JPMB | Journal of Plant Molecular Breeding
Published by Genetics and Agricultural Biotechnology Institute of Tabarestan
2025 | Volume 13 | Issue 1

Exploring SSR patterns and properties in *Ilex cornuta* transcriptome

Hualin Zou

College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei, China

Original paper

Abstract: Based on the transcriptome data of *Ilex cornuta*, the SSR loci were identified and profiled for *I. cornuta* unigenes by using MicroSAtellite (MISA) software. A total of 31335 SSR loci were detected on 24964 Unigene sequences in 124003 Unigenes with a length greater than 500 bp. The frequency of SSR loci was 20.13 %, the frequency of occurrence was 25.27 %, and the average distribution distance was 2.62 kb in *I. cornuta*. A total of 77 repeat motif types were present in the transcriptome of this species. The major repeat types were single nucleotide (52.91%) and dinucleotide (39.47%), while the dominant repeat motifs were (A/T)n and (AG/CT)n, accounting for 50.11% and 28.11% of the total number of SSR loci, respectively. The lengths of the SSR motifs of *I. cornuta* were distributed between 10 and 92 bp with an average length of 13.81 bp, among which the number of SSRs with a length of 10 bp was the largest, with 5847, accounting for 18.66% of the total number of SSR loci. The study provides a theoretical foundation for SSR-based genetic diversity analysis and characterization of genetic resources in *I. cornuta*, while supporting future conservation efforts and molecular-assisted breeding programs.

Keywords: *Ilex cornuta, transcriptome, SSR, sequence characteristics.*

OPEN ACCESS

Edited by

Dr. SH. Hashemipetroudi,
Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University (SANRU), Iran

Date

Received: 13 February 2025
Accepted: 06 April 2025
Published: 21 April 2025

Correspondence

Dr. Hualin Zou
412052145@qq.com

Citation

Zou, H. (2025). Exploring SSR patterns and properties in *Ilex cornuta* transcriptome. *J Plant Mol Breed. 13* (1): 60-70.
doi: 10.22058/jpmb.2025.2053348.1333 .

Introduction

Ilex cornuta Lindl. et Paxt. is an evergreen small tree or shrub of Ilex in Aquifoliaceae, which is a traditional Chinese medicine. The leaves of *I. cornuta* have a long history of application in traditional medicine and is recorded in a variety of Chinese medicine classics, which have the effects of nourishing yin and clearing heat, expelling wind and dampness, calming liver and tonifying kidney (Ye et al., 2021). It has been used to treat rheumatoid arthritis for a long time in folk. The fruit of *I. cornuta* can tonify the liver and kidney, strengthen the tendons and activate the collaterals, with solid astringency and lower energizer; and Ilex cornuta bark water extract is a plant extract to promote bone healing. (Zheng et al., 2022), tonifying the liver and kidney; the root of *I. cornuta* can enhance heart health and blood flow (Balkrishna et al., 2025). Current pharmacological studies have confirmed that *I. cornuta* does play a role in the treatment of antibacterial, lowering blood sugar and blood lipids, dilating coronary arteries, anti-oxidation, immunosuppression, and anti-tumor (Liu et al., 2022). Although many drugs can inhibit the function of the immune system in clinical practice, there are few drugs that can be selected to inhibit the function of the immune system because the decline of the body's immunity when the drug plays a role can easily lead to infection (Ntim et al., 2025). The leaf of *I. cornuta* has two clinical effects of anti-infection and inhibition of immune cell activation, which makes it more and more widely used in clinical practice. Moreover, the leaf of *I. cornuta* has the advantages of abundant sources, low price and less side effects (Mao et al., 2025), indicating its significant development potential and prospect as a traditional Chinese medicine resources.

As a non-model plant, medicinal plants have the characteristics of a lack of genomic data, an unclear genetic background, and slow development (Yang et al., 2016). The analysis of transcriptome distribution and sequence characteristics after sequencing at the transcriptome level plays an important role in protecting the diversity and sustainable utilization of important medicinal plants and endangered medicinal plants (Sharma et al., 2023). Simple sequence repeat (SSR), also known as microsatellite, is a tandem repeat sequence composed of 1-6 nucleotides as the basic unit. SSR markers have significant advantages such as high polymorphism, good repeatability, co-dominance, uniform distribution, wide coverage, and strong species specificity (Sinha et al., 2025). It is one of the most widely used molecular marker technologies. With the rapid development of current high-throughput sequencing methods, the cost of sequencing has also been significantly reduced, providing greater possibilities for the development of SSR molecular markers in the future. Through the analysis of the distribution and characteristics of SSR loci in the transcriptome database after sequencing, it not only provides a more reliable theoretical basis for the design and screening of SSR primers in the future (Liu et al., 2018; He et al., 2020), but also plays a crucial role in the mapping of biological genetic maps, genetic diversity analysis, variety identification, gene mapping, and molecular marker-assisted breeding (Kumar et al., 2024). At present, SSR marker technology has been widely used in plant genetic analysis. The SSR information of Herbs plants such as Kengyilia melanthera (Xiong et al., 2022), *Valeriana jatamansi* Jones (Devi et al., 2025), *Epimedium sagittatum* (Zeng et al., 2010), *Goodyera yunnanensis* (Zhu et al., 2023), woody plants such as *Elaeagnus angustifolia* (Wang et al., 2024), *Populus deltoides* (Gao et al., 2024), *Jatropha Curcas* (Wen et al., 2010), *Paeonia suffruticosa* (He et al., 2020), *Myrica esculenta* (Ginwal et al., 2024) and *Phyllanthus emblica* (Kapoor et al., 2023) were analyzed. Nowadays, there are few reports on the development of SSR marker technology in *I. cornuta*. Therefore, this study used the data obtained from transcriptome sequencing to analyze the distribution and sequence characteristics of SSR in Ilex cornuta, which laid a theoretical foundation for the screening and development of SSR molecular markers, genetic diversity analysis, genetic map construction, variety identification and germplasm resources protection of *I. cornuta* in the future.

Materials and methods

Plant materials

The *I. cornuta* used in the experiment were collected from the West Campus of Yangtze University. The transcriptome data of *I. cornuta* were derived from the sequencing results of the roots, leaves, and fruits

of *I. cornuta* in the early stages of the research group. Transcript dataset is available and was derived from the NCBI Sequence Read Archive (SRA repository), under the accession number PRJNA399054.

SSR locus search

The MISA tool (Microsatellite identification tool) was used to search the SSR loci of 145505 Unigenes with a length of more than 500 bp. The minimum number of repeats of mononucleotide to hexanucleotide repeats was set to 10, 6, 5, 5, 5, and 5, respectively. Excel 2019 software was used to analyze the frequency of SSR loci, motif types, average distribution distance of SSR, composition, and types of repeat motifs in the transcriptome data of *I. cornuta* .

Analysis of SSR repeat motifs and sequence characteristics

SSR's distribution and sequence characteristics in the transcriptome of *I. cornuta* were analyzed by the type and frequency of repeat units, the average distance of SSR distribution, and the composition of SSR motifs. Among them, the frequency of SSR loci = SSR number / total number of Unigene sequences (Tian et al., 2022), the average distance of the SSR distribution = SSR length / total Unigene length.

SSR locus search and primer design

The primers were designed by a Perl script and Primer3.0 software. The parameters were set as follows: the length of the primers ranged from 18 to 27 bp, an optimum length of 20 bp. The expected product band size was 100-300 bp. The annealing temperature (Tm) is 58 °C ~ 65 °C, with an optimum temperature of 60 °C, and the GC content is 40 % ~ 65 %. An attempt was made to avoid primer dimers, hairpin structures, mismatches, etc.

Results

SSR locus information

A total of 124003 non-redundant genes with a length of more than 500 bp were obtained by recombining the transcriptome data of *I. cornuta* , and the total length of the sequence was 81998833 bp. A total of 31335 SSR loci were detected in 24964 Unigenes. The total length of SSR loci was 48.743 kb, and the frequency of SSR loci was 20.13 % and 25.27 %, respectively. Among them, 5124 Unigenes contained more than one SSR locus, accounting for 16.35 % of the total SSR, and 1814 Unigenes contained SSR loci in the form of complexes, accounting for 5.79 % of the total SSR (Table 1). According to the distribution, the average distance of the transcriptome sequence of *I. cornuta* is 2616.85 bp; that is, there is one SSR locus in every 2.62 kb of the sequence.

Distribution of repeat motif types and repeat numbers of SSR loci

Table 2 is the distribution of different types of SSR loci in the transcriptome of *I. cornuta*. In general, the SSR motif types in the *I. cornuta* transcriptome are abundant. Among them, the motif type with the most repetitions is the single nucleotide, with 16580 occurrences; the second was dinucleotides, with 12368 followed by trinucleotides with 2186 occurrences. These account for 39.47 % and 6.98 % of the total SSR loci, respectively. Tetranucleotide (159), pentanucleotide (19) and hexanucleotide (23) accounted for only 0.64 % of the total number of SSR loci. The distribution distance statistics of SSR repeat motifs showed that the average distribution distance of single-nucleotide was the shortest, which was 4.95 kb. This means that a single-nucleotide SSR locus can be detected every 4.95 kb on average, and the distribution density is the largest.

Table 1 Distribution characteristics of SSR in transcriptome of *Ilex cornuta.*

Search type	Search results
Total number of Unigene (bp)	124003
Total size of Unigenes sequences (bp)	81998833
Total number of identified SSR	31335
Number of SSR containing sequences	24964
Number of sequences containing more than one SSR	5124
Number of SSRs present in compound formation	1814

Table 2 Distribution of different motif types of SSR sites in the *Ilex cornuta* transcriptome.

Repeat type	Repeat number								Number of SSR loci	Frequency of occurrence (%)
	5	6	7	8	9	10	11	>11		
Mononucleotide						5847	3306	7427	16580	52.91
Dinucleotide		3352	2344	2483	2684	1283	216	6	12368	39.47
Trinucleotide	1366	550	246	19		2		3	2186	6.98
Tetranucleotide	138	19	1			1			159	0.51
Pentanucleotide	19								19	0.06
Hexanucleotide	11	6	6						23	0.07
Total	1534	3927	2597	2502	2684	7133	3522	7436	31335	100

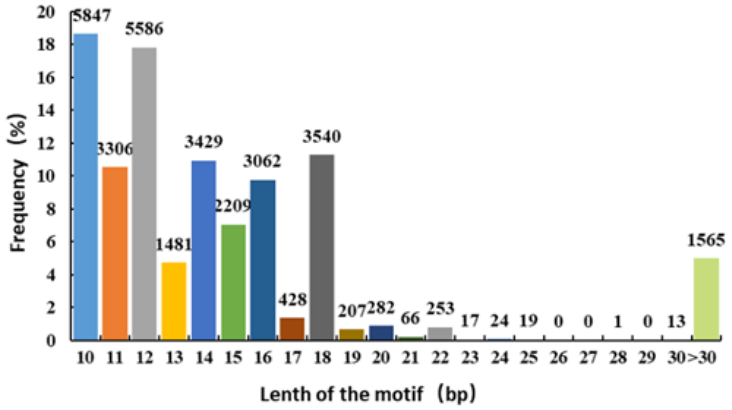

Figure 1. Different SSR motif proportions in *Ilex cornuta*.

The average distribution distance of dinucleotide and trinucleotide was 6.63 kb and 37.51 kb, respectively. The average distribution distances of tetranucleotide and hexanucleotide were 515.72 kb and 3565.17 kb, respectively. The average distribution distance of pentanucleotide was the longest, reaching 4315.73 kb, and the distribution density was the lowest. There were 14796 single nucleotide repeats with 10 ~ 14 repeats, accounting for 89.24 % of all single nucleotides. The number of dinucleotide repeats was mainly concentrated in 6 ~ 10, a total of 12,146, accounting for 98.20 % of all dinucleotides.

The trinucleotide repeats were concentrated in 5, and the number of repeats 5 accounted for 62.49 % of all trinucleotides, The number of repeats of tetranucleotides is the same as that of trinucleotides, which is concentrated in 5, and the proportion of repeat number 5 in all tetranucleotide is as high as 86.79 %. The number of pentanucleotide repeats is 100 % concentrated in 5; the number of repeats of hexanucleotide is 47.83 % concentrated in 5, and the rest is evenly distributed in the number of repeats 6 and 7. After statistics, it was found that among all SSRs, 10 repeats accounted for the most, a total of 7133, accounting for 22.76 % of the total number of SSR loci. The SSR repeat times of different primitive types of *I. cornuta* were mostly concentrated in the lower repeat times of 5-14 times, with a total of 28705, accounting for 91.61 % of the total number of SSR loci.

Characteristics of repeat motif base composition of SSR loci

Considering the principle of base complementary pairing, the statistical data are shown in Figure 1. A total of 77 types of repeat motifs exist in the transcriptome of *I. cornuta* . There are two types of repeat motifs in mononucleotides, of which $(A/T)_n$

is the dominant repeat motif, accounting for 50.11 % of the total number of SSR loci. There were four types of repeat motifs in dinucleotides, of which $(AG/CT)_n$ was the dominant repeat motif, accounting for 28.11 % of the total number of SSR loci. There were 10 types of repeat motifs in trinucleotides, of which $(AAG/CTT)_n$ was the dominant repeat motif, accounting for 1.90 % of the total number of SSR loci. There were 23 types of repeat motifs in tetranucleotides, of which $(AAAC/GTTT)_n$ was the dominant repeat motif, accounting for 0.09 % of the total number of SSR loci. There were 15 motif types in pentanucleotide, of which $(AACCC/GGGTT)_n$ was the dominant repeat motif, accounting for 0.01 % of the total number of SSR loci. There are 23 types of hexanucleotide repeat motifs, and each of them is one, so it is impossible to distinguish their dominant repeat motif types.

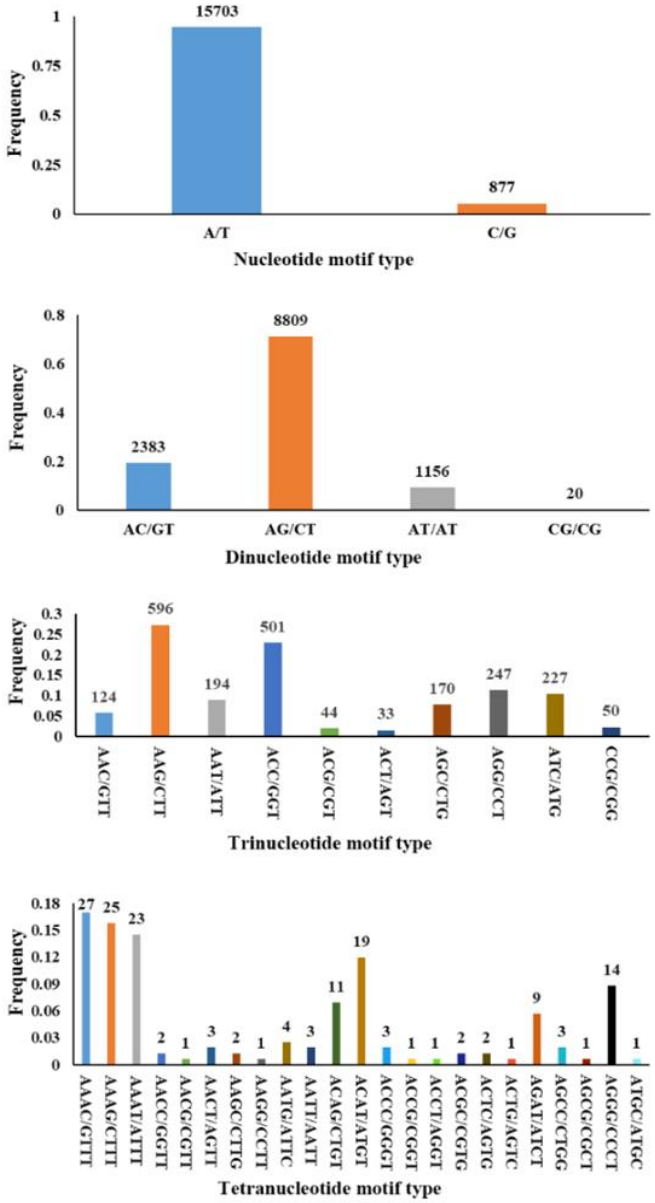

Figure 2. Length distribution of SSR site motifs in Ilex *cornuta* transcriptome.

Distribution of SSR motif length

The length of SSR motifs ranged from 10 to 92 bp, with an average length of 13.81 bp. The number of SSRs with a length of 10 bp was the largest, with 5847, accounting for 18.66 % of the total number of SSR loci. The length of SSR motifs was mainly concentrated in 10 ~ 18 bp, with a total of 29,756, accounting for 92.19 % of the total SSR loci. The average length of SSR from single nucleotide repeat type to hexanucleotide repeat type was 12.07 bp, 15.5 bp, 16.56 bp, 20.65 bp, 25 bp and 34.70 bp, respectively. In general, the number of SSR loci decreased with the increase of SSR motif length (Figure 2).

Primer design of SSR loci

According to the gene sequence obtained by transcriptome sequencing, 31335 SSR primers were designed by Primer 3 software according to the design principle of primers. One pair of primers (Table 3) was designed for each SSR, and 13423 pairs of primers (some primer sequences are attached to Table 4) were obtained, accounting for 42.84 % of the total SSR.

Table 3. SSR primer information in *Ilex cornuta*.

Repeat base type	Number	SSRs that meet primer design requirements	primer length / bp
Mononucleotide	16580	7958	10~22
Dinucleotide	12368	3998	12~92
Trinucleotide	2186	1372	15~51
Tetranucleotide	159	69	20~28
Pentanucleotide	19	12	25
Hexanucleotide	23	14	30~42

Table 4. Partial SSR repeat types and primer sequences in *Ilex cornuta*.

Gene-ID	Repeat base type	Forward primer	Reverse primer	Products length (bp)
c65327_g1	(A)12	GGAAATCAGATTGCTGCCCC	CATCTTTGCGCTTGGAGAAGG	117
c89017_g1	(T)10	TCGCAACAAAGGATATTGAGGG	TCAAACGGTTTTCTCACGCC	114
c92201_g4	(C)11	GACAATGAATTTTGAGTGCCATGG	GGGCTTGCTCAAAGACTTACC	136
c93574_g1	(G)12	GAGTCCCTGGCCAATTACCC	AACTAGCTCAGCTACACGCG	130
c71699_g1	(TG)8	GTTCTGTTCCCAATCCAAGGC	CACAACATTTTCCTTTACACCACG	150
c95624_g1	(AT)6	CATTATGATGGGTCCAAGCGC	ACACTCCCCTGAACTGAACC	152
c24800_g1	(TA)6	GCGTGGTAGGAGAAAACATATTCC	CCATATCACCTGAACAGCACG	102
c69825_g1	(CT)6	TCCACCTCCACTTTTCTCGC	ATGAACACAAACACCTCTCCC	158
c79807_g1	(CGA)5	AGCGCTCATTTTCTTCACCG	CTCTCCCCTGACATACCAGC	132
c89362_g1	(AGC)6	TAGCTACAACAGTGGCAGCC	TCTGAAGCTGCGACTAAGCC	106
c98101_g4	(TTC)5	TCTGTAGAGGGGACAAAGCC	GCCCAATCTGTCCTATTTGTGG	100
c66310_g1	(AGTT)5	GCAACAGAGCTGGTATTTCCC	TGGCTGTGCTGTCTCTTAACC	144
c94671_g3	(CTCC)7	ACAAAGTGTGTTTGGTTGGGG	TGCTTTTGAAGATCTAATGGGGC	141
c85757_g1	(CAAAA)5	CATGTTTGCCCTTGTCCAGC	GCTCTCAAGTACCGTAAGTTGG	172
c98470_g3	(GTTGG)5	AGAGAGACCGTCTGAGAGGG	AGTACTAGCCCACCTTCCCC	130
c96074_g1	(ACCTCG)7	AGCAAGTCCATCTCAGCACC	TTCCCCTGTGAGCAGAAACC	219
c97371_g3	(TCACCA)6	AACGCAATGAGAGGGCTTCC	GGTGTAGATGTGGATGGAGGG	163

Among the SSRs that met the requirements of primer design, there were 7958 SSRs of single nucleotide repeat type (accounting for 59.29 %). There were 3998 dinucleotide repeat SSRs (accounting for 28.78 %). There were 1372 trinucleotide repeat SSRs (10.22 %). There were 69 tetranucleotide repeat SSRs (accounting for 0.51 %). There were 12 (0.09 %) and 14 (0.1 %) SSRs of pentanucleotide and hexanucleotide repeat types, respectively. According to the statistics, a total of 13378 SSR loci that meet the requirements of primer design are concentrated in the range of 10 ~ 22 bp, accounting for 99.66 % of the SSR loci that meet the requirements of primer design.

Discussion

In this study, the SSR locus information was analyzed based on the transcriptome data of *I. cornuta* . A total of 31335 SSR loci were detected in 24964 Unigene sequences from 124003 Unigenes. The frequency of SSR loci was 20.13 %, which was higher than that of *Pinus yunnanensis* (3.07 %) (Cai et al., 2015) and *Zanthoxylum nitidum* (7.82 %) (Zhu, 2023b). It is slightly lower than 24.75 % of *Tussilago farfara* (He, 2019), which is much lower than 35.87 % of *Kengyilia melanthera* (Xiong et al., 2022) and 62.07 % of *Cyclocarya paliurus* (Liu et al., 2024). This may be related to the specificity of the species, the size of the sequence, and the different criteria set for SSR site search (Liu et al., 2023). The average distribution distance of *I. cornuta* SSR loci was 2.62 kb, that is, there was one SSR locus in every 2.62 kb long sequence. It is higher than *Glycyrrhiza uralensis*. (3.23 kb) (Liu et al., 2015), *Angelica biserrata* (4.9 kb) (Liu et al., 2020), *Bletilla striata* (4.41 kb) (Xu et al., 2018) and *Curcuma longa* (14.73 kb) (Joshi et al., 2010). At the same time, it shows that the number of SSR loci in the transcriptome of *I. cornuta* is abundant.

Studies have shown that the presence of a large number of simple repeat motifs in a species indicates a high level of evolution, and vice versa indicates a short evolution time or a low mutation frequency of the species (Harr and Schlötterer, 2000; Velasco et al., 2007). In this study, the largest proportion of SSR repeat types in the *I. cornuta* transcriptome is single nucleotide, accounting for 52.91 % of the total SSR, which is similar to *Goodyera yunnanensis* (Zhu et al., 2023) and *Pseudotaxus chienii* (Xu et al., 2020). Dinucleotide repeats and trinucleotide repeats accounted for 39.47 % and 6.89 % of the total SSRs, respectively. The sum of mononucleotide repeats, dinucleotide repeats and trinucleotide repeats accounted for 99.36 % of the total SSRs. Tetranucleotide, pentanucleotide، and hexanucleotide repeats accounted for only 0.64 % of the total number of SSR loci. Different from *Ilex cornuta*, some plants have the largest proportion of dinucleotide repeats, such as *Sesamum indicum* (Wei et al., 2014) and *Eucalyptus* (Liu et al., 2018). The most abundant repeat types in different plants are different. In lower plants, the dominant types are mainly high-level repeat motifs, while in higher plants, the dominant types are mainly low-level repeat motifs (Liu et al., 2023). In lower plants, such as *Poria cocos* Wolf (He, 2015), the number of hexanucleotide repeats is the largest. The statistical data show that the proportion of low-level repeat motifs in the transcriptome of *I. cornuta* is very large, indicating that *I. cornuta* may have high variability or have a long history of evolution.

Among the 77 repeat motif types of *I. cornuta* , the largest proportion was mononucleotide (A/T)n, accounting for 50.11 % of the total number of SSR loci and 94.96 % of the total number of mononucleotides. The second was dinucleotide (AG/CT)n, (AC/GT)n, (AT/AT)n, accounting for 28.11 %, 7.60 %, and 3.69 % of the total SSR loci, respectively. The dinucleotide dominant repeat motif of *I. cornuta* is the same as (AG/CT)n in some medicinal plants, such as *Epimedium sagittatum* (Zeng et al., 2010), *Mentha piperita* (Kumar et al., 2015), etc. The dominant repeat motif in trinucleotide repeats was (AAG/CTT)n, accounting for 1.90 % of the total number of SSR loci, which was also the same as the dominant repeat motifs of medicinal *Epimedium sagittatum* (Zeng et al., 2010) and *Mentha piperita* (Kumar et al., 2015) in trinucleotide repeats. In addition, this is also the same as the results of dicotyledonous plants with (AAG/CTT)n as the main trinucleotide repeat type (Morgante et al., 2002).

Studies have shown that the polymorphism of SSR is closely related to its length: when SSR < 12 bp, the polymorphism is extremely low; when it was between 12-20 bp, the polymorphism was moderate; when SSR ⩾ 20 bp, the polymorphism was higher. In this study, a total of 9153 SSRs with a

length of less than 12 bp, accounting for 29.21 % of the total SSRs; a total of 20224 SSRs were greater than or equal to 12 and less than 20, accounting for 63.64 % of the total SSRs; there were 1958 SSRs greater than 20 bp, accounting for 7.15 % of the total SSRs. After removing the extremely low polymorphic SSRs with a length of less than 12 bp, the remaining SSRs were re-counted. The results showed that the number of dinucleotide repeat SSRs (12368) > the number of single nucleotide repeat SSRs (7427) > the number of trinucleotide repeat SSRs (2186) > the number of tetranucleotide repeat SSRs (159) > the number of hexanucleotide repeat SSRs (23) > the number of hexanucleotide repeat SSRs (19). The statistical results showed that most of the SSR loci in the transcriptome of *I. cornuta* may have potential polymorphisms.

Conclusion

In summary, the transcriptome of *I. cornuta* species exhibits a substantial diversity and abundance of SSR loci.. A substantial number of SSR loci exhibit potential polymorphisms in this species . However, these findings are based solely on sequence characteristic analysis, and has not been confirmed through experimental verification. This study establishes a foundation for future research to advance the genetic understanding and conservation of *I. cornuta*, supporting SSR-based marker development, molecular breeding, and genetic mapping.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, Formal analysis, Visualization, Writing- original draft, Validation, and Investigation; H.Z.

Funding

This research received no external funding.

Acknowledgments

Conflict of Interest Statement

The author declares no conflict of interest.

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JPMB
Journal of Plant Molecular Breeding
Published by Genetics and Agricultural Biotechnology Institute of Tabarestan
2024 | Volume 13 | Issue 1

مقاله پژوهشی

بررسی الگوها و خصوصیات SSR در ترانسکریپتوم *Ilex cornuta*

هوآلین زو

دانشکده باغبانی، دانشگاه یانگ تسه، جینگژو، هوبی، چین

چکیده: براساس داده‌های ترانسکریپتوم *Ilex cornuta*، جایگاه‌های SSR با استفاده از نرم‌افزار MicroSatellite (MISA) برای ژن‌های *I. cornuta* شناسایی و پروفایل شدند. در مجموع ۳۱۳۳۵ جایگاه SSR برروی ۲۴۹۶۴ توالی یونیژن، از مجموع ۱۲۴۵۰۰ یونیژن بررسی شده‌ای که دارای طول بیشتری از ۵۰۰ باز نوکلئوتیدی بودند شناسایی شدند. فراوانی جایگاه‌های SSR به مقدار ۲۰/۱۳ درصد ، فراوانی وقوع ۲۵/۲۷ درصد و میانگین فاصله توزیع ۲/۶۲ کیلوباز در *I. cornuta* برآورد گردید. در مجموع ۷۷ نوع موتیف تکراری در ترانسکریپتوم *I. cornuta* شناسایی شد که بیشترین آنها توالی تکراری تک نوکلئوتیدی (۵۲/۹۱ درصد) و جفت نوکلئوتیدی (۳۹/۴۷ درصد) بودند. همچنین موتیف‌های تکراری (A/T)n و (AG/CT)n به ترتیب ۵۲/۹۱ درصد و ۵۲/۹۱ درصد از تعداد کل موتیف‌ها را تشکیل می‌دادند. اندازه موتیف‌های SSR در گیاه *I. cornuta* بین ۱۰ تا ۹۲ جفت باز با طول متوسط ۱۳/۸۱ جفت باز متغیر بود که در این میان تعداد ۵۸۴۷ SSR دارای موتیف‌هایی با طول بیش از ۱۰ جفت باز بوده، که ۱۸/۶۶ درصد از تعداد کل مکان‌های SSR را به خود اختصاص می‌دادند. این مطالعه یک مبنای نظری برای استفاده بیشتر از نشانگرهای SSR برای تجزیه و تحلیل تنوع ژنتیکی و شناسایی منابع ژرم پلاسم *I. cornuta* فراهم می‌کند.

کلمات کلیدی: *Ilex cornuta*، رونوشت، SSR، ویژگی های توالی.

ویراستار علمی
دکتر سیدحمیدرضا هاشمی پطرودی،
پژوهشکده ژنتیک و زیست فناوری کشاورزی طبرستان،
دانشگاه علوم کشاورزی و منابع طبیعی ساری

تاریخ
دریافت: ۲۵ بهمن ۱۴۰۳
پذیرش: ۱۷ فروردین ۱۴۰۴
چاپ: ۱ اردیبهشت ۱۴۰۴

نویسنده مسئول
دکتر هوآلین زو
412052145@qq.com

ارجاع به این مقاله
Zou, H. (2025). Exploring SSR patterns and properties in *Ilex cornuta* transcriptome. *J Plant Mol Breed. 13* (1): 60-70.
doi: 10.22058/jpmb.2025.2053348.1333 .