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Developing EST-SSR Markers for Identifying and Evaluating *Asparagus* Germplasm Resources Based on Transcriptome Sequences

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Abstract: *Radix asparagi* is the dried root tuber of the Liliaceae plant *Asparagus cochinchinensis* (Lour.) Merr., which is a major Chinese medicinal herb with high medicinal and edible value in China. The planting area of *A. cochinchinensis* is extensive, and there is blind introduction in various regions, leading to confusion the origin of *Radix asparagi* and impure germplasm. This study conducted morphological and karyotype analyses on cultivated *Asparagus* resources from seven main production areas in China and developed SSR molecular markers suitable for the identification of *Asparagus* germplasm resources based on the transcriptome sequencing results. The morphological results indicate that in addition to *A. cochinchinensis* (Lour.) Merr., recorded in the *Pharmacopoeia of the People's Republic of China*, there are also *A. taliensis* Wang et Tang and *A. lycopodineus* (Baker) Wang et Tang cultivated in China. All the tested *Asparagus* resources were diploid and had 20 chromosomes. A total of 8841 single genes containing SSR loci were identified using transcriptome sequencing of Neijiang *Asparagus*, including 761 SSR loci with trinucleotide repeat units. One hundred pairs of SSR primers were randomly designed from the trinucleotide repeat loci for PCR and polymorphism verification, and ten pairs were selected for identification of *Asparagus* germplasm resources. The genetic diversity results of ten pairs of primers in seven *Asparagus*-producing regions were consistent with the morphological identification. This study provides technical support for the comprehensive evaluation and utilization of *Asparagus* germplasm resources.

Keywords: *Radix asparagi*; traits; chromosomes; transcriptome; SSR marker; genetic diversity

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1. Introduction

Asparagus cochinchinensis (Lour.) Merr (*A. cochinchinensis*). is a perennial vine plant in the Liliaceae family. In traditional Chinese medicine (TCM), its root tubers have the effects of nourishing Yin, moistening dryness, clearing the lungs, and promoting fluid production. It is commonly used to treat dry cough, sticky phlegm, whooping cough, dry throat, thirst, and intestinal dryness with constipation [1]. *Asparagus* contains a large number of chemical components, with more than 90 isolated and identified. These components mainly include carbohydrates, saponins, sterols, lignans, and various other chemical substances [2], which have significant therapeutic effects on asthma, tumors, Alzheimer's disease, gut diseases, inflammatory diseases, and others [3–5]. In addition, *Asparagus* has various applications in health products, food, and cosmetics [6]. The extract of *Asparagus* has whitening and anti-aging effects and can be used as a raw material for fermentation and brewing [7,8], which has great development prospects and research value.

Asparagus plants are distributed in tropical and temperate regions, including China, Japan, South Korea, and Vietnam [9,10]. There are more than 30 *Asparagus* species in China,

mainly including *A. cochinchinensis*, *A. meioclados*, *A. filicinus*, *A. munitus*, *A. lycopodineus*, etc. [11] (http://www.cn-flora.ac.cn/ (accessed on 11 May 2021.)). Currently, the identification of *Asparagus* germplasm mainly relies on morphological methods, which are subjective and uncertain. Experts have not reached an agreement on the identification of the same germplasm, leading to confusion in the cultivation and use of *Asparagus* seeds, arbitrary introduction in some production areas, rampant cultivation of non-pharmacopoeia species, and insufficient promotion of genuine species. Systematically conducting comprehensive identification and evaluation of "morphology cell molecular biology" can effectively reduce the confusion regarding *Asparagus* seeds' cultivation. Cell karyotype and molecular marker identification, as cutting-edge technologies, have played an important role in the systematic identification and evaluation of crop germplasm and are of great significance in ensuring germplasm purity. However, there are currently no mature and effective identification tools applied to the actual research of *Asparagus* resources.

SSR microsatellite markers, also known as simple sequence repeats (SSRs), are abundant short tandem repeat sequences (STRs) that are scattered throughout the genome, representing genomic repeat regions [12,13]. SSR fragments are abundant in the nuclear genome and the organelle genome, and due to their stability and transferability, they have been widely used in systematic germplasm identification and population genetic analysis [14]. SSR molecular marker technology has been widely used for genetic diversity research in plants such as *Lonicera japonica*, *Dendrobium nobile*, *Astragalus membranaceus*, *Hedysarum polybotrys*, *Houpoea officinalis*, and *Ligusticum sinense* [15–19]. In the study of *Asparagus*, Zeng et al. [20] conducted genetic diversity analysis on *Asparagus* in different ecological environments and found that it had rich genetic diversity. Ou et al. [21] established the Inter Simple Sequence Repeat PCR (ISSR-PCR) genetic diversity analysis for *Asparagus*, in which the universal unidirectional primers were utilized; most of them were dominant markers, which cannot distinguish between dominant homozygous and heterozygous genotypes. So far, there have been no reports on the SSR markers of *Asparagus*, and there is a lack of research on its genetic diversity, which has led to serious constraints on the evaluation, identification, protection, and utilization of *Asparagus* resources. Therefore, research on SSR markers and genetic diversity in *Asparagus* is of great significance.

Responding to the current situation of confusing germplasm resources and deficient molecular marker identification technology for *Asparagus*, our study was carried out to develop stable and effective SSR primers for the identification of germplasm resources of *Asparagus* through transcriptome sequencing of *A. cochinchinensis* (the species specified in the Chinese *Pharmacopoeia*) and to identify and evaluate the resources of *Asparagus* in different regions by combining morphological, pharmacological, and chromosomal analyses to reveal the genetic diversity of *Asparagus*. The results of this study will be useful for the identification and evaluation of *Asparagus* species in different regions. This study will provide a molecular basis and technical support for the identification of the germplasm of *Asparagus*, genetic diversity analysis, and the production and promotion of genuine *Asparagus*.

2. Materials and Methods

2.1. Plant Materials and DNA Extraction

The *Asparagus* materials used in this study were locally cultivated from seven areas in five provinces of China. They were staminiferous plants that have been domesticated for more than 20 years. They were uniformly planted in the resource nursery of the Academy of Agricultural Sciences in Neijiang City, Sichuan Province using asexual ramets propagation. Through long-term phenological observation, we found that they had distinct morphological characteristics (Table 1). The root tips used for chromosome observation were collected as needed to ensure the activity of the root tip cells.

Table 1. Provenances of *Asparagus*.

ID	Genotypes	Population	Provenance Location	Sample Size	Longitude (E)	Latitude (N)	Elevation (m)
TD-1	TD1-1	NJ	Neijiang, Sichuan	1	105°8′	29°49′	370
	TD1-2			1	105°22′	29°49′	350
	TD1-3			1	105°7′	29°47′	350
	TD1-4			1	105°12′	29°49′	440
	TD1-5			1	105°10′	29°49′	500
TD-2	TD2-1	WC	Wuchuan, Guizhou	1	107°55′	28°42′	1020
	TD2-2			1	108°09′	28°58′	864
	TD2-3			1	108°06′	28°41′	890
	TD2-4			1	108°24′	28°42′	720
	TD2-5			1	108°13	28°30′	750
TD-3	TD3-1	YL	Yulin, Guangxi	1	110°1′	22°28′	80
	TD3-2			1	110°10′	22°37	75
	TD3-3			1	109°59′	22°32′	80
	TD3-4			1	109°58′	22°29′	80
	TD3-5			1	109°56′	22°31′	85
TD-4	TD4-1	HH	Honghe, Yunnan	1	103°30′	22°51′	1380
	TD4-2			1	101°53′	24°58′	1356
	TD4-3			1	102°44′	23°27′	1983
	TD4-4			1	102°32′	23°29′	1689
	TD4-5			1	102°57′	25°9′	1891
TD-5	TD5-1	CX	Chuxiong, Yunnan	1	101°53′	24°58′	1350
	TD5-2			1	101°55′	25°1′	1324
	TD5-3			1	101°43′	24°38′	1345
	TD5-4			1	102°13′	24°23′	1280
	TD5-5			1	103°2′	24°56′	1320
TD-6	TD6-1	KY	Kaiyang, Guizhou	1	107°13′	26°53′	1080
	TD6-2			1	107°11′	26°58′	1158
	TD6-3			1	106°54′	26°53′	980
	TD6-4			1	106°35′	27°12′	880
	TD6-5			1	106°21′	27°20′	1205
TD-7	TD7-1	ES	Enshi, Hubei	1	109°29′	30°16′	780
	TD7-2			1	108°59′	29°37′	914
	TD7-3			1	109°5′	29°42′	910
	TD7-4			1	109°14′	29°52′	894
	TD7-5			1	108°50′	29°20′	950

The young stem tips of the *A. cochinchinensis* plant line TD-1 were used for the transcriptome sequencing, which. had good traits, with abundant anthocyanins in its tender stems; normal growth of tissue materials; and no disease, pest, or mechanical damage. Three replicates were sampled, slam-frozen in liquid nitrogen, and stored at −80 °C for subsequent RNA extraction and transcriptome sequencing.

The tender stems of *Asparagus* germplasms were used for SSR screening and genetic diversity evaluation. The Plant DNA Extraction Kit (TSINGKE TSP101-200, Beijing, China) was used to extract DNA from seven tender stems of *A. cochinchinensis*. The concentration and purity of the genomic DNA were quantified using NanoDrop 2000 ultra-micro spectrophotometer (Thermo, Dalawie, MA, USA). The qualified genomic DNA was diluted to 20 ng/μL and stored at −20 °C.

2.2. Morphological Examination

With *Flora of China* [11] as the reference, the morphological characteristics of the *Asparagus* plants were examined. The main indicators were plant type, stem length, stem characteristics, number of leafy branches, leafy branch morphology, hard thorn length, pedicel length, flower color, filament attachment, root length to thickness ratio, etc., which were recorded using a Canon EOS R8 digital camera to identify the *Asparagus* plants from

different origins. The characteristics of medicinal materials were described in accordance with the provisions of the *Pharmacopoeia of the People's Republic of China* [22], and the root traits, length, thickness, organoleptic, stone cells, and calcium oxalate needle crystals of the different germplasm resources were compared. The cross sections, stone cells, and needle crystal bundles of calcium oxalate of *Radix asparagi* was observed under an optical photographic microscope (OLYMPUS, BX51, Olympus Inc., Tokyo, Japan).

2.3. Examination of Root Tip Chromosomes

The chromosome preparation and observation was conducted as previously described [23,24]. A 1–2 cm sample of the meristem tissue was taken from the root tip of plant material and put into a moist 5 mL Eppendorf tube. It was treated with nitrous oxide (N_2O) for 2 h, fixed with glacial acetic acid for 5–10 min, and then stored in 70% alcohol at −20 °C. The fixed and preserved root tips were rinsed 1–2 times in dd H_2O, and a 2–3 mm portion of the front end was cut off and put into 10 μL of enzyme solution (cellulase:pectinase = 4:2), which was subject to a 37 °C water bath for 40 min. The root tips were rinsed once with dd H_2O and then twice with anhydrous ethanol. After rinsing, 20–30 μL of glacial acetic acid was added to prepare a cell suspension. A 10 μL sample of the cell suspension was dropped onto a clean glass slide each time; after air-drying, cells were examined with an electron microscope (Leica, DM3000, Leica Camera AG, Wetzlar, Germany) for imaging.

2.4. RNA Extraction and Transcriptome Sequencing

The tender stems of TD-1 were used to extract RNA with the RNAprep Pure Plant Total RNA Extraction Kit (TSINGKE TSP411, Beijing, China); the extracted RNA was subject to DNase digestion. The cDNA library was prepared as previously described [25]; firstly, the eukaryotic mRNA was enriched with Oligo (dT), then the mRNA was decomposed into short fragments by adding interruption reagents, and the interrupted mRNA was used as a template to synthesize single-stranded cDNA using 6-base random primers. The purified double-stranded cDNA was subjected to end repair and A-tailing, fragment size selection, and PCR amplification to construct a sequencing library. Unreferenced transcriptome sequencing was performed in Qingke Biotechnology Co., Ltd. (Beijing, China) to complete the de novo assembly and related analysis of transcriptome data.

2.5. SSR Marker Development and PCR Primers

After obtaining transcriptome data, MISA (Microsatellite Identification Tool) software was used (http://pgrc.ipk-gatersleben.de/misa/misa.html (accessed on 8 July 2021) to perform expressed sequence tag (EST)-SSR loci screening on the Unigene sequence of the *A. cochinchinensis* transcriptome, with screening criteria set as: mononucleotide repeats ≥10 times and di-, tri-, tetra-, penta-, and hexanucleotide repeats ≥5 times. Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA) was used for PCR primer design. In order to obtain better PCR amplification, the following MusaKavas parameters [26] were adopted: PCR product length was 100–300 bp, primer length was 18–23 bp, GC content was 40–60%, annealing temperature was 52–60 °C, and the difference in annealing temperature between forward and reverse primers was <2 °C. The designed primers were subjected to BLAST analysis in the NCBI database to determine target specificity. A total of 100 pairs of primers were designed and synthesized by the Qingke Biotechnology Company (Beijing, China).

2.6. SSR-PCR and Polymorphism Analysis

One hundred pairs of synthesized fluorescent primers were used for PCR amplification of TD-1. The components of the amplification system are shown in Table 2. The amplification procedure followed the previous laboratory research: pre-denaturation at 98 °C for 3 min; 98 °C for 10 s, 62 °C for 10 s, 72 °C for 10 s, for a total of 39 cycles; extend at 72 °C for 5 min; and store at 4 °C. The PCR products were separated with 1% agarose gel electrophoresis, and the specificity of PCR products was investigated using a DL100

DNA marker (Tiangen Biotechnology Co., Ltd., Beijing, China) as the size standard of PCR products. The recovery, purification, and sequencing of the running gel products were performed to verify reproducibility. SSR primers with good specificity and repeatability were selected and synthesized into fluorescent primers. A second round of fluorescence PCR reaction was conducted, with primers containing the adaptor, modified primers of the adaptor, and corresponding reverse primers. The three primers could be used in multiplex PCR to achieve the goal. The final amplified fluorescent PCR products were subject to 3730xl sequencing, and the obtained data were analyzed using Genemapper 5.0 software. Based on the analysis results, whether different primers led to specific fragment polymorphism was judged. Finally, primers with good polymorphism, repetitive sequences, and high specificity were selected for SSR-PCR amplification of seven individuals.

Table 2. Amplification system of SSR-PCR.

Component	Volume
2 × T5 super PCR Mix (PAGE)	10 μL
10 μM Primer F	1 μL
10 μM Primer R	1 μL
Template (genomic DNA)	1 μL
ddH_2O	7 μL
Total	20 μL

2.7. Data Analysis

After screening the SSR primers for polymorphism, the SSR results were evaluated as previously described [27]. The specific bands of each individual were counted as either 0 (absent) or 1 (present). The polymorphic bands generated with different primers present in the sample were recorded as "1" at a polymorphic site, while those that were absent were recorded as "0", thus constructing a matrix of 0 and 1. The data were edited and formatted using GenAlEx version 6.51 software [28]; various genetic diversity indicators of the SSR loci were calculated using Popgen1.32 software, including observed alleles (Na), effective alleles (Ne), heterozygosity (H), Shannon index (I), etc. In the NTSYS 2.1 software, based on the similarity matrix, the unweighted pair group method with arithmetic means (UPGMA) was used to establish individual clustering trees . Finally, based on Nei's genetic distance, principal coordinate analysis (PCoA) was performed on all samples [29].

3. Results

3.1. Plant Morphology

The morphological results indicate that the samples collected from Neijiang (TD-1), Yulin (TD-3), Chuxiong (TD-5), Kaiyang (TD-6), and Enshi (TD-7) were *A. cochinchinensis* (Lour.) Merr. Its stem is smooth, with angular branches and clusters of 3–6 leafy branches, forming an acute triangular shape. The length of the hard thorns on the stem is 2.5–3.5 mm; every two flowers are axillary, light green. The pedicel is 2–4 mm long, and the joints are generally located in the middle. The filaments are not attached to the perianth segments. The plants collected from Wuchuan (TD-2) and Honghe (TD-4) were *A. taliensis* Wang et Tang (Table 3, Figure 1).

Table 3. Germplasm resources of *Asparagus* used in this study.

ID	Plant Type	Average Stem Length (cm)	Stem	Number of Leafy Branches	Leafy Branch Morphology	Length of Leafy Branch (mm)	Hard Thorn (mm)	Pedicel Length (mm)	Flower Color	Filiform Adhesiveness	Root Length: Root Thickness	Botanical Origin
TD-1	Climbing vine	120	Smooth stem with longitudinal lines	3–6	Sharp triangular shape	12	3	2	Pistachio	Filaments are not attached	7:1	*A. cochinchinensis*
TD-2	Climbing vine	180	Stem is smooth and longitudinal lines are not obvious	6–9	Sharp triangular shape	9	4	2.5	Yellow	Middle and lower parts of filament are attached	6:1	*A. taliensis*
TD-3	Climbing vine	140	Smooth stem with longitudinal lines	3–5	Sharp triangular shape	14	3.5	2.5	Pistachio	Filaments are not attached	6:1	*A. cochinchinensis*
TD-4	Climbing vine	210	Stem is smooth and longitudinal lines are not obvious	6–9	Sharp triangular shape	8	4.5	3	Yellow	Middle and lower parts of filament are attached	7:1	*A. taliensis*
TD-5	Climbing vine	130	Smooth stem with longitudinal lines	3–5	Sharp triangular shape	13	3.5	2	Pistachio	Filaments are not attached	6:1	*A. cochinchinensis*
TD-6	Climbing vine	140	Smooth stem with longitudinal lines	3–5	Sharp triangular shape	12	3	2.5	Pistachio	Filaments are not attached	7:1	*A. cochinchinensis*
TD-7	Semi-erect vine	110	Stem is smooth and longitudinal lines are not obvious	2–3	Sickle shape	13	Inconspicuous	No	No	No	5:1	*A. cochinchinensis*

Figure 1. *Asparagus* plants and root characteristics. (**a**,**b**) TD-1 plant morphology and male flowers; (**c**,**d**) TD-2 plant morphology and male flowers; (**e**,**f**) TD-7 plant morphology.

3.2. Microscopic Characteristics of Asparagus Medicinal Materials

The characteristics of the medicinal parts of *Asparagus* medicinal materials are shown in Table 4 and Figure 2. Among all the tested samples, there is a significant difference in the shape of the dry root tubers. The root tubers of TD-2 and TD-4 were longer and larger than those of other materials, which complies with the description of 5–18 cm in the *Pharmacopoeia*; stone cells and calcium oxalate needle crystals were found in all samples under a microscope. In TD-2 and TD-4, the large columns were eye-catching, with a bitter taste and no sweet taste.

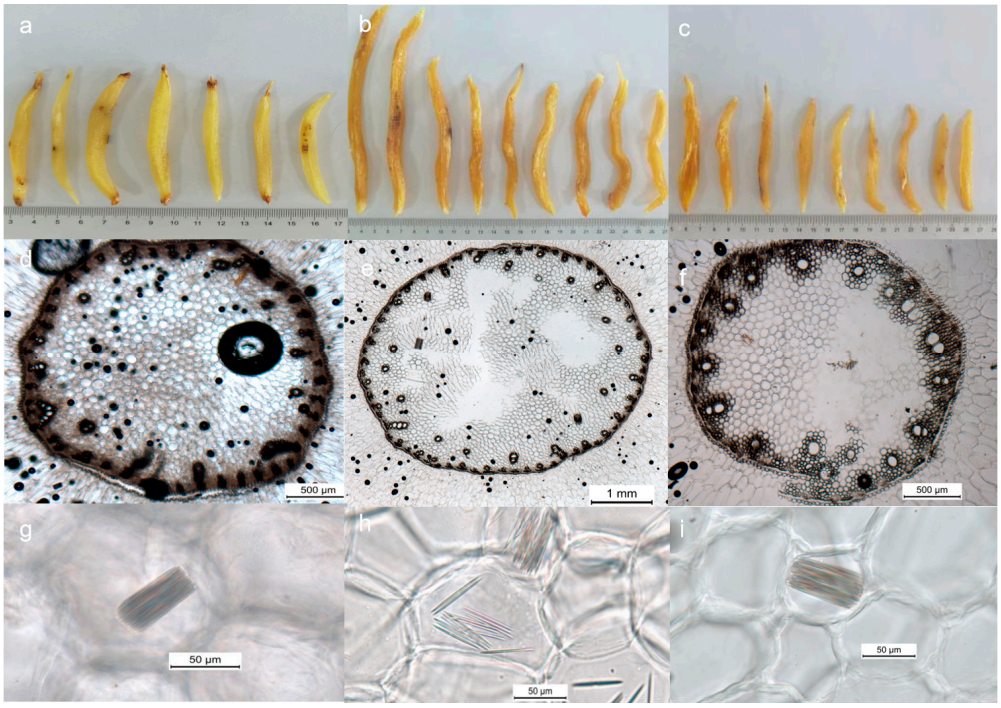

Figure 2. Characteristics of the medicinal parts of *Asparagus* plants. (**a**–**c**) dry root tubers of TD-1, TD-2, TD-7; (**d**–**f**) microscopic cross-section of TD-1, TD-2, TD-7 root tubers; (**g**–**i**) calcium oxalate needle crystals in TD-1, TD-2, TD-7.

Table 4. Dried root tubers of *Asparagus* resources.

ID	Root Shape	Root Length: Thickness	Stone Cell	Calcium Oxalate	Organoleptic
TD-1	Spindle	10:1	Occasionally seen	Yes	Light yellow, transparent, sweet taste
TD-2	Long spindle	12:1	Yes	Yes	Yellow, translucent, bitter
TD-3	Spindle	10:1	Yes	Yes	Yellow, transparent, sweet, slightly bitter
TD-4	Long spindle	13:1	Yes	Yes	Yellow, translucent, bitter
TD-5	Spindle	9:1	Occasionally seen	Yes	Pale yellow, transparent, slightly bitter
TD-6	Spindle	9:1	Occasionally seen	Yes	Pale yellow, transparent, sweet, slightly bitter
TD-7	Short spindle	7:1	Occasionally seen	Yes	Light yellow, transparent, sweet taste

3.3. Root Tip Chromosomes

The chromosomes of the *Asparagus* plants were examined under an electron microscope (Figure 3); there were 10 pairs (20) of chromosomes in samples from all producing regions, which were generally very short (2.2–5.6 μm). All samples were diploid.

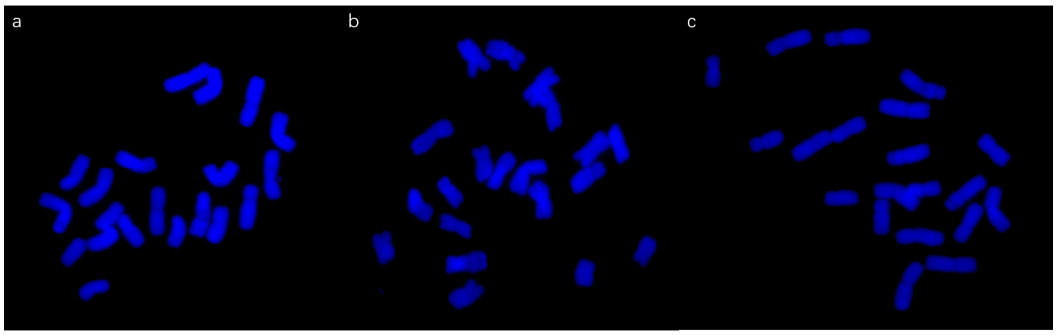

Figure 3. Root tip chromosomes of *Asparagus* plants. (**a**) TD-1; (**b**) TD-2; (**c**) TD-7.

3.4. Transcriptome and Microsatellite Characteristics of Asparagus

In transcriptome sequencing of *A. cochinchinensis*, approximately 6.83 G transcriptome data were obtained. In the MISA search, 8841 Unigenes containing SSR loci were identified, in which 1057 Unigenes had different base repeat units, with a frequency of 11.96%. Among the SSR loci, the trinucleotide repeats the most, i.e., 761 (71.99%), with CGG/CCG (44) and CCG/CGG (32) predominant; dinucleotide repeats took second place with 218 (20.62%), in which AG/CT (39) and AT/AT (31) were predominant. There were 18 tetranucleotide repeats, 20 pentanucleotide repeats, and 40 hexanucleotide repeats, in which no prolific repeating elements were found (Table 5).

Table 5. SSR repeat types in the young stem of *A. cochinchinensis* TD-1.

Type of Repeat	Number	Percentage (%)	Number of Predominant Repeat Motifs	Percentage of Predominant Repeat Motifs (%)
Dinucleotide	218	20.62	AG/CT:39 AT/AT:31	17.89 14.22
Trinucleotide	761	71.99	CGG/CCG:44 CCG/CGG:32	5.78 4.21
Tetranucleotide	18	1.71	TTTC/GAAA:2	11.1
Pentanucleotide	20	1.89	No	No
Hexanucleotide	40	3.78	No	No
Total	1057			

Footer: Each motif of pentanucleotides and hexanucleotides occurred only once.

3.5. Development of SSR Primers for Asparagus

To carry out germplasm resource identification and genetic diversity analysis of *Asparagus*, 100 pairs of SSR primers were randomly designed from the SSR loci identified

from the transcriptome data of TD-1. The first round of PCR amplification showed that 74 of the 100 pairs of primers had specificity (Figure 4), and the sequencing results suggested that 41 PCR products contained SSR sequences. A second round of fluorescence PCR was conducted on seven *Asparagus* samples, and 10 out of 41 pairs of primers displayed high specificity, repeatability, and polymorphism (Figure 5 and Table 6).

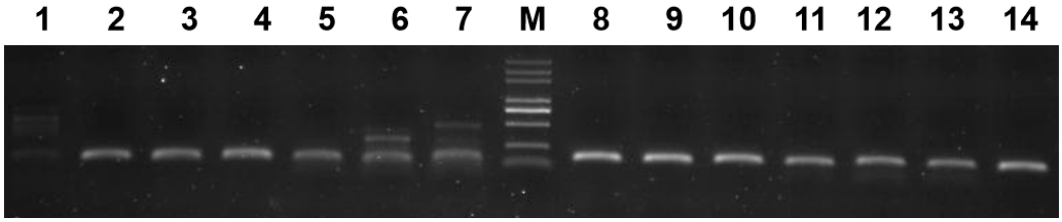

Figure 4. Agarose gel electrophoresis shows the results of the first round of PCR amplification. PCR products from 14 out of 100 pairs of SSR primers are shown. The primer name from left to right is: Asp.1, Asp.7, Asp.8, Asp.13, Asp.29, Asp.34, Asp.36, Asp.47, Asp.61, Asp.64, Asp.87, Asp.88, Asp.95, Asp.99; the genomic DNA of TD-1 was the PCR template.

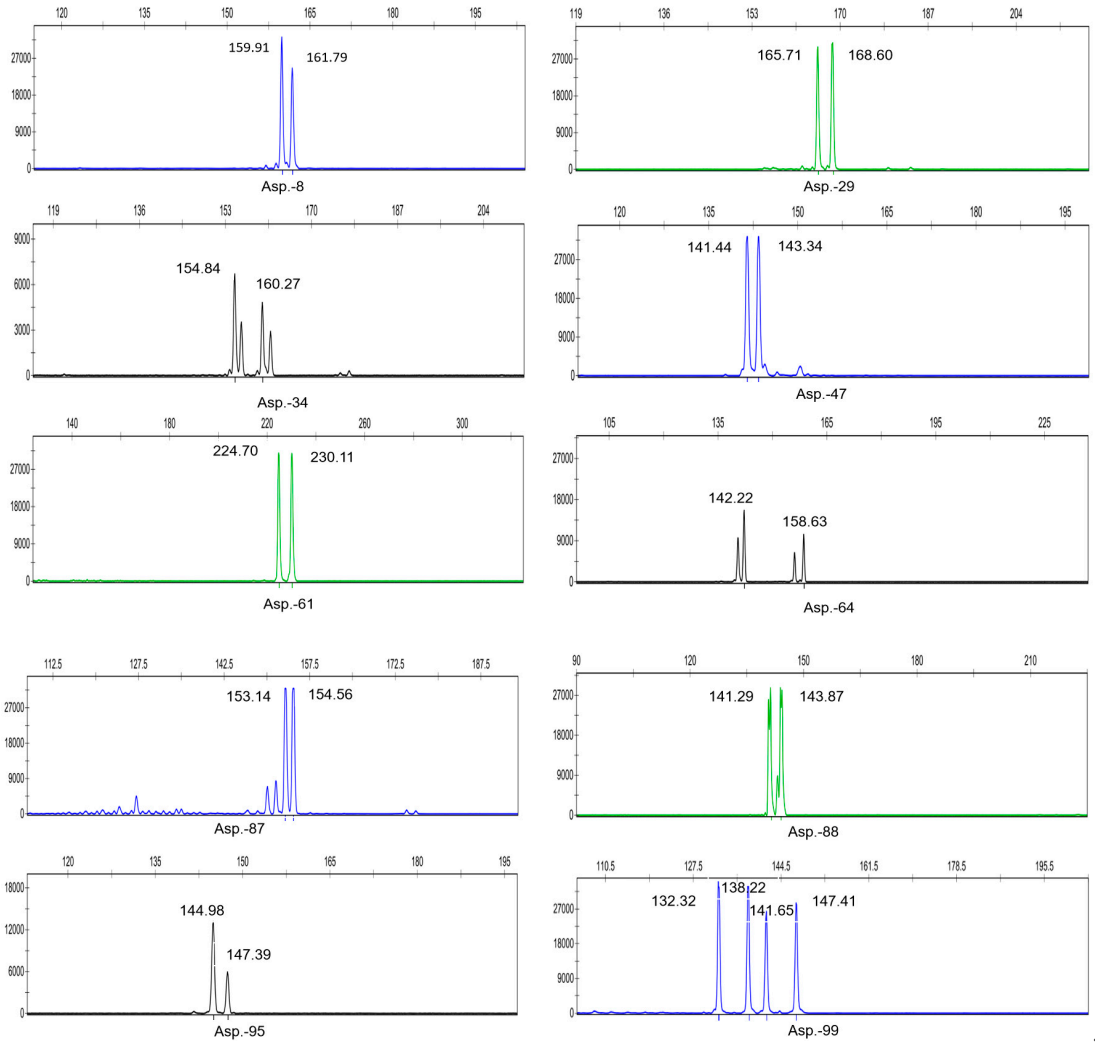

Figure 5. Results of fluorescent capillary electrophoresis using ten SSR primers (taking TD-4 as an example). The x-axis represents fragment size of an amplified microsatellite, and the y-axis represents the fluorescence intensity of the amplified products.

Table 6. Primer screening.

Primer ID	Primer Sequence	Original Repeating Unit	Number of Repeats	Annealing Temperature	Specificity	Polymorphism	PCR Product Size (bp)
Asp.-8	F:AACCTGTACAGCTCGTCGATG R:TGTTGCAGAACATCGCGAAG	CAC (3 × 5)	15	57	Yes	Yes	160
Asp.-29	F:ATGAGGACGTTGGACCAGTAATC R:GACAAGCTAGAGAGGTACAGAGC	CGC (3 × 5)	15	59	Yes	Yes	158
Asp.-34	F:TGACGATGATGAGAGGGATGAAG R:TTCAAAGGGGAAGGGAAAAACTG	CCG (3 × 7)	21	57	Yes	Yes	158
Asp.-47	F:GTCCATGTCTTCCTCCTTCGAC R:GGACTCCGGCATCGAGAAG	CGG (3 × 7)	21	60	Yes	Yes	157
Asp.-61	F:ACAGATCTCAATCATCCCAGGTT R:CTCCTTAATCAGAAGGGCTGTGT	CAT (3 × 8)	24	57	Yes	Yes	155
Asp.-64	F:AGCTACTTATCCGCCACTCTTTC R:TCCCACCTCACTATACAGACCAT	ACC (3 × 7)	21	58	Yes	Yes	155
Asp.-87	F:TTTGAGACTCAAGCAAAAGCACC R:TGCTTAGGAACTCTAAACACTGT	TTA (3 × 8)	24	55	Yes	Yes	151
Asp.-88	F:TGATCCTGTTCAGGAACGAAGAG R:TTCCTTTCTCAAGATCCAGAGCC	TGC (3 × 7)	21	58	Yes	Yes	151
Asp.-95	F:CTCGAGTTCACCGTCCAAAAC R:GAGGAGGACAGGGAGATGCTAT	CGT (3 × 6)	18	59	Yes	Yes	149
Asp.-99	F:GAGTCGCTGAACTTCCATCTGAG R:GATCCCAACCCGAACCCTACTC	CGC (3 × 8)	24	60	Yes	Yes	149

3.6. SSR Primer Diversity

The genetic diversity evaluation results of 10 SSR loci are shown in Table 7. A total of 71 allele loci were detected in 7 samples using 10 pairs of primers. Among them, the primers Asp.-34 and Asp.-95 led to the minimum number of alleles, i.e., 5; Asp.-61 and Asp.-87 led to the maximum number of alleles (9); and the average number of alleles per locus was 7.1000. The total number of effective alleles (Ne) was 13.7362, with a range of 1.2499 (Asp.-87) to −1.5544 (Asp.-99). The average number of Ne per locus was 1.3714. The Shannon index (I) ranged from 0.3337 (Asp.-87) to −0.5191 (Asp.-99), with an average value of 0.4040. The average observed heterozygosity (H) of the loci ranged from 0.1908 (Asp.-87) to −0.3405 (Asp.-99), with an average of 0.2486. These results showed that the 10 selected pairs of SSR primers of *Asparagus* had relatively rich polymorphism.

Table 7. Polymorphism of 10 pairs of SSR primers.

Primer ID	No. of Bands	No. of Polymorphic Bands	Proportion (%)	Na Allele Number	Ne Number of Effective Alleles	H Heterozygosity	I Shannon Index
Asp.-8	14	6	42.86	2.00	1.45	0.28	0.45
Asp.-29	14	8	57.14	2.00	1.33	0.22	0.36
Asp.-34	9	5	55.56	2.00	1.33	0.23	0.37
Asp.-47	12	8	66.67	2.00	1.26	0.20	0.34
Asp.-61	21	9	42.86	2.00	1.44	0.28	0.44
Asp.-64	14	7	50.00	2.00	1.37	0.25	0.41
Asp.-87	13	9	69.23	2.00	1.25	0.19	0.33
Asp.-88	16	7	43.75	2.00	1.43	0.28	0.45
Asp.-95	9	5	55.56	2.00	1.32	0.23	0.39
Asp.-99	22	7	31.82	2.00	1.55	0.34	0.52
Mean	14	7	51.54	2.00	1.37	0.25	0.40
St. Dev				0.00	0.26	0.12	0.15

3.7. Genetic Structure of Asparagus Populations

The cluster analysis was conducted on seven populations of *Asparagus* to construct a UPGMA tree (Figure 6). The genetic distance and correlation of *Asparagus* resources were analyzed using PCoA (Figure 7). When K = 2, SSR markers that can be amplified with 10 pairs of PCR primers divided 7 *Asparagus* germplasms into 2 groups. The first group mainly consisted of TD-2 (Wuchuan, Guizhou) and TD-4 (Honghe, Yunnan), with

significantly longer stems. Their flowers were yellow, with reflexes; there were clusters of 5–7 leaves and large, thick root tubers. The second group included TD-1 (Neijiang, Sichuan), TD-3 (Yulin, Guangxi), TD-5 (Chuxiong, Yunnan), TD-6 (Kaiyang, Guizhou), and TD-7 (Enshi, Hubei). They were low plants, with semi-erect or creeping stems, light yellow-green flowers, clusters of 3–6 leaves, and short root tubers. Among them, TD-7 can be distinguished from other resources and is a separate subgroup. Its leafy branches were flattened and no flowering was observed. The cluster analysis and genetic structure analysis indicated that its genetic background and morphological traits were related to environmental factors.

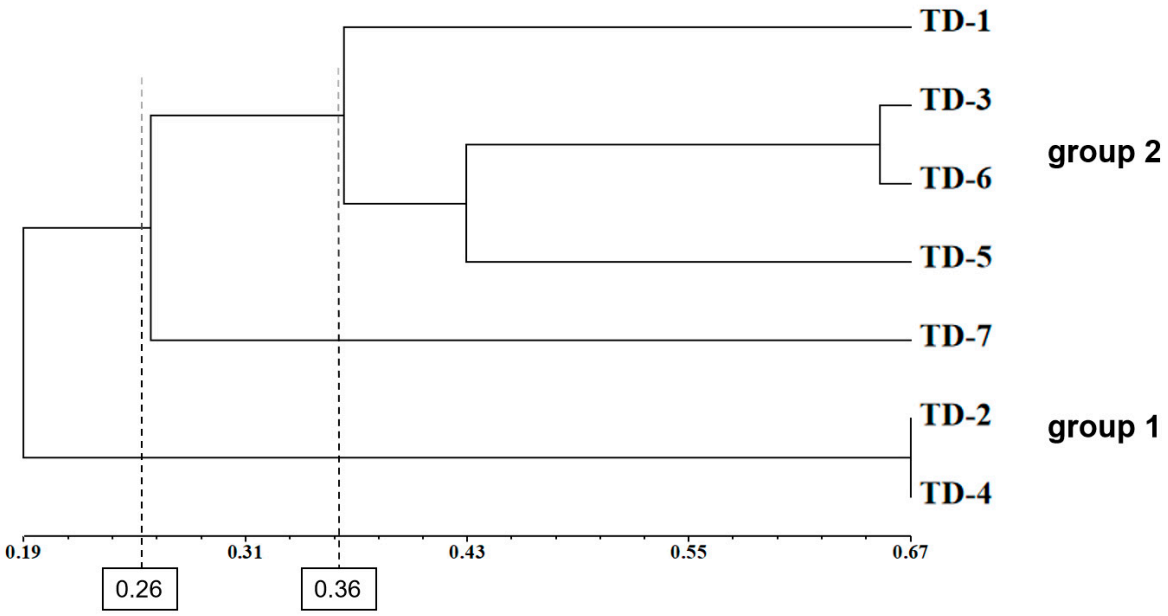

Figure 6. UPGMA clustering analysis of seven *Asparagus* resources.

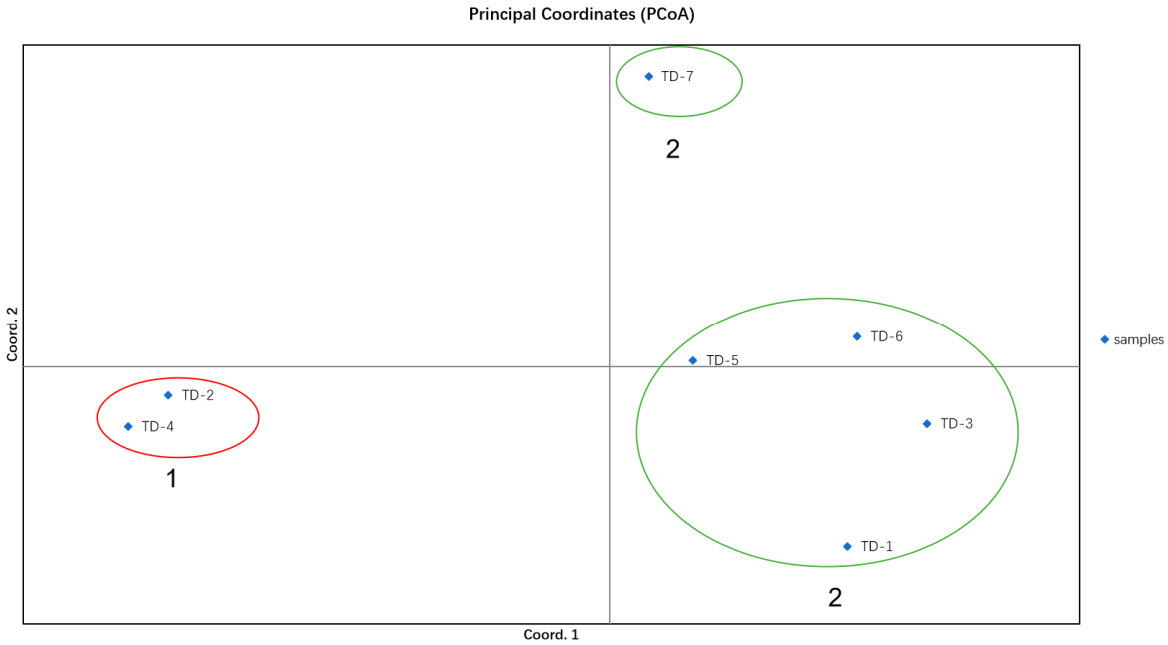

Figure 7. PCoA result of seven *Asparagus* resources. The red circle represents Group 1, and the green circle represents Group 2.

4. Discussion

Asparagus is a high-yield economic crop with medicinal and health values. *Radix asparagi* was first recorded in the "Shennong Materia Medica Classic" [30] and is listed as a top-grade herb. It has been recorded in the Materia Medica of various dynasties and is now one of the commonly used Chinese medicinal herbs. China has a wide variety of *Asparagus* germplasm and a large planting area, but there are also problems such as name confusion, variety degradation, poor quality, and low yield. According to textual research on this herb, there are many cases of mistakenly recognizing *Asparagus meioclados* as "Little *Asparagus*" (Xiao Tian Dong in Chinese) and *Asparagus filicinus* as "Indigenous *Radix stemonae*" (Tubaibu in Chinese) [31]. In the medicinal research on *Asparagus*, it has been found in various regions. Closely related species of the same genus are used as *Radix asparagi* for medicine, and even plants from other families are used as *Radix asparagi* [32]. Therefore, conducting systematic identification and evaluation of *Asparagus* plants is an important means to ensure the purity of germplasm resources and drug safety. This study provides a more accurate identification and classification of *Asparagus* germplasm through the morphological description, cytological examination, and development of transcriptome-based SSR markers, laying a foundation for the identification and evaluation of *Asparagus* germplasm resources, germplasm improvement, resource protection, and sustainable utilization.

4.1. Morphological Examination

This study conducted a morphological investigation of *Asparagus* resources from different production areas based on the *Flora of China*. Three different germplasm types were identified, i.e., *A. cochinchinensis*, *A. taliensis*, and *A. lycopodineus*, and the latter two were non-pharmacopeial resources. The tuber root yield and size of *A. taliensis* in Yunnan and Guizhou provinces are superior to those of *A. cochinchinensis*. The indigenous people prefer local varieties that grow well and are superior in shape and yield for local cultivation. Production process, could be reasonable despite the phenomenon of chaotic planting of *Asparagus*.

4.2. Chromosome Examination

The predominant chromosome number of *Asparagus* is x = 10, and in some cases x = 8, 9, 15. This aneuploidy phenomenon is mainly manifested at the intraspecific level [33], such as x = 9, 10 in *A. dauricus* and x = 8, 9, 10 in *A. filicinus*. This phenomenon has also been observed in other genera such as *Polygonatum* [34] and *Cyananthus* [35]. In this study, all seven *Asparagus* resources had ten pairs of diploid chromosomes (n/2n = 10/20), which is consistent with previous studies [36]. However, further analysis is needed to study their karyotype formula.

4.3. SSR Markers

There are a large number of genetic variations in the *Asparagus* populations, which control traits such as flowering, leaf shape, flower color, stem color, and tuber morphology. The analysis of genetic structure and diversity is crucial for understanding the medicinal properties of *Asparagus*. In genetic feature research, DNA markers can help researchers understand the structure and degree of variation within plant species [37]. The detection of EST-SSR depends on many factors, such as genome structure, tools, and methods [38]. In the transcriptome of *A. cochinchinensis*, 8841 SSRs were detected, containing 1057 different base repeat units, with dinucleotides and trinucleotides being the most common motifs. The analysis results of the SSR sequences are similar to those of *Asparagus officinalis* [39]. In addition, the frequency of SSRs in *A. cochinchinensis* was 11.96%, lower than in *A. officinalis* (13.07%) [40], *Raphanus sativus* (23.8%) [41], and castor (28.4%) [42], but higher than in sesame (8.9%) [43] and *Chrysanthemum morifolium* (2.84%) [44]. The frequency of SSR loci in dicotyledonous plants is generally between 2.65% and 16.82% [38], with an average value of 9.73%. Hence, the transcriptome and SSR data obtained in this study have high

research value. In addition, the repeat sequence length of the SSR loci in this study was mainly between 14 and 24 bp. When the repeat sequence length in SSR loci is greater than or equal to 20 bp [45], there is high polymorphism; there is moderate polymorphism when it is between 12 and 19 bp and low polymorphism when it is below 12 bp. The length of the repeat sequences amplified using SSR primers developed in this study is 15–24 bp, and most of them are greater than 20 bp, indicating that the polymorphism of the SSR markers developed in this study is at a high level.

4.4. Genetic Diversity of SSR in Asparagus

Genetic diversity is the most fundamental condition for the long-term survival and evolution of species [46]. The higher the genetic diversity, the stronger the ability of a species to adapt to the environment [47]. The use of SSR markers to evaluate genetic diversity leads to more accurate analysis within species [48], which is illustrated in wheat [49] and safflower [50], but relevant literature on *Asparagus* is lacking. In genetic diversity research, Ne is a measure of the uniformity of allele distribution in a population. In this study, the number of different alleles for 10 SSRs was 71, with an average of 7.1000 alleles per locus and Ne of 1.3714, which is lower than that of *Quercus acutissima* (4.014) [51]. The average number of alleles per locus is influenced by the number of samples and detection sites, and different values can be obtained in different analyses [52]. Therefore, the low Ne values of *Asparagus* resources in this study may be due to the small sample size and different analysis methods used in the study.

Ho is the proportion of heterozygous individuals in the analysis sample, and expected heterozygosity (He) represents the percentage of the population that belongs to heterozygotes when unexpected hybridization occurs between individuals [52]. The average observed heterozygosity (*H*) and Shannon information index (*I*) are the main indicators for measuring the level of genetic diversity of a species [53], and their values are positively correlated with the richness of genes [40]. In this study, *I* was 0.4040, higher than that of *Festuca arundinacea* (0.401) [54] and lower than that of *A. officinalis* (0.37) [40], *Quercus acutissima* (1.338) [51], and alfalfa (0.553) [55]. In terms of heterozygosity, *H* of each locus in *A. cochinchinensis* is 0.2486, which is lower than that of *A. officinalis* (0.37) [40] and higher than that of *Melia azedarach* [56] and *Toona ciliata* [57]. The genetic diversity of the *Asparagus* resources in this study is moderate, which may be related to the small sample size of this study and the asexual reproduction of *Asparagus*, such as ramet propagation and root tuber propagation.

4.5. Genetic Structure of Asparagus Resources

Genetic variation generates genetic differentiation in spatial distribution, creating the genetic structure of a species population [58]. The genetic structure of the population is largely influenced by human activities, such as artificial cultivation, domestication, and pollination [59]. This study used cluster analysis and PCoA to divide the *Asparagus* populations into two groups and three subgroups, which were consistent with the morphological results. The *Asparagus* resources in Neijiang, Sichuan (TD-1); Yulin, Guangxi (TD-3); Chuxiong, Yunnan (TD-5); and Kaiyang, Guizhou (TD-6) were clustered into one group, mainly distributed in low mountains and hilly areas below 500 m. They were identified as the same species, *A. cochinchinensis*, according to the Chinese *Pharmacopoeia*. Notably, their flowers are light green, the filaments are not attached to the perianth, the root size is moderate, and the taste is sweet and slightly bitter. The resources of Wuchuan (TD-2) in Guizhou and Honghe (TD-4) in Yunnan were clustered into the second group, mainly distributed in mountainous areas above 800 m. Their flowers are yellow, with the middle of their filaments attached to the perianth, and their root tubers are thick and bitter in taste. There is no significant provincial difference between the two groups, but there is a certain relationship with elevation. In addition, *Asparagus* resources exhibited high diversity both in morphology and at the molecular level. However, due to the limited use of *Asparagus* resources in this study, there may be some deviation in diversity analysis. Therefore, it is

necessary to continue conducting multi-dimensional genetic diversity analysis of *Asparagus* from multiple regions.

5. Conclusions

In accordance with the extensive distribution of *Asparagus*, and with high intra- and interspecies diversity, phenotypic and molecular markers are effective tools for detecting *Asparagus* diversity. MISA online software was used to comprehensively analyze the transcriptome data of *A. cochinchinensis*, and 8841 Unigenes containing SSR loci were identified. Ten specific *Asparagus* EST-SSR markers were screened out. In the morphological identification, we found that the number of leafy branches and the filiform adhesiveness of the flowers are important indexes to carry out the identification. Moreover, SSR molecular markers can be used as a supplementary means of morphological identification to strengthen the credibility of the morphological identification. Moreover, genetic diversity analysis showed that there may be two groups and two subgroups in the seven *Asparagus* samples, agreeing with the morphological results. This study classifies and identifies *Asparagus* germplasms based on phenotypic characteristics and molecular markers, which is of great significance for protecting *Asparagus* germplasm resources and promoting *Asparagus* industrialization. Our results not only lay a good foundation for the study of the genetic diversity of *Asparagus*, but also provide technical support for expanding the technical path of germplasm identification of *Asparagus*, for carrying out germplasm improvement and protection, and for utilization of *Asparagus* resources.

Author Contributions: D.L., F.Y. and C.L. contributed equally to this work. M.Y. and X.L. jointly supervised this study. D.L., F.Y., A.C. and J.W. performed transcriptome and gene functional analyses. D.L. and F.Y. prepared the DNA and RNA samples and performed the PCR analysis. D.L. conceived the study and drafted the manuscript. D.L. and F.Y. revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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