Clematis Genetic Diversity and Hybrid Identification using ISSR Markers

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Clematis genetic diversity and hybrid identification using ISSR markers

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Abstract

Background
Clematis taxa are diverse, with high ornamental value. However, these plants have a complicated genetic background and a long growth period. Thus, molecular identifications are necessary to shorten the breeding cycle.

Results
Here, the genetic diversity of 17 parental taxa (five wild species and 12 Texas cultivars) were analyzed using inter simple sequence repeat (ISSR) markers. We obtained 108 alleles using 12 ISSR primers (an average of 9 alleles per primer). Genetic parameters, including the number of alleles (Na), the effective number of alleles (Ne), Nei's genetic diversity (H), and Shannon's diversity (I), suggested that these 17 taxa were highly diverse. Phylogenetic analysis recovered the 17 taxa in two large clades: one cluster included all of the cultivars, as well as *Clematis pinnata, C. brevicaudata,* and *C. tubulosa;* the second cluster included *C. fusca* and *C. reticulata.* The pairwise genetic distances between all cultivars were 0.421–2.368, suggesting that these cultivars may have derived from closely related species. We next performed five crosses between parental taxa and used ISSR markers to validate the authenticity of the 15 hybrid offspring. ISSR primers amplified bands specific to the male parents of each cross. Male parent-specific bands were identified in 11 of the 15 offspring; these 11 offspring were identified as true hybrids. The remaining progeny were considered self-hybrids due to the absence of male parent-specific bands.

Conclusion
Our results demonstrated that ISSR molecular markers may be useful tools for the verification of true *Clematis* hybrids. ISSR-based genetic diversity analyses, early hybrid identification, and marker assisted selection of *Clematis* taxa may improve breeding efficiency, excavate key genes associated with important traits, promote the development of new varieties, and shorten the breeding cycle.
Keywords: Clematis; Wild; Cultivar; ISSR molecular marker; Genetic diversity; hybrid identification

1. Background

There are more than 300 species of *Clematis* L. worldwide. *Clematis*, known as ‘Queen of Fujimoto’ in garden applications, have rich colors, multiple flower shapes, and a long flowering period [1]. Species in this genus are perennial woody or herbaceous vines that are popular ornamental garden plants [2]. *Clematis* have strong resistance to stress and cold, as well as high medicinal value [3]. China is a rich source of *Clematis* diversity, and many Chinese *Clematis* have other desirable traits [2].

A previous report showed that the offspring produced by the interspecific hybridization of *C. tubulosa* and *C. brevicaudata* were morphologically similar to *C. pinnata* [5]. However, the systematic classification of Wang Wencai and Li Liangqian [6], which was based on morphological and palynological characters, placed these three species in different subgroups.

Thus, the relationship among these three species is unclear. Molecular analysis may help to resolve this uncertainty. In addition, the phylogenetic position of *C. reticulata* within *Clematis* has yet to be investigated, although this species is morphologically similar to *C. fusca.* In addition, no molecular or morphological phylogeny of the *Clematis* of China is available. Thus, it is important to investigate the molecular diversity and taxonomic status of the wild Chinese *Clematis* to help to resolve the phylogenetic relationships within this genus.

Over the last several hundred years of *Clematis* breeding and cultivation by horticulturists and botanists, thousands of horticultural varieties of *Clematis*, each with their own distinct characteristics, have been developed [7]. Most of these varieties are composed of multiple different species; some species have been developed through hybridization, while others are domesticated wild species [4]. Due to long-term artificial selection, the genetic backgrounds of *Clematis* varieties, the genetic relationships among varieties, and the relationships between wild species and domestic varieties have become blurred. For some hybrid varieties, it is no longer possible to identify or locate the original hybrid parents. Thus, parental selection for hybrid breeding is often difficult. At the same time, many *Clematis* varieties resemble wild species, with highly similar morphological characters. Thus, the accurate identification of *Clematis* taxa has become increasingly challenging. Finally, *Clematis* phenotypes are strongly affected by environmental factors [8], rendering traditional morphological classification methods ineffective for species identification. Therefore, it is important to develop molecular markers for *Clematis*, both to study genetic diversity and phylogenetic relationships in this confusing genus, and to provide a framework for the classification and identification of *Clematis* varieties at the molecular level.

Hybrid breeding remains the most effective method for the selection and breeding of new *Clematis* [5]. Additionally, hybridization and recombination can be used to improve multiple target traits in *Clematis* [9]. However, the high genetic heterozygosity and long generation cycles of *Clematis* taxa compared with other perennial flowers means that it is difficult to obtain a genetic hybrid population and complete a complete growth cycle [10]. This difficulty hinders *Clematis* genetic map construction, the quantitative trait locus (QTL) mapping of important traits, and map-based cloning [11]. Therefore, it is necessary to verify that seedlings have correctly hybridized at an early stage to ensure the smooth development of breeding programs and the maintenance of stable trait inheritance, as well as to optimize planting time and minimize costs [12].

DNA molecular markers are currently widely used in genetic studies, and phylogenetic analyses based on molecular markers are more objective than traditional morphological comparisons [13,14]. DNA markers can also be used for hybrid identification and seed verification; compared with other methods, DNA marker analyses save both time and labor [15]. Inter Simple Sequence Repeat (ISSR) markers use anchored microsatellite DNA as the primer: 2–4 random nucleotides are added to the 3'- or 5'-end of the SSR sequence. In the PCR reaction, the anchored primer causes annealing at a specific site, leading to PCR amplification of DNA fragments between repetitive sequences with complementary anchor primers. ISSR markers generate more phylogenetically relevant polymorphic fragments than other types of molecular markers, including Simple Sequence Repeats (SSR), Sequence Related Amplified Polymorphisms (SRAP), and Random Amplified Polymorphic DNA (RAPD) [16]. In addition, the genetic data provided by ISSR markers are more consistent

with morphological variability and the evolutionary history of the morphotypes [17]. ISSR markers are also simple to use, have good reproducibility, and can distinguish among similar genotypes [18]. Therefore, ISSR markers are ideal for genotype identification, map construction, gene tagging, and genomic and cDNA fingerprinting [19].

Clematis ISSR markers have been widely used for studies of genetic diversity and evolutionarily relationships [20]. For example, Yu Weijun [21] investigated genetic diversity among wild *Clematis*, horticultural varieties, and local populations using ISSR markers, showing that ISSR molecular markers could be used to identify, and differentiate between, horticultural *Clematis* varieties and wild species. ISSR markers were also used to show that the main parental populations of *Clematis* in China, as well as their derived offspring, had high levels of genetic diversity, and that most of the genetic variation was among varieties [22].

However, due to the long growth cycles of some *Clematis*, there are relatively few studies of hybrid populations, especially artificial hybrid populations. Studies demonstrating the molecular identification of *Clematis* hybrid progeny are also lacking.

To address this knowledge gap, we designated 17 *Clematis* taxa (five wild species and 12 Texas cultivars) as parents and conducted artificial crosses to obtain five hybrid combinations and 15 F1 hybrids. We then used ISSR markers to verify the true *Clematis* hybrids, and to analyze the molecular relationships among the wild species and the cultivars. The specific objectives of this study were: (1) to assess the feasibility of ISSRs for *Clematis* research, (2) to use ISSR markers to quantify genetic diversity among the 17 clematis (5 wild species and 12 cultivars) (3) to use ISSR markers to visualize phylogenetic relationships among species to provide a framework for subsequent classification and analysis of *Clematis* species and varieties, and (4) to use ISSR markers to identify true *Clematis* hybrids. Our results may provide a framework for the development of new, high-quality, highly ornamental *Clematis* varieties via uniparental inheritance. In addition, the use of molecular markers to clarify the genetic relationships among *Clematis* taxa is of great importance for the cultivation of new varieties.

2. Results

2.1. Genetic diversity of the parental taxa

2.1.1. ISSR polymorphism

Out of the 100 primers screened against the 17 parental *Clematis* taxa, 12 primers amplified 108 fragments (100–2000 bp) that produced bright, clear, reproducible bands without smearing: U824, U836, U844, U845, U841, U866, U815, U843, U834, U835, U840, and U899 (Table 1). Each of these 12 primers amplified 6–14 loci (an average of 9); all the amplified loci were polymorphic (Table 4). Primer U836 (Fig. 1) amplified the most loci (14 bands), while primers U844 and U866 amplified the fewest loci (6 per primer). The genetic diversity of 17 parental *Clematis* taxa was relatively high: the number of alleles (Na) was 2.00, the number of effective alleles (Ne) was 1.2808, Nei's gene diversity (H) was 0.1988, and Shannon's diversity information index (I) was 0.3373. Thus, this gene pool was relatively rich, with a broad genetic basis.

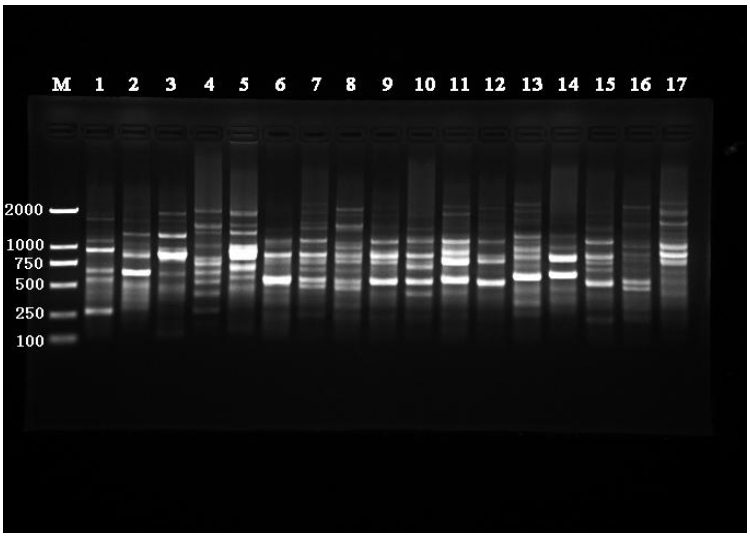

Fig. 1. Electrophoresis gel showing the bands amplified by the ISSR primer U844 from the 17 parental *Clematis* taxa.
Note: Lanes 1–17 are *Clematis fusca*, *C. reticulata*, *C. pinnata*, *C. tubulosa*, *C. brevicaudata*, 'Kaleidoscope,' 'Red Fatty,' 'Rouge Button,' 'Nazawa,' 'Bode,' 'Ultramarine,' 'Peach Cut,' 'Anisa,' 'Sophia,' 'Kawako,' 'Red Echo,' and 'Pascal,' respectively.

Table 1. ISSR primers selected to genotype the 17 parental *Clematis* taxa, showing the number of loci and polymorphic loci amplified by each primer.

Primer Name	Primer Sequence	Annealing temperature (°C)	Loci amplified	Polymorphic loci amplified	Percentage polymorphic loci
U815	CTCTCTCTCTCTCTCTG	54.0	9	9	100
U824	TCTCTCTCTCTCTCTCG	55.4	9	9	100
U834	AGAGAGAGAGAGAGAGYT	57.5	7	7	100
U835	AGAGAGAGAGAGAGAGYC	50.4	8	8	100
U836	AGAGAGAGAGAGAGAGYA	55.4	14	14	100
U840	GAGAGAGAGAGAGAGAYT	55.4	8	8	100
U841	GAGAGAGAGAGAGA	55.4	11	11	100

U843	GAYC CTCTCTCTCTCTCTCT RA	54.0	8	8	100
U844	CTCTCTCTCTCTCTCT RC	55.4	6	6	100
U845	CTCTCTCTCTCTCTCT RG	55.4	13	13	100
U866	CTCCTCCTCCTCCTCC TC	55.4	6	6	100
U899	CATGGTGTTGGTCAT TGTTCCA	51.8	9	9	100

2.1.2. Phylogenetic relationships and genetic distance among the parental Clematis

UPGMA analysis of the 17 parental *Clematis* taxa recovered these taxa in two large clades, herein designated "Branch I" and "Branch II" (Fig. 2). Branch I contained two subgroups. One subgroup included three distinct species (*C. pinnata, C. brevicaudata,* and *C. tubulosa*), with a clade of *C. pinnata* plus *C. brevicaudata* sister to *C. tubulosa*. The second subgroup contained all 12 of the Texas cultivars ('Kaleidoscope,' 'Nazawa,' 'Bode,' 'Kawako,' 'Rouge Button,' 'Red Echo,' 'Red Fatty,' 'Ultramarine,' 'Peach Cut,' 'Anisa,' 'Sophia,' and 'Pascal'). This monophyletic grouping suggested that all the cultivars might derive from closely related species, although the position of cultivar 'Pascal' sister to all other cultivars might indicate that 'Pascal' derives from a more genetically distant species. Branch II contained species with low genetic identify, *C. fusca* and *C. reticulata.*

Pairwise genetic distances between the 17 *Clematis* taxa ranged from 0.421 to 2.368 (average, 1.024), indicating a relatively large degree of genetic distance across all 17 taxa. The lowest genetic distances were recovered between 'Kaleidoscope' and 'Nazawa' (0.421) and between 'Rouge Button' and 'Red Echo' (0.472), while the largest genetic distance was observed between *C. pinnata* and 'Kawako' (2.368). As expected, based on the dendrogram (Table. 2), the pairwise genetic distances between the morphological species (0.818–2.368; average, 1.327) were generally greater than the pairwise genetic distances between the cultivars (0.421–1.408; average, 0.845; Table 5).

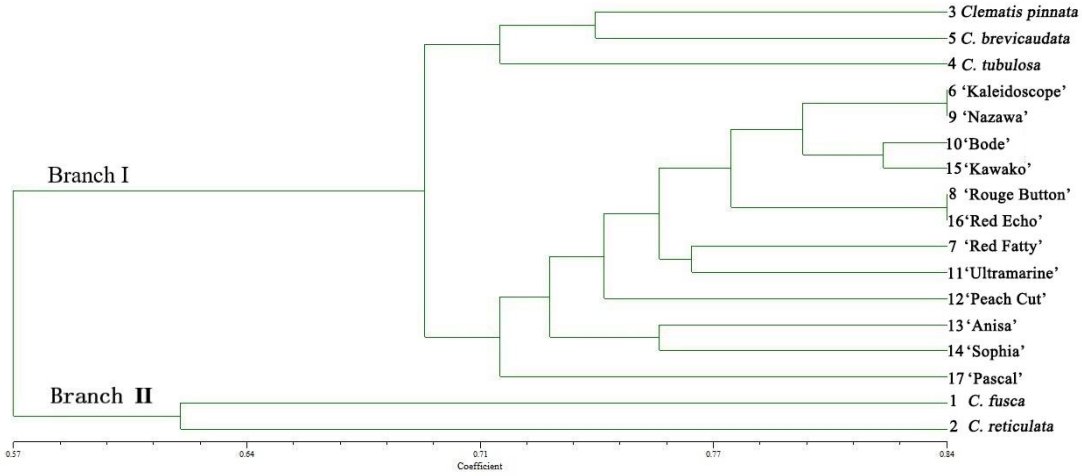

Fig. 2 UPGMA dendrogram generated based on Nei's genetic distances among the 17 parental *Clematis* taxa, scaled by the genetic identify coefficient.
Note: The 17 clematis samples are grouped into two large branches. Branch I includes 15 *Clematis* (ID numbers 3–17) and Branch II includes 2 *Clematis* (ID numbers 1 and 2). The ID numbers correspond to those defined in Table 1.

Table 2. Nei's original measures of pairwise genetic distances among the 17 parental *Clematis* taxa. Integers in the header and first column correspond to the ID numbers given in Table 1.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	0.000																
2	0.875	0.000															
3	1.431	1.682	0.000														
4	1.595	1.030	1.136	0.000													
5	1.479	1.387	0.731	1.250	0.000												
6	0.818	0.860	1.734	1.270	1.020	0.000											
7	1.167	1.209	2.325	1.285	1.323	0.629	0.000										
8	1.251	1.159	1.715	1.145	1.406	0.792	0.977	0.000									
9	0.715	1.209	2.325	1.167	1.205	**0.421**	0.894	0.536	0.000								
10	0.938	0.980	1.941	1.307	1.227	0.577	0.628	0.799	0.548	0.000							
11	0.875	1.273	1.290	1.231	1.387	0.692	0.757	0.936	0.590	0.875	0.000						
12	1.037	1.196	2.040	1.182	1.577	0.749	0.909	0.730	0.647	0.931	0.637	0.000					
13	0.986	1.161	1.361	1.707	1.052	0.949	1.252	1.048	0.846	0.986	1.161	0.861	0.000				
14	1.211	1.119	1.452	1.211	1.048	0.934	1.189	1.021	1.189	1.077	1.001	1.176	0.890	0.000			
15	1.211	1.253	**2.368**	1.328	1.143	0.752	0.832	0.579	0.496	0.518	0.801	0.770	1.008	0.876	0.000		
16	1.144	1.340	2.301	1.144	1.433	0.772	0.988	0.443	0.670	1.011	0.829	0.791	0.823	0.914	0.472	0.000	
17	1.136	1.466	1.511	1.521	1.202	0.993	1.402	0.638	0.891	1.270	0.692	1.117	1.066	0.839	0.839	0.868	0.000

Note: The specific ID numbers (1–17) correspond to those defined in Table 1

2.2. ISSR-based hybrid verification

2.2.1. ISSR analysis

For each hybrid combination (X1–X5; Table 3), we assessed whether the 12 primers selected in section 3.1.1 could be used to determine hybrid purity. For parent combination X1 ('Bode' ♀ × *C. fusca* ♂), 11 of the 12 primers (91.67%; all primers except U824) generated a total of 25 male parent-specific markers; for parent combination X2 (*C. pinnata* ♀ × *C. tubulosa* ♂), all 12 primers (100.0%) generated 56 male parent-specific markers; for parent combination X3 ('Bode' ♀ × *C. reticulata* ♂), 10 primers (83.33%; all primers except U866 and U843) generated 28 male parent-special markers; for parent combination X4 (*C. tubulosa* ♀ × *C. brevicaudata* ♂), all 12 primers (100%) generated a total of 28 male parent-specific markers; and for parent combination X5 ('Anisa' ♀ × *C. reticulata* ♂), 10 primers (83.33%; all primers except U824 and U841) generated a total of 26 male parent-specific markers (Table 3). The following primers amplified male-specific markers in all combinations: U836, U844, U845, U815, U834, U835, U840, U899. Notably, different primers reliably amplified

specific bands for different hybridization combinations (Fig. 3).

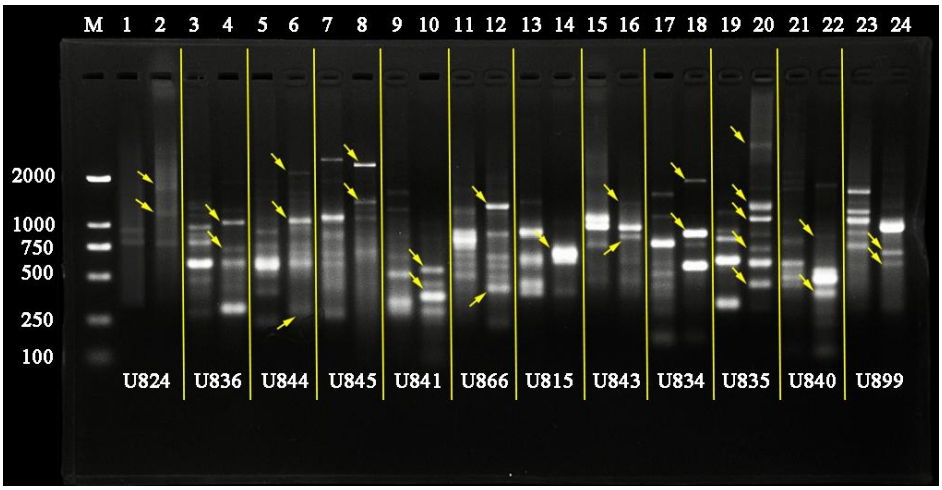

Fig. 3 Male-female polymorphisms between the parent taxa in combination X1 ('Bode' ♀ × *C. fusca* ♂) amplified using ISSR primers.

Note: Arrowheads indicate the polymorphic bands in the male parent (even-numbered lanes are the male parent, odd-numbered lanes are the female parent). Lane M: DL 2000 DNA Ladder; Lanes 1–2: primer U824; Lanes 3–4: primer U836; Lanes 5–6: U844; Lanes 7–8: U845; Lanes 9–10: U841; Lanes 11–12: U866; Lanes 13–14: U815; Lanes 15–16: U843; Lanes 17–18: U834; Lanes 19–20: U835; Lanes 21–22: U840; Lanes 23–24: U899.

Table 3. ISSR primers amplifying male parent-specific markers for each of the parental *Clematis* combinations. Details of the hybrid combinations are given in Table 2.

Parent combination ID	Number of primer combinations amplifying male parent-specific markers	Primers amplifying male parent-specific markers	Total male parent-specific markers	Percentage of primer combinations with male parent-specific markers (%)
X1	11	U824, U836, U844, U845, U841, U866, U815, U843, U834, U835, U840, U899	27	100.0
X2	12	U824, U836, U844, U845, U841, U866, U815, U843, U834, U835, U840, U899	56	100.0
X3	10	U824, U836, U844, U845, U841, U815,	28	83.33

		U834, U835, U840, U899		
X4	12	U824, U836, U844, U845, U841, U866, U815, U843, U834, U835, U840, U899	28	100.0
X5	10	U836, U844, U845, U866, U815, U843, U834, U835, U840, U899	26	83.33

2.2.2. Hybrid identification

For each combination, only the primers that successfully amplified male parent-specific bands were used to verify hybrid purity. Nine primers (U824, U844, U845, U841, U815, U834, U835, U840, and U899) verified that both of the offspring of combination X1 (‘Bode’ ♀ × *C. fusca* ♂), A-75 and A-83, were true hybrids. Similarly, all 12 primers verified that six of the eight offspring of combination X2 (*C. pinnata* ♀ × *C. tubulosa* ♂) were true hybrids due to the presence of 1–3 male parent-specific bands in these progeny: D-105, D-101, E-53, E-51, E-25, and E-31. None of the ISSR primers amplified male parent-specific bands in offspring E-34 and E-52, indicating that these progeny were not true hybrids. Seven primers (U836, U844, U845, U841, U834, U835, and U899) amplified male parent-specific bands in the single offspring of combination X3 (‘Bode’ ♀ × *C. reticulata* ♂), D-81. Two primers (U845 and U841) verified that one of the three progeny of combination X4 (*C. tubulosa* ♀ × *C. brevicaudata* ♂), E-73, was a true hybrid. The other two offspring of this cross, E-64 and E-62, were not true hybrids. Finally, male parent-specific bands were amplified in the single offspring of combination X5 (‘Anisa’ ♀ × *C. reticulata* ♂), E-95, by six primers (U836, U845, U866, U834, U840, and U899).

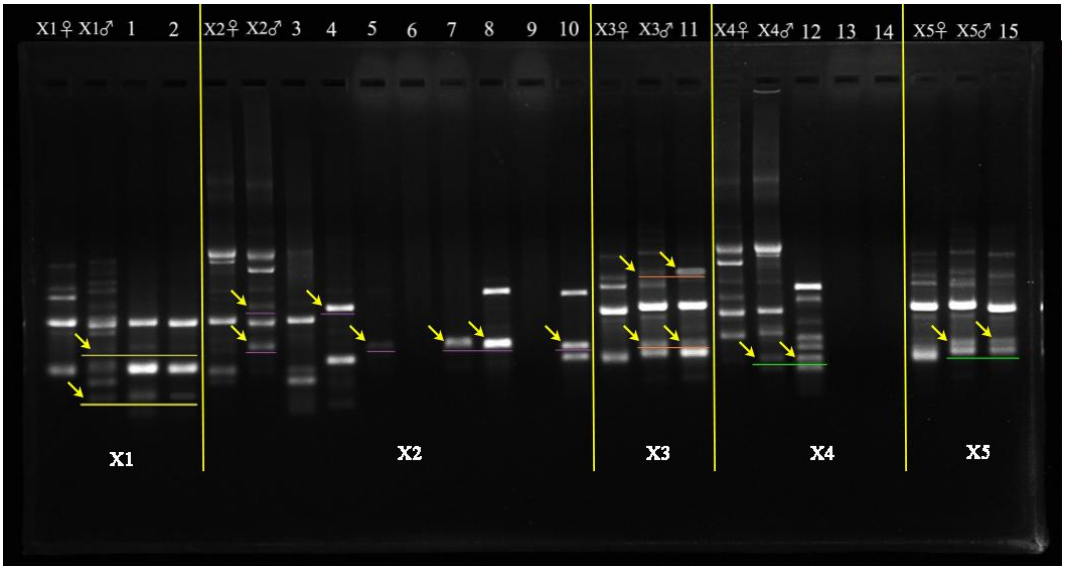

Fig. 4 Bands amplified by the ISSR primer U835 (Table 3) in parent plants and their offspring for all the crosses included in this study (X1–X5; Table 2).
Note:Each parent combination + offspring is grouped in the gel image using vertical lines. In each group, the first two lanes are the female and male parents (as indicated by the symbols ♀ and ♂), and the remaining lanes correspond to the offspring. Combination X1, Lane 1–2: offspring A-83 and A-75, respectively; Combination X2, Lanes 3–10: offspring D-105, D-101, E-53, D-34, D-51, D-25, D-52, and D-31, respectively; Combination X3, Lane 11: offspring D-81; Combination X4, Lanes 12–14: offspring E-73, E-64, and E-62, respectively; Combination X5, Lane 15: offspring E-95. The target bands (i.e., the male parent-specific markers) are underlined with different colors corresponding to the different hybrid combinations.

Table 4. Hybrid identification results

Parent combination ID	Numbers of the hybrids	Primers used	True hybrids	Total number of true hybrids
X1	2	U824	A-75	2
		U844	A-83,	
		U845	A-83, A-75	
		U841	A-83, A-75	
		U815	A-83, A-75	
		U834	A-75	
		U835	A-83, A-75	
		U840	A-75	
		U899	A-83, A-75	
X2	8	U824	D-105, D-101, E-53, E-25	6
		U836	E-25, E-31,	
		U844	D-105, E-25	
		U845	D-105, D-101, E-25	
		U841	D-105, D-101, E-53, E-25, E-31	
		U866	D-105	
		U815	D-105	
		U843	D-105	
		U834	D-105, D-101	
		U835	D-101, E-53, E-51, E-25, E-31	
		U840	D-105, D-101, E-31, E-53	

		U899	D-105	
X3	1	U836	D-81	1
		U844	D-81	
		U845	D-81	
		U841	D-81	
		U834	D-81	
		U835	D-81	
		U899	D-81	
X4	3	U845	E-73	1
		U841	E-73	
X5	1	U836,	E-95	1
		U845	E-95	
		U866	E-95	
		U834	E-95	
		U840	E-95	
		U899	E-95	

3. Discussion

3.1 Phylogenetic positions of the 17 parental Clematis taxa

We successfully used ISSR molecular markers to genotype five wild *Clematis* and 12 *Clematis* cultivars, suggesting that ISSR markers are suitable for genetic clustering and distance analyses in this genus. The five wild clematis were recovered in two distinct clusters: *C. pinnata, C. tubulosa*, and *C. brevicaudata* formed a clade, corresponding to Sect. *Clematis*. *C. fusca* and *C. reticulata,* both of which fall into the *Clematis* subgenus *Urophylla*, also formed a clade. These placements were thus consistent with traditional systematic taxonomy [6]. In combination, molecular and morphological characters may more accurately reflect phylogenetic relationships among taxa.

The species *C. pinnata* belongs to the Subsect. *Pinnatae* of the Sect. *Tubulosae*. C. pinnata is a woody vine with ternately compound leaves or one to two pinnately compound leaves; the sepals of this plant extend obliquely, with tubular blue-white flowers. *C. brevicaudata,* which is also a woody vine, belongs to the Subsect. *Vitalbae* of the Sect. *Clematis* [6]. *C. brevicaudata* has pinnate or twice-pinnate compound leaves, with extended sepals and round white flowers. Both *C. pinnata* and *C. brevicaudata* fall into the Subgen. *Clematis* [6]. In contrast, the status of *C. pinnate* is unclear as this plant is morphologically ambiguous. Xie et al [28] pointed out that “erect vs. spreading sepals” is not a stable character in *C. pinnata*, because in this plant the sepals are first erect but later become spreading or bent backwards. Thus, this character cannot be used to distinguish *C. pinnata*. Although *C. pinnata* is morphologically unlike *C. brevicaudata,* a recent revision placed *C. pinnata* in the Sect. *Tubulosae* [29], which was consistent with our results, as well as those of a previous molecular phylogenetic study [30]. In addition, *C. crotula,* which, like *C. pinnata,* is a semi-shrub with blue-purple sepals and tube-shaped flowers, also falls into the European

Clematis subgenus and has also been placed in the Sect. *Tubulosae*. This result was consistent with the conclusion of Shi Jinghua [31] that *C. pinnata* is the product of interspecific hybridization.

C. fusca and *C. reticulata* are woody vines [32]. C. *fusca,* which falls into the Sect. *Viorna*, has pinnately compound leaves, sepals covered with brown hairs, and lilac, bell-shaped flowers. *C. reticulata* has yet to be systematically classified. This species, which has pinnately compound leaves and white-purple bell-shaped flowers, is morphologically very similar to *C. fusca*. This suggests that *C. reticulata* may fall into in Sect. *Viorna* with *C. fusca*. Thus, our results were consistent with systematic taxonomy based on morphological characters.

All 12 of *Clematis* cultivars included in this study belong to the Texas group, with morphologically similar bell-shaped flowers. Consistent with the morphological characters, our molecular results suggest that these cultivars are closely related. Here, 12 *Clematis* from the Texas group clustered with the three wild *Clematis* from Sect. *Clematis*, indicating a close genetic relationship and hinting that the hybrid parents may originate from Sect. *Clematis*, providing a theoretical basis for the cultivation and breeding of new varieties.

3.2 Identification of true Clematis *hybrids*

When breeding hybrid *Clematis,* it is very important to verify hybrid authenticity early in the growth process, as *Clematis* grow slowly and are difficult to keep alive. Early identification of true hybrids helps to prevent wasted time and effort. In addition, it is critical to accurately predict whether offspring are more likely to favor the male or female parent in order to determine whether or not to continue a given breeding program. Traditionally, hybrids in a variety of plant groups have been identified based on phenotype [33]. However, phenotyping is time-consuming and requires extensive growing areas. In addition, morphological characteristics are easily affected by external environmental factors, compromising phenotype-based identifications [34]. Thus, there is a need for hybrid-verification methods that are more sensitive, less affected by external factors, and applicable earlier in the growth process.

To address this need, several studies have investigated DNA-based molecular markers in a variety of plants [35,36,37]. However, studies using ISSR markers to authenticate true *Clematis* hybrids are rare. Here, we used ISSR molecular markers to authenticate 15 offspring of five *Clematis* crosses; 11 of the 15 offspring (73.33%) exhibited male parent-specific bands and were thus identified as true hybrids. Our results demonstrated that ISSR molecular markers can be used to rapidly and simply verify true *Clematis* hybrids, which can then be used for map construction and further cross-breeding programs.

We found that the ISSR primer U845 successfully identified true hybrids in every combination we tested. Thus, U845 might represent a potential universal primer for the identification of true *Clematis* hybrids.

3.3 Conclusions

This study is the first to use ISSR molecular data to authenticate true *Clematis* hybrids. Our results showed that ISSR markers are a powerful and efficient approach to hybrid identification in this genus. The simple, low-cost molecular method of true *Clematis* hybrid verification demonstrated herein might be applicable to *Clematis* breeding programs

worldwide, while the specific ISSR primers developed for this study might be useful for the identification, registration, and protection of *Clematis* taxa. Finally, by classifying the 17 *Clematis* taxa included in this study and resolving the genetic relationships among the wild *Clematis* species, between the wild species and the cultivars, and among the cultivars, our results help to clarify the phylogenetic positions of *Clematis* taxa within the genus and provide reliable background data for future hybrid breeding programs. Genetic diversity analysis, early hybrid identification, and marker-assisted selection in *Clematis* using ISSR markers may help to improve breeding efficiency, excavate key genes associated with important traits, perform gene cloning and transgenesis, breed new varieties, and shorten the breeding cycle.

4. Materials and methods

4.1. Plant materials and treatments

A total of seventeen *Clematis* accessions (five wild and 12 cultivars) and their offspring were used in this study (Tables 1 and 2). The five wild *Clematis* had different geographical origins, and were genetically and morphological diverse [20]. The 12 cultivars used were all bell-shaped varieties in the Texas group.

In our preliminary work, we performed many forward and backward crosses among 5 wild *Clematis* species and 12 horticultural varieties. However, the long breeding cycle of this genus and the low survival rate of the hybrids presented severe challenges. Finally, 5 hybrid combinations were successfully obtained. Due to their slow growth rates, the 15 hybrid progeny required 6 months of cultivation before they could be accurately identified. Single genotypes from each parental accession were hand pollinated between June and August 2020 to generate the F1 hybrids. A total of 15 F1 individuals were derived from five cross (Table 2). F1 seeds were harvested from the female parents in October 2020. The F1 individuals were grown in a greenhouse at approximately 22°C under a 16 h photoperiod for 12 weeks and then transplanted into an experimental field at the Agricultural University, Baoding, Hebei, China (115.49058, 38.817921).

Table 5. The 17 *Clematis* taxa included in this study.

ID number	Name	Status	ID number	Name	Status
1	*Clematis fusca*	Wild	10	‘Bode’	Cultivar
2	*C. reticulata*	Wild	11	‘Ultramarine’	Cultivar
3	*C. pinnata*	Wild	12	‘Peach Cut’	Cultivar
4	*C. tubulosa*	Wild	13	‘Anisa’	Cultivar
5	*C. brevicaudata*	Wild	14	‘Sophia’	Cultivar
6	‘Kaleidoscope’	Cultivar	15	‘Kawako’	Cultivar
7	‘Red Fatty’	Cultivar	16	‘Red Echo’	Cultivar
8	‘Rouge Button’	Cultivar	17	‘Pascal’	Cultivar
9	‘Nazawa’	Cultivar			

Table 6. Parental *Clematis* combinations that successfully generated progeny.

Hybrid combination ID	Female parent ID	Male parent ID	Offspring IDs	offspring
X1	10	1	A-83, A-75	2
X2	3	4	D-105, D-101, E-53, D-34, D-51, D-25, D-52, D-31	8
X3	10	2	D-81	1
X4	4	5	E-73, E-64, E-62	3
X5	13	2	E-95	1

Note: Parental IDs correspond to the plants listed in Table 1. Offspring IDs shown in italics were *not* true hybrids (as determined using ISSR primers)

4.2. DNA extraction

DNA was extracted from the 17 *Clematis* parental taxa and young leaves of the 15 6-month-old F1 offspring using plant genomic DNA extraction kits (CW0531S, Kangwei Century, Beijing China), following the manufacturer's instructions. The quality and quantity of the genomic DNA were estimated by measuring the A260/A280 ratio using a UV spectrophotometer and by performing gel electrophoresis [23]. DNA concentrations were adjusted to 40 ng/μL to facilitate polymerase chain reaction (PCR) amplification. DNA samples were stored at −20°C until use.

4.3. Primer selection and PCR amplification

After preliminary screening and re-screening of 100 pairs of ISSR primers (previously developed by our research group; [20]), effective polymorphic ISSR primers were selected and used to genotype the 17 parental taxa and the 15 F1 offspring. We tested which of the selected ISSR primers amplified characteristic paternal bands. We then used the identified primers to amplify the DNA of the 15 F1 plants; plants with characteristic paternal ISSR bands were identified as true hybrids [24].

All primers were synthesized by Invitrogen (Shanghai, China). Each 25-μL PCR volume contained 2 μL DNA template (40–50 ng • μL^{-1}), 12.5 μL Master Mix (Invitrogen, Shanghai, China), 2 μL each primer (6 μmol • L^{-1}; Invitrogen, Shanghai, China), and 8.5 μL ddH_2O. The reactions ran in a 96-well thermal cycler (Applied Biosystems Inc., Foster City, CA, USA), with the following cycling conditions: 94°C for 5 min; 36 cycles of 94°C for 50 s,50.4–57.5°C for 1 min (Table 3), and 72°C for 1 min; and a final elongation step at 72°C for 10 min. The PCR products were separated on an 1.5% agarose gel using electrophoresis at 120 V for 45 min.

Table 7. Primers used in Inter Simple Sequence Repeat (ISSR) analysis.

Primer Name	Primer Sequence	Annealing temperature/°C
U815	CTCTCTCTCTCTCTCTG	54.0
U824	TCTCTCTCTCTCTCTCG	55.4
U834	AGAGAGAGAGAGAGAGYT	57.5

U835	AGAGAGAGAGAGAGAGYC	50.4
U836	AGAGAGAGAGAGAGAGYA	55.4
U840	GAGAGAGAGAGAGAGAYT	55.4
U841	GAGAGAGAGAGAGAGAYC	55.4
U843	CTCTCTCTCTCTCTCTRA	54.0
U844	CTCTCTCTCTCTCTCTRC	55.4
U845	CTCTCTCTCTCTCTCTRG	55.4
U866	CTC CTC CTC CTC CTC CTC	55.4
U899	CATGGTGTTGGTCATTGTTCCA	51.8

4.4. Data analysis

Several genetic diversity metrics, as well as genetic distance, were calculated for the 17 clematis using Popgene 1.32 and NTSYS 2.10 [25]. including the observed number of alleles (Na), effective number of alleles (Ne), Shannon's information index (I), and expected heterozygosity (He). Unweighted pair-group method with arithmetic means (UPGMA) cluster analysis of the 17 clematis was performed based on Nei's genetic distance data using NTSYS 2.10. We determined and analyzed the molecular weight of each amplified DNA fragment based on the gel images [26].

5. Abbreviations

ISSR: Inter simple sequence repeat
Na: The number of alleles
Ne: The effective number of alleles
H: Nei's genetic diversity
I: Shannon's diversity
QTL: Quantitative trait locus
SSR: Simple Sequence Repeats
SRAP: Sequence Related Amplified Polymorphisms
RAPD: Random Amplified Polymorphic DNA

6. Declarations

6.1. Ethics Approval and Consent to participate

Consent

6.2. Consent for publication

Consent

6.3. Availability of supporting data

Not applicable

6.4. Competing interests

Not applicable

6.5. Funding

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6.6. Authors' contributions

WANG Xin Complete all experiments, perform data analysis and wrote the manuscript;

LI Mingyang helped perform the analysis with constructive discussions;

TIAN Lin performed the experiment;

LIU Dongyun contributed to the conception of the study.

6.7. Acknowledgements

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