resulting in significant improvements in sugarcane breeding and genetics [105]. For this introgression, plants of the F_1 and first backcross (BC_1) generations receive diploid gametes from a female *S. officinarum*, and haploid gametes from male *S. spontaneum*. Individuals in the second backcross generation (BC_2) receive haploid gametes from both parents [106].

Sugarcane breeders classify cross-progenies into three groups based on cultivar-specific SSRs [11,107]. (1) The Hybrid (H) group progeny possess no non-parental alleles, and show at least one male-specific SSR; (2) the Off-type (O) group possess at least one non-parental allele; and (3) the Self/Hybrid (S/H) group progeny possess at least one female-specific allele. Identification of H- or O- progeny is straightforward, but identifying the H- progeny within the S/H group is difficult without the possibility of segregation analysis of SSR markers.

Pan et al. [69] addressed this type of question by investigating the segregation of SMC336BS, a multi-allelic sugarcane SSR marker in 92 single pollen grains of the sugarcane cultivar L99–233, and 162 F_1 progenies from a bi-parental cross between L 99–233 (male) and HoCP 00–950 (female). In the pollen grains, 22 genotypes were detected ranging from 1.08–11.83% frequency, and among the F_1 progenies 33 genotypes were detected at 0.62–8.64% frequency. Edmé et al. [58] investigated the *S. spontaneum* (IND, $2n \approx 7x \approx 56$) x *Saccharum officinarum* (Green German, $2n \approx 11x \approx 110$) interspecific cross, resulting in F_1 progeny from 169 full-sibs and 193 SSR loci. Of these loci, 78% segregated in a Mendelian pattern, while the remaining 22% were distorted. Lu et al. [68] collected 964 single pollen grains and 288 self-progenies (S_1) of sugarcane cultivar LCP 85–384, and used capillary electrophoresis- and fluorescence-based DNA fingerprinting with PCR to examine segregation. Of the 20 SSR alleles described, eleven (55%) segregated in a Mendelian manner, while six segregated in 3:1 ratios, and were thus indicative of simplex markers.

4.8. SSR-Assisted Identification of Wild Cross Hybrids

Morphology cannot be used to identify sugarcane hybrids: true hybrids are often indistinguishable from plants resulting from self-progeny or pollen impurity [108]. SSRs have been used in sugarcane and other species to distinguish true hybrids [109], and can detect outcrossing events and thus estimate rates of selfing [110]. Genus-specific Alu-like sequences can distinguish between hybrids including *Saccharum* × *Erianthus* and *Saccharum* × *Miscanthus* [111]. 5S rDNA markers can identify hybrids of *E. arundinaceus* and *S. officinarum* [108,112]. In recent studies, genus-specific SSRs could even recognize backcross progeny of a fertile *S. officinarum* × *E. arundinaceus* F_1 individual [109]. In all cases studied with genome in situ hybridization, chromosome eradication was observed in all hybrids [108].

In order to widen the currently limited genetic base of sugarcane, Gao et al. [70] combined genes from the wild germplasm by making crosses of *Saccharum* spp. × *Erianthus arundinaceus* × *S. spontaneum*. Progeny from this cross of sugarcane and the intergeneric hybrid were identified with SSR and sequence-related amplified polymorphism (SRAP) markers. A study by Cai et al. [109] used identified true *Saccharum* spp. BC_1 progeny by using SSRs with 5S rDNA PCR to screen both the F_1 clones from crosses of *Saccharum officinarum* × *E. arundinaceus*, and the BC_1 populations from crosses between F_1 clones and sugarcane (*Saccharum* spp.). Liu et al. [113] used three pairs of SSR primers and five pairs of SRAP markers to examine the hybrid GXAS07-6-1 resulting from the cross of *Erianthus arundinacius* (Retz.) Jesws. × *Saccharum spontaneum* L., and found that GXAS07-6-1 was a true hybrid. Liu et al. [71] used nine pairs of SSR primers for identification of 57 progenies of *Saccharum* spp. × *Narenga porphyrocoma* (Hance) Bor. Of these, 14 progenies showed the parental bands and were thus identified as genuine hybrids.

5. Concluding Remarks and Future Perspectives

SSR markers are widely used for measurement of the diversity of the sugarcane germplasm resource and commercial varieties. Their particular advantages include the low cost of assays and equipment, and high levels of throughput and ease of use. As discussed above, different levels of throughput are available for use in sugarcane. Therefore, a suitable marker system can be designated according to need.

In recent years, the acceptance and use of SSR-based markers has increased significantly in studies and breeding of sugarcane. Advances in SSR marker application made possible the direct prediction of phylogenetic relationships and genetic variability across sugarcane genotypes without use of faulty pedigree records or the confounding influence of environmental factors. For these reasons SSR markers have been developed, isolated, and characterized across a wide range of plant species, including crop species like cereals, legumes, vegetables, fruits, and spice and beverage crops, as well as other economically and ecologically important species, such as conifers and forest trees. SSR markers are additionally utilized for extensive genetic analyses, including paternity analysis, hybrid testing, studies of genetic diversity, and eco-evo analyses. They are an integral part of fundamental research, including genome and QTL analysis, gene mapping, and MAS. The type of molecular markers used for a particular study must be established based on genetic factors, including mode of inheritance, procedural considerations including complexity, and economic considerations such as time and cost.

Current sugarcane research continually produces prodigious amounts of DNA sequence data, which requires map-based and sequencing tools to cross-reference genes and genomes. Because sugarcane presents a limitation for analysis of sugarcane, SSRs are an invaluable tool, especially for comparative mapping of genomes, as demonstrated in graminoids and legumes. Future research will benefit from a public database of sugarcane EST-SSR primer pairs that enable amplification of loci that are orthologous across species and genera. Ideally, these would be uniformly dispersed across the genomes of sugarcane, maize, rice (*Oryza sativa*), tall fescue (*Festuca arundinacea*), sorghum, and arabidopsis genomes, and would prove beneficial to geneticists and breeders. Furthermore, identification of allele-specific SSRs for genes central to agronomic traits will advance plant breeding

molecular technology. Thus, SSRs provide a valuable addition to SNPs as molecular marker systems which identify functional gene polymorphisms.

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