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Development of SSR molecular markers based on the published genome in seashore paspalum

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Abstract

Seashore paspalum (*Paspalum vaginatum* Swartz) is a widely utilized turfgrass in subtropical and tropical regions. To assist germplasm evaluation and breeding new varieties, a study was carried out to identify Simple Sequence Repeats (SSR) sites in the seashore paspalum reference genome and develop primers using bioinformatics software. This study revealed a total of 94,748 SSR sites retrieved in the seashore paspalum reference genome. The average length of SSRs is 14.79 bp, and the number of bases in SSRs accounts for 0.22% of the entire genome. The relative abundance of SSRs is 147.17 loci/Mb, and the relative density of SSRs is 2,175.97 bp/Mb. In addition to single-base repeat units, the most common di-base repeat units are AT, AG, and AC. The most common tri-base repeat units are AGG, ACG, and AAG. The most common quad-base repeat unit, penta-base repeat unit, and hexa-base repeat unit are ATAC, AAAAG, and ATATAC, respectively. In the flanking region of the SSR sites in the reference genome, 85,955 pairs of primers were successfully designed, accounting for 90.72% of the SSR sites identified in the entire genome. Fourteen pairs of SSR marker primers were designed for genetic diversity analysis, which amplified 45 polymorphic sites in 33 seashore paspalum accessions. The Ne values ranged from 1.08 to 2.34, the Shannon diversity index I ranged from 0.18 to 1.03, and Nei's index ranged from 0.08 to 0.57. The 33 collections were classified into six categories. The genetic variation among the 33 collections of seashore paspalum is high and polymorphic-rich. The results of this study provide a theoretical foundation for breeding new varieties of seashore paspalum.

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Introduction

Seashore paspalum (*Paspalum vaginatum* Swartz) is a perennial grass in the paspalum genus of the Gramineae family, with a chromosome number of 2n = 20[1]. Seashore paspalum produces both rhizomes and stolons, and has a medium to fine texture, good color density, and tolerance to low mowing[2]. It can be used as a turfgrass in sports fields, home lawns, as well as golf courses in subtropical and tropical regions[3–5]. Seashore paspalum has excellent salt tolerance and can survive well in tidal flats with a salinity level above 470 mmol/L NaCl[6].

Simple sequence repeats (SSRs), also known as microsatellite DNA, are commonly present in plant genomes. As a type of molecular marker, SSRs have many advantages, such as being scattered in the genome, high abundance, co-dominant inheritance, high polymorphism, and good repeatability[7,8]. SSR molecular markers are widely used in areas involving plant genetic diversity analysis, parent identification, and genetic linkage map development[9].

There are mainly two methods to develop SSR molecular markers using bioinformatics technology, which are based on expressed sequence tags (Expressed sequence tag, EST) to develop EST-SSR primers (eSSR), and based on genome sequence to develop genome SSR primers (gSSR), respectively[7,9]. Because the eSSR primers are derived from highly conserved DNA transcription regions, the polymorphism revealed by them is theoretically lower than that of gSSR primers. In addition, the sensitivity of eSSR markers in distinguishing closely related genotypes is not as high as gSSR markers. Genomic SSR markers are highly polymorphic and tend to be widely distributed throughout the genome, resulting in better map coverage than eSSR[10,11]. However, the development of gSSR markers is limited but increasing due to the complexity associated with genomic sequencing, especially in polyploid species[12,13]. The development of gSSRs in plants such as soybeans (*Glycine max* L.), grapes (*Vitis vinifera* L.), and bayberries (*Morella rubra* Lour.) has been previously reported[9,14,15], the genomes of some warm-season turfgrasses have also been published, like bermudagrass, zoysiagrass, St. Augustinegrass[16–18], but there are fewer gSSR studies of them, and similarly, there are fewer reports on the development of gSSRs in seashore paspalum[19].

In this study, based on the recently published genome information of seashore paspalum (GCA_026573395.1)[20], bioinformatics tools were used to identify SSR loci, reveal their characteristics, design primers, and evaluate the polymorphism using those primers. The SSR developed in this study may facilitate further research on genetic diversity analysis, genetic linkage mapping, marker-assisted selection, and identification of hybrids in seashore paspalum.

Materials and methods

Experimental materials

Thirty-three germplasm sources of seashore paspalum were planted at the Turfgrass Research Institute of Nanjing Agricultural University in Jurong, Jiangsu Province (China) for molecular marker validation and genetic diversity analysis. The sources of the germplasms are presented in Table 1, and individual plants were planted using sprigs.

Genomic DNA extraction

Leaf samples of actively growing seashore paspalum were collected for DNA extraction using the modified CTAB (Cetyltrimethyl Ammonium Bromide) method[21]. The quality of the DNA was

verified by 0.8% agarose gel electrophoresis and the DNA concentration was determined by NanoReady (Hangzhou LifeReal Biotechnology Co., Ltd., Hangzhou, China). The extracted DNA was diluted to a concentration of 50 ng/L and stored at −20 °C.

Genome source of seashore paspalum

The reference genome sequence of seashore paspalum (GCA_026573395.1) was downloaded from the National Center for Biotechnology Information database (www.ncbi.nlm.nih.gov). The assembled seashore paspalum genome contains 10 chromosomes, 2,211 scaffolds, with a total genome size of 646.9 Mb.

SSR site mining and primer design

The downloaded seashore paspalum genome sequence was imported into the software Krait[22] for SSR site search. The minimum repeat numbers for mono-, di-, tri-, tetra-, penta-, and hexanucleotide motifs were set to 10, 6, 5, 5, 5, and 5, respectively. The flanking sequences within 100 bp of the identified SSR sites were used for primer design. The parameters for primer design were set as the default settings in Krait software, with a product size range of 100–400 bp, melting temperature (TM) range of 58–65 °C, a GC content range of 40%–80%, and a primer length range of 18–27 bp.

SSR primer screening and polymorphism detection

Multiple SSR sites with different types of repeats (excluding mononucleotide repeats) were randomly selected. The designed primers for these SSR sites were screened using the ePCR (electronic PCR) plugin in TBtools software[23]. Primers showing specificity were selected for subsequent synthesis and non-denaturing polyacrylamide gel electrophoresis for specificity and polymorphism detection. Fourteen pairs of specific primers were selected based on ePCR results, and these primers were used for genotypic analysis of the 33 seashore paspalum germplasm accessions. All primers were synthesized by Beijing Tsingke Biotechnology Co., Ltd. (Beijing, China).

The PCR amplification was performed in a 10-μL reaction, including 1 μL DNA template, 0.5 μL each of upstream and downstream primers, 3 μL of ddH_2O, and 5 μL of 2 × PCR Mix (Beijing Tsingke Biotechnology Co., Ltd., Beijing, China). The PCR amplification was performed under the following conditions: initial denaturation at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 60 °C, and 30 s at 72 °C, and a final extension at 72 °C for 10 min. The PCR products were then subjected to a 9% non-denaturing polyacrylamide gel electrophoresis. After electrophoresis, the gel was silver stained and photographed, and the presence or absence of alleles was recorded as 1 or 0 for further primer polymorphism analysis, respectively. Popgene 3.2 software was used to calculate the number of alleles (Na), effective number of alleles (Ne), Shannon information index (I), and Nei's gene diversity (H). Genetic distances were calculated for the 33 seashore paspalum germplasm sources according to Dice[24] using NTSYS-pc. The genetic similarity coefficient (GS) was calculated, and dendrograms were constructed using the unweighted pair-group method with arithmetic averages (UPGMA) by the NTSYS-pc computer software.

Table 1. Information of 33 germplasm sources of seashore paspalum.

No.	Germplasm ID	Type or cultivar	Source
1	SP001	Seaspray	Hainan, China
2	SP002	Salam	Hainan, China
3	SP004	Sealsle 2000	Zhejiang, China
4	SP005	Breeding material	Radiation mutagenesis
5	SP006	Adalayd-2	Guangdong, China
6	SP019	Mauna key	USA
7	SP022	HI 26	USA
8	SP035	AM3554	USA
9	SP036	Temple 1	USA
10	SP041	Grif 15191	Brazil
11	SP107	Breeding material	USA
12	SP052	Breeding material	Radiation mutagenesis
13	SP053	Breeding material	Radiation mutagenesis
14	SP061	Breeding material	Anhui, China
15	SP083	Breeding material	Hainan, China
16	SP084	Breeding material	Hainan, China
17	SP089	Breeding material	Hainan, China
18	SP090	Breeding material	Hainan, China
19	SP091	Breeding material	Guangxi, China
20	SP092	Breeding material	Guangxi, China
21	SP093	Breeding material	Hainan, China
22	SP094	Breeding material	Guangxi, China
23	SP095	Breeding material	Fujian, China
24	SP096	Breeding material	Anhui, China
25	SP097	Breeding material	Zhejiang, China
26	SP098	Breeding material	Shanghai, China
27	SP099	Breeding material	Fujian, China
28	SP100	Breeding material	Hainan, China
29	SP102	Breeding material	USA
30	SP103	Breeding material	Hainan, China
31	SP106	Breeding material	Jiangsu, China
32	SP108	Breeding material	Hainan, China
33	SP110	Breeding material	Jiangsu, China

Results

Distribution and characteristics of SSR in seashore paspalum genome

A published seashore paspalum genome was used for developing SSR markers, which comprised 1,902 assembled sequences (Table 2). The total length of these sequences was 646.89 Mb, with the effective sequences accounting for 643.81 Mb. Additionally, there were 3.09 Mb of unknown bases, and the CG content of the genome sequence was 45.9%.

A total of 94,748 SSRs in the seashore paspalum genome were detected (Table 3). These SSRs spanned a total of 1,400,913 base pairs (bp), with an average length of 14.79 bp per SSR. On average,

Table 2. Summary of the genomic information of seashore paspalum.

Item	Description	Number
Total number of sequences	Counts	1,902
Total length of sequences	A + T + C + G + N (bp)	646,899,508
Total valid length of sequences	A + T + C + G (bp)	643,809,508
Unkown bases (Ns) in sequences	Bp	3,090,000
Percentage of unkown bases	Percentage (%)	0.48
GC content	Not include Ns (%)	45.9

Table 3. Frequency information of SSRs in the reference genome of seashore paspalum.

Item	Description	Number
Total number of perfect SSRs	Counts	94,748
Total length of perfect SSRs	Bp	1,400,913
The average length of SSRs	Total SSR length/total SSR counts (bp)	14.79
SSRs per sequence	Total SSR counts/sequence counts	50
The percentage of sequence covered by SSRs	Total SSR length/total sequence length (%)	0.22
Relative abundance	Total SSRs/total valid length (loci/Mb)	147.17
Relative density	Total SSR length/total valid length (bp/Mb)	2,175.97

each sequence contained 50 SSR sites. The number of bases in the SSRs accounted for 0.22% of the entire genome. The relative abundance of SSRs was calculated as 147.17 loci per megabase (Mb), while the relative density of SSRs was 2,175.97 bp per Mb. For more specific information, please refer to Table 3.

The distribution of detected SSR repeat types is shown in Table 4 and Fig. 1. Among the 94,748 SSR sites detected in the seashore paspalum genome, the number of SSRs with mononucleotide repeats is the highest (53,595), accounting for 56.57% of all SSRs, followed by dinucleotide repeats (22,044), trinucleotide repeats (16,047), tetranucleotide repeats (2,086), and pentanucleotide repeats (579), which account for 23.27%, 16.94%, 2.2%, and 0.61% of the total SSRs in the genome, respectively. The least is hexanucleotide repeats (397), which account for 0.42% of the total SSRs in the genome.

The types of SSR repeat motifs in the seashore paspalum genome are shown in Fig. 2. The most common repeat motif is A (42,895), accounting for 45.27% of all SSRs in the seashore paspalum genome. The next most common repeat motif is C (10,700), accounting for 11.29%. In addition to mononucleotide repeat motifs, AT (8,801), AG (7,522), and AC (4,203) are the most common in dinucleotide repeats. In trinucleotide repeats, AGG (2,859), ACG (2,306), and AAG (1,588) are the most common. In tetranucleotide repeats, ATAC (353) is the most common. In pentanucleotide repeats, AAAAG (75) is the most common. In hexanucleotide repeats, ATATAC (60) is the most common.

Through the analysis of the annotated files of the seashore paspalum genome, the distribution of SSRs in different regions is shown in Fig. 3. Approximately 86% of SSRs appear in intergenic regions, 7.96% of SSRs occur in exon regions, and 6.07% of SSRs occur in coding sequence (CDS) regions.

Primer design

For the discovered SSRs markers, Krait software successfully designed 85,955 primer pairs in the flanking region of the SSRs site in the seashore paspalum genome, accounting for 90.72% of the SSRs sites mined from the whole genome. The lack of primer coverage on the remaining sites might be due to insufficient flanking sequences or not meeting the appropriate primer design parameter settings. The distribution of seashore paspalum genome SSR primers on different chromosomes is shown in Table 5.

Table 4. Description of different types of SSRs in the reference genome of seashore paspalum.

Type	Counts	Length (bp)	Percent (%)	Average length (bp)	Relative dbundance (loci/Mb)	Relative density (bp/Mb)
Mono	53,595	621,084	56.57	11.59	83.25	964.7
Di	22,044	409,090	23.27	18.56	34.24	635.42
Tri	16,047	282,576	16.94	17.61	24.93	438.91
Tetra	2,086	55,620	2.2	26.66	3.24	86.39
Penta	579	16,835	0.61	29.08	0.9	26.15
Hexa	397	15,708	0.42	39.57	0.62	24.4

Mono, Di, Tri, Tetra, Penta, and Hexa refer to the types of simple sequence repeats (SSRs) with one, two, three, four, five, and six nucleotide repeats, respectively. The same abbreviations will be used throughout.

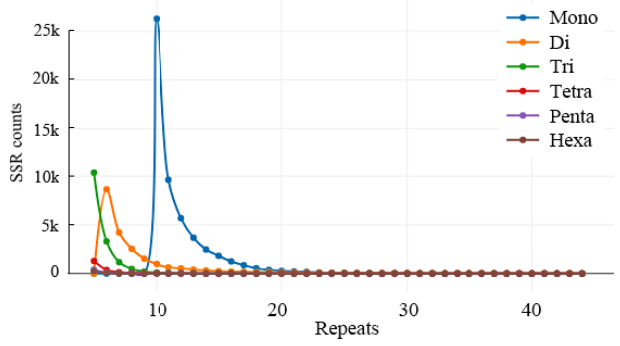

Fig. 1 The distribution and quantity information of different repeat types of SSRs in the reference genome of seashore paspalum.

Polymorphism verification and genetic diversity analysis of SSR primers

Based on the ePCR electronic simulation results of the mined SSR sites and the designed primers, this study designed 60 pairs of gSSR primers. After preliminary screening, 14 pairs of gSSR primers were determined for the genetic diversity analysis of 33 seashore paspalum collections. The results showed that 45 polymorphic loci were amplified at the predicted position by 14 pairs of gSSR primers, with an average of 3.21 loci amplified per pair of primers (Fig. 4). The primer Tm value range and the predicted amplification product information are shown in Table 6.

Using Popgene software to analyze the polymorphism of the 14 selected primers, the observed Na ranged from 2 to 4, with an average Na of 2.21; Ne ranged from 1.08 to 2.34, with an average Ne of 1.66; Shannon's genetic diversity index (I) ranged from 0.18 to 1.03,

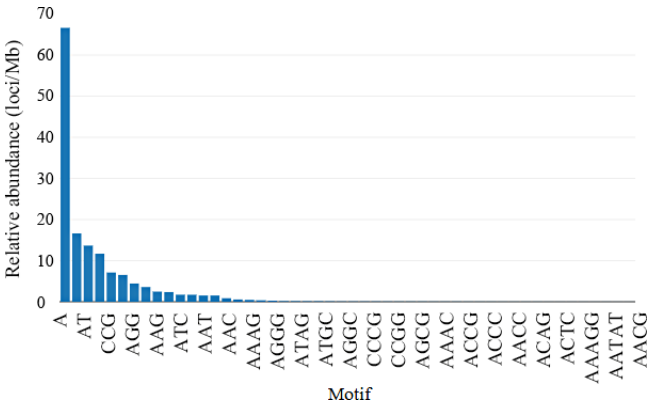

Fig. 2 The most abundant motif categories in the reference genome of seashore paspalum.

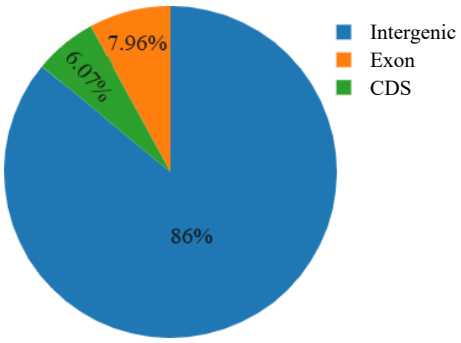

Fig. 3 Distribution of perfect SSRs in different region of seashore paspalum reference genome.

Table 5. Distribution of SSR primers in the chromosomes of the seashore paspalum reference genome.

Chromosome	Maker mapped	Chromosome	Maker mapped
CM.08	10,591	CM.14	5,991
CM.09	8,841	CM.15	4,498
CM.10	8,849	CM.16	6,282
CM.11	7,670	CM.17	6,635
CM.12	7,676	All	85,955
CM.13	6,399		

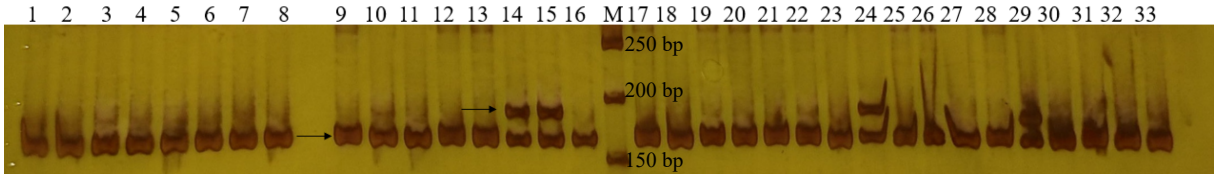

Fig. 4 Amplification of 33 collections of seashore paspalum with the SSR primers SPR75. M: 500-bp DNA marker; 1–33: SP001–SP110.

Table 6. Information of marker and genetic diversity.

Marker	Repeat motif	Forward primer	Reverse primer	Tm (°C)	Product size (bp)	Na	Ne	I	Nei's
SPR5	(GGA) 8	GAGAAGGTGACGAGGAGGC	TTCAAAAGTTTGCGGACCGG	59	219	2.00	1.98	0.69	0.50
SPR6	(CT) 9	CGATCGCTTCATGGGAGTGG	GCCTAGCTAGCTCTGCATCC	60	188	2.00	1.98	0.69	0.50
SPR10	(GA) 10	CCTTCGTTCAGTTCAGCTGC	GACCAAATTCAAGGCCCACC	59	144	2.00	1.98	0.69	0.50
SPR11	(TG) 11	TGGGCTAAGCAAGGATGAGG	AGTGATGTACAGCTGCTGCC	59	136	2.00	1.50	0.52	0.33
SPR15	(ATCT) 20	ACCAGAAACTAGCGAGGTAGC	AAGATGATCAGCAGTGCCCC	59	187	2.00	1.84	0.65	0.46
SPR16	(GAG) 11	ACTACACTAATCGGCGGTCG	TGGACTTTGGACGTCGTTCC	59	171	2.00	1.50	0.52	0.33
SPR18	(AT) 26	TCTGTAGTGGTATGCGCACG	CACTTTTACAGTTGCGGGGC	60	172	4.00	2.34	1.03	0.57
SPR26	(AT) 25	GGAGGCACTGTCTAGTATGTGG	GCGACTGAATGTTCTGGACC	59	188	2.00	2.00	0.69	0.50
SPR27	(AT) 28	GGACTGCAGGAACTTTTGAGC	CGATCCACTCTCTTTTCTTTCTCC	59	197	3.00	2.06	0.76	0.51
SPR28	(AGC) 7	GAATGGCGCTTGAGTTGAGC	CAGGATCCCCAAGGTGAGC	60	144	2.00	1.35	0.43	0.26
SPR31	(AAG) 20	ATGCCCGTACTTAGGAAGGC	TTAATCCACGGTAGGGGTGC	59	235	2.00	1.46	0.50	0.32
SPR57	(CGGC) 7	TAACTCGTCGTCGTCTCACG	ACTGACGAAGGATGGATGCG	59	135	2.00	1.10	0.18	0.09
SPR63	(TTAGA) 7	CGTGCTACATGTCCGTGGG	TTGCGCTTAATGATGGCTCC	60	135	2.00	1.08	0.17	0.08
SPR75	(GGAT) 5	CCCATGCGTGCTCAATAACG	ACATACACCACACCGTACCG	59	167	2.00	1.13	0.23	0.11
Average						2.21	1.66	0.55	0.36

with an average I value of 0.55; Nei's genetic diversity index ranged from 0.08 to 0.57, with an average value of 0.36.

Germplasm cluster analysis of seashore paspalum based on SSR

According to the UPGMA clustering results of the above 14 pairs of SSR primers on 33 collections (Fig. 5), 33 collections were divided into six categories (I, II, III, IV, V, and VI) at a genetic similarity coefficient of 0.71. Among them, categories II, V, and VI only contained a single material, which were SP083, SP096, and SP102, respectively. Category III included five collections, which were SP052, SP053, SP061, SP098, and SP097. Category IV contained two collections, which were SP95 and SP099, and the other 23 collections were classified as category I.

Discussion

SSR molecular markers can be used for plant genetic diversity analysis, construction of plant DNA fingerprinting maps, etc.[25,26]. There are various approaches to developing SSR molecular markers, including constructing gene libraries[27], and developing SSR markers through bioinformatics databases[28], etc. Liu et al.[29] used the method of constructing a gene library to develop SSR molecular markers in seashore paspalum, discovering 54 positive clones out of 13,000 clones. Jia et al.[30] successfully excavated 3,010 potential SSR molecular markers in seashore paspalum using transcriptome sequencing technology. With the rapid development of sequencing technology, the genomic information of several plants has been improved[31–33]. Genomic sequence information can be used to develop SSR molecular markers, and the number of SSR molecular

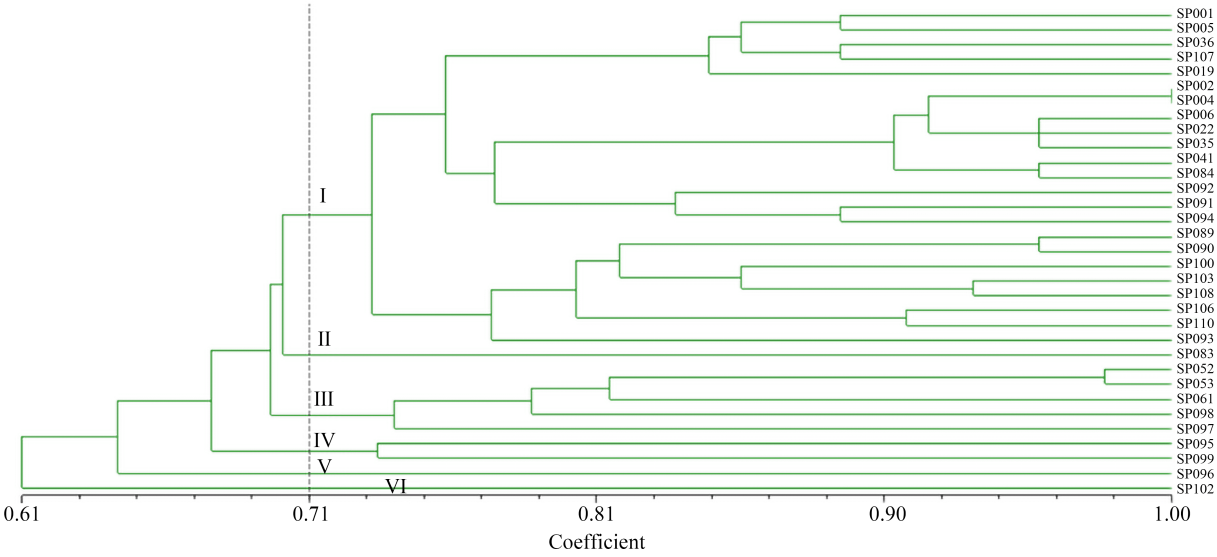

Fig. 5 UMPGA genetic clustering of 33 collections of seashore paspalum based on SSR molecular markers.

markers that can be obtained will be much higher than other methods[34].

Based on the recently published seashore paspalum reference genome information, this study carried out SSR loci mining and primer design and validation analysis. A total of 94,748 SSRs were detected (Table 3), which greatly increased the number compared to the EST-SSR number (11,568) mined by Jia et al.[30] in the 'Adalady' transcriptome of seashore paspalum. This study and their research summary on SSR mining standards are consistent, indicating that more comprehensive SSR information can be obtained by mining the seashore paspalum genome for SSR.

In the seashore paspalum reference genome, excluding mononucleotide repeats, dinucleotide and trinucleotide repeats accounted for the most, making up 23.27% and 16.94% of the total 94,748 (Table 4), respectively, which is consistent with previous studies[35–38] on the SSR genome analysis results of various plants. Although mononucleotide repeat SSRs are the most abundant and widely distributed in the genome, they are difficult to analyze after PCR amplification and electrophoresis detection. Therefore, mononucleotide repeats are rarely used as utilizable SSR markers. The most common dinucleotide repeat SSR is the AT repeat, with 8,801 occurrences, accounting for about 40% of the dinucleotide repeat type (Fig. 2), which is consistent with the finding of Powell et al.[39].

Through the analysis of the reference genome, the results show that the most number of SSRs in the seashore paspalum genome occurs in the gene interval area, accounting for as much as 86% of the SSRs, indicating that SSRs are often present in the non-coding regions of genes (Fig. 3). In addition, only 6.07% of SSRs appear in the gene coding area (Fig. 3), and the degree of polymorphism of SSR markers in the gene coding area has a greater impact on the phenotyping of plants. Therefore, the development of SSRs located in the gene coding area also has a greater impetus for plant breeding work. In this study, 14 pairs of SSR primers were utilized to analyze the genetic diversity of 33 seashore paspalum collections, in which, the observed Na ranged from 2 to 4 and Ne ranged from 1.08 to 2.34 (Table 6). The lower values of Na and Ne might be due to the close genetic backgrounds of the 33 seashore paspalum collections, indicating by results that 23 of these collections were classified into cluster I according to the cluster analysis (Fig. 5). In future studies, the agronomic traits of the 33 seashore paspalum collections would be added to investigate whether these SSR markers are associated with agronomic traits. This study designed primers for the SSR loci mined from the seashore paspalum reference genome. Primers were successfully developed from over 90% of the SSR loci (Table 5), indicating that the software used in this experiment could batch design primers for the corresponding loci of the genome, and the loci mined could be used in the subsequent SSR marker development.

Conclusions

This research used bioinformatics software including Krait and TBtools to mine SSR information from the reference genome of seashore paspalum. The distribution and quantity information of SSR sites were comprehensively obtained from the reference genome. In addition, 14 new SSR markers were successfully developed. The newly developed primers supplemented the SSR markers of seashore paspalum. In future research, these new SSR markers can be used for the genetic diversity analysis of seashore paspalum germplasm resources. Primer development methods and these primers can also be utilized for kinship analysis, molecular marker-assisted breeding, the construction of genetic linkage maps, and the localization of important traits. This approach will provide molecular technological support for breeding the new varieties in seashore paspalum.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Yang Z; funding acquisition: Yang Z, Yu J; data collection: Chen Q, Yu J; analysis and interpretation of results: Chen Q, Yu J, Wang M; draft manuscript preparation: Chen Q, Yu J. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

Dates

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