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Comparative and Phylogenetic Analysis of *Anthurium andraeanum* Hybridization Based on Molecular and Morphological Traits

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Abstract: Hybridization is considered an important mode of species evolution, but the genetic evolutionary process of *Anthurium andraeanum* hybridization is still poorly characterized. In order to provide the molecular and morphological basis for phylogenetic analysis in *A. andraeanum* hybridization, we analyzed the morphological, nuclear genomic, and chloroplast genomic data of five *A. andraeanum* cultivars and explored the correlations between different traits and nuclear and chloroplast genome characteristics. *A. andraeanum* hybrid 1 is an *A. andraeanum* 'Baron' (♀) × *A. andraeanum* 'Spice' (♂) cross, and *A. andraeanum* hybrid 2 is an *A. andraeanum* 'Cheers' (♀) × *A. andraeanum* hybrid 1 (♂) cross. The *A. andraeanum* hybrids reflected their parents' heterozygous features in their morphologies, nuclear genomes, and chloroplast genomes. The morphological traits in the F1 generation were widely separated, showing continuous variation. Based on cluster analysis, the five *A. andraeanum* cultivars could be divided into two groups. The ISSR analysis results were highly correlated with the spathe color. Among the five *A. andraeanum* cultivars, the composition and structure features of chloroplast genomes were completely the same or highly similar, respectively. Phylogenetic analysis based on complete chloroplast genome data showed that the genetic stability of the chloroplast is high in *A. andraeanum*, manifested as uniparental maternal inheritance, where the chloroplast genome composition and structural features of hybrids are highly similar to those of the maternal parent.

Keywords: phylogenetic analysis; chloroplast genome; hybridization; *Anthurium andraeanum*

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1. Introduction

Anthurium andraeanum is a perennial herb in the *Anthurium* genus of the Araceae family. Due to its brilliant spathe and spadix color, many species in this genus have been cultivated as ornamental houseplants and for cut flowers since the nineteenth century. It is especially appealing as a cut flower material with a long flowering period. The characteristics of the spathes, spadices, and leaves are essential to the commercial quality of *A. andraeanum*. Therefore, these components are favorable targets for selective breeding. For example, spathe coloration is an important feature of *A. andraeanum* and is mainly determined by anthocyanins. The *A. andraeanum* genus is the largest in Araceae, including approximately 950 species distributed in the Neotropics [1,2]. Reproductive disorders are still prevalent in *A. andraeanum* plants, and significant differences in pollen-to-ovule ratios have been observed between self-pollinating and non-self-pollinating species in the Araceae family [3]. The genetic background related to the kinship and genetic diversity of germplasm is the basis for successfully breeding *A. andraeanum*, which is a cross-pollinated plant with significant trait segregation in the progeny. *A. andraeanum* has high genetic diversity due to hybridization and wide morphological variation. However, basic research on *A. andraeanum* genetics has been limited, and the genetic mechanisms of important

traits remain unclear. To date, only a few related investigations have been conducted on *A. andraeanum*.

Traditional plant classification is based on the morphological characteristics of plants. With the development of molecular biotechnology, phylogenetic genomics, a new discipline of plant classification based on molecular technology, has been gradually developed and established. Twenty genotypes of *A. andraeanum* were characterized using DNA fingerprinting and SPAR markers [4]. The ISSR molecular marker technique can be applied to the identification of *A. andraeanum* cultivars. The genetic diversity and genetic relationships among 26 cultivars of *A. andraeanum* were analyzed by using ISSR molecular marker technology [5]. The sequencing assembly of the genome and transcriptome of the major tissue organs of *A. andraeanum* has provided new fundamental information on the genetic basis of ornamental traits, determinants of morphology and function, and plant evolution in the Araceae family [6–9]. Related studies have reported chloroplast genome sequencing in the Araceae family [9–11]. Chloroplast phylogenetic analyses indicate that *A. andraeanum* is a member of Pothoideae and provide insight into the molecular evolution in the Araceae family [12,13]. However, the effect of chloroplasts on the traits of cytoplasmic hybrid progeny is not yet clear.

The genetic diversity analysis of closely related phenotypic and ornamental traits can be used for the classification of germplasm resources and core germplasm construction. Studying plant phylogeny from the perspective of the whole chloroplast genome has become a hotspot in current plant genetic and taxonomic research. In this study, in order to provide the molecular and morphological basis for phylogenetic analysis in *A. andraeanum* hybridization, *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* 'Cheers' with different traits were directionally selected as parents, which are suitable for planting in a local area with popularization value for industrialization. *A. andraeanum* hybrid 1 is an *A. andraeanum* 'Baron' (♀) × *A. andraeanum* 'Spice' (♂) cross, and *A. andraeanum* hybrid 2 is an *A. andraeanum* 'Cheers' (♀) × *A. andraeanum* hybrid 1 (♂) cross. We analyzed the morphological, nuclear genomic, and chloroplast genomic data of the five *A. andraeanum* cultivars to study the correlations between different traits and nuclear and chloroplast genome characteristics. This study provides references for the selection of elite cultivars and high-quality *A. andraeanum* parents, research on crossbreeding, and an understanding of the genetic mechanism of *A. andraeanum*.

2. Materials and Methods

2.1. Plant Material and DNA Extraction

A. andraeanum 'Baron' (voucher number HNAN012), *A. andraeanum* 'Spice' (voucher number HNAN011), and *A. andraeanum* 'Cheers' (voucher number HNAN004) were sourced from the Post-Entry Quarantine Station for Tropical Plants, Haikou Customs, located in Wenchang City, Hainan Province. The comprehensive index of biological characteristics and horticultural characters performed best in *A. andraeanum* 'Spice' and *A. andraeanum* 'Cheers'. The spathe color of *A. andraeanum* 'Spice' was red, with a few green areas on the edge; the spadix color was rose; and its tip was orange-brown. The spathe color of *A. andraeanum* 'Cheers' was pink, the spadix color was white, and its tip was green. The spathe color of *A. andraeanum* 'Baron' was pink, with a few green areas on the edge; the spadix color was rose; and its tip was green. The characteristics of *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* 'Cheers' are suitable for selective breeding.

A. andraeanum hybrid 1 (voucher number HNAN-HF030) is an *A. andraeanum* 'Baron' (♀) × *A. andraeanum* 'Spice' (♂) cross, and *A. andraeanum* hybrid 2 (voucher number HNAN-HF017) is an *A. andraeanum* 'Cheers' (♀) × *A. andraeanum* hybrid 1 (♂) cross. Fresh leaves of the *A. andraeanum* plants were collected, immediately frozen in liquid nitrogen, and then stored at −80 °C. Total genomic DNA was extracted from the leaves using the Plant Genomic DNAKit (TIANGEN, Biotechnology, Beijing, China) according to the instructions. The quality and quantity of DNA were confirmed using 1% agarose gel electrophoresis and a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). The DNA samples were

diluted to 60 ng/μL for polymerase chain reaction (PCR) amplification. High-quality DNA was used for further sequencing.

2.2. Measurement of Major Morphological Traits

Thirty healthy and strong plants from each cultivar were randomly selected to determine morphological traits, with one sample taken per plant. All plants were planted in planting troughs and placed in glasshouse with shading, cooling, and heating functions. The leaves corresponding to the largest normal spathe with 1/2–2/3 pistil of the spadix were mature. The spathe length and width were measured in the flower of largest normal spathe with 1/2–2/3 pistil of the spadix mature. The morphological traits were measured during same growth and development periods, and mean and standard deviation (SD) of the quantitative characteristics of each *A. andraeanum* cultivars were statistically calculated. The morphological parameters of these samples include plant height, leaf length and width, spathe length and width, spathe color, spadix color, and spathe shape. For each of the five *A. andraeanum* cultivars, a phenetic analysis was performed using five variables: plant height, leaf length and width, and spathe length and width. The measurement method is as follows:

Plant height: Measure the height of plants with a ruler.

Leaf length: Flatten the blade and measure the length from the blade tip to the base using a ruler.

Leaf width: Flatten the blade and measure at the widest position using a ruler.

Spathe length and width: Flatten the spathe and measure the length from the spathe tip to the base, recorded as spathe length; measure the broadest position recorded as spathe width using a ruler.

Spathe and spadix color: Quantify the spathe and spadix color according to the RHS color chart (Royal Horticultural Society Color Chart, RHSCC).

2.3. ISSR-PCR Amplification

Using the five collected *A. andraeanum* cultivars as experimental materials, polymorphic primers were screened from the universal primers, and the universal primers were used to construct the *A. andraeanum* ISSR (inter-simple sequence repeat) molecular marker system. One hundred primers (Set No. 9, Nos. 801-900) published by the University of British Columbia (UBC) and *A. andraeanum* ISSR primers, which have been reported, were selected as the screening primers for this study [5]. The primers (annealing temperature of 48 °C: ISSR10 and ISSR15; annealing temperature of 50 °C: ISSR04, ISSR06, ISSR807, ISSR810, and ISSR873; annealing temperature of 52 °C: ISSR07, ISSR817, and ISSR825; annealing temperature of 54 °C: ISSR02, ISSR16, ISSR811, ISSR843, and ISSR895; annealing temperature of 56 °C: ISSR850, ISSR854, ISSR857, and ISSR899; annealing temperature of 58 °C: ISSR03) were the informative primers used for distinguishing the *A. andraeanum* cultivars.

The 25 μL PCR reaction solution for ISSR markers contained 60 ng of DNA template, 1.0 U of Taq polymerase, 2.5 μL of 10×reaction buffer, 2.0 mM of $MgCl_2$, and 0.2 mM of dNTP and 0.5 μM of ISSR primer. The PCR reaction involved initial pre-denaturation for 5 min at 94 °C, denaturation for 45 s at 94 °C, 45 sec of annealing temperature (48–58 °C), and elongation for 1.5 min at 72 °C with 40 cycles, extended at 72 °C for 7 min, and stored at 4 °C. The PCR amplified product of ISSR primers was detected using 1.6% agarose gel electrophoresis, using the 2 kb DNA ladder marker (TaKaRa, Biotechnology, Dalian, China) as the control. The electrophoretic patterns of the PCR amplified products were recorded digitally using an Ultra-Violet Products Gel-Doc imaging analysis system (UVP, Upland, CA, USA). The DNA bands were scored for the presence and absence. The PCR amplification results were analyzed via UPGMA clustering analysis using the common primers obtained from the screening.

2.4. Chloroplast Genome Sequencing and Analysis

Fresh leaves from five samples of *A. andraeanum* were collected, and libraries were constructed and sequenced using high-quality DNA and the Illumina NovaSeq system. Raw sequence reads were processed for quality control using Fastp 0.19.7 [14]. Subsequently, high-quality reads were assembled into the chloroplast genome using the de novo assembler SPAdes v.3.14.1 software [15]. Finally, *Acorus tatarinowii* (MN536753) was used as the reference genome, and five complete plastid genomes were annotated with PGA [16]. The chloroplast genome sequences of *A. andraeanum* 'Spice', *A. andraeanum* 'Baron', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2 were deposited in GenBank for the first time under accession numbers PP959702-PP959706, respectively. cpDNA genome sequences of *A. andraeanum* samples were physically mapped using OGDRAW version 1.3.1 [17].

2.5. Characterization and Comparative Analyses of Chloroplast Genomes

The frequency of amino acid use and relative synonymous codon usage (RSCU) were determined using CodonW 1.4.2 [18]. Oligonucleotide repeat sequences were determined using REPuter (https://bibiserv.cebitec.uni-bielefeld.de/reputer/, accessed on 21 June 2024) with a minimum repeat size of $\geq$30 [19]. The chloroplast genome was aligned using MAFFT v7.429 [20]. The chloroplast genome structures and gene contents of *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2 were compared with five previously reported chloroplast genomes of Araceae: *A. andraeanum* 'Alabama', *Pothos scandens*, *Stenospermation multiovulatum*, *Epipremnum amplissimum*, and *Spathiphyllum patulinervum*. The gene contents and rearrangements of genomes based on covariate block analysis were compared using integrated Mauve alignment 2.3.1 [21]. IR contraction and expansion in these cultivars were studied using IRscope V0.1.R [22].

2.6. Phylogenetic Analysis

The DNA banding patterns obtained by ISSR PCR amplification were analyzed for phylogenetic interpretation, which was based on constructing a binary matrix. Bands that migrated to identical positions on the agarose gel were scored as present, '1', and those without were scored as absent, '0'. A characterization matrix was used to count the total number of ISSR bands and polymorphic bands of the amplification products and to calculate the percentage and number of polymorphic bands for individual primers. The variable data matrix was standardized. UPGMA dendrograms were generated for these taxa based on the polymorphic bands.

The complete cpDNA sequences of *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2 were obtained and compared with the sequences of related species available. Seventy-five chloroplast sequences of the Araceae family, including *A. andraeanum* 'Alabama' (NC_072558), *Epipremnum amplissimum* (NC_047232), *Pothos scandens* (MN046891), *Spathiphyllum patulinervum* (NC_047233), and *Stenospermation multiovulatum* (NC_047235), were downloaded from NCBI. *Acorus tatarinowii* (MN536753) was selected as the outgroup. The orthologous genes in each sample with a copy number of 1 were identified using OrthoFinder v2.3.14 and ligated to construct a phylogenetic tree [23]. After linkage, genes were aligned using muscle v3.8.1551 and cleaned using Gblocks v0.91b [24,25]. Maximum likelihood phylogenies based on the best-fit model (HIVb + I + G + F) were conducted using IQ-TREE v1.6.12 with the bootstrap value set to 1000 [26].

3. Results

3.1. Morphological Comparison

The main morphological characteristics of *A. andraeanum* 'Baron' (Figure 1a), *A. andraeanum* 'Spice' (Figure 1b), *A. andraeanum* 'Cheers' (Figure 1c), *A. andraeanum* hybrid 1 (Figure 1d), and *A. andraeanum* hybrid 2 (Figure 1e) are shown in Figure 1 and Table 1.

The morphological traits in the F1 generation were widely separated, showing continuous variation. The plant height, leaf length and width, spathe length and width, spathe color, spadix color, and spathe shape were variable. The spathe length and width of the five cultivars were 14.3–19.7 cm and 12.0–16.5 cm, respectively. Due to parental trait effects, the hybrids showed an obvious inheritance tendency of *Anthurium* cut flower cultivars. The horticultural characters of spathe and spadix performed better and showed some hybrid advantage. The spathe color of *A. andraeanum* hybrid 1 was red/pink with a few green areas on the edge; the spadix color was rose. The spathe color of *A. andraeanum* hybrid 2 was pink with a few green areas on the edge, the spadix color was pink, and its tip was green. The morphological characteristics of *A. andraeanum* hybrid 1 were highly similar to those of the paternal parent, and those of *A. andraeanum* hybrid 2 were highly similar to those of the maternal parent, such as the spathe and spadix.

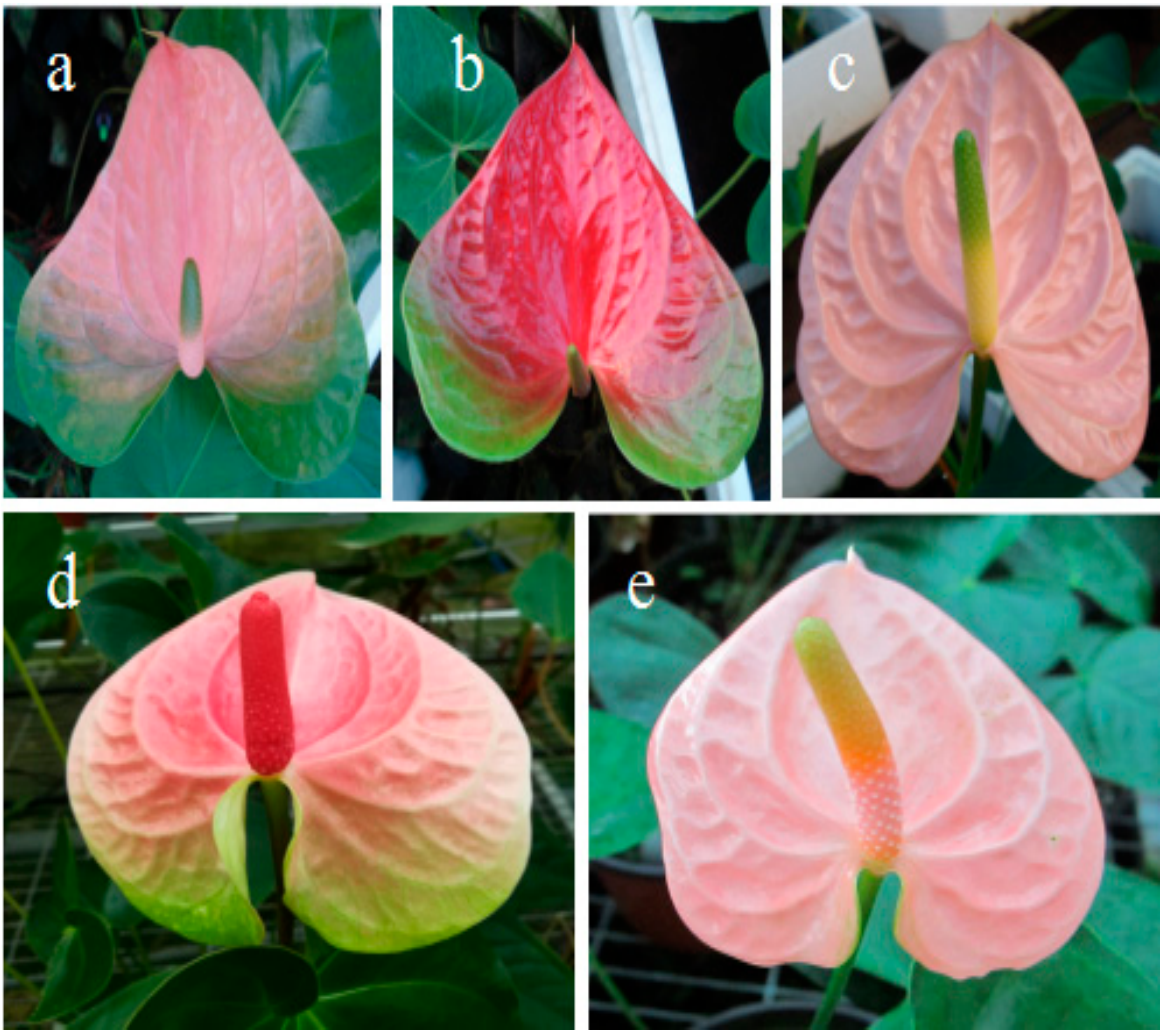


Figure 1. The spathe and spadix of the five *A. andraeanum* cultivars investigated in this study. (**a**) *A. andraeanum* 'Baron'; (**b**) *A. andraeanum* 'Spice'; (**c**) *A. andraeanum* 'Cheers'; (**d**) *A. andraeanum* hybrid 1; (**e**) *A. andraeanum* hybrid 2.

Table 1. The morphological characteristics of the five *A. andraeanum* cultivars.

Cultivars	Plant Height (cm)	Leaf Length (cm)	Leaf Width (cm)	Spathe Length (cm)	Spathe Width (cm)	Spathe Color	Spadix Color	Spathe Shape
A. andraeanum 'Baron'	65.5 ± 5.8	29.5 ± 2.8	20.8 ± 1.9	18.6 ± 1.7	15.7 ± 1.3	Pink Green	Rose Green	Broad Ovate
A. andraeanum 'Spice'	59.7 ± 5.5	26.7 ± 2.6	19.2 ± 1.8	19.7 ± 1.9	16.5 ± 1.5	Red Green	Rose Orange Brown	Broad Ovate
A. andraeanum 'Cheers'	79.1 ± 6.9	23.9 ± 2.3	21.0 ± 2.0	14.3 ± 1.4	12.0 ± 1.1	Pink	White Green	Broad Ovate
A. andraeanum hybrid 1	66.1 ± 6.0	31.2 ± 3.0	21.3 ± 2.1	19.2 ± 1.8	16.3 ± 1.4	Red/Pink Green	Rose	Broad Ovate
A. andraeanum hybrid 2	59.2 ± 5.6	25.6 ± 2.4	18.9 ± 1.7	17.5 ± 1.6	14.8 ± 1.2	Pink Green	Pink Green	Broad Ovate

3.2. Chloroplast Genome Sequencing and Analysis of Basic Features of the Five Chloroplast Genomes of A. andraeanum

The circular chloroplast genomes of the five *A. andraeanum* cultivars were 162,560–162,658 bp in size (Figure 2), and each one consisted of a large single-copy region (LSC, 88,814–88,912 bp), a small single-copy region (SSC, 22,856 bp), and a pair of inverted repeats (IR, 25,445 bp) (Table 2). We found that the number of protein-coding genes and tRNAs of *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2 were similar to those of a congener, *A. andraeanum* 'Alabama'. Their chloroplast genomes all contained 130 genes, including 85 PCG genes, 37 tRNA genes, and 8 rRNA genes. Of these, *A. andraeanum* hybrid 1 differed slightly from *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', and *A. andraeanum* hybrid 2 in the size of its LSC region. The G/C content ratio of *A. andraeanum* hybrid 1 was different from those of the other *A. andraeanum* cultivars. The overall GC content of *A. andraeanum* hybrid 1 was 36.33% (18.46% + 17.87%), and it was 36.33% (18.47% + 17.86%) in other *A. andraeanum* cultivars. In *A. andraeanum* hybrid 1, the GC content of the LSC region was 36.67%, with an observed increase of 0.01% compared to the other four *A. andraeanum* cultivars.

The five cultivars shared genes that were functionally identical, including 52 genes related to photosynthesis (e.g., *psaA*, *psbA*, *rbcL*, *petA*, *pafI*, *atpA*, and *ndhA*), 50 genes related to transcription and translation (e.g., *rpl2*, *rps2*, *rpoA*, *rrn5*, and *trnA-UGC*), 4 other genes (*accD*, *cemA*, *ccsA*, and *infA*), and 2 genes with unknown functions (*ycf1* and *ycf2*). The LSC region included *psaA*, *psbA*, *rbcL*, *petA*, *pafI*, *atpA*, *ndhA*, *rps2*, *rpl14*, *trnC*, *infA*, *rpoA*, *clpP*, *cemA*, *accD*, and *matK*. Some of these genes, such as *rps7*, *rps12*, *trnM*, *trnL*, *trnV*, *trnI*, *trnA*, *trnR*, *rrn16*, *rrn23*, *rrn4.5*, *rrn5*, *ycf2*, and *ndhB,* usually exist as single copies in the IRA/B regions. The IRB region has more *rps19*, *rpl2*, and *rpl23* genes than the IRA region. In contrast, the SSC region included *ndhF*, *rpl32*, *trnL*, *ccsaA*, *ndhD*, *psaC*, *ndhE*, *ndhG*, *ndhI*, *ndhA*, *ndhH*, *rps15*, and *ycf1*.

Compared to the parents (*A. andraeanum* 'Baron' and *A. andraeanum* 'Spice'), *A. andraeanum* hybrid 1 had insertions of 1 bp and 97 bp InDel special marker sites in the LSC region. The 1 bp InDel special marker site was located between the *ndhC* and *trnV*-UAC genes, and the 97 bp InDel special marker site was located between the *rbcL* and *accD* genes. Our analysis revealed a higher evolutionary rate of *A. andraeanum* hybrid 1 genes compared to *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', and *A. andraeanum* hybrid 2, with observed differences of 0.11% in the LSC region. The most significant evolutionary rate discrepancy was noted in the LSC region, potentially attributed to the translocation of the InDel insertion to the LSC region.

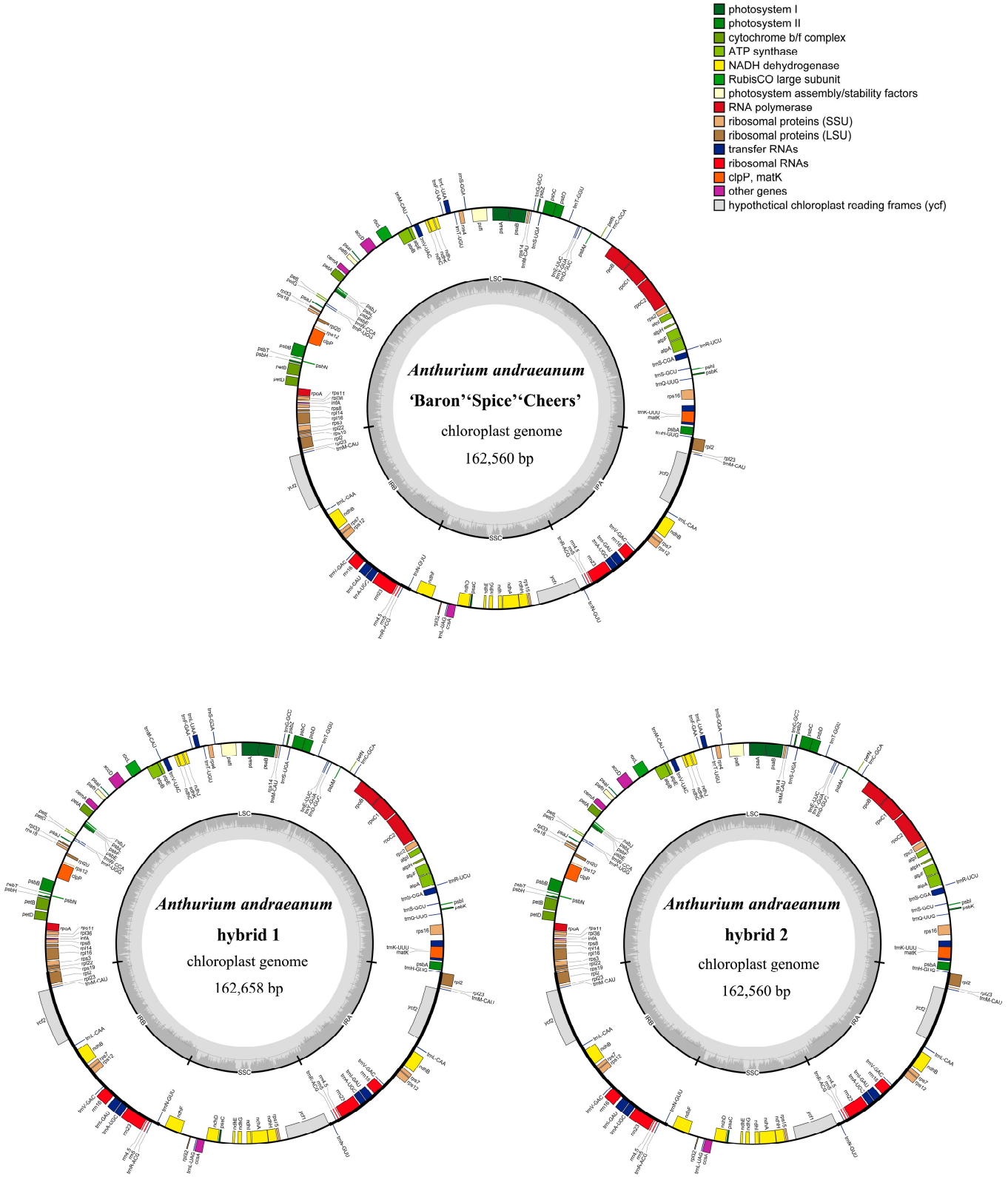


Figure 2. Chloroplast genome map of the five *A. andraeanum* cultivars.

Table 2. Comparison of the genomic characteristics among the Araceae chloroplast genomes. LSC, large single copy; SSC, small single copy; IR, inverted repeat; PCG, protein-coding gene.

Cultivars	Size	Genome Size (bp)			Number of Genes				GC (%)
		LSC	SSC	IR	PCG	tRNA	rRNA	Total	
A. andraeanum 'Baron''Spice''Cheers'	162,560	88,814	22,856	25,445	85	37	8	130	36.33
A. andraeanum hybrid 1	162,658	88,912	22,856	25,445	85	37	8	130	36.33
A. andraeanum hybrid 2	162,560	88,814	22,856	25,445	85	37	8	130	36.33
A. andraeanum 'Alabama'	162,560	88,814	22,856	25,445	85	37	8	130	36.33
Epipremnum amplissimum	163,335	89,714	22,053	25,784	84	37	8	129	36.14
Pothos scandens	164,719	102,956	6779	27,492	90	36	8	134	35.36
Spathiphyllum patulinervum	164,751	91,841	21,600	25,655	84	37	8	129	35.95
Stenospermation multiovulatum	163,822	90,512	21,448	25,931	84	37	8	129	36.11

3.3. Amino Acid Frequency and Codon Usage

The amino acid frequency and codon usage in the chloroplast genomes of *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2 were similar. *A. andraeanum* hybrid 1 had two InDel special marker site inserted in the LSC non-coding region, but it did not affect the amino acid frequency and codon usage. The codons most frequently used were UUA and AGA (one of six codons encoding Leucine and Arginine), reaching an RSCU of 1.89, followed by GCU (one of four codons encoding Alanine) with an RSCU of 1.78. Codon preference was observed for all amino acids except for Methionine and Tryptophan, both of which had 1 codon with no preference, while 30 codons had an RSCU of >1.00, 29 codons had an RSCU of <1.00, and the lowest RSCU, 0.34, was for the codon AGC encoding Serine (Figure 3).

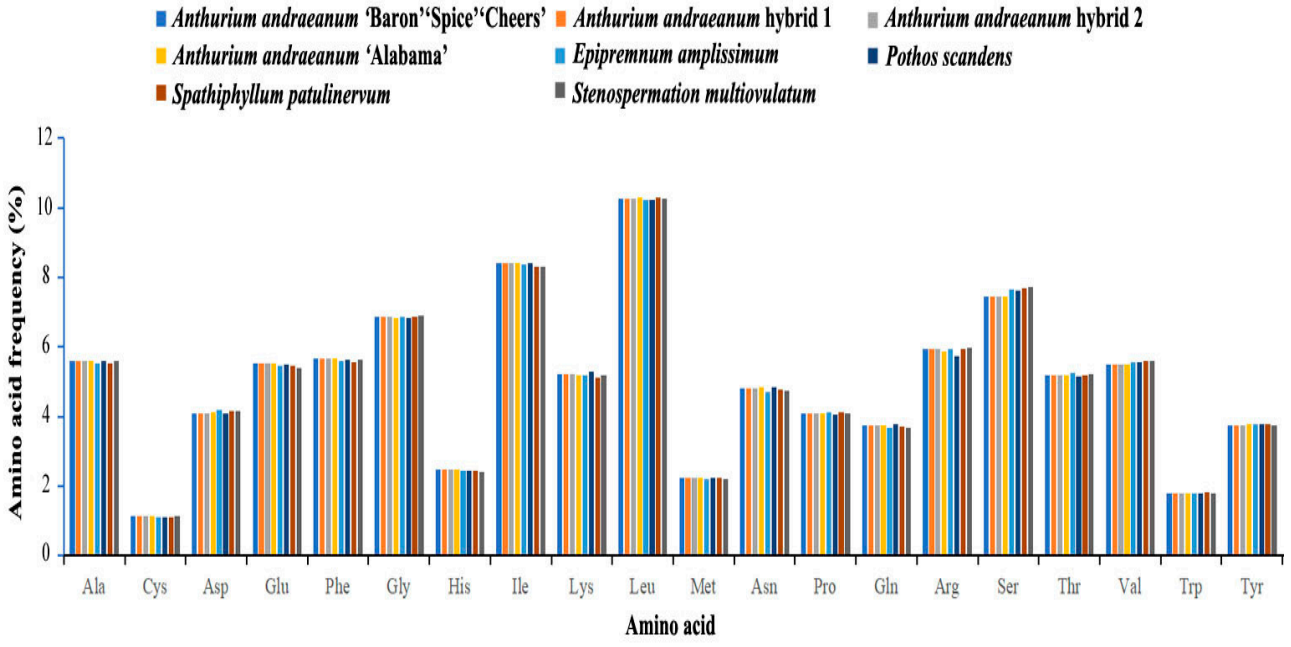


Figure 3. Amino acid frequency among the Araceae chloroplast genomes. The X-axis shows amino acids, whereas the Y-axis shows the amino acid frequency as a percentage.

3.4. Repeat Analyses

REPuter was used to detect oligonucleotide repeats in the five chloroplast genomes of *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2, but the number and type of repeats varied considerably between the five cultivars. We observed 16 repeats in *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* 'Cheers'; 19 repeats in *A. andraeanum* hybrid 1; and 16 repeats in *A. andraeanum* hybrid 2. We observed 10 forward, 5 palindromic, and 1 reverse repeat in *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* 'Cheers'. *A. andraeanum* hybrid 1 had 13 forward, 5 palindromic, and 1 reverse repeat, and *A. andraeanum* hybrid 2 had 10 forward, 5 palindromic, and 1 reverse repeat. Most of the repetitive sequences appeared in the LSC region. In *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* 'Cheers', seven repeats were found. A total of 10 repeats were found in *A. andraeanum* hybrid 1, and 7 repeats were found in *A. andraeanum* hybrid 2. Most repeats in the five cultivars were between 30 and 34 bp or ≥45 bp in size, and the repeat size ≥ 45 bp was found in *A. andraeanum* hybrid 1 more often than in *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', and *A. andraeanum* hybrid 2 (Table 3).

Table 3. Repeat analyses among the Araceae chloroplast genomes. F, forward; P, palindromic; C, complementary; R, reverse; LSC, large single copy; SSC, small single copy; IR, inverted repeat. LSC/SSC and LSC/IR represent shared repeats between regions.

Cultivars	Types of Repeats				Distribution of Repeats			The size of Repeats			
	F	P	C	R	LSC	SSC	IR	30–34	35–39	40–44	≥45
A. andraeanum 'Baron''Spice''Cheers'	10	5	0	1	7	4	5	9	0	1	6
A. andraeanum hybrid 1	13	5	0	1	10	4	5	9	0	1	9
A. andraeanum hybrid 2	10	5	0	1	7	4	5	9	0	1	6
A. andraeanum 'Alabama'	10	5	0	1	7	4	5	9	0	1	6
Epipremnum amplissimum	9	3	0	3	7	3	5	9	2	0	4
Pothos scandens	5	5	1	3	3	0	11	4	7	1	2
Spathiphyllum patulinervum	17	10	1	32	10	41	9	33	20	2	5
Stenospermation multiovulatum	15	21	8	12	4	0	52	49	4	0	3

3.5. Gene Arrangement and Inverted Repeat Contraction and Expansion

The arrangement of genes in *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2 was similar; it did not feature gene rearrangement and inversion. The gene arrangement was found to be structurally identical to *A. andraeanum* 'Alabama', as shown in the colinear block of the Mauve alignment. The arrangement of genes did not change due to expansions of the SSC region, and *A. andraeanum* hybrid 1 had two InDel special marker site inserted in the LSC non-coding region, but it did not affect the gene arrangement. However, the genes in the LSC, SSC, and IR regions in *Pothos scandens* chloroplasts varied in their arrangement. The genes in the LSC and IR region were relatively long. The arrangement of genes in the SSC region in *Pothos scandens* chloroplasts changed due to the expansion of the LSC and IR region (Figure 4).

The circular structures of the cpDNA genomes of the five *A. andraeanum* taxa formed four boundaries between the LSC, SSC, and IR regions: LSC-IRB, IRB-SSC, SSC-IRA, and IRA-LSC. Among the five chloroplast genomes, the boundary expansions or shrinkages at the LSC-IRB, IRB-SSC, SSC-IRA, and IRA-LSC borders were the same. In the five *A. andraeanum* samples, the LSC-IRb border was within the *rps19* gene (with 22 bp of the gene in the LSC region), the IRb-SSC border was within the *trnN* gene (with 353 bp of the gene in the IRb region), the SSC-IRa border was within the ycf1 gene (with 38 bp of the gene in the SSC region), and the IRa-LSC border was within the *trnH* gene (with 1 bp of the gene in the IRa region) (Figure 5). However, several features of the chloroplast genome differed between *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* hybrid

1, including forward repeats, InDel marker sites, etc. The chloroplast genomic features of *A. andraeanum* 'Cheers' and hybrid 2, including IR-LSC/SSC boundaries, SSRs, and SNPs, were structurally identical.



Figure 4. Gene arrangement analysis based on Mauve alignment. White boxes represent protein-coding genes, red boxes represent rRNA, black boxes represent tRNA, green boxes represent intron-containing tRNA, the line between two white boxes indicates intron-containing protein-coding genes, and the vertical line on the collinearity analysis graph indicates the collinear region among the Araceae chloroplast genomes.

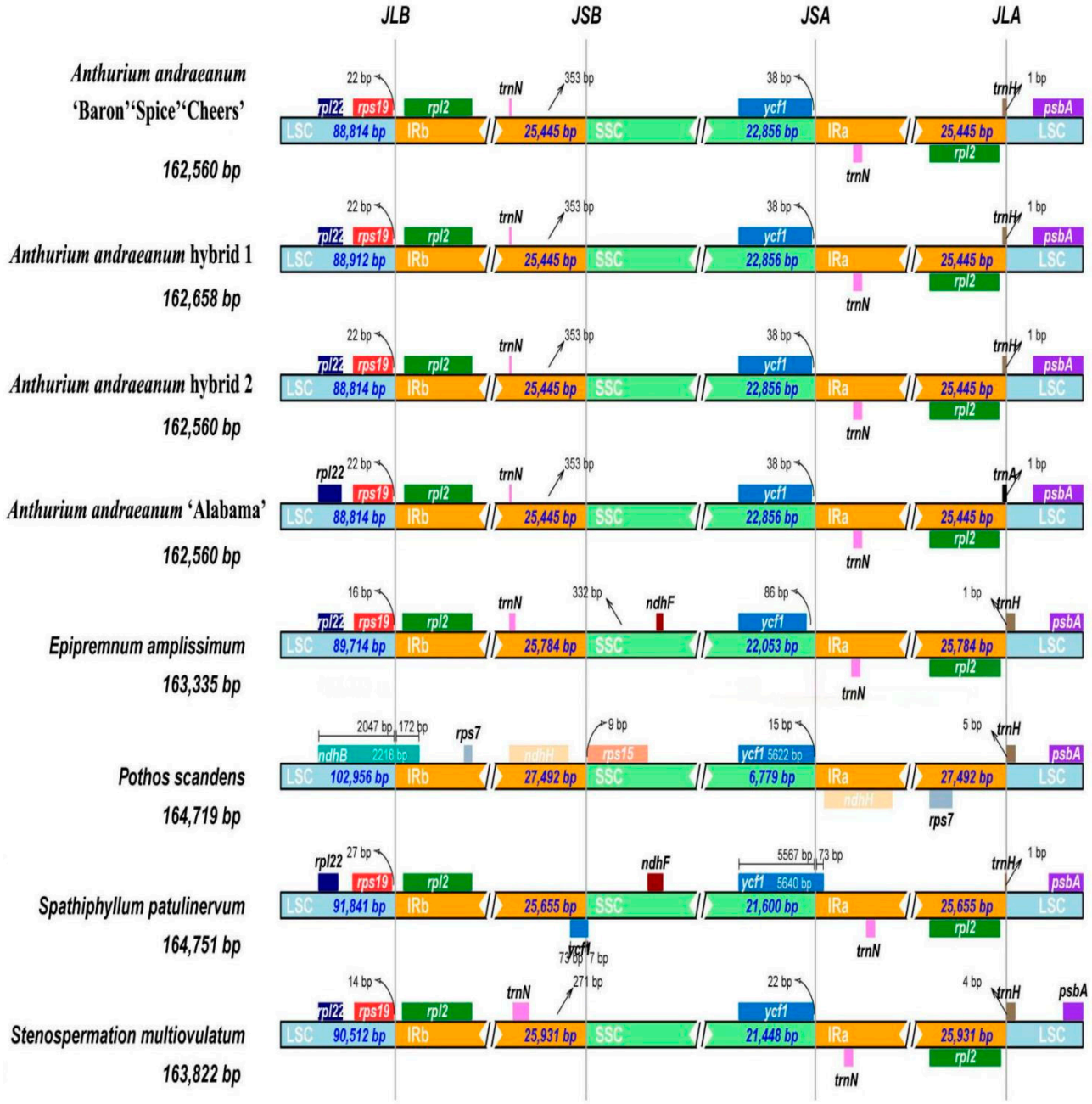


Figure 5. Comparison of the borders of large single-copy (LSC), small single-copy (SSC), and inverted-repeat (IR) regions among the Araceae chloroplast genomes.

3.6. Phylogenetic Analysis

The primers selected for the PCR amplification of *A. andraeanum* germplasm DNA resulted in 97 amplified bands, of which 90 were polymorphic, with a polymorphism ratio of 92.78%. UPGMA dendrograms were generated for the five *Anthurium* cultivars based on the average Euclidean distance. As shown in Figure 6, the differences between taxa ranged from 0.74 to 0.94. According to the cluster analysis chart, the five *A. andraeanum* cultivars were divided into two categories. Of these, *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', and *A. andraeanum* hybrid 1 clustered into one clade, and *A. andraeanum* 'Cheers' and *A. andraeanum* hybrid 2 clustered into another clade, suggesting closer relationships between *A. andraeanum* hybrid 1 and *A. andraeanum* 'Spice' and between *A. andraeanum* hybrid 2 and *A. andraeanum* 'Cheers'. As shown in Figure 1, the spathe colors of *A. andraeanum* hybrid 1 were highly similar to those of the paternal parent, and those of *A. andraeanum* hybrid 2 were

highly similar to those of the maternal parent. The cluster analysis showed that the ISSR analysis results were highly correlated with the spathe color (Figure 6). The ISSR molecular marker methodology is applicable to the characterization of *A. andraeanum* cultivars.

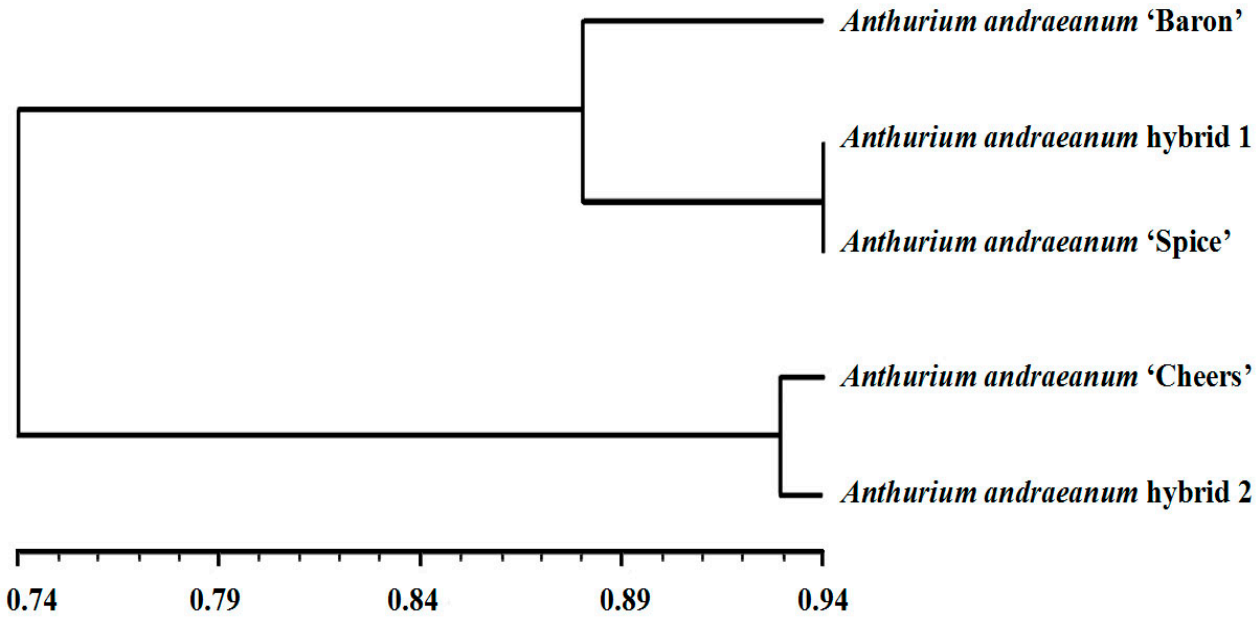


Figure 6. UPGMA clustering analysis of the five *A. andraeanum* cultivars based on ISSR markers.

Based on the complete chloroplast genomes of the *A. andraeanum* cultivars, a phylogenetic tree was constructed using the maximum likelihood method to explore the interspecific relationships between *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* hybrid 1, and *A. andraeanum* hybrid 2, using *Acorus tatarinowii* (MN536753) as the outgroup. As shown in the tree, fourteen closely related species were clustered into two clades, with *Monstera adansonii*, *Monstera deliciosa*, *Epipremnum amplissimum*, *Spathiphyllum cannifolium*, *Spathiphyllum kochii*, *Spathiphyllum patulinervum*, and *Stenospermation multiovulatum* forming the first clade alone. *A. andraeanum* and *Pothos scandens* were clustered into the second clade, indicating that these *A. andraeanum* cultivars are more closely related to the Pothos family. In particular, *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* 'Alabama', and *A. andraeanum* hybrid 2 can be divided into a small branch in the second clade, indicating that the interspecific relationships between *A. andraeanum* 'Baron', *A. andraeanum* 'Spice', *A. andraeanum* 'Cheers', *A. andraeanum* 'Alabama', and *A. andraeanum* hybrid 2 are more closely related in *A. andraeanum*. *A. andraeanum* hybrid 1 is allocated to a small branch in the second clade (Figure 7). The genetic stability of the chloroplast is high in *A. andraeanum*, and the chloroplast genome composition and structural features of the hybrids are highly similar to those of the maternal parent. *A. andraeanum* hybrid 1 contained 1 bp and 97 bp InDel special marker sites inserted in the LSC region, which increased its evolutionary efficiency.

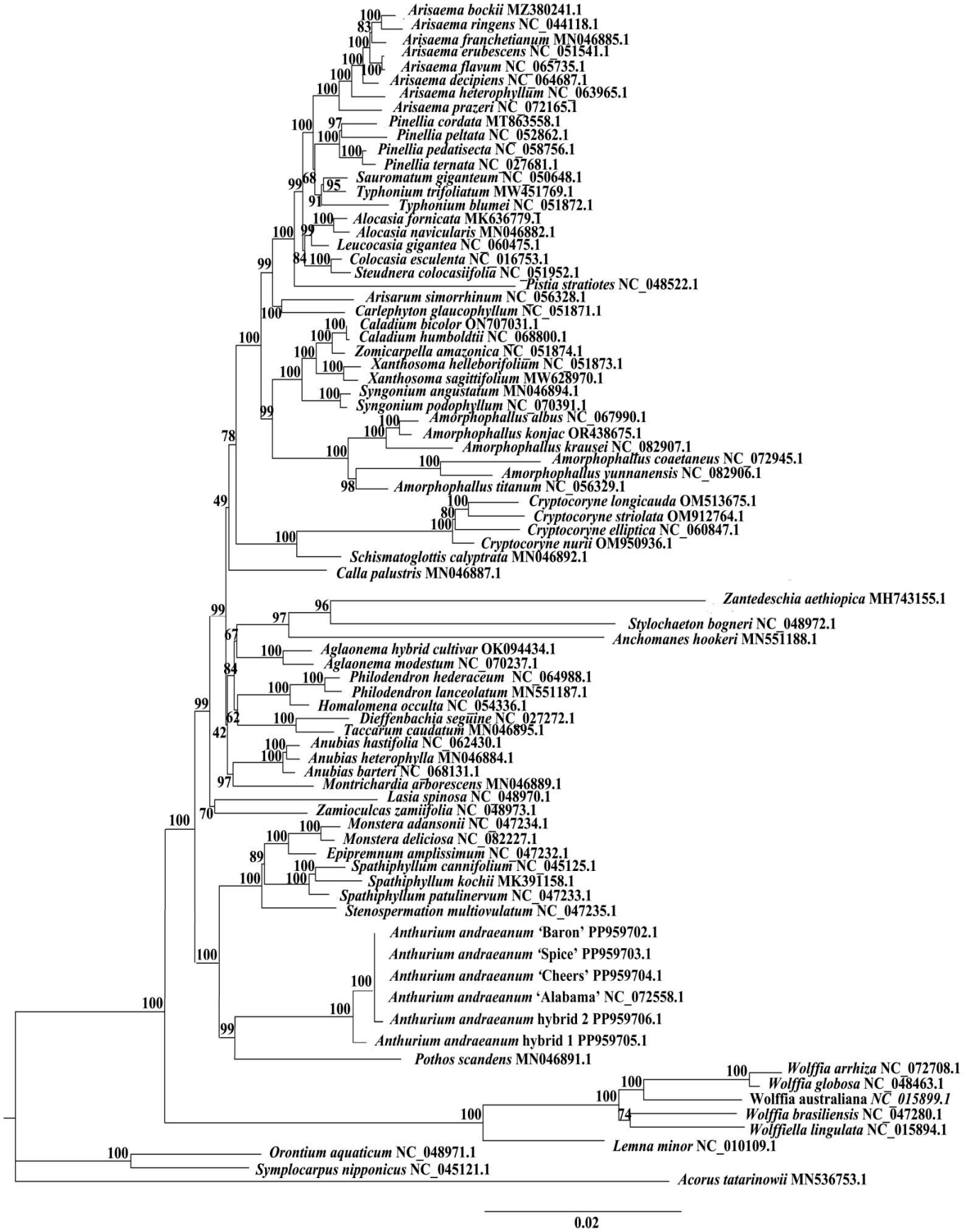


Figure 7. Maximum likelihood phylogenetic tree of the Araceae family reconstructed from chloroplast genome data.

4. Discussion

A. andraeanum is a cross-pollinated plant with significant trait segregation in the progeny. Its morphological characteristics are crucial because they significantly impact its ornamental value and commercial properties. The results of this study showed that the morphological traits in the F1 generation were widely separated, showing continuous variation. Due to parental trait effects, the hybrids showed an obvious inheritance tendency of *Anthurium* cut flower cultivars. The spathe length and width of *A. andraeanum* hybrid were 17.5–19.2 cm and 14.8–16.3 cm, respectively (Figure 1; Table 1). Morphological markers are highly influenced by the environment, and the genetic basis of various phenotypes may be the same.We analyzed the morphological, nuclear genomic, and chloroplast genomic data of the five *A. andraeanum* cultivars to study the correlations between different traits and nuclear and chloroplast genome characteristics. Earlier ISSR analysis revealed that the most closely related cultivars shared common characteristics [5]. Our cluster analysis results further proved that ISSR analysis was highly correlated with the spathe color (Figure 6). The variability and distribution patterns of the *A. andraeanum* cultivars showed that the quantitative characteristics did not follow a normal distribution, except the spadix thickness at the middle and the spathe size [27]. The results of phylogenetic analysis based on complete chloroplast genome data showed that the chloroplast genome has a comparatively less effect on the spathe color (Figure 7). The chloroplast genome encodes many key proteins involved in photosynthesis and other metabolic processes [28]. The results of a previous study indicate that light intensity is a key environmental factor influencing plastid development and leaf color formation in *A. andraeanum* [29]. Despite the enormous potential of molecular markers, their implementation in breeding programs has been limited by the lack of information on the stability of QTL across different genetic backgrounds. The high-density linkage map construction and QTL analysis for morphological traits offer valuable insights into the genetic basis in *A. andraeanum* breeding [30].

The chloroplast possessed an independent genome inherited from the maternal parent; it was relatively stable and genetically conserved [28,31]. The chloroplast shows uniparental inheritance, which is maternal in most angiosperms and paternal in some gymnosperms [32]. Similar to most angiosperms, the chloroplast phylogenetic analysis results further proved that the chloroplast manifested as uniparental maternal inheritance in *A. andraeanum* (Figure 7). The five studied chloroplast genomes proved highly similar in their overall size (162,560–162,658 bp), structure, gene order, and content. Nucleotide diversity analysis showed that the variation in the non-coding region was higher than that in the coding region, and the variation in the SC region was higher than that in the IR region (Figure 2; Table 2). This pattern is present in other angiosperm plastid genomes [33,34]. *A. andraeanum* hybrid 1 contained two InDel special marker sites inserted in the LSC region: a 1 bp InDel special marker site between the *ndhC* and *trnV*-UAC genes and a 97 bp InDel special marker site between the *rbcL* and *accD* genes. The InDel special marker sites did not affect the amino acid frequency or codon usage, indicating their location in non-coding regions (Figures 3–5; Table 3). Repeats play an important role in the generation of InDels and SNPs. Error-prone DNA polymerases cause numerous mutations due to errors in replication [35,36]. Genome evolution in *Anthurium* may also involve mechanisms such as genome expansion and contraction and chromosome recombination/loss [37]. The complete chloroplast genomes of *Anthurium huixtlense* and *Pothos scandens* (Pothoideae, Araceae) show inverted repeat expansions and contractions, affecting evolutionary rates [12]. A pairwise comparison of sequence divergence values for all introns and intergenic spacers revealed that the *ndhF-rpl32*, *psbM-trnD*, and *trnS-trnG* regions were the fastest-evolving regions [31].

The spathe is the main ornamental organ, and its color variation is mainly due to its anthocyanin content. The results obtained from this study indicate that the morphological traits are controlled by multiple genes. To further understand these results, it is necessary to investigate the anthocyanin biosynthesis pathway that leads to the spathe colors in *Anthurium*. Transcriptome profiling lays the foundation for elucidating the regulatory

mechanism of anthocyanin biosynthesis in *A. andraeanum* [6]. Inheritance studies of major spathe colors identified three dominant genes, O, R, and M, that control various color phenotypes in *Anthurium* [38]. The expression of the transcription factors AaMYB2 and R2R3-MYB was associated with anthocyanin biosynthesis in spathes [39,40], and the differential expression of AN2 and UFGT may result in anthocyanin-loss mutations [41]. F3'H expression may be a key control point associated with anthocyanin biosynthesis [42]. In addition, the chalcone isomerase (CHI) gene was associated with color differences [43]. MicroRNAs (miRNAs) play an important role in the coloration of plant leaves, flowers, and fruits and mediate the genetic regulation of *A. andraeanum* spathe coloration and the cold stress response [44–46]. Altogether, our comparative and phylogenetic analysis of *A. andraeanum* reciprocal natural hybridization based on molecular and morphological traits will serve as a foundation for future research and provide a reference for breeding new cultivars, improving existing cultivars, and managing the germplasm of new cultivars of *A. andraeanum*.

5. Conclusions

A. andraeanum hybrids reflected their parents' heterozygous features in their morphologies, nuclear genomes, and chloroplast genomes. Based on cluster analysis, the five *A. andraeanum* cultivars could be divided into two groups, and the ISSR analysis results were highly correlated with the spathe color. Among the five *A. andraeanum* cultivars, the composition and structure features of chloroplast genomes were completely the same or highly similar, respectively, and InDel special marker sites were inserted into the chloroplast genome during hybridization. Phylogenetic analysis based on complete chloroplast genome data showed that the genetic stability of the chloroplast is high in *A. andraeanum*, manifested as uniparental maternal inheritance; the chloroplast genome composition and structural features of hybrids are highly similar to those of the maternal parent; and the chloroplast genome has comparatively less effect on the spathe color. Taken together, the comprehensive analysis of the morphological, nuclear genomic, and chloroplast genomic data of five *A. andraeanum* cultivars offers valuable insight and information for understanding their phylogeny and the development of molecular markers for the cultivars.

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