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Development of SSR Markers Based on Transcriptome Sequencing and Verification of Their Conservation across Species of Ornamental *Pennisetum* Rich. (Poaceae)

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Abstract: *Pennisetum* species have importance in foraging, agriculture, energy-production, the environment, and landscaping. To promote the preservation and utilization of ornamental *Pennisetum* resources, we developed simple sequence repeat (SSR) markers from the *Pennisetum setaceum* cv. 'Rubrum' transcriptome and verified their conservation in 38 sources. Our transcriptome sequencing efforts generated 58.91 Gb of clean data containing 55,627 unigenes. We functionally annotated 30,930 unigenes, with functions enriched in translation and ribosomal structure and biogenesis. Database comparisons indicated that the closest relative of *P. setaceum* cv. 'Rubrum' is *Setaria italica*. Over five thousand SSR markers were detected in the transcriptomic data. We selected 38 pairs of highly polymorphic SSR markers from 50 randomly selected SSR markers. Based on genetic diversity analysis of 38 ornamental *Pennisetum* sources, we obtained 312 polymorphic bands, with an average of 8.21 alleles per primer. Principal coordinate analyses and generation of a, which proved that *Pennisetum* has moderate genetic diversity. In addition, fingerprint maps were constructed to improve *Pennisetum* identification. The transcriptome data generated by our study enhances the transcriptional information available for *P. setaceum*. This study lays the foundation for the collection and utilization of ornamental *Pennisetum* resources and provides a basis for future breeding projects using this species.

Keywords: *Pennisetum*; illumina sequencing; genetic diversity; SSR molecular marker; transcriptome

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1. Introduction

The genus *Pennisetum* belongs to the grass family (Poaceae), represent annual or perennial herbs distributed in tropical and subtropical regions [1]. *Pennisetum* species have importance in foraging [2], agriculture [3], environment [4] and energy production [5], and are also widely used in ornamental gardening [6]. *Pennisetum* ornamental grasses have elegant stalks, beautiful inflorescences, and colorful leaves. *Pennisetum* can be used for diverse landscaping needs, as they can be planted as a single plant, in clusters, in pieces, or in rows [7]. In addition to their ornamental value, they also have the advantage of being adaptable, and resistant to drought, and requiring low maintenance [8]. One member of this family, *P. setaceum* cv. 'Rubrum' has purple leaves throughout most of its growth stages, with high ornamental value, is widely used all over the world [9] and is lovingly referred to as "purple fountain grass" [10].

Molecular markers that have been developed to date include inter-simple sequence repeat (ISSR) [11], random amplified polymorphism DNA (RAPD) [12], restriction fragment length polymorphism (RFLP) [13], amplified fragment length polymorphism (AFLP) [14],

simple sequence repeat (SSR) [15] and simple nucleotide polymorphism (SNP) [16]. Among these marker types, the use of SSR markers is advantageous as they are abundant, multi-allelic, highly polymorphic, and co-dominant. As such, SSRs are an ideal tool to examine the genetic diversity of plants [17]. The key to utilizing SSR molecular markers lies in their development. Early SSR primer development methods includes cDNA library construction and clone sequencing [18], as well as by searching for expressed sequence tags in public databases [19]. Transcriptome sequencing has become a popular method for developing SSR markers. Transcriptome sequencing (i.e., RNA-seq) represents a high-throughput technology for sequencing cDNAs obtained by reverse transcription of mRNA transcribed in a specific tissue or cell at a certain developmental stage or functional state [20]. It is an effective tool to identify SSR markers in species without reference genome and non-model organisms [20]. Furthermore, it has been shown that SSRs obtained from one species can be used to detect the diversity of related species and even other genera of the same family [21].

Zhou et al. [22] conducted genetic diversity analysis on 35 sources of *Pennisetum* from China and the United States. They verified the cross-species reactivityof *Hemarthria* EST-SSR markers. Wang et al. [23] developed 83,706 SSR markers from *Pennisetum purpureum* Schum 'Zise' and identified 28 pairs of polymorphic markers. In the past, our group has screened 15 *Pennisetum*-specific SSR markers by magnetic bead enrichment technology, and 147 polymorphic alleles were used to further verify the traditional phylogenetic classification of *Pennisetum* [6]. However, the sources of *Pennisetum* used in these studies were mostly forage-type *Pennisetum* or *Pennisetum* crops, and research on ornamental *Pennisetum* remained minimal. In terms molecular information on ornamental *Pennisetum* grasses, there are only a few SSR markers that have been developed. In addition, trancriptome data from ornamental *Pennisetum* species are lacking.

In our previous research, we constructed the full-length transcriptome of *P. setaceum* cv. 'Rubrum and revealed the molecular mechanism underlying anthocyanin accumulation [24]. In the present study, we used this Illumina transcriptome data to: (1) develop SSR markers for *P. setaceum* cv. 'Rubrum' and enhance the transcriptional information available for *Pennisetum*, (2) verify whether these SSR markers are conserved among various *Pennisetum* species, and (3) analyze the genetic diversity of 38 *Pennisetum* accessions, investigate their genetic relationships, and establish genetic fingerprints. Thus, our study will provide a basis for functional genetic analyses, resource identification, and cultivation of new varieties of ornamental *Pennisetum*.

2. Materials and Methods

2.1. Plant Material and DNA Extraction

The *P. setaceum* cv. 'Rubrum' selected for transcriptome sequencing was collected from the National Precision Agriculture Research Demonstration Base in 2020 (116°28′ E, 39°94′ N). The 38 sources of *Pennisetum* included some widely used *Pennisetum* varieties and different mutants cultivated by our group. The original materials were collected from Beijing, Anhui and Kunming in China (Table 1). Meanwhile, *Setaria viridis* and *Panicum virgatum* were selected as experimental controls. The experimental materials consisted of fresh leaves or stems that were sampled and stored at −80 °C. Genomic DNA was extracted from the 40 plant samples using the CTAB method [25]. The concentration of the extracted DNA samples were measured using a Nanodrop 2000 (NanoDrop Technologies, Wilmington, DE, USA), uniformly diluted DNA to 20 ng/μL and stored at −20 °C until further use. PCR amplifications were optimized according to our previously reported protocols with minor modifications [21].

Table 1. The information of 38 *Pennisetum* and 2 control materials.

Sample ID	Sample Name	Sampling Location
1	*P. alopecuroides*—A	Beijing
2	*P. alopecuroides*—B	Beijing
3	*P. alopecuroides*—C	Beijing
4	*P. alopecuroides*—D	Beijing
5	*P. alopecuroides*—E	Beijing
6	*P. purpureum* Schum. —A	Beijing
7	*P. purpureum* Schum. —B	Beijing
8	*P. purpureum* Schum. —C	Beijing
9	*P. purpureum* Schum. —D	Beijing
10	*P. purpureum* Schum. —E	Beijing
11	*P. purpureum* Schum. —F	Beijing
12	*P. purpureum* Schum. —G	Beijing
13	*P. alopecuroides*—F	Anhui
14	*P. alopecuroides* cv. 'Ziguang'	Beijing
15	*P. alopecuroides* cv. 'Liren'	Beijing
16	*P. alopecuroides* cv. 'Baijian'	Beijing
17	*P. alopecuroides* cv. 'Wucai'	Beijing
18	*P. alopecuroides* variation 'Wucai' —A	Beijing
19	*P. alopecuroides* variation 'Wucai' —B	Beijing
20	*P. villosum*	Beijing
21	*P. setaceum* cv. 'Rubrum'	Beijing
22	*P. purpureum*	Beijing
23	*P. orientale* cv. 'Xuerong'	Beijing
24	*P. setaceum*	Beijing
25	*P. alopecuroides* cv. 'Changsui' —A	Beijing
26	*P. alopecuroides* cv. 'Changsui' —B	Beijing
27	*P. alopecuroides* cv. 'Aizhu' —A	Beijing
28	*P. alopecuroides* cv. 'Aizhu' —B	Beijing
29	*P. alopecuroides* cv. 'Little Bunny'	Beijing
30	*P. alopecuroides* cv. 'Hameln'	Beijing
31	*P. purpureum* schumab cv. Red	Kunming
32	*P. alopecuroides* cv. 'Ziguang'	Kunming
33	*P. clandestinum*	Kunming
34	*P. alopecuroides* cv. 'Little Bunny'	Kunming
35	*P. alopecuroides* cv. 'Purple'	Kunming
36	*P. alopecuroides* cv. 'Fire Works'	Kunming
37	*P. alopecuroides* cv. 'Baimeiren'	Kunming
38	*P. villosum*	Kunming
39	*P. virgatum*	Beijing
40	*S. viridis*	Beijing

2.2. Transcriptome Sequencing of *P. setaceum* cv. 'Rubrum'

Total RNA was extracted from *P. setaceum* cv. 'Rubrum' using the Trizol method as per the manufacturer's specifications. The constructed cDNA libraries were then sequenced using the Illumina NovaSeq 6000 high-throughput sequencing platform (San Diego, CA, USA). Sequencing and quality control processes were performed according to the standard workflow of the Biomarker Corporation (Beijing, China). After obtaining a large amount of high-quality sequencing data, sequence assembly was performed using Trinity software [26]. The resulting unigenes were aligned with the Non-Redundant Protein (NR) [27], Swiss-Prot [28], Clusters of Orthologous (COG) [29], Eukaryotic Orthologous Groups (KOG) [30], eggNOG4.5 [31], and Kyoto Encyclopedia of Genes and Genomes (KEGG) [32] databases using the DIAMOND software [33]. SSR analysis was performed on unigenes that were longer than 1kB using MISA software [34]. SSR loci with dinucleotide to hexanucleotide repeat types are preferentially selected. The number of SSR markers selected for different repeat types is determined by their proportions. Using Primer3 (http://primer3.sourceforge.net/releases.php) (accessed on 3 December 2021) for each SSR primer design.

2.3. SSR Prediction, Selection, and Primer Design

Eight materials with different phenotypic traits including *P. alopecuroides*—A *P. purpureum Schum.* —D *P. alopecuroides* cv. 'Wucai', *P. villosum*, *P. alopecuroides* cv. 'Little Bunny',

P. alopecuroides cv. 'Fire Works', *P. virgatum*, and *S. viridis* were selected for use in screening 50 randomly selected molecular markers that were generated from the RNA-seq data. The primers that produced clear bands and detected polymorphism were used in subsequent experiments. All materials were amplified by PCR using primers synthesized by Ruibiotech Company (Beijing, China). The concentration of primers is 10 μmol/L. Our optimized 10 μL PCR reaction system consisted of 5 μL 2 × Taq Master Mix, 0.6 μL forward primer, 0.6 μL reverse primer, 1 μL DNA, and 2.8 μL ddH_2O. The PCR cycling conditions were as follows: pre-denaturation at 94 °C for 3 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 48 °C for 30 s and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The resulting PCR products were electrophoresed on an 8% non-denaturing polyacrylamide gel at 170 V 70 min. A 100 bp ladder (TransGen Biotech, Beijing, China) was used as a size marker. After electrophoresis, the PCR amplicons were by silver staining and photographed.

2.4. Genetic Diversity Analysis

To establish the original matrix, the bands visualized on the non-denaturing polyacrylamide gel were manually read. Due to the complex genetic background of *Pennisetum*, aneuploidy and polyploidy exist widely in *Pennisetum*, and it is difficult to detect the peak value of each locus, so we analyzed SSR markers as binary dominant. We used the molecular markers of co-dominant inheritance as dominant markers. Clear bands were recorded as "1", whereas samples were assigned a "0" if the bands were difficult to distinguish or non-existent. POPGENE v1.3.2 [35] was used to analyze genetic distance, observed allele number (Na), effective allele number (Ne), gene diversity index (H), and Shannon information index (I), and to analyze genetic diversity. The formula PIC = $1 - \sum_j^i {P_{ij}}^2$ (P_i and P_j are the frequencies of the *i*th and *j*th genes at a locus) [21], was used to calculate the polymorphism information content (PIC) of the primers.

2.5. Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Clustering Analysis and Principal Component Analysis (PCoA) of 38 Accessions

NTSYS v2.1 [36] was used to evaluate the similarity of 38 *Pennisetum* samples, and to establish UPGMA and PCoA. A UPGMA cluster diagram was drawn by the SSR data similarity matrix. PCoA was performed according to anastomotic differences between binary genotypic profiles. Distance and covariance were both standardized [21].

2.6. Construction of Fingerprints

Fingerprints were constructed by reading the banding information obtained following gel electrophoresis of the PCR products. At positions of the same length, positions with a band were marked in white, and positions without a band were marked in black, with their binary identities represented as "1" and "0", respectively. Binary identities were converted to decimal identities.

3. Results

3.1. Illumina Sequencing and De Novo Transcriptome Assembly

A total of 58.91 Gb of clean data was obtained from the transcriptome sequencing of *P. setaceum* cv. 'Rubrum'. Clean data from each sample reached a maximum of 6.15 Gb, and the percentage of Q30 bases was over 94.22%. After assembly, a total of 55,627 unigenes were obtained, and their N50 was 1637 bp in length. The assembly integrity qualified the transcriptome for subsequent analysis. The transcriptome data has been deposited in the NCBI database under accession number "PRJNA744323".

3.2. Functional Gene Annotation

Functional annotation of the unigenes was performed by aligning them to the NR, Swiss-Prot, KEGG, COG, KOG, GO, eggNOG, and Pfam databases. In general, a total of 30,930 unigene annotations were obtained (Table 2). The COG classification statistics

showed that the most genes were classified into "translation, ribosomal structure, and biogenesis", followed by "posttranslational modification, protein turnover, and chaperones" (Figure 1a). The eggNOG classification statistics showed that the genes were widely distributed in "signal transduction mechanisms posttranslational modification, protein turnover chaperones" (Figure 1b). The results of NR protein sequence alignment showed that the closest relative species to *Pennisetum* was *Setaria italica*, followed by *Setaria viridis*, *Panicum hallii*, *Panicum miliaceum* (Figure 1c), with homologous percentages of 37.10%, 23.50%, and 7.00%, respectively.

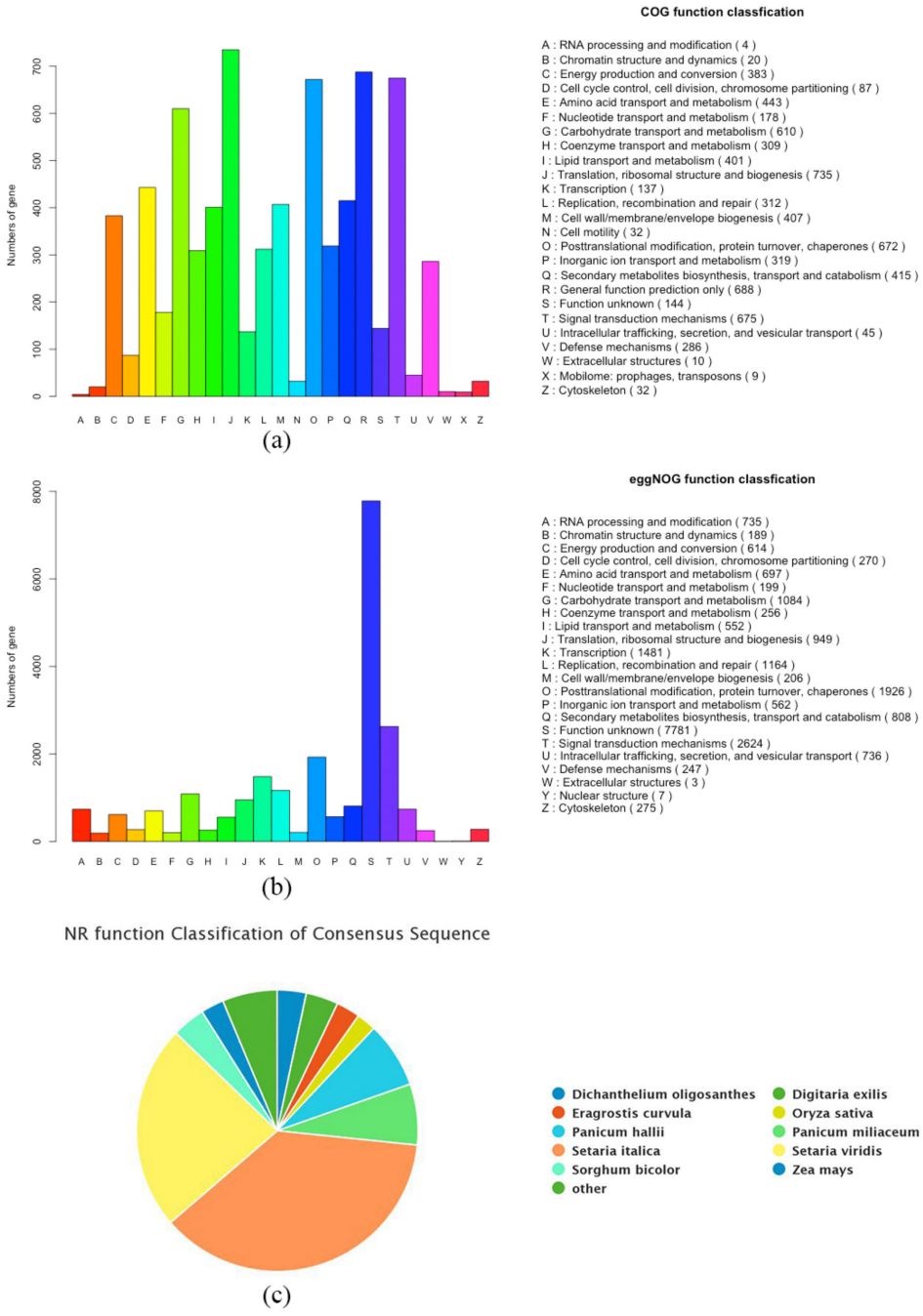

Figure 1. The gene annotation results of *P. setaceum* cv. 'Rubrum' based on COG, NOG and NR databases. (**a**) COG gene annotation results. (**b**) NOG gene annotation results. (**c**) NR function classification of consensus sequences.

Table 2. Unigene annotation statistics.

Database	Annotated Number	300 ≤ Length < 1000	Length ≥ 1000
COG Annotation	6606	1553	5053
GO Annotation	22,845	8859	13,986
KEGG Annotation	17,384	5855	11,529
KOG Annotation	13,498	4152	9346
Pfam Annotation	18,502	5282	13,220
Swissprot Annotation	16,727	5347	11,380
eggNOG Annotation	23,365	8827	14,538
Nr Annotation	30,020	12,302	17,718
All Annotated	30,930	12,999	17,931

3.3. Development of Novel SSRs and Analysis of Their Conservation

A total of 5122 SSR loci were obtained from the 55,627 unigenes using MISA software, and the distribution frequency of SSR loci was 9.21%. There were six SSR types, including mononucleotide repeats, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, pentanucleotide repeats, and hexanucleotide repeats (Table 3). The most abundant type of SSR was trinucleotide repeats (41.94%), followed by mononucleotide repeats (39.26%), and dinucleotide repeats (14.70%). The number of tetranucleotide repeats, pentanucleotide repeats, and hexanucleotide repeats was low, with the number of hexanucleotide repeats (0.04%) being the least (only two) (Table 3). Fifty pairs of SSR primers were randomly selected from the 5122 SSR loci (Table 4), and eight materials with obvious phenotypic differences were utilized to further select polymorphic SSRs. We selected 38 pairs of primers with higher polymorphisms to perform PCR amplification of 40 experimental materials. The raw band data are presented in Table S1. The amplification results of representative polymorphic primers in 40 samples are shown in Figure S1.

Table 3. The distribution and quantity of different SSR types.

Type	Number	Proportion
Mono nucleotide	2011	39.26%
Di nucleotide	753	14.70%
Tri nucleotide	2148	41.94%
Tetra nucleotide	42	0.82%
Penta nucleotide	11	0.21%
Hexa nucleotide	2	0.04%
c [1]	153	2.99%
c* [2]	2	0.04%
Total number of identified SSRs	5122	

[1] "c" means contains at least two SSRs, and the distance between them is less than 100bp without overlap. [2] "c*" means there is overlap between composite SSRs.

Table 4. The information of 50 pairs of SSR makers.

Primer ID	Polymorphism	Forward Primer Sequence (5–3)	Reverse Primer Sequence (5–3)
PaSSR—1	Yes	TATACTTGGTTGCCACGGGT	TTCATGGTGATGCGTCATTT
PaSSR—2	Yes	AACCCCTAGCAGTCTCTCCC	GCGGTACTCGTACTGCTTGA
PaSSR—3	No	TCCATGGAGTACCCGAAGAG	ACATCAACCACTGCAACCAA
PaSSR—4	No	AAAATTAGGTCCGCTTGCCT	GACCGATTCCAATTCCGTTA
PaSSR—5	No	CCCCTTTTTCTCTCACTCCC	CCACCAATTTGCCTTTCAGT
PaSSR—6	No	AAAGAAAGAAAAGAAAACGCACA	CCTAGCTTGTCTGCCTCCTG
PaSSR—7	Yes	GCGAGGAGATTCAGAGATCG	GGACGAACAAAGAGACCGAG
PaSSR—8	Yes	TATGGGTTGCTCCTCGAATC	ATTGAACAGCTTCTGCGGAT
PaSSR—9	Yes	TGGATGGAGGACAGTGATGA	ACGACCAGGAAAGCCTTACA

Table 4. *Cont.*

Primer ID	Polymorphism	Forward Primer Sequence (5–3)	Reverse Primer Sequence (5–3)
PaSSR—10	Yes	TGTTCCGATATGCCTGTTTT	CTGCAACATTCTGCATGGAC
PaSSR—11	Yes	AGCTAGGCACAAAGAAGGCA	CTAGCTTCATGATGCACGGA
PaSSR—12	Yes	CTTTACCCAAACAGCCCCTC	TCTGGATTAACCACTTCGGC
PaSSR—13	Yes	TGGTCAGTTGTCGACTCAGG	ACGCACTTGTACTGTGGCTG
PaSSR—14	Yes	GTCCACGAGAGAGGGAAGAG	GTAGCATATCCCGCCTGTGT
PaSSR—15	No	GGCTCAATTTGGTGCATTCT	TATTAAACCAGGGTGGCTGC
PaSSR—16	Yes	AGCAGCAACAACTGCAACAG	GCTACAGGGTTTGCCACATT
PaSSR—17	Yes	GACCAGTCGCTCTCGACC	TAATCCACCTTCCAAGCCAG
PaSSR—18	Yes	CTCAGAAGGGTGGGTACGAA	TGTGCCAATGCAGAGAAGTC
PaSSR—19	No	TCAACCAGGCCAGATCATAA	ACGAGGCCTCTACGACAGAA
PaSSR—20	No	ACCTCTGCGTGGTGAAGAAT	CTCCAGAAGTAGCAGCAGCA
PaSSR—21	Yes	GCTCTCGCAGTACATCTCCC	GCCACTTGACCTTCTCCTTG
PaSSR—22	No	TCGTGGTCAAACTGATAGCG	CTCCAGAAGTAGCAGCAGCA
PaSSR—23	No	CAGCAAATGCAGCCTATCAA	CTGTTGGTCACTGGTCCCTT
PaSSR—24	Yes	AAGGGACCAGTGACCAACAG	CCAGATTCACGAACTGACCA
PaSSR—25	No	GACAAAACTACGGGGGTCAA	CGGTGGGGAAGAAAGAAAAT
PaSSR—26	Yes	AGACGAGCGGAGAGGAAAC	TCCGCTCCTTGATCTTTCTG
PaSSR—27	Yes	GCACCACCACCTCTCTTCTT	CGAGGAGGAAGATCTCGATG
PaSSR—28	Yes	AACCTCTTCGCTTCTCTCCC	CAGCAGGCACAACTTCCAT
PaSSR—29	Yes	TTCGATTGCTTGTATGCTGC	CCGCACGTAGTTGTGAGTGT
PaSSR—30	Yes	TTCTTCTTCGCCGTACGAAT	GATCGAGATGGCGACAAAAT
PaSSR—31	Yes	GTTCCCCTCTGTATCTGGGC	GCTGGGGAAGGAAGACCTC
PaSSR—32	Yes	AGTACGGCTGCCTCGTCTAC	TAGTTGCGGTCGAGAAGGAT
PaSSR—33	Yes	ATCAGGTCGGTGGTGAGAAC	CCCATCTGATGCTCCAACTT
PaSSR—34	Yes	TGCAGAGAAACCAATTGCAG	CCGGTTCATAAGCTGGTGTT
PaSSR—35	Yes	ATGCTCTATGCACTCCCACC	TGAACCCTGATTTGAGGTCC
PaSSR—36	Yes	CCGCTGTAACTCTCAGCCAC	CACTCCTTCACTCAGCCTCC
PaSSR—37	No	CGCACCTCGTTCGATTTTT	GAACAGGTGCACAGGAGGAC
PaSSR—38	Yes	TTACCCTCCCAGATTGCTTG	CGTGAAAAGAATAGTCGTCCG
PaSSR—39	Yes	CACCACCACCTCTCCTCTTC	GAGAAGCTCATGTCGACGG
PaSSR—40	Yes	TTCCACATCTCCGCTTCTCT	CCTTGAACTTCTCCTCGTCG
PaSSR—41	Yes	TCGGGAAGAAAGCTGAAAAA	CTCGCCTCCTCTCCTCTCTT
PaSSR—42	Yes	GAGGCCTCTCCCTCTCTCTC	GACCAAACCCAAACCCAAC
PaSSR—43	Yes	CTCCGCTCATCCTACCCTC	TGGGTTCTAGGGTTCTGTCG
PaSSR—44	Yes	CCAAATTTTCCAAGCCAAAA	ACTGGTGGATCTGCGCCT
PaSSR—45	Yes	GCTCTTCATCATAGCGGTGG	AGACCGAGGACGTAGAGCAG
PaSSR—46	Yes	AAATGCCATGACAACTGCTG	CAAGAACGCAGACGACAAAA
PaSSR—47	Yes	CGGATTTCCTACAGCGAGAG	ATACCGACAAAAACCCGACA
PaSSR—48	Yes	GTGCGTCTCACACACCACA	CCAAGTGGGGATGAACAGAG
PaSSR—49	No	TAGACTTCGGTCGACTCGCT	AACGAACACCTGGCGTAGAT
PaSSR—50	Yes	CAGGGTGCAGTTAAGGGTTC	CCATCTGTGTTCATATGGCG

3.4. Genetic Diversity Statistics

A total of 312 polymorphic bands were amplified using 38 pairs of SSR markers, with an average of 8.21 bands per marker. The PIC ranged from 0.16 (PaSSR—2) to 0.44 (PaSSR—47), with an average of 0.35 (Table S2). 37 locus was medium level polymorphism ($0.25 < PIC < 0.5$), one locus was low level ($PIC < 0.25$), and not have high level polymorphism ($PIC > 0.5$) locus. SSRs displayed wide genetic variation among accessions. The average Na was 2.00. The Ne ranged from 1.19 (PaSSR—2) to 1.81 (PaSSR—47), with an average of 1.60. The H ranged from 0.16 (PaSSR—2) to 0.44 (PaSSR—47), with an average of 0.35. The I values were between 0.16 (PaSSR—2) and 0.63 (PaSSR—47), with an average of 0.52 (Table S2). The genetic similarity coefficient between accessions (Table S3) ranged from 0.39 to 1.00 with an average of 0.58 (Table S3).

3.5. Cluster Analysis of 38 Pennisetum Accessions

UPGMA cluster analysis was performed on 40 samples according to the genetic distance, and the genetic relationships between all samples were obtained by combining the clustering results. Based on the control samples, *S. viridis* and *P. virgatum* verified the reliability of the test results. *S. viridis* was singly branched at a genetic similarity coefficient of 0.43, and the switchgrass *P. virgatum* was classified into a single class at a genetic similarity coefficient of 0.44 (Figure 2). When the genetic distance was 0.50, the 38 *Pennisetum* samples were divided into five categories. The *Pennisetum* variants were divided into one category at a genetic similarity of 0.89. When the genetic similarity was 0.81 (Figure 2), the *P. purpureum* Schum. variants were classified into one category. *P. alopecuroides* 'Baimeiren' from Kunming, Yunnan, and *P. setaceum* from Beijing are classified into one category, indicating that the two are closely related and could be distinguished when the genetic similarity is 0.47. The phenotypes of these variant materials have different degrees of variation, and it is impossible to accurately classify them only by phenotypic characteristics. The results confirmed their genetic relationship and further verified the reliability of the clustering results. *P. clandestinum* was branched separately at a genetic similarity of 0.48, indicating that it is distantly related to other *Pennisetum* samples. *P. alopecuroides* cv. 'Little Bunny' and *P. alopecuroides* cv. 'Hameln' have similar phenotypes at a certain growth stage, thus it is difficult to distinguish the two based on their phenotype. UPGMA clustering revealed that they branched at a genetic similarity of 0.48 (Figure 2). These results demonstrate that this clustering method can effectively distinguish similar materials.

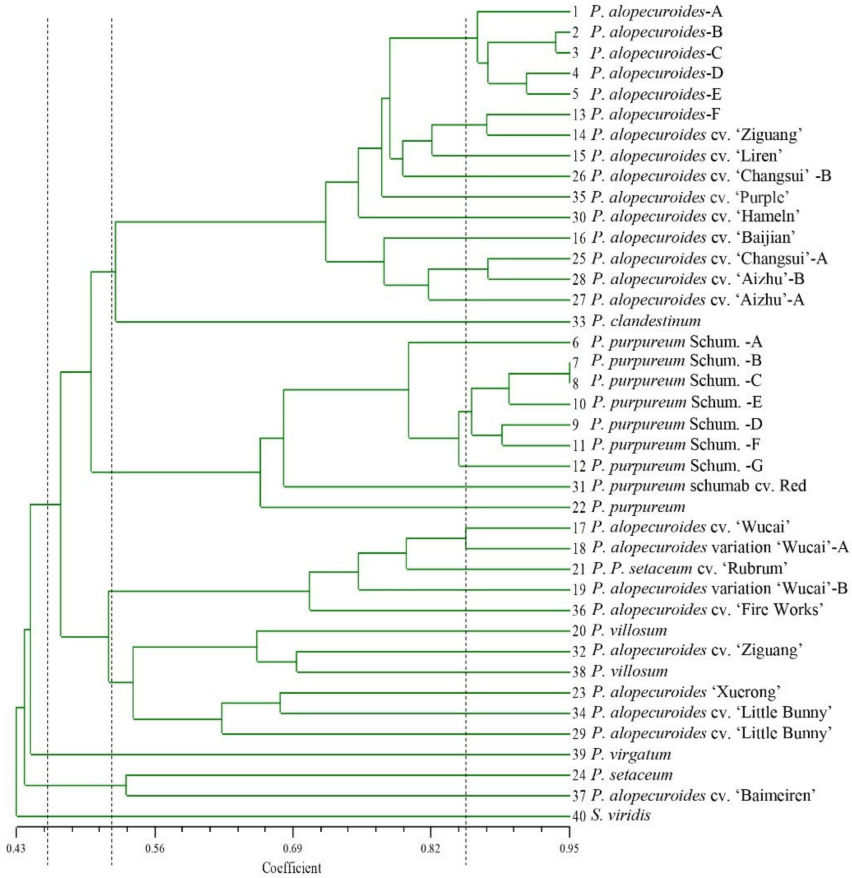

Figure 2. UPGMA clustering analysis of 40 samples. The number on the horizontal axis in the figure represents the genetic coefficient, and the number on the vertical axis represents the samples number from 1 to 40.

3.6. PCoA of 38 Pennisetum Accessions

PCoA was performed on 38 *Pennisetum* materials according to genetic distance (Figure 3), and the 38 *Pennisetum* samples were divided into five parts, consistent with the UPGMA clustering tree. *P. clandestinum* was classified as a separate category. Samples 1–5, 13–16, 25–28, 30, and 35 were clustered into one category. Sample 24 (*P. setaceum*) and sample 37 (*P. alopecuroides* cv. 'Baimeiren') were found to be closely related. It may be because their genetic backgrounds are more consistent. Samples 23 (*P. orientale* cv. 'Xuerong'), 29 (*P. alopecuroides* cv. 'Little Bunny' (Beijing, China)), and 34 (*P. alopecuroides* cv. 'Little Bunny' (Kunming, Yunnan)) were classified into one category. These findings suggest that these species are closely related, genetic relationship is less related to region, and there is less gene exchange with local varieties.

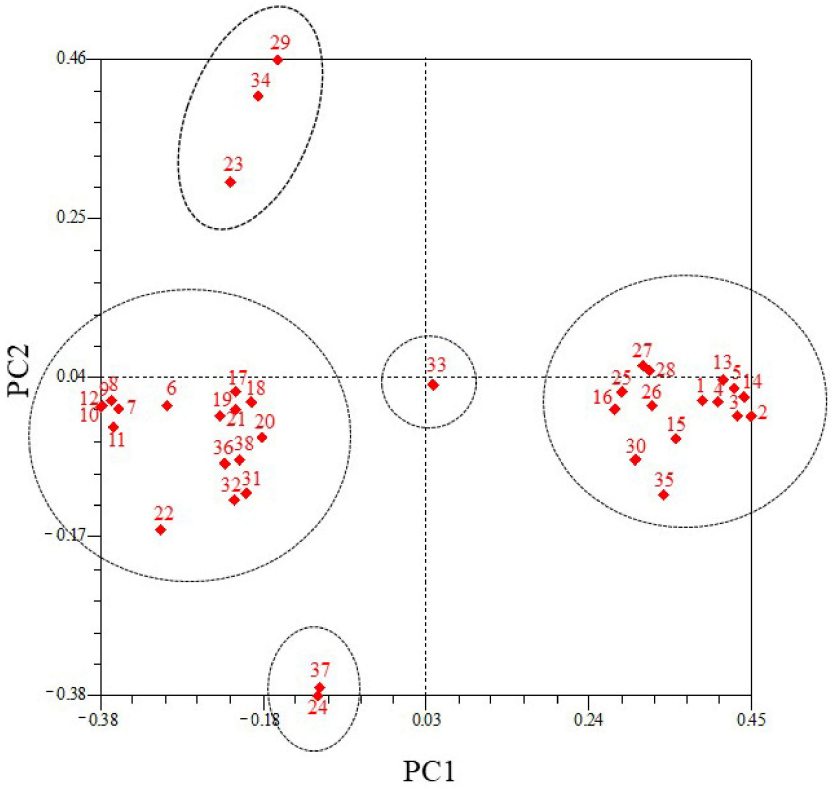

Figure 3. PCoA analysis of 38 *Pennisetum* materials. The numbers on the horizontal and vertical coordinates in the figure represent the genetic coefficient, and the numbers represent the sample numbers from 1 to 40.

3.7. Fingerprint of Pennisetum Varieties with Similar Genetic Background

PCR analysis of the SSR markers revealed that the PaSSR-11 primer could effectively distinguish five strains of *Pennisetum* with the same genetic background, including samples 1–5 (*P. alopecuroides*—A, *P. alopecuroides*—B, *P. alopecuroides*—C, *P. alopecuroides*—D, and *P. alopecuroides*—E. Fingerprints were established for the identification of variants (Figure 4). In addition, the PaSSR-1 and PaSSR-8 primers could distinguish between *P. purpureum* Schum. samples (samples 6–12).

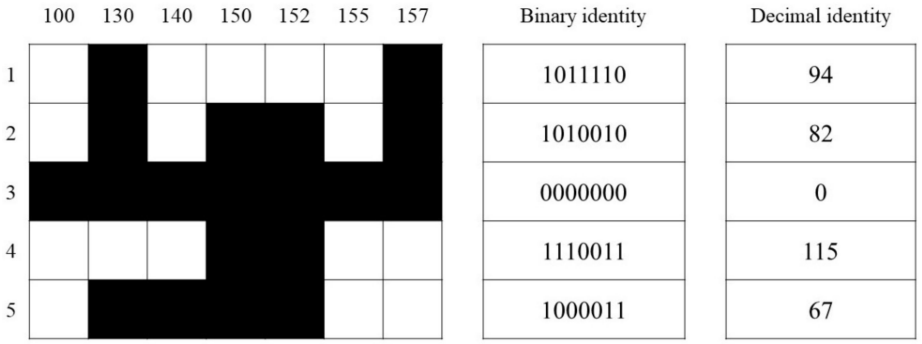

Figure 4. Identification fingerprint of 5 *Pennisetum* varieties by primer PaSSR11. The numbers 1–5 on the ordinate represent *Pennisetum* samples (No. 1–5), and the numbers on the abscissa represent the length of alleles; the white squares represent amplified bands, and the black squares represent non-amplified bands. The binary identity is represented as "1" and "0" respectively; the decimal identity is converted from the binary identity.

4. Discussion

As an ornamental *Pennisetum* species with purple appearance, *P. setaceum* cv. 'Rubrum' has high ornamental value, strong drought tolerance, low maintenance cost, and landscaping value. In this study, 58.91 Gb of clean data was obtained via second-generation transcriptome sequencing of *P. setaceum* cv. 'Rubrum'. The N50 of unigenes was 1637 bp, which was higher than those of two *P. purpureus* samples (N50 of 586 bp and 583 bp) [37] but lower than two *P. glaucum* samples (1860 bp and 1691 bp) [38]. The percentage of Q30 bases in *P. setaceum* cv. 'Rubrum' transcriptome was over 94.22%, which was higher than that of two kinds of *P. glaucum* (92.2% and 83.5%) [38]. A total of 55,627 unigenes were obtained after sequencing and assembly of the *P. setaceum* cv. 'Rubrum' transcriptome, which was more than the 6799 and 1253 unigenes identified in the two kinds of *P. glaucum* [39]. Compared with the transcriptome data of *Pennisetum* above, the assembly integrity of the transcriptome data and number of unigenes detected in this study is higher. This may be related to the genome size of the species itself and the quality of the sequencing. The NR database comparison showed that the species most closely related to *Pennisetum* was *S. italica*, followed by *S. viridis*. According to the records of Flora of China, both *S. italica* and *S. viridis* belong to the genus *Setaria*, and both *Pennisetum* and *Setaria* belong to the Trib. Paniceae R. Br. *Pennisetum* was not present in the top ten species with according to protein sequence alignment, which may be due to the lack of *Pennisetum* information currently available in the database. Therefore, it is very important to generate additional *Pennisetum* transcriptome information.

Previous studies obtained 4493 SSR markers through the transcriptome sequencing of *Medicago sativa*, with an SSR distribution frequency of 8.28% [40]. A total of 3745 SSR loci were obtained from transcriptome sequencing of *Pinus kesiya var. langbianensis*, with an SSR distribution frequency of 6.28% [41]. These values are lower than the number of SSR markers (5122) we obtained from the *P. setaceum* cv. 'Rubrum' transcriptome in this study. Among the SSR markers we identified, trinucleotide repeats (41.94%) were the most abundant, followed by mononucleotide repeats (39.26%). The main SSR type observed in the *Pinus kesiya var. langbianensis* transcriptome was the trinucleotide repeat (49%), followed by the dinucleotide repeat (24%) [41]. Among the SSR markers developed by transcriptome sequencing of purple elephant grass, trinucleotide repeats (59,368) were the most abundant, followed by dinucleotide repeats (15,524) [23]. Mononucleotide repeats (64.93%) were the most abundant in the *Carex breviculmis* transcriptome, followed by trinucleotide repeats [21]. This indicates that there are differences in the distribution of SSR repeat types among different species.

The ability to use the same SSR primers across species determines the value of these primers. In our previous studies, 42 SSRs from *C. breviculmis* were found to be conserved between species and within *Carex* species [21]. Li et al. [42] developed 18 polymorphic SSR

markers for *P. glaucum*, which amplified polymorphic bands from 40 *Pennisetum* sources. Seventy-eight SSRs from *Triticum aestivum* were conserved from *Triticum* to *Hordeum* [43]. In the present study, 50 SSR markers were randomly selected from the 5122 SSR markers we identified, of which 38 pairs of SSR primers showed polymorphism. Those primers were used in PCR among different *Pennisetum* species and demonstrated that the corresponding SSRs were conserved. The SSR marker amplification results of the 38 *Pennisetum* samples in this study showed that the average PIC value was 0.35, which indicates a moderate level of polymorphism [44]. The PIC in our study was lower than the average PIC value of the SSR markers of the 128 *Camellia* sinensis varieties (0.704) [45], and *Prunus avium* (0.59) [46], but higher than *Carex* (0.259) [21]. This suggests that the PIC value, reflecting the degree of variation in SSR loci, may be related to germplasm. The genetic diversity in the collected *Pennisetum* samples was found to be moderate, which may be due to the materials having been collected in a relatively concentrated place where the genetic background difference may not be particularly large. In addition, based on the results of UPGMA clustering and PCoA analysis, we were able to determine the genetic background of different *Pennisetum* plant materials. And the results showed that the genetic relationship of *Pennisetum* samples was not closely related to the geographical distribution location. The phylogeny of *P. clandestinum* was classified into a separate category in UPGMA clustering and PCoA analysis. *P. clandestinum* is native to Africa and, thus, it perhaps not expected to have genetic similarity to other materials [47]. In addition, two kinds of *P. alopecuroides* cv. 'Little Bunny' materials from Beijing and Yunnan were clustered as one category, indicating that they are closely related. However, *P. alopecuroides* cv. 'Ziguang' from Beijing and *P. alopecuroides* cv. 'Ziguang' from Kunming, Yunnan, were classified into two categories. We speculated that this may be because they can cross with other local varieties when applied in different places.

Fingerprint maps can be used to identify different varieties, which is an important step for furthering *Pennisetum* breeding applications. We constructed fingerprints based on the genetic relationships and SSR results of 38 samples. In previous studies, the Ctcp016 primer was able to identify 12 samples of *Carex* based on genetic diversity, and the MtTFSSR-10 primer could distinguish seven varieties of alfalfa. In a study on *Chrysanthemum* × *morifolium* Ramat., researchers identified 480 traditional Chinese chrysanthemum varieties and established their fingerprints [48]. The fingerprints generated in the present study have important reference value in *Pennisetum*, which are notoriously difficult to distinguish. It is worth noting that the PaSSR-11 primer can distinguish strains of *P. alopecuroides*, and a combination of the PaSSR-1 and PaSSR-8 primers can distinguish seven kinds of *P. purpureum Schum. P. alopecuroides* cv. 'Hameln' and *P. alopecuroides* cv. 'Little Bunny' are similar in appearance and cannot be distinguished based on phenotypic characteristics. In this study, we succeeded in distinguishing them at the gene level using the PaSSR-1 and PaSSR-2 primers. The SSR markers developed in this study have greatly improved our ability to identify *Pennisetum* species.

5. Conclusions

Second-generation transcriptome sequencing of *P. setaceum* cv. 'Rubrum' provided abundant transcriptome data for *Pennisetum*. Thirty-eight of 50 randomly selected SSRs were conserved among the *Pennisetum* species studied herein, demonstrating the feasibility of developing SSR markers using RNA-seq in *Pennisetum*. Genetic diversity analysis of 38 *Pennisetum* samples was performed, revealing a high level of genetic diversity among *Pennisetum* species. Through UPGMA clustering and PCoA analysis, 38 *Pennisetum* samples were genetically classified, and a fingerprint map was developed to improve *Pennisetum* identification. This study lays the foundation for the subsequent collection and utilization of ornamental *Pennisetum* resources.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12071683/s1, Figure S1: Representative SSR amplification

results of *Pennisetum* varieties coded from 1 to 40; Table S1: Band Statistics Raw Data; Table S2: Genetic diversity parameters of 38 pairs of polymorphic primers in the 38 *Pennisetum* materials; Table S3: The genetic similarity coefficient between accessions.

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