Abstract: Trait tagging through molecular markers is an important molecular breeding tool for crop improvement. SSR markers encoded by functionally relevant parts of a genome are well suited for this task because they may be directly related to traits. However, a limited number of these markers are known for *Musa* spp. Here, we report 35136 novel functionally relevant SSR markers (FRSMs). Among these, 17,561, 15,373 and 16,286 FRSMs were mapped in-silico to the genomes of *Musa acuminata*, *M. balbisiana* and *M. schizocarpa*, respectively. A set of 273 markers was validated using eight accessions of *Musa* spp., from which 259 markers (95%) produced a PCR product of the expected size and 203 (74%) were polymorphic. In-silico comparative mapping of FRSMs onto *Musa* and related species indicated sequence-based orthology and synteny relationships among the chromosomes of *Musa* and other plant species. Fifteen FRSMs were used to estimate the phylogenetic relationships among 50 banana accessions, and the results revealed that all banana accessions group into two major clusters according to their genomic background. Here, we report the first large-scale development and characterization of functionally relevant *Musa* SSR markers. We demonstrate their utility for germplasm characterization, genetic diversity studies, and comparative mapping in *Musa* spp. and other monocot species. The sequences for these novel markers are freely available via a searchable web interface called Musa Marker Database.

Keywords: *Musa* spp.; comparative mapping; functional domain; SSR markers

1. Introduction

Banana is one of the most consumed and commercially important fruit crops. Approximately 90% of the world’s bananas are produced in tropical and sub-tropical regions. Banana fruit significantly contributes to the export revenue and food security of these regions, and other banana plant parts are
used locally for food and fibre. The annual global banana production in 2017–2019 was 116 million
tons with an approximate value of USD 31 billion [1]. Most of the cultivated banana varieties are
triploid, while some are diploid and have either a *Musa acuminata* (A) genome or are hybrids of the
*Musa acuminata* and *Musa balbisiana* (B) genomes. The different levels of ploidy and chromosome
numbers of banana have led to complexity in taxonomy [2] and are associated with parthenocarpy,
leading to female sterility, seedless fruit and non-viable seeds. As a result, cultivated bananas are mainly
reproduced asexually, with a consequent narrow genetic base. Genomic abundance, co-dominant
inheritance, assay simplicity, and hyper variability make microsatellite markers (simple sequence
repeats or “SSRs”) highly useful for genetic studies. The SSR markers located within protein-coding and
the associated untranslated regions (UTRs) of genes may be directly linked with traits. Consequently,
such functionally relevant SSR markers (FRSMs) can be useful to rapidly build marker–trait linkages
and to discover genes/quantitative trait loci (QTLs) related to traits of agronomic importance in crop
plants [3–6]. A number of studies have shown that functionally associated molecular markers are more
powerful than anonymous markers for marker-assisted selection, marker-trait association [7], genetic
diversity [4], comparative mapping [3] and the construction of transcript maps [8], and as anchor
markers for evolutionary studies in plant species [3–5,8]. For banana, several hundred SSR markers
have been developed from EST sequences [9–12], from BAC end sequences [13,14] and from A and B
genome sequences [15]. However, only a small proportion of these markers have been experimentally
validated and used for genotyping in banana. For example, Christelova et al. [16] used 19 SSR marker
for genotyped 695 banana accessions. In addition, many of these banana SSR markers are not publicly
available, and their chromosomal location, degree of polymorphism and functional characteristics
have not been estimated which has limited the application of banana SSR markers in genotyping,
association studies and fine mapping of important agronomic traits in banana breeding. Due to these
limitations, there is a need for a well-curated SSR marker database based on information from in-silico
data mining, including the genomic or chromosomal positions of SSRs, functional association and gene
affinity, together with information based on experimental validation of SSRs, such as polymorphisms,
transferability and allelic variations. This resource can accelerate various applications of genomics,
genetics and breeding in bananas.

The application of molecular markers to banana breeding programs has included RAPD [17–19]
(randomly amplified polymorphic DNA), ISSR [20–23] (inter simple sequence repeat), AFLP [24]
(amplified fragment length polymorphism), DArT [25] (Diversity Arrays Technology), SSR [15] and
SNP [26] (Single Nucleotide Polymorphism) and ASSP (Single Amino Acid Polymorphism) [27–30].
A recent review of the application of various molecular marker techniques in banana breeding showed
RAPD, ISSR and AFLP marker techniques to be generally less effective compared to the SSR markers,
due to low reproducibility, less allelic variation within single loci, and lack of co-dominance [31].
More recently developed NGS technology-based markers including RADseq, DARTseq and GBS
provide high genome coverage and accuracy for genetic analysis compared to SSR markers. However,
NGS approaches come at higher costs and require expertise for downstream application, while SSR
genotyping is relatively simple assay and reasonable. An SSR genotyping approach is also able to
systematically add new information to existing data sets. Moreover, SSR multi-allelic while SNP
bi-allelic, so SSRs are more likely to detect polymorphism. As a result, banana breeders as well as other
plant breeders still rely on SSR markers.

A large number of the transcript sequences from *Musa* spp. that are publicly available have not yet
been mined for SSR markers. Recently, four whole genome sequences of the banana have been
published and are available in the public domain. The total length of the *Musa acuminata* genome
(A genome) sequence assembly [27,28] is 439 Mb representing 84% of the estimated size (523 Mb) of the
DH-Pahang genome, which is distributed among 11 chromosomes. Similarly, a high quality 430 Mb
(87%) draft genome of *M. balbisiana* [29] (B genome) was assembled into 11 chromosomes. The 525 Mb
draft genome of *M. schizocarpa* [30] (S genome) was anchored to 11 groups, while the 462.1 Mb draft
genome assembly of *M. itinerans* [31] covers 75.2% of the 615.2 Mb genome. The availability of these
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Genome assemblies facilitates the extraction of geneic regions and the development and in-silico mapping of SSR markers. Transcriptome sequences are another valuable resource for identifying transcription factor (TF)-encoding gene products and TF-functional domains that are useful for developing functionally relevant molecular markers. The use of transcript sequences to identify SSR markers associated with TF-derived genes or functional domains has been reported in only a few plant species, including sugarcane [3], chickpea [32], tomato and pepper [33] but not previously for Musa spp.

In view of the above, the present study was conducted to (i) develop functionally relevant novel SSR markers from Musa transcript sequences and identify their loci, (ii) assess the cross-taxon transferability of developed markers, (iii) estimate the usefulness of functionally relevant Musa SSR markers for comparative mapping, (iv) evaluate the potential of these SSR markers for large-scale genotyping applications in Musa spp., (v) apply the SSRs in genetic diversity and population structure analysis of Musa germplasm and (vi) develop a searchable freely accessible SSR marker database.

2. Materials and Methods

2.1. Plant Material Collection and DNA Extraction

Eight genotypes (‘Prata’, ‘Kluai namwa khom’, ‘FHIA21’, ‘Dwarf Cavendish’, ‘FHIA 17’, ‘BITA2’, ‘BGY3’, ‘FHIA-03’) that represent major groups of banana and plantain (details of these listed in Supplementary Table S15) were used for the preliminary selection and transferability analysis of FRSMs (functionally relevant SSR markers). To estimate genetic diversity, population structure and phylogenetic relationships among Musa and related species (Ensete sp.), a total of 50 accessions were used. All the plant materials were collected from the core collection of Musa germplasm, maintained at FTRI, Guangzhou, PR China. Total genomic DNA was extracted from young fresh leaf samples using the CTAB method as previously described by Gawel and Jarret [34] with minor modifications.

2.2. Data Processing, SSR Mining, Marker Development, In-Silico Characterization, Physical Mapping and Functional Annotation

A total of ~0.1 Gb EST sequences of Musa spp. were acquired from public databases (EST Tool Kit and NCBI). All transcript sequences were combined into a single FASTA file with the aid of an in-house Perl script. After combining the sequences, the est_trimmer.pl (http://pgrc.ipk-gatersleben.de/misa/download/est_trimmer.pl) script was used to remove low-quality sequences, poly A/T and low-complexity regions at the 5’ and 3’ ends. The remaining high-quality sequences were assembled with CAP3 (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::cap3) with default parameters. Then mRNA sequences were extracted from genome assembly of recently published 4 musa genomes (A, B, S and I genomes) using perl script. SSR-containing mRNA (transcripts) and EST sequences were harvested using modified MISA script, then all the SSR-containing sequences were clustered with CDHIT using default parameters. The assembled non-redundant transcript sequences were searched for microsatellites using MISA (micro satellite identification tool, http://pgrc.ipk-gatersleben.de/misa/), restricting the output to perfect mono-, di-, tri-, tetra-, penta- hexa-, hepta-, octa-, nano- and deca-nucleotide motifs with a minimum of 10, 6, 5, 5, 5, 5, 5, 4, 4, 4 and 4 repeat units, respectively. Identified SSRs were characterized based on (i) the length of repeat motifs (Class I > 20 bp, Class II ≤ 20 bp) and (ii) the nucleotide composition of repeat motifs (AT-rich, GC-rich and AT-GC balance). Forward and reverse primers for the identified SSRs were designed with primer3 software using default parameters. A custom Perl script called duplicate marker finder (http://mmdb.genomicsres.org/mumdb_Download.html) was used to remove redundant primer sets from the primer database. Further, non-redundant primer set compare with all the published Musa SSR markers and overlap markers were removed from the database. If more than one set of primers was generated for the same transcript sequence, one primer set was chosen randomly for further analysis. SSR-containing transcripts were analysed with the ORF (open reading frame) finder Perl script using default parameters to predict the longest ORF within the
transcript. Finally, the localization of SSR motifs in either UTR or CDS/intron regions was determined by comparing the ORF position with the SSR position within the transcript sequence. The genome assembly of *M. acuminata* (DH Pahang), *M. balbisiana* (Pisang Klutuk Wulung -PKW), *M. schizocarpa* and *M. itinerans* were downloaded from Musa Genome Hub (https://banana-genome-hub.southgreen.fr/species-list), and FRSMs were mapped onto the eleven chromosomes of these genome assemblies with the ePCR strategy. ePCR results were verified by BLAST searches of the markers against the whole-genomic sequences. Marker positions on the chromosomes were recorded and added to the database. A physical map was drawn using MapChart [35] software (Version 2.2). The specific in-silico-generated amplicons from these four genomes were compared with the expected amplicon size of each marker, and size differences were recorded. If an amplicon size differed by at least 2 bp, the SSRs were classified as polymorphic; otherwise, amplicons were considered monomorphic.

The putative function of each FRSM was determined by Blast2GO analyses. Flanking regions (200 bp up and 200 bp downstream) of the FRSM loci were searched against the non-redundant protein database in GenBank using BLASTX [36] with an E-value threshold of 10\(^{-10}\). Based on the functional annotation results, FRSMs were classified according to three GO terms: biological process, molecular function and cellular process [37-39]. The KEGG database (http://www.genome.jp/kegg/pathway.html) was used to annotate the pathways for these FRSMs. To identify transcription factor (TF)-associated and TF-functional domain-associated FRSM markers, we conducted a BLAST search of SSRs against the plant TF database (http://planttfdb.cbi.pku.edu.cn/) with an E-value threshold of 0.001 and a query coverage of 65%.

2.3. In-Silico Comparative Genome Mapping

The flanking sequences of FRSM loci were searched with BLAST against the genome sequences of *M. acuminata*, *M. balbisiana*, *M. schizocarpa*, sorghum, foxtail millet and rice to obtain marker-based syntenic relationships among the species. A bit score threshold of 54.7 and E-value threshold of 10\(^{-5}\) were considered as significant for this BLAST analysis. To check the presences of SSRs in the orthologous region of the target species, we chose the flanking sequences from orthologous region of the target species, found the SSRs using the MISA script and compared the repeat patterns and lengths with their corresponding FRSM loci. The marker-based syntenic relationships were finally visualized with Circos 0.55 (http://circos.ca).

2.4. Evaluation of PCR Amplification and Genetic Marker Potential

To assess the amplification efficiency of the in-silico-developed FRSM primer, a subset of 273 primer pairs was selected based on the in-silico results including, transferability, polymorphism, known gene functions and the physical distance between two primer sets should be 1.5 to 2 mb. The primers were synthesized by Sangon Biotech Co., Ltd., Shanghai, China; PCR amplifications were carried out for eight *Musa* accessions (Supplementary Table S15) representing diverse genomic groups of the core collection of the *Musa* germplasm, maintained at FTRI, Guangzhou, PR China. The FRSM primers displaying clear and reproducible amplicons in a gel-based assay were selected for the genotyping of 50 accessions of the *Musa* germplasm collection (Supplementary Table S15). PCR amplification was carried out in 25 μL containing the following: 50 ng of genomic DNA, 1 μL of each primer pair (concentration, 10 mM), 12.5 μL of PCR-Mix (Takara), 1.0 U of Taq DNA polymerase enzyme and 8.5 μL of dH₂O. PCR amplification was carried out in a MJ-PTC-200 tm thermal controller (MJ Research, Waltham Mass) using the following programme: 94 °C for 3 min, 35 cycles at 94 °C for 30 s, 55–60 °C (according to the primer annealing temperature) for 30 s, 72 °C for 45 s, followed by a final step at 72 °C for 7 min. The amplified PCR products were resolved on a 3% agarose gel, and the band size was measured with the aid of a 100 bp DNA ladder. PCR products of selected primer pairs were resolved on a denaturing 6% polyacrylamide gel, and bands were visualized by silver staining.
2.5. Data Collection and Statistical Analysis

The PCR amplification result was recorded for each marker. The SSR bands of all genotypes were scored as band present (1) or absent (0). Allele frequencies were used to calculate the polymorphism information content (PIC) using the following formula: 

$$PIC = \sum p_i^2$$

where $p_i$ is the proportion of the $i$th allele. PIC values and allele frequencies were calculated using Power Marker software. The NEI72 module (genetic distance data module) of the NTSYSpc software package (version 2.1 [40]) was used to estimate genetic similarity among genotypes. Based on the NEI72 similarity matrix, UPGMA dendrograms were constructed using the SAHN module. The FIND module was employed to identify all trees that could result from tied similarity values. Mantel test statistics were used to assess the reliability of the clustering by comparing the cophenetic matrix [40] and the similarity matrix. The effective number of alleles ($ne$), Shannon’s information index ($I$), expected homozygosity ($Ho$), expected heterozygosity ($He$), Nei’s expected heterozygosity, Wright’s fixation index ($Fis$) and the Ewens-Watterson Test for Neutrality were calculated using Popgene software [41].

2.6. Population Structure

The population structure of 50 banana accessions was estimated using STRUCTURE 2.3.4 software [42]. The admixture model was used with a burn-in period length of 10,000 and 100,000 MCMC iterations. Five independent runs were performed for each $k$ from 1 to 10. The best value of $k$ was estimated based on the Delta $k$ method using Structure Harvester software [43]. Barplots of the Q matrix were drawn using DISTRUCT software [44]. Principal coordinate analysis (PCoA) for population separation was carried out using the dissimilarity matrix data of each individual (genotype) and using GenAlEx software [45].

2.7. Functional SSR Marker Database

In order to maximize the utility and ensure the availability of developed novel functional Musa SSR markers, here we developed a searchable database using HTML, CSS, JavaScript, PHP and MySQL based coding language. PHP script was used to bridge the search interface and database. JavaScript, PHP and HTML use for results visualization, and download in XLS or CSV format. First all the marker information (29 attributes) were organized in a table with a unique marker id. Then all these markers with their corresponding attributes stored in a mySQL database. The database and database search interface hosted in the published web site called Musa marker database (http://mmdb.genomicsres.org/index.html).

3. Results and Discussion

3.1. FRSM Marker Development, In-Silico Characterization, Chromosomal Distribution, Physical Mapping and Genome Coverage

A total of 68,716 SSR-containing transcript sequences were extracted using MISA (with perfect mono-, di-, tri-, tetra-, penta- hexa-, hepta-, octa-, nano- or deca-nucleotide motifs with a minimum of 10, 6, 5, 5, 5, 4, 4, 4 and 4 repeat units, respectively) from 222,124 transcripts sequences that were originally obtained from four published Musa genome sequences and ~0.1 Gb EST sequences (Supplementary Table S1). Subsequently those SSR-containing transcripts were assembled into 52,453 unigene sequences and searched for simple sequence repeats (SSRs) to design functionally relevant SSR markers for Musa. The type and frequency distribution of SSR motifs are presented in Table 1. The proportion of SSR-bearing Musa unigenes was 31% (68,716 out of 222,124), which is higher than those reported for coffee, cassava, and cereals [46–49] and lower than those reported for iris and sugarcane [3,50]. The frequency of SSRs in Musa unigenes was one per 2.2 kb, which is higher than that reported for sugarcane transcript SSRs (ISSR/10.9 kb) [3], but similar to reports for rice (ISSR/3.6 kb), sorghum (ISSR/5.9 kb) and barley (ISSR/8.9 kb) [51,52]. The proportion of SSR-bearing unigenes was much higher in this study compared to earlier observations based on EST sequences of Musa [53]. This difference may be due to the variation in search criteria, size of data sets and
software tools used in each study, as suggested previously for similar comparisons [5,54–59]. Out of 51,814 SSR-bearing unigenes, 30,780 (59.4%) contained more than one SSR motif. Among the different repeat units, di-nucleotide (51.1%), mono-nucleotide (31.5%) and tri-nucleotide (14.6%) repeat motifs were more frequent than other repeat motifs. In addition, tri-nucleotide repeats were more frequent in CDS (coding sequences) regions (see Supplementary Table S2). The same trend was also reported for Musa EST-SSRs and SSRs in cereals and legumes [51,60]. The high abundance of tri-nucleotide repeat motifs in the coding region of most genomes can be explained by the fact that length-altering mutations do not change the reading frame. Based on the repeat motif length, 59.6% SSRs were characterized as Class II type (12–20 bp), and the remaining 40.4% were classified as Class I type (>20 bp). Backiyaran et al. [9] also found more class II SSRs than class I SSRs in Musa EST sequences. From 113,480 identified SSRs (di to hexa repeat SSR use for marker development), reverse and forward primers could be designed for 80,899 (71.3%) SSRs. For the remaining 28.7%, primer design failed due to insufficient flanking regions or due to insufficient sequencing quality in the genomic region of interest. Similar results have been reported for many other plant species [57,61,62]. Primer redundancy is a major problem in SSR primer design projects, especially if a large number of primers are designed for a large set of sequences [63]. Primer redundancy may be the consequence of chromosomal or gene duplications. Alternatively, some of the EST sequences possess multiple SSR tracts, in which case multiple primer sets might be generated from the same flanking sequences. To filter redundant and multiple sets of primers from the same flanking sequences, a Perl script called Duplicate Marker Finder (http://mmdb.genomicsres.org/mumdb_Download.html) was used to reduce the SSR primer set number to 66,246. In order to develop novel SSR markers, non-redundant markers were compiled with the published Musa SSR markers and 35,136 (43.4%) novel Musa SSR markers were found. These primer pairs were then characterized by both in-silico and wet lab methods. Out of the 35,136 primer pairs, 1104 (1.7%) were characterized as transcription factor-associated primers (Table 1).

| Characters under Study                      | Unigenes |
|--------------------------------------------|-----------|
| Total number of sequences examined         | 52,453    |
| Total size of examined sequences (bp)      | 359,005,912 |
| Total number of identified SSRs             | 166,503   |
| Number of SSR-containing sequences         | 51,814    |
| Number of sequences containing > 1 SSR     | 30,780    |
| Number of SSRs present in compound form    | 47,796    |
| SSR density (1 SSR per bp)                 | 2156      |
| Class II SSRs (12–20 nucleotides)         | 99,231    |
| Class I SSRs (>20 nucleotides)             | 67,272    |
| AT rich SSRs                               | 112,614   |
| GC rich SSRs                               | 17,073    |
| AT/GC balance                              | 36,616    |
| Mononucleotide repeats                     | 52,440    |
| Dinucleotide repeats                       | 85,090    |
| Trinucleotide repeats                      | 24,330    |
| Tetranucleotide repeats                    | 2884      |
| Pentanucleotide repeats                    | 413       |
| Hexanucleotide repeats                     | 763       |
| Heptanucleotide repeats                    | 180       |
| Octanucleotide repeats                     | 238       |
| Nanonucleotide repeats                     | 28        |
| Decanucleotide repeats                     | 137       |
| No. of primers designed                    | 80,899    |
| Non-redundant primers                      | 66,246    |
| Novel SSR primer identified                | 35,136    |
| TF-associated SSRs                         | 1104      |
| Transferable to other Musa sp.              | 18,209    |
| Genome specific                            | 16,927    |
| In-silico polymorphic                      | 8605      |
| No. of SSRs in CDS                         | 13,223    |
| No. of SSRs in 5’ UTR                       | 11,718    |
| No. of SSRs in 3’ UTR                       | 10,099    |

Table 1. Distribution of microsatellites in transcript sequences and functionally relevant SSR marker development.
Among the TF-associated FRSM, 11%, 8% and 7% were related to the transcriptome factor families ERF, NAC and C2H2 (Figure 1). In total, 13,223 (37.6%) primers were found to originate from CDS, 11,718 (33.4%) from 5' UTR and 10,099 (28.7%) from 3' UTR regions. Expansion or contraction of SSR repeats in the 5' UTR region of a gene may affect transcription and/or translation, while SSR repeat unit variation in the 3' UTR region may be responsible for gene silencing or transcriptional slippage. SSR repeat polymorphisms in CDS regions may lead to the activation or deactivation of gene function by truncating or extending proteins [5]. Therefore, SSR markers derived from different regions (CDS, 5'UTR and 3'UTR) of genes may have different characteristics. Scott et al. [64] observed polymorphism differences among SSR markers derived from different regions of genes in the grape genome. The 5' UTR-derived SSR markers are mostly polymorphic between cultivar and species, while the 3' UTR-derived SSR markers are polymorphic at the cultivar level and CDS-derived SSR markers are polymorphic between species and genera. In terms of polymorphism detection, 3'-UTR-derived SSR markers are superior to those derived from 5'-UTRs [4] for cultivar identification and genetic diversity analysis. In view of the association of SSR from different gene regions with variation at different taxonomic levels, from previous studies [5,64], we propose that SSR markers derived from different regions of Musa genes should be used for different applications in banana breeding programmes; for example, 3'UTR-derived Musa SSR markers may be most suitable to distinguish banana cultivars.

Figure 1. Functional annotation of transcript sequences carrying microsatellites in their functional domains. A total of 1104 SSR-bearing transcript sequences were associated with functional domains of TF.
The genomic distribution and physical localization of the 35,136 SSR markers on the A, B and S genomes of banana are presented in Table 2 and in Supplementary Figures S1–S3. A total of 17,561 (50.0%), 15,373 (43.8%) and 16,286 (46.4%) markers mapped on the eleven chromosomes of the genomes of *M. acuminata* (A genome), *M. balbisiana* (B genome) and *M. schizocarpa* (S genome), respectively. All physically mapped markers are available in the *Musa* Marker Database (http://mmdb.genomicsres.org/mumdb_Download.html). The chromosome-wide distribution and frequency of the physically mapped markers revealed the highest frequency on chromosome 4 for the A and S genomes (2203 markers (6.3%) for the A genome; 2080 markers (5.9%) for the S genome), while for the B genome, the highest number of markers mapped on chromosome 8 and the lowest frequency on chromosome 1 (1176 markers (3.3%) for the A genome; 1227 markers (3.5%) for the S genome). The highest and lowest marker densities were found on chromosome 4 and chromosome 9, respectively, in both the A and S genomes. The physical map constructed in this study is highly dense compared to the physical map reported for foxtail millet [62]. The high-density SSR marker-based physical map constructed in this study will be helpful for the selection of suitable genome-wide SSR markers across the eleven chromosomes for various applications in improved banana breeding programmes, including large-scale genotyping, comparative genome mapping, QTL analysis and population genetic studies.

### Table 2. Summary of chromosomal distribution and average density of SSR markers mapped on the genomes of *M. acuminata* (A genome), *M. balbisiana* (B genome) and *M. schizocarpa* (S genome).

| Genome | No of Markers Mapped | Density (1 Primer/Mb) |
|--------|----------------------|-----------------------|
|        | A (%) | B (%) | S (%) | A (%) | B (%) | S (%) |
| Chr1   | 1176  | 3.3   | 1227  | 3.5   | 1098  | 3.1   | 24.72 | 17.96  | 32.80  |
| Chr2   | 1215  | 3.5   | 1276  | 3.6   | 1151  | 3.3   | 24.29 | 13.60  | 32.35  |
| Chr3   | 1731  | 4.9   | 1155  | 3.3   | 1639  | 4.7   | 20.23 | 20.92  | 27.33  |
| Chr4   | 2203  | 6.3   | 1001  | 2.8   | 2080  | 5.9   | 16.84 | 24.63  | 23.67  |
| Chr5   | 1566  | 4.5   | 1512  | 4.3   | 1478  | 4.2   | 26.73 | 15.64  | 35.19  |
| Chr6   | 1982  | 5.6   | 1547  | 4.4   | 1649  | 4.7   | 18.97 | 17.99  | 26.06  |
| Chr7   | 1464  | 4.2   | 1276  | 3.6   | 1377  | 3.9   | 23.93 | 17.41  | 34.13  |
| Chr8   | 1824  | 5.2   | 1567  | 4.5   | 1696  | 4.8   | 24.61 | 17.66  | 32.35  |
| Chr9   | 1486  | 4.2   | 1232  | 3.5   | 1428  | 4.1   | 27.80 | 21.02  | 36.93  |
| Chr10  | 1498  | 4.3   | 1564  | 4.5   | 1443  | 4.1   | 25.15 | 16.13  | 30.01  |
| Chr11  | 1258  | 3.6   | 1204  | 3.4   | 1170  | 3.3   | 22.22 | 17.21  | 31.42  |
| ChrUn  | 158   | 0.4   | 812   | 2.3   | 77    | 0.2   |       |        |        |
| Total Mapped | 17,561 | 50.0 | 15,373 | 43.8 | 16,286 | 46.4 |        |        |        |
| Unmapped | 17,575 | 50.0 | 19,763 | 56.2 | 18,850 | 53.6 |        |        |        |

3.2. Functional Annotation and Association of Transcription Factor

To identify the functional significance of the 35,136 novel microsatellites markers, a BLASTX analysis was performed against the non-redundant protein database using the Blas2Go tools. A total of 14,312 (41%) were functionally annotated due to their high similarity (e value < e$^{-10}$) with at least one protein in the non-redundant protein database (Figure S4a). The remaining 20,824 (59%) did not show a significant similarity to known protein sequences in the databases and therefore were not annotated. According to the Gene Ontology (GO) scheme, functionally annotated FRSMs were classified into three categories: cellular components (CC), molecular function (MF) and biological process (BP) (Figure S4e–g). The sum of these FRSMs per category did not add up to 100% because some FRSMs were classified into more than one category. A total of 12,120 FRSMs were annotated with 44,279 annotations for the three main GO categories, while 3891FRSMs were annotated with all three GO categories and 4148 were assigned at least two GO annotation categories (Figure S4d, Tables S3 and S4). Among the three categories, BP annotations were most frequent, followed by CC and MF (Figure S4 and Table S4). SSR-bearing transcript with CC annotations were mainly annotated as intracellular organelle (26%), cytoplasm (22%), membrane (21%) and cell periphery (5%) (Figure
S4e,h). Under the MF category, most of the SSR-bearing transcripts were annotated as organic cyclic compound binding (26%), followed by heterocyclic compound binding (26%), transferase activity (16%), and hydrolase activity (13%) (Figure S4f). Among the SSR-bearing transcripts with BP annotations, annotations suggested that the proteins are involved in primary metabolic process (20%), nitrogen compound metabolic process (17%), cellular metabolic process (16%) and biosynthetic process (11%) (Figure S4g,h).

3.3. In-Silico Comparative Genomic Mapping between Musa and Non-Musa spp.

Comparative mapping of the FRSMs among the three Musa genomes is illustrated in Figure 2a–d and revealed that 5938 (16.9%) of the FRSMs were common among the four Musa genomes, while only 11 (0.03%) were found to be common in the genomes of four Musa and four non-Musa species (Figure 2a,b). A total of 17,403 FRSMs physically mapped onto the eleven chromosomes of M. acuminata. We compared their physical location on the Musa genome with their location on the chromosomes of the related monocot plant genomes of foxtail millet, rice and sorghum (Figure 2c,d, Table 3). In-silico comparative genomic mapping showed a considerable proportion of sequence-based orthology and syntenic relationships, with SSR markers distributed over eleven Musa chromosomes with the chromosomes of foxtail millet (~1%, 240), rice (~1%, 264) and sorghum (~2%, 268) (Figure 2, Supplementary Table S5–S7). Many of these markers show syntenic relationships with more than one chromosome of foxtail millet, sorghum and rice. This result hints at segmental duplication events among the chromosomes of the respective genomes, similar to the observations reported by Pandey et al. [62], that many of the physically mapped foxtail millet SSR markers have syntenic relationships with more than one chromosome of sorghum, maize and rice.

Table 3. A summary of functionally related microsatellite marker-based comparative mapping showing maximum syntenic relationships of the chromosomes of Musa acuminata with those of foxtail millet, rice and sorghum.

| Musa Chromosomes | Foxtail Millet Chromosomes | Rice Chromosomes | Sorghum Chromosomes |
|------------------|---------------------------|-----------------|--------------------|
| Chr 01           | F03 (10; 42%)             | R01 (13; 42%)   | S01 (11; 41%)      |
| Chr 02           | F04 (3; 23%)              | R01 (5; 25%)    | S01 (6; 29%)       |
| Chr 03           | F09 (4; 16%)              | R01 (5; 16%)    | S02 (9; 27%)       |
| Chr 04           | F03 (9; 27%)              | R01 (13; 46%)   | S02 (7; 21%)       |
| Chr 05           | F02 (6; 24%)              | R01 (5; 25%)    | S06 (4; 20%)       |
| Chr 06           | F08 (5; 22%)              | R03 (6; 20%)    | S02 (6; 21%)       |
| Chr 07           | F02 (5; 28%)              | R01 (6; 30%)    | S03 (5; 24%)       |
| Chr 08           | F04 (6; 19%)              | R01 (8; 27%)    | S01 (6; 23%)       |
| Chr 09           | F04 (3; 21%)              | R07 (4; 16%)    | S01 (3; 18%)       |
| Chr 10           | F04 (6; 33%)              | R02 (4; 24%)    | S01 (5; 19%)       |
| Chr 11           | F09 (5; 33%)              | R01 (3; 25%)    | S04 (4; 27%)       |

Note: % represent the number of Musa microsatellite markers mapped on the chromosome of related plant species.

In-silico comparative mapping between Musa and foxtail millet genomes demonstrated a syntenic relationship of 240 SSR marker loci distributed on eleven chromosomes of the Musa genome with 480 genomic regions on 9 chromosomes of the foxtail millet genome (Figure 2d; Supplementary Table S5). The higher frequency of SSR marker-based syntenic relationships with foxtail millet and Musa was (10; 42%) found between Foxtail millet chromosome 3 and Musa chromosome 1 (Table 3). A total of 264 SSR marker loci that physically mapped onto the eleven chromosomes of Musa showed synteny with the 12 chromosomes of rice with an average frequency of 9%, which is lower than what we found for the foxtail millet and sorghum chromosomes (Figure 2d; Supplementary Table S6). The 264 Musa SSR marker loci showed significant matches with 436 genomic regions of 12 rice chromosomes. Overall, rice chromosome 1 showed a high frequency of syntenic relationships with most of the Musa chromosomes, except Chromosomes 1, 4 and 6. The syntenic relationship between the Musa and
sorghum genomes revealed that 268 SSR marker loci have significant matches with 656 genomic regions on 10 chromosomes of sorghum. Maximum syntenic relations were found between *Musa* chromosome 1 and sorghum chromosome 1 (11, 41%) (Figure 3; Supplementary Table S7).

**Figure 2.** Functionally related microsatellite marker-based genomic relationships of Musa with other plant species. (a) Venn diagram representing the common FRSM markers among the three Musa genomes. (b) Venn diagram representing the common (transferable) FRSM markers among the Musa and non-musa genomes. (c) FRSM-based syntenic relationship among the three *Musa* genomes. (d) FRSM-based syntenic relationship among the *Musa* and non-*Musa* genomes.

In-silico mapping often retains the orthologous region of the target species without SSR regions, and this phenomenon may limit the applicability of SSR markers in other species, especially distant relatives. Therefore, we checked all orthologous regions from the target species for the presence of SSR repeats. Our results showed that 83.6% (for *M. acuminata*), 93.2% (for *M. balbisiana*) and 91.9% (*M. schizocarpa*) of the homologs (Table S8) may have either the same or shorter SSR lengths than the reference SSR motifs. However, a small percent of the orthologous regions of the foxtail, rice and sorghum genomes contain SSR motifs. These findings indicated that homologous regions may exist in the targeted species, but some of them lacked SSR motifs.
well as between foxtail millet and maize. As foxtail millet, sorghum and maize species belong to the
same subfamily, *Panicoideae*, these are more closely related than the species compared in our current
study. The similarities found among the genomes are in line with their evolutionary distances and
conform to the expectation that syntenic relationships among plant species decrease with increasing
phylogenetic distance [62,66]. Our findings show that microsatellite marker-based comparative genome
mapping of *Musa* and other plant species (monocots) provides a first picture of genome conservation
among Musa, foxtail millet, rice and sorghum. Furthermore, these comparative maps will be useful in
map-based gene cloning from Musa, which is agronomically important for obtaining marker-based
genotyping information from other related plant species.

3.4. PCR Amplification Efficiency, Polymorphism, Transferability and Genetic Marker Potential

A sub-set of 273 selected FRSMs was tested for PCR amplification efficiency, polymorphism,
transferability and genetic marker potential (Figure 3; Supplementary Table S9). Among these markers,
259 produced a clear, reproducible PCR amplification product of the expected size. A total of 249 (91%) markers
produced single bands, whereas 24 markers had more than one band. Furthermore, 36 markers
produced either longer or shorter fragments than the expected size. Overall, 203 (74%) of the markers
showed polymorphism in the wet lab (PCR) assay among eight accessions of *Musa*, and a total of

![Figure 3. Polymorphic and monomorphic marker distribution among different classes based on in-silico and wet lab analyses.](image-url)
715 different alleles were detected among the eight accessions. The number of alleles per marker ranged from 2 to 12 (Supplementary Table S9 and Figure S5), with an average of 3.5 alleles. Trinucleotide repeat motif-containing markers showed the highest percentage of polymorphism both from in-silico and wet lab assays (Figure 3). It should be noted that the proportions of markers found to be monomorphic and polymorphic in the in-silico and in the wet lab assays differed substantially, most likely because a larger number of accessions (Supplementary Table S10) were used for the PCR experiments, while genomic sequences were not available for all accessions for the in-silico polymorphism assay. Therefore, only three (A, B and S genomes) available genomes were used for the in-silico polymorphism assay.

Transferability to closely related species is one of the most important potential features of SSR markers, offering the opportunity to use markers developed from one species to be applied to multiple species. This is particularly useful if little is known about the genomic sequence of the species of interest. Transferable markers are a good source for detecting orthologous loci between two species and are useful for incorporation into genetic maps. To estimate the cross-species transferability of the markers developed in this study, a set of 273 markers were analysed for the ability to amplify genomic DNA of eight banana species representing different genomic groups (Supplementary Table S10). A total of 194 (71%) markers produced amplicons in at least three species among the eight Musa species. From the literature, it was found that few markers are transferable to less closely related species; for example, on average, 25% of the Centella asiatica EST-SSR markers were shown to be transferable to the family Apiaceae, while 40% of the barley EST-SSR markers were found to be transferable to rice [46,69]. Higher levels of SSR marker transferability have been reported for other plant species, including citrus, millet and sugarcane [57,62,66]. The transferability of the SSR markers between species or families greatly relies on the evolutionary distance between the species [66]. In this study, we tested the SSR marker transferability between Musa species (M. acuminata and M. balbisiana and between hybrids of the two) while the above-mentioned studies by Thiel et al. [46], Sahu et al. [69] examined marker transferability at the genus and family levels. Consequently, our marker transferability rate is expected to be higher than that found by Thiel et al. [46] and Sahu et al. [69]. The high transferability rate of FRSMs between banana species supports their utility in comparative mapping studies and for the identification of markers associated with important agronomic traits.

3.5. Assessment of Genetic Diversity and Population Structure

One of the most important applications of SSR markers is the assessment of genetic diversity within natural populations, among core collections of germplasm and among breeding lines. Such genetic variation information is vital for efficient conservation and for the use of genetic resources for crop improvement programmes. Consequently, a core set of 50 Musa accessions and 15 FRSMs was used to determine the effectiveness of the selected FRSMs for the assessment of genetic diversity in Musa spp. In total, 49 alleles were identified in 15 loci with an average of 3.3 alleles per locus (Supplementary Table S11). The PIC (polymorphic information content) values for individual loci ranged from 0.26 to 0.66 with a mean of 0.50 and the effective number of alleles per locus varied from 2.02 to 3.44. The Shannon information index (I) ranged from 0.74 to 1.44, with a mean of 0.94. The average PIC value found in this study was comparable with that reported for SSR markers in citrus species by Biswas et al. [57] and higher than that reported for SSR markers in Musa by de Jesus et al. [70]. As PIC values are greatly influenced by the number of genotypes and genetic background of the genotypes, this explains the discrepancy between the current study and the findings reported by de Jesus et al. [70], which used different sets of germplasm. The average observed and expected heterozygosity were 0.71 and 0.62, respectively (Supplementary Table S12). The Nei’s average genetic diversity [71] ranged from 0.51 to 0.71, with an average of 0.62. The fixation index ($F_{IS}$) was found to be positive for four loci (C01P3A00134, A2M000167, AB2M006866 and NovelTSSR001634) and negative for the remaining eleven loci (Supplementary Table S12). In this collection of loci, the majority were homozygous in the tested population and thus should be informative for population genetic studies in banana. The overall mean value of the fixation index was 0.17, revealing an excess of heterozygosity present in
the studied population. Several factors can result in an excess of heterozygosity in plant populations, including a wide range of cross-compatibility and polyploidy and a high proportion of loci under selection and allele-scoring biases. Most likely, the excess heterozygosity observed in this study is due to the polyploidy of the banana accessions studied, which are representative of commonly grown cultivars. Since population genetics applications require selectively neutral markers to avoid misleading results [72], neutrality tests have been advocated, for example by Kim et al. [73]. Therefore, in this study, we performed the Ewens-Watterson test for the neutrality of 15 markers and observed frequency values ranging from 0.291 to 0.494 (Supplementary Table S13). At all loci, the mean expected homozygosity value was higher than the observed value.

The clustering of subpopulation based on k-value (Figure 4a), UPGMA tree (4b) and admixture model base population structure was assed to test the strength of the FRSMs. Phylogeney analysis showed that 50 banana accessions (Figure 4b) clustered into two major groups according to their genomic background, with cluster II including all genotypes with only the A genome, while the AB genomic composition genotypes grouped in cluster I. According to the genome composition and ploidy level, cluster I and cluster II were further divided into two and five sub-groups, respectively. Most of the accessions of sub-group 1 were diploids and triploids. Sub-group 2 was composed of diploids and triploids; while sub-group 3 included most of the accessions from the Musa sub-group Mutika/Lujugira and in this sub-group the members were diploid, triploid and tetraploid. In sub-group4, most of the members were triploid Cavendish banana. As expected Ensete sp. was located in a distinct clade outside of the Musa species. Cluster analysis clearly showed that diploid A accessions share sub-clusters with other triploid and tetraploid accessions of the A genomic group. Similar results were also reported by earlier studies [74]. These observations may suggest that the variation within subspecies of M. acuminata is complex and that cultivars of M. acuminata have a wide partition from their fertile diploids rather than their cultivated AAA relatives. In this study we noticed that most of studied genotypes were different from each other with few exceptions. For example, we cannot distinguish genotype “Nyamwihogora” and “Intokatoke”, both are triploid and composed with A genome. Our SSR marker was unable to differentiate “Igitsiri” and “Ingagara”. We were also unable to distinguish two cavendish cultivars “Dwarf Parfitt” and “Formosana”. This finding is due to the genomic similarity of these genotypes. Such as triploid A genome type cultivars like cavendish are known to be less diverse cultivars. Different types of molecular markers such as PCR-RFLP, SSR and ITS failed to distinguish completely all the triploid A genome type cultivar [74]. Considering our findings and previous evidence, we recommended SNP or DART types of molecular markers may be suitable for distinguishing triploid A genome type cultivars of banana. To verify the adjustment between similarity matrices and respective dendrogram-derived matrices, we estimated the cophenetic correlation coefficient (r), and the significance of the correlation was determined by the Mantel matrix correspondence test. The correlation coefficient was statistically significant (Supplementary Table S14), demonstrating that the FRSMs are capable of distinguishing the A and AB genotypes of Musa spp.

A Bayesian clustering model-based analysis estimated the distribution of 49 alleles at 15 SSR loci among 50 accessions of banana. The number of subpopulations (k) was estimated to range between 2 and 10. To identify the approximate number of subpopulations, we estimated the maximum value of the logarithm of likelihood (LnP(k)). However, we did not find the LnP(k) value to achieve a clear plateau, and the value continued to increase together with the variances between the tested k. Under these conditions, the k value was anticipated to be between 5 and 7 (Figure 4a). We noticed that there was no longer variation for the population grouping at the k values of 5, 6 and 7 (Figure 4a). In this situation, the highest peaked value of k is more suitable to estimate the number of subpopulations than the lowest peak value [75]. This approach fit well to identify the sub-population in banana [70]. Therefore, we adopted this approach for identifying the number of sub-populations, and the highest k value (k = 7) was chosen for 50 banana accessions distributed in six subpopulations. This subpopulation clustering clarifies the underlying genetic structure among the studied banana accessions. A Principal Coordinates analysis (PCoA) estimate of the efficiency of tested FRSMs grouped the 50 banana accessions into
two major categories according to their genome composition (Supplementary Figure S6). Therefore, PCoA analyses are also able to differentiate banana accessions according to the main genotypes. Phylogenetic clustering methods for detecting genomic compositions were also reported for bananas by Ning et al. [74]. Our STRUCTURE analysis demonstrated clear separation of the 50 banana germplasm accessions according to their genomic background and provided further evidence of the utility of our FRSMs for the characterization of banana germplasm.

Altogether, we were able to show that FRSMs are promising markers for successful large-scale genotyping analyses, germplasm characterization, genetic diversity, and population structure studies in *Musa.*

![Figure 4. Phylogenetic relationship and population structure of 50 Musa accessions using 15 FRSMs; (a) plot of delta K values for different numbers of population assumed; (b) UPGMA dendrograms constructed based on the NEI72 similarity matrix; (c) sub-populations represented by distinct colours. Each column represents each accession (the name of the accession are presented in Table S15) that can be fractionated into segments, and the whole size has the potential to estimate membership fractions (q) in k clusters. The genomic background of each accession was based on morphological descriptions recorded in the Musa germplasm database.](image-url)
3.6. Novel Functional Musa SSR (NFMS) Marker Database Developments

All the developed (35,136) novel functional Musa SSR markers with 29 features have been placed in the freely searchable marker database. This marker database contains eight flexible search fields including SSR Type, SSR Class, SSR Motif rich with, SSR Polymorphism, SSR Transferable to other plant species and SSR associated with gene function (Figure 5a–c). The search results display as a list of markers with Marker ID, forward- and reverse-primer sequences, and the sequence id, with links to additional information including all the 29 features such as transferability, polymorphism, genome position, flanking sequences and functional annotations. Query results can be downloaded in XLS and CSV file formats for subsequent use.

Figure 5. The flow of a database search in NFMS. (a) marker search interface (b) query result (c) details information of each marker.

Identifying the most suitable SSR markers from a large data set is both challenging and time consuming. The availability of a well-structured and searchable SSR marker database facilitates the selection of desirable SSR markers for breeders, as well as reducing the time, cost and labour. Hence, we built the banana SSR marker database to store and to share the novel functional Musa SSR markers identified by our study. SSR marker databases have been developed for several plant species including rice [76], maize, sorghum, soybean [77], tomato [78], chickpea [79], pigeon pea [80], and also previously for banana [81]. The existing online genomic resources for Banana including the Banana Genome Hub (http://banana-genome-hub.southgreen.fr/home1) (BGH), TropGENE Database (http://tropgenedb.cirad.fr/tropgene/JSP/index.jsp) and BanSatDb database (http://webtom.cabgrid.res.in/bansatdb/index.php) also contain molecular marker information. Among these three databases, BGH and TropGENE store only Musa SNP markers, while BanSatDb was developed for Musa SSR markers. BanSatDb allows the design SSR primers from the genomes of three Musa species: *M. acuminata*, *M. balbisiana*, and *M. itinerans*, but not for *M. schizocarpa*. There are some other important limitations of the BanSatDb database including the very limited and inflexible search parameters:
It does not permit a user to develop primers from the genome; it cannot provide the primer redundancy, transferability, or information on polymorphism to the other Musa and non Musa species; and it does not provide SSR motif information in the designed primers. Overall, there is no unique identifier for the designed primers which can affect reproducibility in downstream application of the markers by the same or different researchers. The NFMS data base, by comparison, is more user friendly, flexible and contains a much larger volume of information for each developed marker: Each marker entry has 29 different features to assist users to easily select the most suitable marker pair from their query result. Users are able to download the entire search result in a single XLS or CVS format file within a single click. These unique features, the volume of the information and the quality of data in the NFMS database provide a user friendly, unique and versatile novel Musa marker database.

4. Conclusions

Here, we present the first set of functional SSR markers (FRSMs) developed for Musa spp. together with a freely searchable database. A large number of FRSMs were mapped onto the Musa A, B and S genomes. The markers were characterized by both in-silico and wet lab techniques, and their utility for genetic diversity studies was estimated. Our results demonstrate the utility of FRSMs for large-scale banana germplasm characterization, comparative mapping and genomic-associated studies among Musa and non-Musa spp.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/12/1479/s1, Figure S1: Physical map of M. acuminata genome specific FRSMs. A genomic distribution of 2871 functionally relevant SSRs marker on the eleven chromosomes of M. acuminata. Left bar represents chromosome length (Mb). Figure S2: Physical map of M. balbisiana genome specific FRSMs. A genomic distribution of 2754 functionally relevant SSR markers on the eleven chromosomes of M. balbisiana. Left bar represents chromosome length (Mb). Figure S3: Physical map of M. schizocarpa genome specific FRSMs. A genomic distribution of 2591 functionally relevant SSR markers on the eleven chromosomes of M. schizocarpa. Left bar represents chromosome length (Mb). Figure S4: Functional annotation and GO classification of FRSM markers. Distribution of FRSM markers among three GO categories. Top 20 GO terms of the GO Distribution, Figure S5: Allelic variation represent by FRSM markers (Marker: A2M000167 and AB2M002966). Len 1 to 50 represent the genotypes (Name of the genotype and genomic composition listed in Table S15) and M = 100 bp Marker. Figure S6: Principal coordinate analysis (PCoA) of 50 individuals based on genotypic information from 49 alleles obtained from 15 SSR markers. Here Cluster I represent AB genomic composition genotypes and Cluster II represent genotypes with only the A genome. Table S1: Summary of transcript sequences assembly, Table S2: Distribution of different types of SSR within UTR and CDS (%), Table S3: FRSMs annotated by GO category, Table S4: Distribution of unique FRSMs among three GO categories, Table S5: Comparative mapping between M. acuminata (A genome) and Foxtail millet, Table S6: Comparative mapping between M. acuminata (A genome) and Rice, Table S7: Comparative mapping between M. acuminata (A genome) genome and Sorghum, Table S8: Percentages of the orthologous regions from the targeted species having SSR motifs, Table S9: Wet lab assay summary, Table S10: Percent transferability of FRSM from different Musa spp., Table S11: Summary of genic variation statistics for all Loci, Table S12: Summary of heterozygosity statistics for all Loci, Table S13: The Ewens-Watterson test for neutrality, Table S14: 2-way Mantel (1967) method test results., Table S15: List of accessions used in this study.

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References
1. FAO. Banana Market Review; FAO: Rome, Italy, 2020; p. 7.
2. D’Hont, A.; Paget-Goy, A.; Escoute, J.; Carreel, F. The interspecific genome structure of cultivated banana, Musa spp. revealed by genomic DNA in situ hybridization. Theor. Appl. Genet. 2000, 100, 177–183. [CrossRef]
3. Parida, S.; Pandit, A.; Gaikwad, K.; Sharma, T.; Srivastava, P.; Singh, N.; Mohapatra, T. Functionally relevant microsatellites in sugarcane unigenes. BMC Plant Biol. 2010, 10, 251. [CrossRef] [PubMed]
4. Parida, S.; Dalal, V.; Singh, A.; Singh, N.; Mohapatra, T. Genic non-coding microsatellites in the rice genome: Characterization, marker design and use in assessing genetic and evolutionary relationships among domesticated groups. BMC Plant Biol. 2009, 10, 140. [CrossRef] [PubMed]
5. Varshney, R.K.; Graner, A.; Sorrells, M.E. Genic microsatellite markers in plants: Features and applications. Trends Biotechnol. 2005, 23, 48–55. [CrossRef]
6. Li, Y.-C.; Korol, A.B.; Fahima, T.; Nevo, E. Microsatellites within genes: Structure, function, and evolution. Mol. Biol. Evol. 2004, 21, 991–1007. [CrossRef]
7. Ramalingam, J.; Savitha, P.; Alagarasan, G.; Saraswathi, R.; Chandrababu, R. Functional marker assisted improvement of stable cytoplasmic male sterile lines of rice for bacterial blight resistance. Front. Plant Sci. 2017, 8. [CrossRef]
8. Gujaria, N.; Kumar, A.; Dauthal, P.; Dubey, A.; Hiremath, P.; Bhanu Prakash, A.; Farmer, A.; Bhide, M.; Shah, T.; Gaur, P.; et al. Development and use of genic molecular markers (GMMs) for construction of a transcript map of chickpea (Cicer arietinum L.). Theor. Appl. Genet. 2011, 122, 1577–1589. [CrossRef]
9. Backiyarani, S.; Uma, S.; Varatharaj, P.; Saraswathi, M.S. Mining of EST-SSR markers of Musa and their transferability studies among the members of order the Zingiberales. Appl. Biochem. Biotechnol. 2013, 169, 228–238. [CrossRef]
10. Passos, M.A.; de Oliveira Cruz, V.; Emediato, F.L.; de Camargo Teixeira, C.; Souza, M.T.; Matsumoto, T.; Rennó Azevedo, V.C.; Ferreira, C.F.; de Alencar Figueiredo, L.F.; et al. Development of expressed sequence tag and expressed sequence tag–simple sequence repeat marker resources for Musa acuminata. AoB Plants 2012, 2012, 1. [CrossRef]
11. Li, W.J.; Ma, H.; Li, Z.H.; Wan, Y.M.; Liu, X.X.; Zhou, C.L. Thirty-four Musa (Musaceae) expressed sequence tag–derived microsatellite markers transferred to Musella lasiocarpa. Genet. Mol. Res. 2012, 11, 4. [CrossRef]
12. Passos, M.A.N.; de Cruz, V.O.; Emediato, F.L.; Teixeira, C.C.D.; Miller, R.N. Analysis of the leaf transcriptome of Musa acuminata during interaction with Mycosphaerella musicola: Gene assembly, annotation and marker development. BMC Genom. 2013, 14, 78. [CrossRef] [PubMed]
13. Cheung, F.; Town, C.D. A BAC end view of the Musa acuminata genome. BMC Plant Biol. 2007, 7, 29. [CrossRef] [PubMed]
14. Miller, R.N.; Passos, M.A.; Menezes, N.N.; Souza, M.T.; do Carmo Costa, M.M.; Azevedo, V.C.R.; Amorim, E.P.; Pappas, G.J.; Ciampi, A.Y. Characterization of novel microsatellite markers in Musa acuminata subsp. burmannicoides, var. Calcutta 4. BMC Res. Notes 2010, 3, 148. [CrossRef] [PubMed]
15. Biswas, M.K.; Liu, Y.; Li, C.; Sheng, O.; Mayer, C.; Yi, G. Genome-wide computational analysis of Musa microsatellites: Classification, cross-taxon transferability, functional annotation, association with transposons & miRNAs, and genetic marker potential. PLoS ONE 2015, 10, e0131312.
16. Christelová, P.; De Langhe, E.; Hřibová, E.; Cížková, J.; Sardos, J.; Hušáková, M.; Sutanto, A.; Kepler, A.K.; Swennen, R.; Roux, N. Molecular and cytological characterization of the global Musa germplasm collection provides insights into the treasure of banana diversity. Biodivers. Conserv. 2017, 26, 801–824. [CrossRef]
17. Ray, T.; Dutta, I.; Saha, P.; Das, S.; Roy, S. Genetic stability of three economically important micropropagated banana (Musa spp.) cultivars of lower Indo-Gangetic plains, as assessed by RAPD and ISSR markers. Plant Cell Tissue Organ Cult. 2006, 85, 11–21. [CrossRef]
18. Dhanapal, S.; Sekar, D.S.; Satheesh, P.M. Efficiency of RAPD, SSR and ISSR markers in evaluating the genetic fidelity for micropropagated Musa acuminata plant exposed to coal extracted humic acid and commercially available products. Int. J. Agric. Sci. Res. IJASR 2014, 4, 77–86.
19. Bhat, K.; Jarret, R. Random amplified polymorphic DNA and genetic diversity in Indian Musa germplasm. Genet. Resour. Crop Evol. 1995, 42, 107–118. [CrossRef]
20. Venkatachalam, L.; Sreedhar, R.; Bhagyalakshmi, N. Genetic analyses of micropropagated and regenerated plantlets of banana as assessed by RAPD and ISSR markers. *Vitro Cell. Dev. Biol. Plant* 2007, 43, 267–274. [CrossRef]

21. Mou, H.; Lin, G.; Zhou, Y.; Li, X.; Li, C.; Wei, H.; Wu, D.; Zhang, J.; Wang, Q.; Pan, Y. Genetic diversity analysis of banana (*Musa spp.*) based on ISSR molecular marker. *Southwest China J. Agric. Sci.* 2010, 23, 1206–1210.

22. Wang, Q.; Qin, Y.; Chen, H.; Hu, G. Establishment of an inter-simple sequence repeats reaction system (ISSR) for banana (*Musa AAA*). *J. South China Agric. Univ.* 2010, 31, 13–16.

23. Khatri, A.; Bibi, S.; Dahot, M.U.; Khan, I.A.; Nizamani, G.S. In vitro mutagenesis in banana and variant screening through ISSR. *Pak. J. Bot.* 2011, 43, 2427–2431.

24. Ude, G.; Pillay, M.; Nwakanma, D.; Tenkouano, A. Analysis of genetic diversity and sectional relationships in Musa using AFLP markers. *Theor. Appl. Genet.* 2002, 104, 6. [CrossRef] [PubMed]

25. Risterucci, A.M.; Hippolyte, I.; Perrier, X.; Xia, L.; Caig, V.; Evers, M.; Huttner, E.; Kilian, A.; Glaszmann, J.C. Development and assessment of Diversity Arrays Technology for high-throughput DNA analyses in Musa. *Theor. Appl. Genet.* 2009, 119, 10. [CrossRef] [PubMed]

26. Till, B.J.; Jankowicz-Cieslak, J.; Sägi, L.; Huynh, O.A.; Utsushi, H.; Swennen, R.; Terauchi, R.; Mba, C. Discovery of nucleotide polymorphisms in the *Musa* gene pool by EcoTilling. *Theor. Appl. Genet.* 2010, 121, 10. [CrossRef]

27. D’Hont, A.; Denoeud, F.; Aury, J.-M.; Baurens, F.-C.; Carrel, F.; Garsmeur, O.; Noel, B.; Bocs, S.; Droc, G.; Rouard, M. The banana (*Musa acuminata*) genome and the evolution of monocotyledonous plants. *Nature* 2012, 488, 213. [CrossRef]

28. Martin, G.; Baurens, F.C.; Droc, G.; Rouard, M.; Cenci, A.; Kilian, A.; Hastie, A.R.; Doležel, J.; Aury, J.; Alberti, A. Improvement of the banana “Musa acuminate” reference sequence using NGS data and semi-automated bioinformatics methods. *BMC Genom.* 2016, 17, 243. [CrossRef]

29. Wang, Z.; Miao, H.; Liu, J.; Xu, B.; Yao, X.; Xu, C.; Zhao, S.; Fang, X.; Jia, C.; Wang, J. *Musa balbisiana* genome reveals subgenome evolution and functional divergence. *Nat. Plants* 2019, 5, 810–821. [CrossRef]

30. Belser, C.; Istace, B.; Denis, E.; Dubarry, M.; Baurens, F.-C.; Falentin, C.; Genete, M.; Berrabah, W.; Chièvre, A.-M.; Delourme, R. Chromosome-scale assemblies of plant genomes using nanopore long reads and optical maps. *Nat. Plants* 2018, 4, 879. [CrossRef]

31. Wu, W.; Yang, Y.-L.; He, W.-M.; Rouard, M.; Li, W.-M.; Xu, M.; Roux, N.; Ge, X.-J. Whole genome sequencing of a banana wild relative *Musa itinerans* provides insights into lineage-specific diversification of the Musa genus. *Sci. Rep.* 2016, 6, 31586. [CrossRef]

32. Kujur, A.; Bajaj, D.; Saxena, M.S.; Tripathi, S.; Upadhayaya, H.D.; Gowda, C.L.; Singh, S.; Jain, M.; Tyagi, A.K.; Parida, S.K. Functionally relevant microsatellite markers from chickpea transcription factor genes for efficient genotyping applications and trait association mapping. *DNA Res.* 2013, 20, 355–374. [CrossRef]

33. Yu, J.-K.; Paik, H.; Choi, J.; Han, J.-H.; Choe, J.-K.; Hur, C.-G. Functional domain marker (FDM): An in silico demonstration in Solanaceae using simple sequence repeats (SSRs). *Plant Mol. Biol. Rep.* 2010, 28, 352–356. [CrossRef]

34. Voorrips, R. MapChart: Software for the graphical presentation of linkage maps and QTLs. *J. Hered.* 2002, 93, 77–78. [CrossRef]

35. Altschul, S.F.; Madden, T.L.; Schaffer, A.A.; Zhang, J.; Zhang, Z.; Miller, W.; Lipman, D.J. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 1997, 25, 3389–3402. [CrossRef] [PubMed]

36. Ness, R.W.; Siol, M.; Barrett, S.C. De novo sequence assembly and characterization of the floral transcriptome in cross- and self-fertilizing plants. *BMC Genom.* 2011, 12, 298. [CrossRef] [PubMed]

37. Shi, C.Y.; Yang, H.; Wei, C.L.; Yu, O.; Zhang, Z.Z.; Jiang, C.J.; Sun, J.; Li, Y.Y.; Chen, Q.; Xia, T.; et al. Deep sequencing of the *Camellia sinensis* transcriptome revealed candidate genes for major metabolic pathways of tea-specific compounds. *BMC Genom.* 2011, 12, 131. [CrossRef] [PubMed]

38. Wang, X.W.; Luan, J.B.; Li, J.M.; Bao, Y.Y.; Zhang, C.X.; Liu, S.S. De novo characterization of a whitefly transcriptome and analysis of its gene expression during development. *BMC Genom.* 2010, 11, 400. [CrossRef]
40. Rohlf, F.J. *NTSYSpc Numerical Taxonomy and Multivariate Analysis System Version 2.0 User Guide*; Applied Biostatistics Inc.: New York, NY, USA, 1998.
41. Yeh, F.C.; Yang, R.C.; Boyle, T. *POPGENE Software Package Version 1.31 for Population Genetic Analysis*; University of Alberta: Edmonton, AB, Canada, 1999.
42. Pritchard, J.K.; Stephens, M.; Donnelly, P. Inference of population structure using multilocus genotype data. *Genetics* **2000**, *155*, 945–959.
43. Earl, D.A. STRUCTURE HARVESTER: A website and program for visualizing STRUCTURE output and implementing the Evanno method. *Conserv. Genet. Resour.* **2012**, *4*, 359–361. [CrossRef]
44. Rosenberg, N. *Documentation for Distruct Software: Version 1.1*; University of Michigan: Ann Arbor, MI, USA, 2007.
45. Smouse, R.P.; Peakall, R. *GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research—An update*. *Bioinformatics* **2012**, *28*, 2537–2539.
46. Thiel, T.; Michalek, W.; Varshney, R.K.; Graner, A. Exploiting EST databases for the development and characterization of gene-derived SSR-markers in barley (*Hordeum vulgare* L.). *Theor. Appl. Genet.* **2000**, *106*, 411–422. [CrossRef] [PubMed]
47. Raji, A.; Anderson, J.; Kolade, O.; Ugwu, C.; Dixon, A.; Ingelbrecht, I. Gene-based microsatellites for cassava (*Manihot esculenta* Crantz): Prevalence, polymorphisms, and cross-ta taxa utility. *BMC Plant Biol.* **2009**, *9*, 118. [CrossRef] [PubMed]
48. Poncet, V.; Rondeau, M.; Tranchant, C.; Cayrel, A.; Hamon, S.; de Kochko, A.; Hamon, P. SSR mining in coffee tree EST databases: Potential use of EST-SSRs as markers for the *Coffeea* genus. *Mol. Genet. Genom.* **2006**, *276*, 436–449. [CrossRef]
61. Biswas, M.K.; Chai, L.; Qiang, X.; Deng, X. Generation, functional analysis and utility of *Citrus grandis* EST from a flower-derived cDNA library. *Mol. Biol. Rep.* 2012, 39, 7221–7235. [CrossRef] [PubMed]

62. Pandey, G.; Misra, G.; Kumari, K.; Gupta, S.; Parida, S.K.; Chattopadhyay, D.; Prasad, M. Genome-wide development and use of microsatellite markers for large-scale genotyping applications in foxtail millet (*Setaria italica* (L.)). *DNA Res.* 2013, 20, 197–207. [CrossRef]

63. Kraemer, L.; Beszteri, B.; Gäbler-Schwarz, S.; Held, C.; Leese, F.; Mayer, C.; Pöhlmann, K.; Frickenhaus, S. STAMP: Extensions to the STADEN sequence analysis package for high throughput interactive microsatellite marker design. *BMC Bioinform.* 2009, 10, 1. [CrossRef]

64. Scott, K.D.; Eggler, P.; Seaton, G.; Rossetto, M.; Ablett, E.M.; Lee, L.S.; Henry, R.J. Analysis of SSRs derived from grape ESTs. *Theor. Appl. Genet.* 2000, 100, 723–726. [CrossRef]

65. Dida, M.; Gale, M. Comparative analyses reveal high levels of conserved colinearity between the finger millet and rice genomes. *Theor. Appl. Genet.* 2007, 115, 489–499. [CrossRef] [PubMed]

66. Kumari, K.; Muthamilarasan, M.; Misra, G.; Gupta, S.; Subramanian, A.; Parida, S.K.; Chattopadhyay, D.; Prasad, M. Development of eSSR-markers in *Setaria italica* and their applicability in studying genetic diversity, cross-transferability and comparative mapping in millet and non-millet species. *PLoS ONE* 2013, 8, e67742. [CrossRef] [PubMed]

67. Nei, M.; Takezaki, N. Estimation of genetic distances and phylogenetic trees from DNA analysis. In Proceedings of the 5th World Congress on Genetics Applied to Livestock Production, University of Guelph, Guelph, ON, Canada, 7–12 August 1983; Volume 21, pp. 405–412.

70. de Jesus, O.N.; Silva Sde, O.; Amorim, E.P.; Ferreira, C.F.; de Campos, J.M.; Silva Gde, G.; Figueira, A. Genetic diversity and population structure of *Musa* accessions in ex situ conservation. *BMC Plant Biol.* 2013, 13, 41. [CrossRef]

72. Ford, M.J. Applications of selective neutrality tests to molecular ecology. *Mol. Ecol.* 2002, 11, 1245–1262. [CrossRef]

73. Kim, K.S.; Ratcliffe, S.T.; French, B.W.; Liu, L.; Sappington, T.W. Utility of EST-derived SSRs as population genetics markers in a beetle. *J. Hered.* 2008, 99, 112–124. [CrossRef]

74. Ning, S.P.; Xu, L.B.; Lu, Y.; Huang, B.Z.; Ge, X.J. Genome composition and genetic diversity of *Musa* germplasm from China revealed by PCR-RFLP and SSR markers. *Sci. Hortic.* Amst. 2007, 114, 281–288. [CrossRef] [PubMed]

76. Zhao, Z.; Guo, C.; Sutharzan, S.; Li, P.; Echt, C.S.; Zhang, J.; Liang, C. Genome-wide analysis of tandem repeats in plants and green algae. *G3 Genes Genomes Genet.* 2014, 4, 67–78. [CrossRef] [PubMed]

78. Doddamani, D.; Katta, M.A.; Khan, A.W.; Agarwal, G.; Shah, T.M.; Varshney, R.K. CicArMiSatDB: The chickpea microsatellite database. *BMC Bioinform.* 2014, 15, 212. [CrossRef]
80. Arora, V.; Iquebal, M.; Rai, A.; Kumar, D. PIPEMicroDB: Microsatellite database and primer generation tool for pigeonpea genome. *Database* 2013, 2013, bas054. [CrossRef]

81. Arora, V.; Kapoor, N.; Fatma, S.; Jaiswal, S.; Iquebal, M.A.; Rai, A.; Kumar, D. BanSatDB, a whole-genome-based database of putative and experimentally validated microsatellite markers of three *Musa* species. *Crop J.* 2018, 6, 642–650. [CrossRef]

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