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Development of expressed sequence tag-simple sequence repeat markers for *Chrysanthemum morifolium* and closely related species

H. Liu, Q.X. Zhang, M. Sun, H.T. Pan and Z.X. Kong

Beijing Key Laboratory of Ornamental Plants Germplasm Innovation and Molecular Breeding, National Engineering Research Center for Floriculture, Beijing Laboratory of Urban and Rural Ecological Enviroment, College of Landscape Architecture, Beijing Forestry University, Beijing, China

Corresponding author: Q.X. Zhang
E-mail: zqxbjfu@126.com

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ABSTRACT. With the development of chrysanthemum breeding in recent years, an increasing number of wild species in genera related to *Chrysanthemum* were introduced to extend the genetic resources and facilitate the genetic improvement of chrysanthemums via hybridization. However, few simple sequence repeat (SSR) markers are available for marker-assisted breeding and population genetic studies of chrysanthemum and closely related species. Expressed sequence tags (ESTs) in public databases and cross-species transferable markers are considered to be a cost-effective means for developing sequence-based markers. In this study, 25 EST-SSRs were successfully developed from *Chrysanthemum* EST sequences for *Chrysanthemum morifolium* and closely related species. In total, 4164 unigene sequences were assembled from 7180 ESTs of chrysanthemum in GenBank, which were subsequently used to screen for the presence of microsatellites with the

SSRIT software. The screening criteria were 8, 5, 4, and 3 repeating units for di-, tri-, tetra-, and penta- and higher-order nucleotides, respectively. Moreover, 310 SSR loci from 296 sequences were identified, and 198 primer pairs for SSR amplification were designed with the Primer Premier 5.0 software, of which 25 SSR loci showed polymorphic amplification in 52 species and varieties belonging to *Chrysanthemum*, *Ajania*, and *Opisthopappus*. The application of EST-SSR markers to the identification of intergeneric hybrids between *Chrysanthemum* and *Ajania* was demonstrated. Therefore, EST-SSRs can be developed for species that lack gene sequences or ESTs by utilizing ESTs of closely related species.

Key words: Expressed sequence tag-simple sequence repeat; *Chrysanthemum morifolium*; Hybrid identification

INTRODUCTION

Chrysanthemum (*Chrysanthemum morifolium*) is an economically important ornamental species that comprises a large proportion of the flower industry in many Southeast Asian and European countries. The demand for different chrysanthemums, including cut-flower, garden, potted, and ground-cover types, increases each year (Zhang et al., 2011). Many new varieties are bred each year, including cut chrysanthemum, pot chrysanthemum, and ground-cover chrysanthemum. The breeding of new germplasms depends mainly on conventional crossings in the genus *Chrysanthemum*. *Chrysanthemum* species and commercial cultivars, ground cover chrysanthemums, which were bred from species *C. vestitum*, *C. indicum*, *C. zawadskii*, *C. lavandulifolium*, *C. nankingense*, and *C. chanetii* and cultivars, compose the primary gene pool of chrysanthemum (Fukai, 2003).

With the development of chrysanthemum breeding in recent years, an increasing number of wild species in genera related to *Chrysanthemum* were introduced to extend the genetic resources and facilitate the genetic improvement of chrysanthemums via hybridization. Many wild species in *Chrysanthemum* and closely related genera can be successfully crossed with modern chrysanthemums, which represent important genetic resources for introducing new characteristics to florists' chrysanthemums (Zhao et al., 2009). Reciprocal crosses between chrysanthemum and 10 wild chrysanthemum species (*C. articum* ssp. *maekawanum*, *C. lavandulifolium*, *C. crassum*, *C. indicum*, *C. japonicum*, *C. ornatum*, *C. occidental-japonense* var. ashizuriense, *C. yoshinaganthum*, *C. chanetii*, *and C. pacificum* [*Ajania pacifica*]) were possible when the appropriate parental genotypes were carefully chosen (Fukai et al., 2000). *Ajania* and *Chrysanthemum* show some cross-compatibility (Fukai, 2003), and a number of *Chrysanthemum* and *Ajania* hybrids have been successfully obtained (Abd El-Twab et al., 1999). Breeding programs for cut flowers have been reported using *Ajania* species, such as *A. pacifica* (Shibata et al., 1988) and *A. shiwogiku* (Douzono and Ikeda, 1998). Five intergeneric hybrids between the chrysanthemum cultivar Zhongshanjingui (as female) and *A. przewalskii* (as male) were obtained with the help of embryo culture (Deng et al., 2011). The number of natural and artificial intergeneric hybrids between *Chrysanthemum* and *Ajania* is so vast that Ohashi and Yonekura (2004) proposed that *Ajania*, *Arctanthemum*, and *Phaeostigma* should be combined into the genus

of *Chrysanthemum* (Zhao et al., 2009).

In addition, intergeneric hybrids have been reported between *Chrysanthemum* and related species from the same subtribe Artemisiinae (*Crossostephium chinense*) (Kondo et al., 2003). Twenty-three hybrid seedlings have been chosen from the distant hybridization of Taihang chrysanthemum, *Chrysanthemum*, and *Ajania* (Hu and Zhao, 2008). Therefore, the species in genera related to *Chrysanthemum*, such as *Ajania* and *Opisthopappus*, are a secondary gene pool for chrysanthemum breeding, and the closely related genera might be increasingly exploited to genetically improve chrysanthemums via hybridization.

Molecular markers have become an indispensable tool for breeding and cultivar development in many crops (Varshney et al., 2005). Markers are frequently required to correctly identify cultivars, accurately assess genetic relationships and diversity, efficiently tag and map desirable genes, and select superior genotypes early (Gong and Deng, 2010). Among the molecular markers, simple sequence repeats (SSRs) have several advantages, such as their relative abundance and random distribution in the genome, their high rates of polymorphism (multiallelic nature), and their Mendelian codominant inheritance (Morgante and Olivieri, 1993). The development of SSRs via the *de novo* sequencing method is a costly and time-consuming endeavor (Zane et al., 2002). One possible solution to these types of problems would be to exploit publicly available genomic resources for the development of gene-based SSR markers that are more likely to be transferable across taxonomic boundaries (Ellis and Burke, 2007). The rapid and inexpensive development of SSRs from expressed sequence tag (EST) databases has been shown to be a feasible option for obtaining high-quality nuclear markers (Gupta et al., 2003; Bhat et al., 2005). In general, EST-SSRs have been found to be significantly more transferable across taxonomic boundaries than traditional "anonymous" SSRs (Chagné et al., 2004; Gutierrez et al., 2005; Pashley et al., 2006). This transferability is particularly true in plants, where transferability among economically important crop taxa has been demonstrated on a number of occasions (Decroocq et al., 2003; Thiel et al., 2003; Bandopadhyay et al., 2004; Saha et al., 2004; Varshney et al., 2005). Recent research has revealed that ESTs are a potentially rich source of SSRs that reveal polymorphisms not only within the source taxon but also in related taxa (Ellis and Burke, 2007).

However, few SSR markers have been used and developed in *Chrysanthemum*, and no SSR markers have been exploited in its closely related species or genera, such as *Ajania* and *Opisthopappus*. Moreover, few gene sequences or ESTs have been available in these closely related genera of *Chrysanthemum*.

Thus, our objective was to develop SSR markers from EST databases of *Chrysanthemum* for *Chrysanthemum morifolium* and closely related species and genera, which can be employed in molecular marker-assisted breeding, genetic diversity analysis, and other applications.

MATERIAL AND METHODS

Plant materials and DNA extraction

In this study, a total of 52 wild species and varieties of *Chrysanthemum*, *Ajania*, and *Opisthopappus* were employed to test the amplification and polymorphism of primer pairs (Table 1). These materials were collected from 14 provinces in the northeast and northwest of China, central China, eastern China, and southern China. Among these samples, *Ajania*

pacifica is native to the Taiwanese area of China and Japan. Genomic DNA was isolated from young leaves using an improved cetyltrimethylammonium bromide method (Liu et al., 2003).

Table 1. List of *Chrysanthemum*, *Ajania*, and *Opisthopappus* materials used in this study.

Genus	Species	Variety	Origin	Notes
Chrysanthemum	*C. morifolium*	Quanxiangchongtian	Beihai Park in Beijing	Large chrysanthemum
		Baiou	Beihai Park in Beijing	Large chrysanthemum
		Lvyishang	Beihai Park in Beijing	Large chrysanthemum
		Shiyongju	Beihai Park in Beijing	Large chrysanthemum
		Pubu	Beihai Park in Beijing	Large chrysanthemum
		Fanxing	Beihai Park in Beijing	Escarpment chrysanthemum
		Junziyu	Beihai Park in Beijing	Escarpment chrysanthemum
		Lvmudan	Beihai Park in Beijing	Large chrysanthemum
		Huige	Beihai Park in Beijing	Large chrysanthemum
		Meihualu	Beihai Park in Beijing	Large chrysanthemum
		Xuetao	Beihai Park in Beijing	Large chrysanthemum
		Feiliuqianchi	Beihai Park in Beijing	Large chrysanthemum
		Taohongliulv	Beihai Park in Beijing	Large chrysanthemum
		Fenghuangzhenyu	Beihai Park in Beijing	Large chrysanthemum
		Jinjihongling	Beihai Park in Beijing	Large chrysanthemum
		Huangkuilong	Beihai Park in Beijing	Large chrysanthemum
		Fenditan	Beijing Forestry University	Ground-cover chrysanthemum
		Jingqi	Beijing Forestry University	Ground-cover chrysanthemum
		Pudifendai	Beijing Forestry University	Ground-cover chrysanthemum
		Jinguangwanzhang	Beijing Forestry University	Ground-cover chrysanthemum
		Ziyunqinfang	Beijing Forestry University	Ground-cover chrysanthemum
		Fanhuasijin	Beijing Forestry University	Ground-cover chrysanthemum
		Zhaoyanghong	Beijing Forestry University	Ground-cover chrysanthemum
		Jinzhu	Beijing Forestry University	Ground-cover chrysanthemum
		Nongfenzhaoxia	Beijing Forestry University	Ground-cover chrysanthemum
		Baisha	Beijing Forestry University	Ground-cover chrysanthemum
		Danhanfen	Beijing Forestry University	Ground-cover chrysanthemum
		Maoxiangyu	Beijing Forestry University	Ground-cover chrysanthemum
		Xiangfei	Beijing Forestry University	Ground-cover chrysanthemum
		Mibaizao	Beijing Forestry University	Ground-cover chrysanthemum
		Pudidanfen	Beijing Forestry University	Ground-cover chrysanthemum
		Qinglian	Beijing Forestry University	Ground-cover chrysanthemum
		Yaohong	Beijing Forestry University	Ground-cover chrysanthemum
		Yangguangxiaoj	Beijing Forestry University	Ground-cover chrysanthemum
		Yulong	Beijing Forestry University	Ground-cover chrysanthemum
Chrysanthemum	*C. indicum*		Korea	
Chrysanthemum	*C. lavandulifolium*		Anhui Province	
Chrysanthemum	*C. eticuspe*		Hubei Province	
Chrysanthemum	*C. chanetii*		Beijing	
Chrysanthemum	*C. mankingense*		Jiangsu Province	
Chrysanthemum	*C. indicum*		Hubei Province	
Chrysanthemum	*C. indicum* var. *aromaticum*		Hubei Province	
Chrysanthemum	*C. zawadskii*		Heilongjiang Province	
Ajania	*A. fruticulosa*		Qinghai Province	
Ajania	*A. potaninii*		Sichuan Province	
Ajania	*A. achilloides*		Inner Mongolia	
Ajania	*A. scharnhorstii*		Xinjiang Province	
Ajania	*A. khartensis*		Yunnan Province	
Ajania	*A. purpurea*		Tibet	
Ajania	*A. acificum*		Taiwan	
Opisthopappus	*O. taihangensis*		Henan Province	
Opisthopappus	*O. longilobus*		Hebei Province	

Identification of SSR-containing ESTs and primer design

In February 2012, all of the 7180 ESTs of *C. morifolium* were downloaded from Gen-

Bank (up to February 2012) (http://www.ncbi.nlm.nih.gov/Genbank/). A total of 4164 unigene sequences (908 contigs and 3256 singlets) were generated using the CAP3 software. All contigs and singlets were then used to screen for the presence of microsatellites with the SSRIT software (http://www.gramene.org/db/markers/ssrtool). The screening criteria were 8, 5, 4, and 3 repeating units for di-, tri-, tetra-, and penta- and higher-order nucleotides, respectively. Three hundred ten SSRs from 296 sequences were identified. One hundred ninety-eight primer pairs in the flanking region of SSRs were successfully designed using the Primer Premier 5.0 software with a length of 18 to 24 bp, amplification product size of 100 to 500 bp, annealing temperature (Ta) of 40 to 60°C, and GC content between 40 and 60%.

Polymerase chain reaction (PCR) amplification and fragment analysis

PCR was performed in a total volume of 25 μL, which contained approximately 40 to 60 ng template DNA, 2.5 μL 10X PCR buffer, 2.5 μL 25 mM $MgCl_2$, 0.4 μL 10 μM of each primer, 0.5 μL 10 mM dNTPs, 2 U Taq polymerase (Promega, USA), and sterile distilled water. The PCR amplification was performed on a TECHNE instrument (TC-512, UK) with the following program: 94°C for 4 min (initial denaturation); 30 cycles of 94°C for 30 s, the appropriate annealing temperature (Table 2) for 30 s, and 72°C for 1 min; and a final extension for 5 minutes at 72°C.

The PCR products were confirmed on a 2% agarose gel with a constant voltage of 100 V (85 mA) for 40 minutes using a Bio-Rad electrophoresis system (USA). The amplicons were analyzed using an ABI3130xl genetic analyzer (Applied Biosystems, USA). The allele size was determined as base pairs using the GeneMapper software v3.7 (Applied Biosystems, USA).

RESULTS

Of these primer pairs, only 25 revealed polymorphism (Table 2) among 52 wild species and varieties of *Chrysanthemum*, *Ajania*, and *Opisthopappus*. The remaining primer pairs did not amplify PCR products or produced complex band patterns, indicating non-specific amplification. Table 2 lists these working EST-SSR markers, repeat motif, optimal annealing temperature, and annotation of putative functions. Each EST-SSR marker name consisted of the prefix CEM (*Chrysanthemum* EST-derived microsatellite). Nei's gene diversity ranged from 0.0960 to 0.2968, with an average of 0.2290; the average Shannon's information index was 0.2626 in 52 germplasms.

Basic local alignment search tool with translated nucleotide query (BLASTX) searches against the nr protein database (National Center for Biotechnology Information, NCBI) revealed that 7 SSR marker-targeted ESTs have putative gene functions: 1 EST encodes a putative disease-resistance protein, 3 ESTs encode stress-resistance relative proteins, 1 EST encodes a fruit development control protein, 1 EST encodes a growth stage regulatory protein, and 1 EST encodes a DNA repair protein.

Utility of EST-SSRs

The EST-SSR markers CEM2, CEM4, and CEM9 were randomly selected to identify intergeneric hybrids (Figure 1) of the *C. morifolium* Pudidanfen (male parent, hexaploid) and *A. pacifica* (female parent, decaploid), as well as that of *C. morifolium* Yulong (female parent, hexaploid) and *A. pacifica* (female parent).

Table 2. Characteristics of expressed sequence tag-simple sequence repeat (EST-SSR) markers.

Primer name	Motif	No. of repeats	Expected fragment size (bp)	TM	H	I	Primer sequences	Putative gene function
CEM1	ca	8	226-352	58	0.1263	0.2206	F: TTCAAGGGCTGGATGCCTTATT R: GTGTTTGCGATGTTCCATTCTC	Resistance to disease and pest
CEM2	ggt	7	361-385	60	0.2015	0.3231	F: TTCGCTCACTCACTCACTCACA R: CACATAACCACCACTACCACCA	
CEM3	gca	9	252-276	58	0.2825	0.421	F: AGGTTGAGATTCTTGACCAGGGTT R: CTCTTTCTTCTGCTGGCGTTGA	
CEM4	cat	6	255-285	58	0.2968	0.444	F: CCGGTCCCTTTAAGGTTAGGTT R: TTGGAATTTCAGATGGCGTTGT	
CEM5	aaaatg	3	298-340	58	0.1292	0.2266	F: ACTTCAATCCCATTCCTAAACC R: TCTCCCTATCCTCACTCACCTC	
CEM6	ca	11	450-480	55	0.2687	0.4265	F: ATTCCCAACCTTCTTTAACCAT R: ATCAGCTCCATTATCAAGCAAC	
CEM7	tttat	5	365-380	58	0.1538	0.2356	F: CTGTTGACTCGTCCCTTCGTCG R: CCTCATCCTTGACCTTCGTGCT	Stress resistance
CEM8	aag	6	364-400	58	0.2564	0.3662	F: TCCACCGAAGAACCAGAACCAC R: TGTCCCAACCTAACCCATGAAC	Growth stages regulatory factors
CEM9	ca	11	194-218	58	0.2949	0.4504	F: AAACCTCAGAAACCCAAACTCT R: TTTGTAGGAACTGGTGCGGTAG	Fruit development, carbohydrate Metabolism, and cell wall extension
CEM10	tc	21	779-929	58	0.096	0.1874	F: CACTCCCACCGAGATCATACAA R: CTCCAACCCACGCATAAACCAC	Cold resistance
CEM11	aaag	6	324-348	58	0.1896	0.2178	F: TTGTTTCTCCGTCAATCAGTCA R: CACTCCTCCAACTCCTTGTACTTT	
CEM12	caggtg	5	446-736	56	0.2953	0.2532	F: CAACCAGCAATCAACAGAGGAA R: GACCGATTATGACGAAACCAAA	Heat shock protein-encoding gene
CEM13	caa	7	261-279	58	0.1692	0.1987	F: CACCACCACCACTCCTCACCAC R: GGAAGCGACATCATTTCATGTTTAT	
CEM14	caa	7	379-421	56	0.1925	0.2217	F: CTACGGGTTTACGACTTGCTTT R: TGCCTTATCCGCTTCAACTTCT	
CEM15	aaccct	3	415-427	60	0.1768	0.1981	F: CTTTAGCCACCACCGCCTACTTT R: CCTGTTCCCAATCATCCGTCCA	
CEM16	ctaact	4	364-388	58	0.1809	0.2128	F: CAACAACCACCGTCACAACCAT R: AAATATCTCCAGCAGCGGCATC	
CEM17	cagaac	3	464-530	58	0.235	0.3724	F: AATTGCTTCTATGTGGGCTTGG R: ATCGTGGCGAGTCGGGTTGAGT	
CEM18	ata	6	279-291	56	0.1637	0.2139	F: ACAATGGCTGTTCAAGCACAAT R: AAGCAAGTCGTAAACCCGTAGA	
CEM19	caa	7	379-427	56	0.2532	0.2875	F: CTACGGGTTTACGACTTGCTTT R: TGCCTTATCCGCTTCAACTTCT	
CEM20	caa	5	279-291	58	0.2012	0.2114	F: GACCCGAATACCAACAAGTCCA R: TAGGCGGTGAAACATTTAGCAG	
CEM21	cctgca	3	356-374	60	0.1857	0.1958	F: CCCCAAAGGCACCTCAGACAAG R: CAGCACGAAGAGCACGGACAAC	DNA repair protein
CEM22	catcct	3	410-438	56	0.1754	0.189	F: GGGACCCAAGCAGTAAGAATGA R: ACCAAACAACCCTCCAAACCAT	
CEM23	cct	6	260-278	54	0.1805	0.1782	F: GAGAAGACCTTCCCATTGCTAC R: CTGCCATTGGAGATTTCATTTT	
CEM24	atg	6	255-270	54	0.1736	0.1865	F: ACTTCCACTGTCTTCTTCATCC R: CTGCCATTGGAGATTTCATTTT	
CEM25	ac	8	216-232	58	0.1698	0.1867	F: AGCCAGCCAACCATTGTCCATT R: GCGAACTCCACGTCTCAAATCC	

TM = primer melting temperature; H = Nei's (1973) gene diversity; I = Shannon's information index (Lewontin and Krakauer, 1973).

All 3 intergeneric hybrids (AP1, AP2, and AP3) of *C. morifolium* Pudidanfen and *A. pacifica* and the 3 intergeneric hybrids (AY1, AY2, and AY3) of *C. morifolium* Yulong and *A. pacifica* showed specific bands of the male and female parent (Table 3). AP1, AP2, and AP3 presented new bands, b5 and c5, which were absent from their parents, *A. pacifica* and Pudidanfen.

Figure 1. *Ajania pacifica*, *Chrysanthemum morifolium* Pudidanfen, *C. morifolium* Yulong, and intergeneric hybrids. **A.** *A. pacifica.* **B.** *C. morifolium* Pudidanfen. **C.** *C. morifolium* Yulong. **D.** Intergeneric hybrid between *A. pacifica* and *C. morifolium* Pudidanfen 1 (AP1). **E.** AP2. **F.** AP3. **G.** Intergeneric hybrid between *A. pacifica* and *C. morifolium* Yulong 1 (AY1). **H.** AY2. **I.** AY3. The figures have been supplied in JPEG format which need to be shown at the end of the manuscript.

Table 3. Bands of intergeneric hybrids and parents.

Marker	Parents			Intergeneric hybrids					
	Ajania pacifica	Yulong	Pudidanfen	AY1	AY2	AY3	AP1	AP2	AP3
CEM2	a_5, a_6	a_2, a_3, a_4, a_7	a_1, a_2, a_5, a_6	a_3, a_4, a_5	a_3, a_4, a_6, a_7	a_2, a_4, a_5, a_7	a_2, a_3, a_4, a_5	a_2, a_3, a_4, a_5, a_6	a_1, a_2, a_3, a_4, a_5
CEM4	b_2, b_4, b_6	b_1, b_2, b_4, b_7	b_1, b_2, b_3, b_4	b_1, b_2, b_3, b_4, b_7	b_2, b_4	b_1, b_2, b_4	b_1, b_2, b_4, b_5	b_2	b_2, b_4, b_5, b_7
CEM9	c_1, c_2, c_4, c_6, c_7, c_9	c_2, c_4, c_8, c_9	c_3, c_7, c_8	c_1, c_2, c_4, c_6, c_8, c_9	c_1, c_2, c_4, c_7, c_9	c_2, c_4, c_6, c_7, c_9	c_2, c_4, c_7, c_9	c_2, c_4, c_5, c_6, c_7	c_1, c_2, c_3, c_4, c_7

Band size: a_1: 361 bp, a_2: 364 bp, a_3: 367 bp, a_4: 370 bp, a_5: 373 bp, a_6: 376 bp, a_7: 379 bp, b_1: 255 bp, b_2: 261 bp, b_3: 267 bp, b_4: 273 bp, b_5: 279 bp, b_6: 276 bp, b_7: 285 bp, c_1: 194 bp, c_2: 196 bp, c_3: 200 bp, c_4: 202 bp, c_5: 204 bp, c_6: 206 bp, c_7: 210 bp, c_8: 212 bp, and c_9: 214 bp.

DISCUSSION

EST-SSRs are not without their drawbacks, but they offer a number of clear benefits, including rapid and inexpensive development and high levels of cross-taxon portability (Ellis and Burke, 2007). In previous studies, EST-SSR markers were developed, and the transitivity to related species was tested. However, we sought to develop EST-SSR markers for *Chrysanthemum* and its closely related species and genera in this study to identify intergeneric hybrids of *C. morifolium*, *Ajania*, and *Opisthopappus*. Few gene sequences have been determined in *Ajania* and *Opisthopappus*. Thus, the ESTs of *Chrysanthemum* were exploited, and 25 EST-SSRs revealed polymorphism among the wild species and varieties of *Chrysanthemum*, *Ajania*, and *Opisthopappus*. Nei's gene diversity (0.0960-0.2968) and Shannon's information index (0.1782-0.4504) of the EST-SSRs showed a broad range, which indicated that the genetic diversity revealed by each EST-SSR was different. EST-SSRs could be exploited to facilitate evolutionary analyses in a wide variety of taxa. This approach may constitute an effective method for the analysis of species with only limited resources, such as some closely related genera of *Chrysanthemum*. These EST-SSR markers could be used to identify cultivars, accurately assess genetic relationships and diversity, efficiently tag and map desirable genes, and select superior genotypes early in *Chrysanthemum* and closely related species.

BLASTX searches against the nr protein database (NCBI) revealed that 7 SSR marker-targeted ESTs have putative gene functions, such as disease-resistance and stress-resistance, which indicated that ESTs could be utilized to develop functional SSR markers. The application of EST-SSR markers to the identification of intergeneric hybrids was demonstrated and proved to be a valuable tool for the breeding of *Chrysanthemum* and *Ajania* plants.

The intergeneric hybrids of *C. morifolium* (Yulong and Pudidanfen) and *A. pacifica* showed specific bands of the male and female parent; these bands were consistent with their inheritance of acquired characteristics. For example, the hybrids all exhibited ray flowers that were inherited from *C. morifolium* because the inflorescence of *A. pacifica* is only composed of disc flowers. The thin and leathery leaf blades during pubescence were consistent with *A. pacifica*. Furthermore, a number of hybrids presented new bands that were absent in their parents, which indicated that the intergeneric hybrids generated a new genotype. Therefore, EST-SSRs can be developed for species that lack gene sequences or ESTs by utilizing ESTs of closely related species.

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