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Availability Evaluation and Application of MNP (Multiple Nucleotide Polymorphism) Markers in Variety Identification of Chrysanthemum

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Abstract: In China, PBR (Plant Breeder's Right) applications of chrysanthemum rank first among all of the applications of ornamental crops in China due to the plant's significant ornamental, edible, and medicinal values. However, issues of variety infringement and disputes have become increasingly prominent, and traditional molecular markers are difficult to use due to the high heterozygosity and complex ploidy of chrysanthemum. Our study explored the availability of MNP (Multiple Nucleotide Polymorphism) markers in this regard. In total, 30 representative varieties of five types were selected for the screening of MNPs, and another 136 varieties were selected for validation of the screened MNPs. Based on ddRAD-seq (Double Digest Restriction site-associated DNA sequencing) of the 30 varieties, 26,147 SNPs were screened for genetic analysis, and 487 MNPs were screened with a length from 139 to 274 bp, an average of 6.6 SNPs individually, and a repeatability rate of 99.73%. Among the 487 MNPs, 473 MNP markers were found to cover all 27 chromosomes of chrysanthemum. Performance of our MNPs in the 136 varieties was similar to those in the 30 varieties, where the average Ho (observed heterozygosity) was 71.48%, and the average DP (discriminative power) was 82.77%, preliminarily indicating the stability of the 487 MNPs. On the other hand, clustering results based on the 487 MNPs were also generally consistent with those based on the 26,147 SNPs, as well as those based on phenotypic traits, and initial grouping, likewise, further indicating the robust capability of our MNPs in variety discrimination, which is similar to their correspondence with numerous SNPs. Therefore, our MNP markers have great potential in the accurate and rapid identification of chrysanthemum varieties, and, accordingly, in fostering breeding innovation and promoting chrysanthemum marketing.

Keywords: chrysanthemum; variety identification; single nucleotide polymorphism (SNP); multiple nucleotide polymorphism (MNP); phenotypic traits

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1. Introduction

Chrysanthemum [*Chrysanthemum morifolium* Ramat. (*C. grandiflorum* Ramat.), *C. pacificum* Nakai (*Ajania pacifica* Bremer and Humphries), and hybrids between them] is very popular due to its rich colors and diverse shapes of inflorescence. As an important horticultural crop, chrysanthemum was introduced from China to Japan in the 12th century, and then spread throughout the world [1], becoming one of the four largest cut-flower crops in the world in terms of cut-flower marketing, ranking only behind roses [2]. Before its ornamental application, the unique nutritional and bioactive components of some chrysanthemum varieties have made them edible, drinkable, available as medicine, and listed

in the 'Chinese Pharmacopoeia' [3]. In China, cultivation of chrysanthemum has lasted for more than 1000 years. The long history of cultivation has promoted later breeding, accordingly [4]. There are around 3000 varieties recorded in China, which can be divided into five groups, including Chinese traditional varieties, edible varieties, potted varieties, a cut-flower type with small inflorescence, and a cut-flower type with large inflorescence [5].

Chrysanthemum L. was included in the first batch of the 'List of Protected Genera and Species of Agricultural Plants in the People's Republic of China', in 1999. To more effectively establish a plant identification and protection system, China joined UPOV (International Union for the Protection of New Varieties of Plants) as the 39th member country in 1999, adhering to the 1978 Convention text and implementing a new plant variety protection system to safeguard breeders' rights [6]. Under the UPOV Convention, cooperation in legal and technical aspects among member countries has been promoted, the breeding and production speed of new plant varieties has been accelerated, the establishment of an international plant intellectual property system has been expedited, and the sustainable development and innovation of agriculture and forestry have been maintained [7]. Since the acceptance of agricultural plant variety rights applications began, the number of applications in China has steadily increased each year. PBR (Plant Breeder's Right) applications of chrysanthemum rank first among all of the applications of ornamental crops during recent decades in China, and more than 1000 varieties were subject to a DUS (Distinctness, Uniformity, and Stability) test. However, along with the advancement of breeding and production of chrysanthemum, marketing chaos of chrysanthemum has become increasingly prominent, as problems of 'different names corresponding to the same variety' and 'different varieties with the same name' emerged gradually, and are showing an increasing trend. In the course of the handling of PBR infringements and disputes, variety discrimination based on phenotypic traits is time-consuming and labor-intensive, and has risk in wrong identification, since phenotypic differences may be caused by environmental variations, rather than internal genetic disparity.

DNA markers are based on the difference in nucleic acid sequences, reflecting genetic diversity at the DNA level. Compared to morphological markers, cytological genetic markers, and biochemical markers, DNA markers have the advantages of wide distribution, high polymorphism, and good accuracy. DNA marker technology is subject to fixed and easy operation, and free of influence from an external environment. It has been widely used in variety identification, genetic diversity analysis, phyletic tree studies, marker-assisted breeding, and trait-associated gene mapping [8–10]. There have been some reports on the application of DNA markers in chrysanthemum. Zhang et al. used SRAP (sequence-related amplified polymorphism) to analyze the genetic characteristics of flowering traits in two chrysanthemum hybrid varieties [11]. Klie et al. used AFLP (amplified fragment length polymorphism) to study the genetic regulation of bud branching in two chrysanthemum populations [12]. Zhang et al. revealed the genetic diversity based on the DNA fingerprintings of 480 varieties of Chinese traditional chrysanthemum using 20 SSR (Simple Sequence Repeat) markers [13]. Fan et al. developed 25 polymorphic EST-SSR tags (expressed sequence tag simple sequence repeat) for genetic diversity analysis of 59 varieties, and analyzed the genetic model in 192 F1 hybrid varieties [14]. SNP markers have also been applied in studying the genetic diversity of five types of chrysanthemum varieties based on SNP (Single Nucleotide Polymorphism) and mapping the trait-associated genes [5,15,16]. However, there are relatively few reports on the application of DNA molecular markers in variety identification of chrysanthemum [13].

In terms of variety identification, SNP and SSR are two commonly used DNA markers [17]. SSR markers based on PCR amplification and gel electrophoresis detection have a high number of PCR amplification cycles during the detection process, which can easily cause slippage [18]. Additionally, the resolution of gel electrophoresis detection technology is limited, making it difficult to accurately distinguish between true genotypes and erroneous genotypes caused by slippage [19]. Although SNP chips have a large number of markers, their biallelic nature limits their ability to distinguish loci, and their lack of

polymorphism makes it difficult to handle the complex allelic genotypes of polyploid species. Furthermore, the development and use of SNP chips is costly [20,21]. The MNP (multiple nucleotide polymorphism) technology, which combines the GenoBaits and GenoPlex systems, overcomes the shortcomings of SSR and SNP marker technologies while incorporating their advantages [19,22]. The basic principle of the MNP marker method is to utilize multiple SNP loci present in the genome and distinguish DNA sequence differences between individuals by combining the allelic genotypes of these different SNP loci [23]. MNP detection is performed through multiplex PCR, high-throughput sequencing, and bioinformatics analysis. The multiplex PCR with fewer cycles avoids slippage during amplification, and can enrich thousands of marker loci [24]. Additionally, the detection of MNP markers does not require specific expensive equipment, and can be adapted to the three current mainstream sequencing platforms—Illumina, Ion Torrent, and MGI—making it more cost-effective compared to SNP chips [22]. In China, MNP has been applied as a national standard for variety identification in 13 crops, including rice, soybean, rapeseed, eggplant, corn, and tomato [25], and there have been reports on the development and application of MNP in grapes [22], shiitake mushrooms [26], cassava [27], oyster mushrooms [28], and king oyster mushrooms [29]. Furthermore, at the 18th Biochemical and Molecular Biotechnology Working Group meeting held in Hangzhou, China, in 2019, MNP marker-related content was reported by researchers for the first time. MNP is considered an excellent method for constructing variety fingerprint databases, and the technology has garnered attention from attendees.

At present, there is no research or report on MNP marker technology in chrysanthemum. To address the issue of 'different names for the same variety' and 'same name for different varieties' in the current chrysanthemum market, we leveraged the advantages of MNP in variety identification. We used 30 representative chrysanthemum varieties to develop suitable MNP markers and applied them to 136 new varieties. This study lays the foundation for chrysanthemum variety identification, and the establishment and management of corresponding variety databases. It also provides technical support for chrysanthemum intellectual property protection, breeding innovation, and industry development.

2. Materials and Methods

2.1. Materials

In this study, 30 varieties with wide origin sources and diverse phenotypic characteristics were used, which belong to 5 representative groups. They were collected from the Netherlands and several provinces in China (Henan, Jiangsu, Jiangxi, Anhui, Hebei, Beijing, Hubei, and Yunnan) (Table 1). The seedlings were provided by the National Chrysanthemum Germplasm Resource Bank in Nanjing Agricultural University, and were grown in greenhouses in 2022, in Kunming, Yunnan. They were planted in 17 cm × 20 cm pots. The row spacing of cut-flower and bushy varieties was 20 cm × 20 cm and 20 × 30 cm, respectively. In addition, another 136 chrysanthemum varieties were also grown for MNP evaluation and the construction of DNA fingerprintings. These 136 chrysanthemum varieties covered potted varieties, traditional varieties, cut-flower varieties with small flowers, cut-flower varieties with large flowers, 15 pairs of bud mutation varieties, and 2 pairs of candidate varieties under a DUS test (Table S1).

2.2. Phenotypic Analysis

Based on the 'Guidelines for the conduct of tests for distinctness, uniformity and stability-*C. morifoliu* Ramat.' [30], DUS tests were conducted with 36 characteristics collected, including 3 plant characteristics, 1 stem characteristic, 9 leaf characteristics, and 23 flower characteristics (Table S2). According to the DUS test guidelines, the 36 selected traits were all phenotypically assessed. Data collection occurred during the full bloom period of chrysanthemums, specifically before the anthers of single and semi-double varieties split, and when the terminal capitulum of double varieties was fully open. For leaf traits,

observations were made on leaves located at the upper third of the stem. For capitulum traits, the terminal capitulum was observed. For ray floret traits, the outermost florets were observed. For tubular floret traits, the outermost tubular florets were observed before the anthers split. The Shannon–Weaver diversity index (*H*′) was calculated, and the correlation coefficients among the 30 varieties were analyzed using the WGCNA package in R language (Version 4.2.2).

Table 1. Information of the 30 varieties of chrysanthemum.

Group Code	Group Description	Variety Code	Variety Name	Origin
G1	Variety with edible quality	C-1	Hangbaiju	Henan, China
		C-2	Jiuyueju	Jiangsu, China
		C-3	Jinsihuangju	Jiangxi, China
		C-4	Qiyuebai	Anhui, China
G2	Potted variety	C-5	Zhongshanjinyang	Jiangsu, China
		C-6	Zhongshanguanghui	Jiangsu, China
		C-7	Zhongshanzisongguo	Jiangsu, China
		C-8	Xixiahongyi	Jiangsu, China
G3	Chinese traditional variety	C-9	Jinlongzhua	Tianjing, China
		C-10	Fenshiba	Hebei, China
		C-11	Yupantuogui	Hebei, China
		C-12	Jinfenghuangchao	Beijing, China
		C-13	Panlongbiyu	Hubei, China
G4	Cut-flower variety with small inflorescence	C-14	Aladuo	Netherlands
		C-15	Ruiduositebai	Netherlands
		C-16	Kelongxiangshui	Netherlands
		C-17	Luomajiari	Netherlands
		C-18	Yinyang	Netherlands
		C-19	Tesileizi	Netherlands
		C-20	Nannongyuzhu	Jiangsu, China
		C-21	Nannonghengchun	Jiangsu, China
		C-22	Nannongxiazhu	Jiangsu, China
		C-23	Nannongnianluo	Jiangsu, China
G5	Cut-flower variety with large inflorescence	C-24	Nannonghuangfengwo	Jiangsu, China
		C-25	Songyue	Yunnan, China
		C-26	Chengsongyue	Yunnan, China
		C-27	Fenanna	Yunnan, China
		C-28	Danlvtianzan	Yunnan, China
		C-29	Qiuhuang	Yunnan, China
		C-30	Jiemo	Jiangsu, China

2.3. Genetic Analysis

2.3.1. Library Construction

Leaves of 30 varieties were taken in the juvenile stage for DNA extraction using the Plant genomic DNA Extraction Kit (Tiangen Biotech (Beijing) Co., Ltd., Beijing, China). The quality and concentration of DNA were controlled based on electrophoresis detecting and a high-throughput ultra-micro spectrophotometer (Implen NanoPhotometer® N120, Munich, Germany) with the required absorbance ratio > 2.0 (260 nm:230 nm), and the absorbance ratio between 1.7 and 1.9 (260 nm:280 nm).

DNA samples were normalized to achieve consistent concentration, and then digested with Mse I and EcoR I-HF. Corresponding adapters were added, and different libraries were mixed with 5 μL each for electrophoresis. Fragments with 200 to 400 bp were recycled for library amplification. The purified library was sent to Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) for sequencing.

2.3.2. Genetic Analysis

After sequencing, the reads were trimmed and filtered using the fastx_toolkit package (version 0.0.14), with quality scores less than 20. Stacks software (version 2.61) [31] was used for assembling from scratch, and SNP filtering was used with the following standards: at least 80% of samples having this locus, the minor allele frequency of this locus greater than 0.05 (MAF > 5%), and at least 3 reads supporting this locus.

ARLEQUIN 3.5 software was used to calculate the fixation index (Fst), and to analyze genetic diversity and genetic differentiation [32]. The preliminary selected SNPs were filtered with $p > 0.05$, based on the Hardy–Weinberg Equilibrium Law. Principal component analysis was performed using Plink software (version 1.90b6.17), and sample clustering was plotted using the ggplot2 package in R language (version 4.2.2). The ggtree package (version 3.2.1) was used to draw the phylogenetic tree. Population genetic structure analysis was conducted with the order of assumed subgroups (K) from 2 to 10, based on the 5-fold cross-validation. According to the output results, the K value corresponding to the lowest cross-validation error rate was selected as the optimal subgroup classification.

2.4. MNP Screening, Evaluation, and Application

2.4.1. MNP Screening

The reported genome of chrysanthemum nankingense was used as the reference genome [33]. The genomic data of the 30 varieties were aligned with the reference genome, with a sliding length of 1 bp and a window length of 120 bp. Discrimination value, '*D*', of the individual window was calculated as follows:

$$D = 1 - \frac{\sum_1^k C_{b_i}^2}{C_a^2}$$

where 'a' is the total number of varieties in which the MNP marker was detected, 'bi' is the number of varieties with i genotype, and 'k' is the number of genotypes containing more than one variety.

The top 600 windows with the highest *D* values and conservative boundaries were preliminarily selected as MNP markers for variety identification of chrysanthemum. Primers were designed to ensure the amplification length was less than 250 bp.

To further screen, DNA of the 30 chrysanthemum varieties above was extracted, and the qualified DNA was used as a template to construct a high-throughput sequencing library by two rounds of PCR amplification, where the first round enriched the target fragment, and the second round introduced Illumina sequencing corresponding adapters and barcodes. The first round of the amplification system included primer mixture: 4 μL (10 μmol L^{-1}); DNA template (20–200 ng): X μL; GenoPlexs 3 × T Master Mix: 10 μL; and ddH_2O: (16-X) μL. The PCR reaction program covered 95 °C for 15 min, followed by 15 cycles (95 °C denaturation for 30 s, 60 °C annealing and extension for 4 min), 70 °C extension for 4 min, and final cooling to 4 °C. After the reaction was complete, the PCR product was purified using magnetic beads. The second round of the amplification system included adapter primers (5 μmol L^{-1}): 2 μL each; first round PCR purified product as a DNA template: 16 μL; and GenoPlexs 3×T Master Mix: 10 μL. The PCR reaction program covered 95 °C for 3 min, followed by 8 cycles (95 °C denaturation for 15 s, 58 °C annealing for 15 s, 70 °C extension for 30 s), 72 °C final extension for 5 min, and final cooling to 4 °C. Libraries with concentration > 10 ng μL^{-1} and a single band (about 300 bp) were sent to Novogene Bioinformatics Technology Co., Ltd. for sequencing.

Bowtie2 (version 2.1.0) was applied to perform the sequencing data alignment [34], and the detection rate of MNP markers was concluded. After optimization, final MNP markers with an average detection rate of 95% were determined. The script 'perl' was used to calculate the number of allelic genotypes of each MNP marker, observed heterozygosity (*Ho*) in a given population, and discriminative power (DP) [27].

2.4.2. MNP Evaluation

To evaluate the accuracy of the selected MNP markers, we randomly selected 8 varieties from the tested chrysanthemums for reproducibility experiments (two independent experiments conducted by different personnel, using different batches of reagents, and different instruments). Varieties C-1 to C-8 were chosen for the reproducibility experiments. Repeatability was calculated as $R = m/M$, where m indicates the number of MNPs with the same genotypes between replicates, while M is the number of MNPs commonly detected in both replicates [27]. Based on repeatability, accuracy was concluded as accuracy = $1 - (1 - R)/2$ [27].

Genetic similarity (GS) between 2 varieties was calculated as follows, and visualized using the WGCNA package in R language.

$$GS = \frac{n_{ij}}{N_{ij}} \times 100\%$$

where n_{ij} is the number of MNP markers with the same genotyping detected in the 2 given varieties, and N_{ij} is the total number of MNP markers.

2.4.3. MNP Application

The screened MNPs were applied in another 136 varieties of chrysanthemum. The methods for MNP library construction and high-throughput sequencing were described in Section 2.4.2. Perl scripts [27] were used to analyze allele genotypes. Heterozygosity and discriminative power of the MNP markers were also evaluated.

3. Results

3.1. Phenotypic Analysis

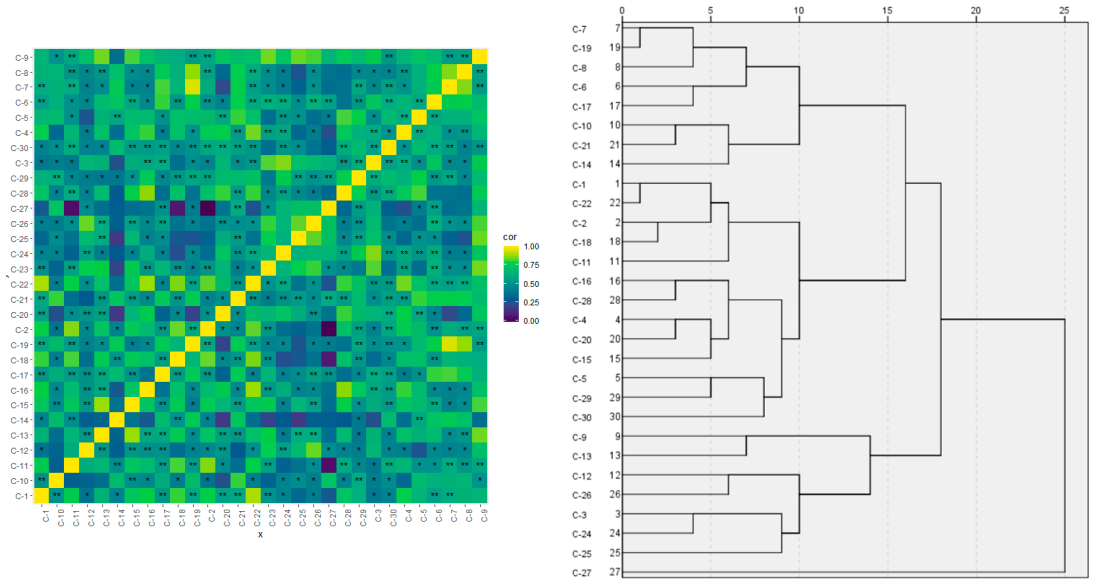
Based on the analysis of 36 phenotypic characteristics collected from 30 varieties (Table S1), a total of 146 variations were detected, with an average of 3.9 variations per characteristic, and with the *H′* from 0.150 to 1.936. The top three characteristics with the highest '*H*' were 'corolla tube length of ligulate floret (Char24)', 'cross-sectional shape of floret (Char25)', and 'main color of inner side of ligulate floret (Char31)', indicating our experimental materials had relatively great diversity in the organ of inflorescence. The pairwise comparison of 30 chrysanthemum varieties across 36 traits showed a minimum difference of 7, a maximum difference of 32, and an average difference of 18.7, indicating significant phenotypic variation among these varieties. Figure 1a showed that the average correlation coefficient between any two varieties was 0.567, with the maximum correlation coefficient of 0.953 and the minimum of 0.037, indicating our experimental materials were relatively independent. In addition, 30 varieties were divided into four categories at the distance of 15 (Figure 1b). The first category included three potted varieties (C-6, C-7, C-8). The second category included three varieties with edible quality (C-1, C-2, C-4). The third category included three Chinese traditional varieties (C-9, C-12, C-13). In general, the clustering basically conforms to variety grouping.

3.2. Genetic Analysis

3.2.1. Library Evaluation

There were 9.50×10^8 clean reads, with an average Q20 value of 100%, an average Q30 value of 96.1%, and GC content of around 38%, revealing the good quality of sequencing data. After assembling, 3,893,161 contigs were obtained. By comparing the samples with consistent contig tags, the average number of sample loci per sample was 836,185.3, the average coverage of the sample to the consistent sequence was 11.3, and the average coverage adjusted for the number of samples per locus was 14.2 (Table S3). All SNP loci were filtered based on a missing rate of <20% and a minor allele frequency of >5%, resulting in an initial set of 31,448 SNP loci. The mutation type statistics for the SNP genotyping results showed that A/G transitions were the most common (9354), followed

by C/T transitions (9250), and that C/G transversions were the least common (1753). These three types accounted for 29.7%, 29.4%, and 5.6% of all base mutations, respectively. The transition-to-transversion ratio was 1.45 (18,604/12,844) (Figure S1).



(**a**) (**b**)

Figure 1. Cluster and correlation analysis of 30 varieties based on 36 phenotypic characteristics: (**a**) correlation analysis; (**b**) cluster correlation analysis. Note: C-1~C-30: variety code (Table 1); *, **: significant correlation with $p < 0.05$, $p < 0.01$, respectively.

3.2.2. Analysis of Population Genetic Differentiation

The Hardy–Weinberg equilibrium refers to the principle that genetic variation in a population will remain constant from one generation to the next, in the absence of disturbing factors. It is generally considered that a population is in equilibrium when the Hardy–Weinberg test *p*-value is greater than 0.05. Therefore, 26,147 SNP loci with *p*-values greater than 0.05 were used for population genetic diversity analysis. Fixation index (Fst) between populations ranged from 0.04328 to 0.0870253, among which only the Fst value between subgroups four and five was less than 0.05, and Fst values between other subgroups were greater than 0.05 (Table S4). Fst is generally used to measure the genetic distance among populations, where Fst value of '0–0.05' indicates small genetic differentiation, while '0.05–0.15' and '0.15–0.25' indicate medium and large genetic differentiation, respectively [35]. Therefore, among the five subgroups, there were medium genetic differentiations, except for subgroups four and five.

3.2.3. Phylogenetic and Population Structure Analysis

Results of the phylogenetic tree showed that varieties of G1 (edible varieties: C-1, C-2, C-3, and C-4), G3 (Chinese traditional varieties: C-9, C-10, C-11, C-12, and C-13), and G2 (potted varieties: C-5, C-6, C-7, and C-8) were clustered, respectively, into different branches, while the 16 other varieties (cut-flower varieties: G4 and G5) were gathered (Figure 2a). Variety clustering of G1, G2, and G3 in the phylogenetic tree was identical to phenotypic grouping (Table 1), while G4 and G5 varieties were difficult to be divided.

Principal component analysis (PCA) also showed generally similar results to the phylogenetic tree, where varieties of G2 (C-5, C-6, C-7, and C-8) were clearly distinguished from other groups by PC1, together with PC2, while varieties of G1 were narrowly distinguished from G3 varieties by PC1 (Figure 2b). Similarly, G4 varieties and G5 varieties were difficult to be discriminated.

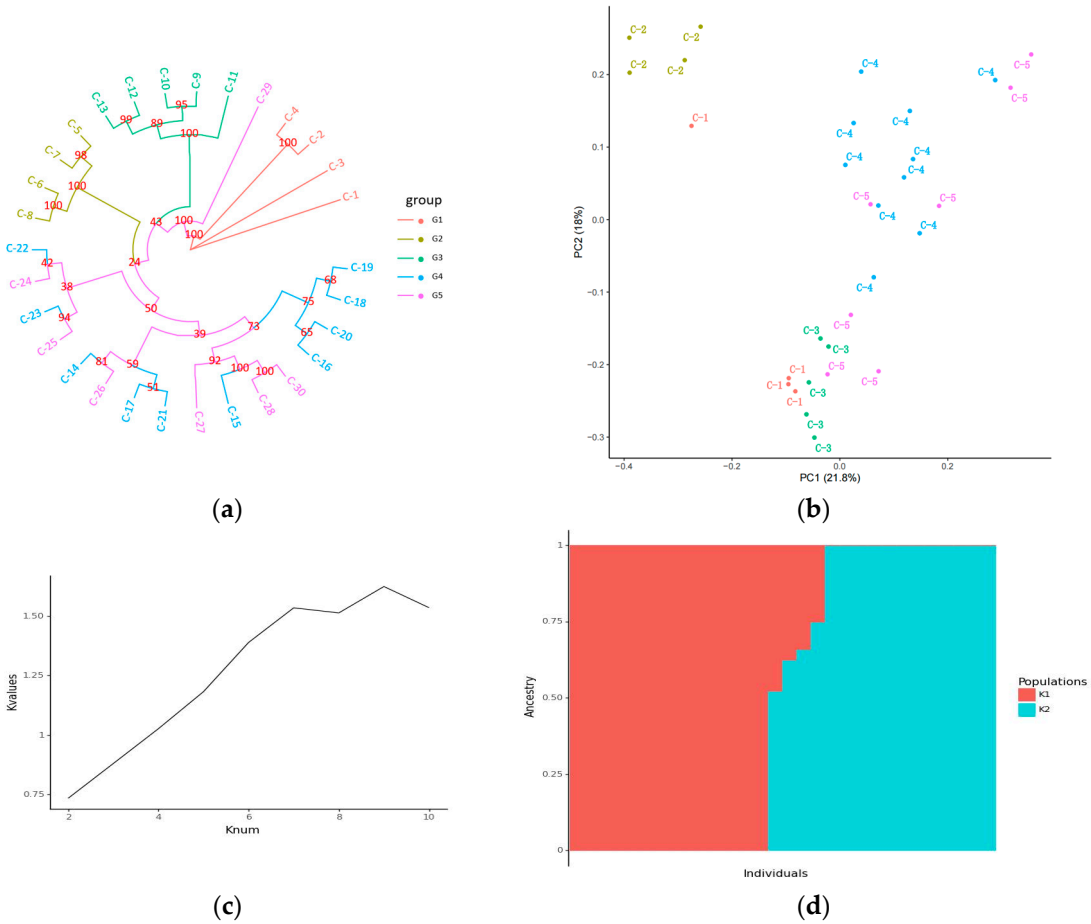


Figure 2. Genetic analysis of 30 varieties based on 31,448 SNPs concluded from simplified genome sequencing data: (**a**) phylogenetic tree; (**b**) principal component analysis; (**c**) line chart of error rate of cross-validation; (**d**) population structure diagram (K = 2). Note: C-1~C-30: variety code; G1–G5: group code (Table 1).

Based on the filtered SNP, admixture software was used for population genetic structure analysis in the order of assumed subgroup (K) number, from two to five. K = 2 was determined as the optimal number (Figure 2c), indicating that the 30 varieties can be mainly divided into two subgroups. Meanwhile, the population structure diagram (K = 2) showed that the two-subgroup-dividing suits most varieties, and only four varieties cannot be divided clearly (Figure 2d).

3.3. MNP Marker Screening, Evaluation, and Application

3.3.1. MNP Marker Screening

Based on simplified genome sequencing data of 30 varieties, 561 MNP markers were obtained. Based on the improved detection rate from 83.42% to 95%, a total of 487 MNP markers were retained, with an amplification length from 139 to 274 bp (Figure 3a). Each MNP marker contained 1 to 10 SNPs with MAF > 5%, with an average of 6.60 (Figure 3b). We aligned the 487 MNP markers with the newly released reference genome (https://doi.org/10.6084/m9.figshare.21655364.v2 accessed on 27 March 2024) of chrysanthemum [36], among which 473 MNP markers were found to cover the entire genome, and were found to be distributed on 27 chromosomes (Figure 3c).

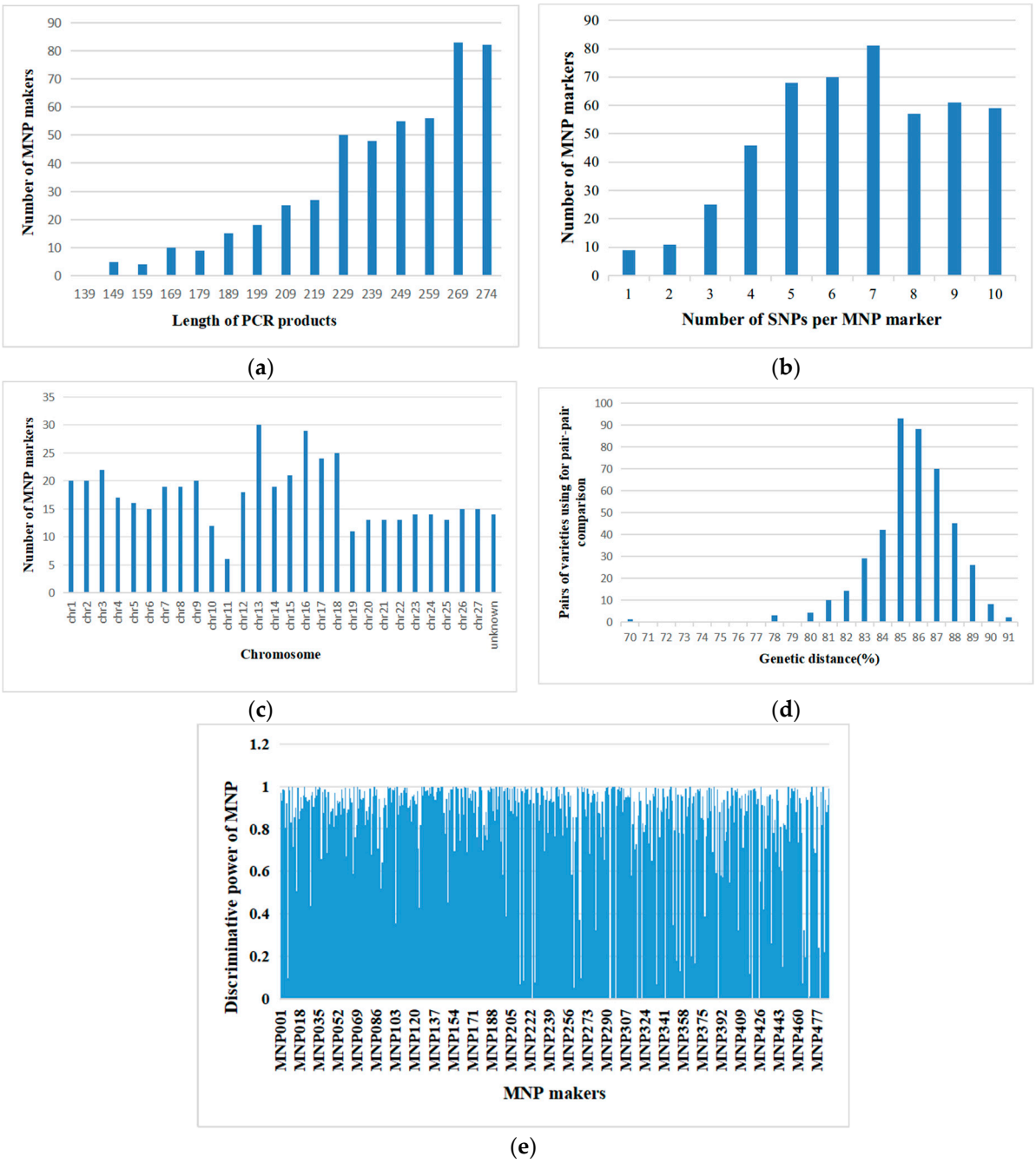


Figure 3. Features of the 487 MNP(multiple nucleotide polymorphism) markers of chrysanthemum. (**a**) Statistical analysis of 487 MNP marker lengths after primer amplification; (**b**) Statistics of the number of MNP markers corresponding to the number of high-frequency SNPs contained in each of the 487 MNP markers; (**c**) Statistical analysis of the distribution of 487 MNP markers on chromosomes; (**d**) Compare the genetic distance of 30 varieties pairwise based on 487 MNP markers and perform logarithmic statistics; (**e**) Evaluate the discriminative ability of each MNP (MNP001-MNP487 represent the numbering of 487 MNP markers respectively).

3.3.2. MNP Marker Evaluation

In the 30 varieties, the number of allele genotypes detected at each MNP marker ranged from 1 to 133, with an average of 13.85 genotypes, and 438 MNP contained more than 5 allele genotypes. Observed heterozygosity (*Ho*) of MNP markers was 71.19% on average, indicating the great genetic diversity of the 30 varieties. A total of 435 pairwise comparisons were made, and percentage of MNP markers with different genotyping between any two

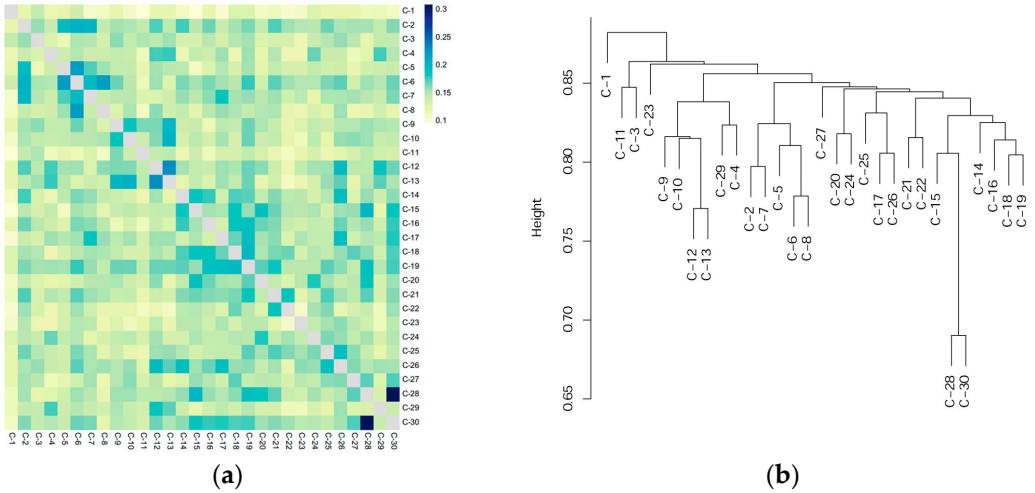
varieties was from 69.03% to 90.23%, with an average of 85.15% (Figure 3d). Furthermore, discriminative power (DP) of the individual MNP marker was evaluated (Figure 3e), and the average DP was 84.25%, with 26 markers having a DP value of 100%. All of the above results revealed the extremely robust discriminative power of the screened MNP markers.

A total of 3710 MNP markers from sixteen libraries of eight varieties were detected in two independent experiments (Table 2). Among them, 3700 markers were repeatable, with a repeatability rate of 99.73%, revealing the high repeatability, stability, and accuracy of the 487 MNP markers.

Table 2. Repeatability and accuracy of the 487 MNP markers in eight varieties of chrysanthemum.

Variety Code	Number of MNP Commonly Detected in Both Replicates	Number of MNPs with Different Genotypes between Replicates	Repeatability (%)	Accuracy (%)
V1	471	1	99.79	99.89
V2	460	0	100.00%	100.00%
V3	471	2	99.58%	99.79%
V4	459	1	99.78%	99.89%
V5	469	1	99.79%	99.89%
V6	465	2	99.57%	99.78%
V7	452	0	100.00%	100.00%
V8	463	3	99.35%	99.68%
sum	3710	10	99.73%	99.87%

The heat map of genetic similarity (Figure 4a) showed that the GS values between any two of the thirty varieties ranged from 9.71% to 30.97%, and varieties with the highest GS value were C-28 and C-30. UPGMA clustering analysis was conducted based on the results of 487 MNP markers from 30 chrysanthemum varieties (Figure 4b). Clustering results indicated that the four varieties (C-7, C-5, C-6, C-8) belonging to potted varieties were clustered together, while the four varieties (C-9, C-10, C-12, C-13) of Chinese traditional variety were clustered together. The clustering results were generally consistent with the evolutionary tree clustering results based on SNP markers.



(**a**) (**b**)

Figure 4. Genetic relationship analysis of 30 chrysanthemum varieties. (**a**) Heat map of genetic similarity between any two of the thirty varieties. (**b**) Phylogenetic tree of chrysanthemum based on 487 MNP sequences from 30 varieties. Note: C-1~C-30: variety code (Table 1).

3.3.3. MNP Marker Application

In the case of the application of 487 MNP markers in 136 varieties, the number of allele genotypes detected at each MNP marker ranged from 1 to 236, with an average of 18.09 genotypes, and 450 markers contained more than 5 allele genotypes. The average *Ho* was 71.48%. In addition, a total of 9180 pairwise comparisons were made between any 2 of the 136 varieties, and the percentage of MNP markers with different genotyping between any 2 varieties was 83.97% on average, and the average DP was 82.77%. Details of genetic features of each of the 487 MNP markers in the 30 varieties, as well as in the 136 varieties, were shown in Table S5. Based on the results of 487 MNP markers from 136 chrysanthemum varieties, UPGMA cluster analysis was performed (Figure 5). The clustering results showed that MNP could cluster most varieties according to their cultivation types. Furthermore, we observed that when the genetic similarity is greater than or equal to 98%, there are 25 pairs of varieties clustering together. These paired varieties exhibit a difference in the number of variant loci equal to or less than seven. Analyzing these clustered varieties, we found that there is one distinct phenotypic trait between paired varieties, primarily related to the main color of the flowers and the shape of the tongue-like small flower tips. These clustered varieties mainly include bud mutants (such as C-153 and C-154, C-163 and C-164, C-155 and C-156, C-143 and C-144, C-145 and C-146, C-166 and C-165), varieties applied for actual DUS testing and their similar counterparts (such as C-135 and C-136, C-115 and C-160, C-133 and C-134), and series-related varieties (such as the Rosa Anna series with C-121, C-122, and C-123). Notably, the genetic similarity of 99.74%, and only one variant locus difference between C-115 and C-115, suggests no significant phenotypic variation between them, despite their different origins, possibly indicating synonymous varieties. Overall, the results from 136 varieties are similar to those of the 30 representative varieties, demonstrating the representativeness of these 30 varieties. Additionally, the proportion of phenotypic differences correlates positively with the number of molecular variant loci, indicating that our MNP markers are feasible for distinguishing chrysanthemum varieties.

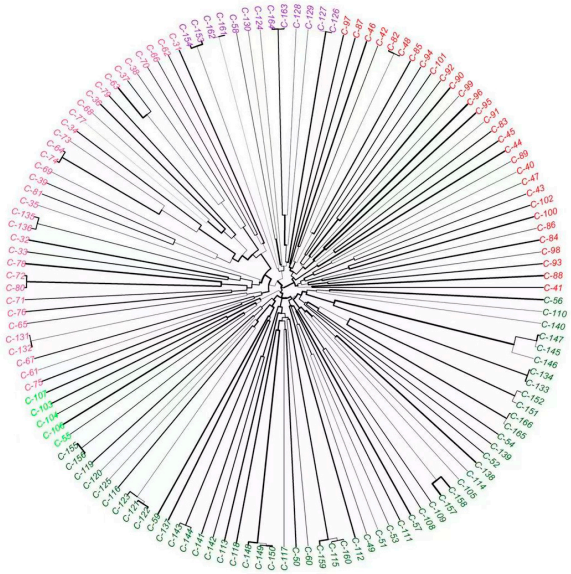


Figure 5. Phylogenetic tree of 136 chrysanthemum varieties based on 487 MNP sequences. Note: C-31~C-166: variety code (Table S1). The same color represents the gathering of varieties together.

4. Discussion

Chrysanthemum has a long evolution history, and its cultivation can be traced back to 3000 years ago. It was initially used as food or medicine, and gradually for ornamental purposes [2], and inflorescence is always the focus, whatever its role is. Therefore, chrysanthemum breeding mainly focuses on inflorescences, with the aim of developing varieties with desired inflorescence traits. Zhang et al. analyzed the diversity of 735 varieties of

Chinese traditional chrysanthemum based on 16 morphological traits, and found that clustering was mainly based on floret type and inflorescence type [13,37]. In our study, characteristics associated with inflorescence and floret generally had high diversity indexes, and were also considered as important indicators for variety grouping and identification. According to the results of phylogenetic trees concluded from both SNP and MNP markers, varieties from G4 and G5 were clustered as a whole, indicating cut-flower type, whether with small inflorenscences or large ones, and had a similar genetic background, which was consistent with previous research [5].

Regarding molecular markers, there is quite a consensus that great care must be taken in varieties for marker screening purpose. Quantity and representativeness of the varieties merit intensive consideration. Wang et al. screened 45 core SNPs based on genome resequencing of 139 tea varieties [38]. Park et al. identified SNPs with the aid of GBS (genotyping by sequencing) of 90 lettuce (*Lactuca sativa*) varieties [39]. Nguyen et al. used 48 varieties to develop pumpkin SNPs [40]. Wan et al. screened 623 MNP markers based on the published SNP information of 241 cassava materials [27]. Ling et al. analyzed 188 varieties of the data of whole genome sequencing, and developed 501 MNP markers for *Lentinula edodes* [28]. Wei et al. obtained 507 MNP markers via Illumina sequencing from 32 varieties of *Pleurotus eryngii* [29]. Liu et al. identified 582 MNP markers based on 31 representative grape varieties [22]. In our study, 30 varieties were selected for marker screening. The 30 varieties represent five types of chrysanthemum, and their expression states of the 36 characteristics covered almost all of the states listed in chrysanthemum DUS test guidelines [30]. Additionally, their GS values ranged from 9.71% to 30.97%, and their average *Ho* value reached 71.19%, indicating that there is rich diversity in 30 varieties. Furthermore, values of *Ho* and DP of the screened MNPs detected in the 30 varieties were close to those in the 136 varieties. Taken together, the 30 varieties were representative and adequate for MNP developing.

So far, MNP markers have been applied in variety identification of rice, soybean, rapeseed, eggplant, maize, tomato, and other crops [25,41]. The MNP method was reported as a more efficient and more accurate alternative over SSRs and SNPs. However, compliments on the accuracy of MNPs were mainly concluded in a theoretical manner, and performance of MNPs was mostly revealed in the population which was used for MNP screening. Robustness of MNPs in practical application is less discussed. In our study, another population with 136 varieties was utilized for further confirmation. Similar results, i.e., *Ho*, DP, et al., between the two populations preliminarily elucidate the stability of the 487 MNPs. Furthermore, clustering results based on the 487 MNPs were generally consistent with those based on the 26,147 SNPs, as well as those on phenotypic traits and initial grouping likewise. Since the 487 MNPs were screened from the 26,147 SNPs and corresponded to 3214 SNPs (individual MNP contained 6.6 SNPs on average), it can be concluded that the screened MNPs can be expected as substitutes for their original SNPs. In addition to this, they have robust capability in variety discrimination. Given the functionality of MNP, similar to other molecular markers such as SNP and SSR, MNP can effectively complement phenotype descriptions. It has particularly promising applications in DUS testing, such as identifying approximate varieties based on genetic relationships and determining relationships between two different varieties.

Given all of that, our MNP markers have great potential in accurate and rapid identification of chrysanthemum varieties, and, accordingly, to foster breeding innovation and to promote chrysanthemum marketing. However, like most previous studies, our research did not explore the detailed GS threshold of MNP in distinguishing varieties, particularly in determining EDV (Essentially Derived Varieties), which is a globally concerned issue. The UPOV 1991 Convention defines the criteria for Essentially Derived Varieties (EDVs). These criteria include the following: (1) an EDV is predominantly derived from the initial variety, or from a variety that is itself predominantly derived from the initial variety, while retaining the expression of essential characteristics resulting from the genotype or combination of genotypes of the initial variety. (2) an EDV must be clearly distinguishable from the initial

variety. (3) except for differences resulting from the act of derivation, an EDV should conform to the initial variety in the expression of essential characteristics resulting from the genotype or combination of genotypes of the initial variety [42]. Due to the proliferation of EDV varieties, genuinely new varieties are becoming increasingly scarce. This reduction in breeding innovation can lead to a vicious cycle [43]. As a member of UPOV, China has established an EDV system through modifications to the "Seed Law of the People's Republic of China" in 2022. Correspondingly, advancements in EDV detection technologies, such as high-density molecular markers, are essential to keep pace with these developments. To fully leverage the role of MNP markers in variety identification, further research based on 487 MNP markers is needed. This research aims to collect more chrysanthemum varieties, especially those within the same pedigree, to establish their genetic fingerprints. By doing so, we can determine the GS threshold and create a fingerprint database based on MNP markers. This database will be used for managing chrysanthemum varieties, including screening similar varieties for DUS testing, and addressing PBR issues related to infringement and disputes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae10080845/s1, Table S1. Information of the 136 varieties of chrysanthemum; Table S2. Grades and distribution of quality characteristics; Table S3. Data for quality control of sample comparison results; Table S4. Index of genetic differentiation (Fst) between populations; Table S5. Details of genetic features of each of the 487 MNP markers in chrysanthemum; Table S6. Information of MNP markers; Figure S1. Base mutation type and number of SNPs.

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