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Isolation and characterization of microsatellite markers from *Clematoclethra scandens* (Actinidiaceae)[1]

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- *Premise of the study:* Microsatellite makers were developed for *Clematoclethra scandens* to investigate its population genetics and speciation.
- *Methods and Results:* A total of 36 microsatellite markers were isolated using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) method. Their polymorphisms were assessed in two natural populations. The results showed that 30 markers displayed prominent polymorphisms and six markers were monomorphic.
- *Conclusions:* These microsatellite loci will facilitate further studies on population genetics and speciation of *C. scandens*.

Key words: Actinidiaceae; *Clematoclethra scandens*; microsatellite markers; polymorphism; population genetics.

Clematoclethra (Franch.) Maxim. (Actinidiaceae) is a genus endemic to the subtropical and temperate regions of central and western China. This genus is characterized by possessing 10 stamens instead of numerous stamens as in the two other genera in Actinidiaceae (Li et al., 2007). Species delimitation within *Clematoclethra* has been highly controversial due to overlapping variation in major taxonomic characters, e.g., pubescence, leaf shape, floral and fruit morphology, and geographic distribution. Liang (1984) recorded 20 species and four varieties. Tang and Xiang (1989), however, recognized only one polymorphic species (*C. scandens* Maxim.) with four subspecies. Li et al. (2007) followed the latter taxonomic treatment in the *Flora of China*. Therefore, this genus has been proposed as a dynamic group with ongoing speciation (Wu et al., 2005).

Population genetic research is critical to provide a better understanding of the genetic structure and speciation of *C. scandens*. In this study, we developed and characterized 36 microsatellite markers for *C. scandens* using the Fast Isolation by AFLP of Sequences COntaining repeats (FIASCO) method (Zane et al., 2002). These markers can be used for further studies of population genetics and speciation.

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METHODS AND RESULTS

Genomic DNA samples of *C. scandens* were extracted from silica gel–dried leaves using a modified cetyltrimethylammonium bromide (CTAB) method (Doyle and Doyle, 1987). The extracted DNA was dissolved in 30 μL TE buffer. The FIASCO method was performed in this study (Zane et al., 2002). Total genomic DNA (~250–500 ng) was completely digested with 2.5 U of *Mse*I restriction enzyme (New England Biolabs, Beverly, Massachusetts, USA), and then ligated to an *Mse*I AFLP adapter (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) using T4 DNA ligase (Fermentas International Inc., Burlington, Ontario, Canada). The digested-ligated fragments were diluted (1 : 10), and 5 μL was used in a PCR reaction with adapter-specific primers (5′-GATGAGTCCTGAGTAAN-3′/5′-TTACTCAGGACTCATCN-3′). PCR amplifications were conducted under the following conditions: 95°C for 3 min; followed by 26 cycles at 94°C for 30 s, 53°C for 1 min, and 72°C for 1 min; and a final extension step at 72°C for 7 min. The amplified DNA fragments (200–800 bp) were enriched by magnetic bead selection with a 5′-biotinylated $(AG)_{15}$ and $(AC)_{15}$ probe. The recovered DNA fragments were reamplified with *Mse*I-N primers. The PCR products were purified using the E.Z.N.A. Gel Extraction Kit (Omega Bio-Tek, Guangzhou, China), ligated into pBS-T II vector (Tiangen, Beijing, China), and then transformed into *Escherichia coli* strain DH5α competent cells (TaKaRa Biotechnology Co., Dalian, Liaoning, China). To identify cloned fragments that possibly contained simple sequence repeats (SSRs), PCR reactions were performed with vector-specific primers T7/SP6, T7/$(AC)_{10}$, and SP6/$(AG)_{10}$, respectively. All PCR reactions were performed using the same conditions: 95°C for 3 min; followed by 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension step at 72°C for 8 min. The positive clones were captured for sequencing using an ABI PRISM 3730XL DNA sequencer (Applied Biosystems, Foster City, California, USA). Sequences that contained SSRs and enough flanking regions were selected for primer design using the PRIMER 5.0 software package (Clarke and Gorley, 2001).

The designed primer pairs were assessed in 24 individuals of *C. scandens* from two natural populations collected in China: SNJ (Shennongjia, Hubei: 31°33′12.51″N, 110°21′28.91″E, elevation 1747 m) and EMS (Emeishan, Sichuan: 29°32′38.81″N, 103°19′27.29″E, elevation 2328 m). Voucher specimens (Shennongjia population: KUN1216590; Emeishan population: KUN1216591) are deposited at the Herbarium of the Kunming Institute of Botany (KUN). The PCR reactions were performed in 20 μL reaction volumes containing 10–50 ng genomic DNA, 0.5 μM of each primer, and 10 μL 2× *Taq* PCR MasterMix (0.1 U *Taq* polymerase/μL, 0.5 mM each dNTP, 20 mM Tris-HCl [pH 8.3], 100 mM

Table 1. Characteristics of 36 microsatellite loci in *Clematoclethra scandens.*

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
CLE007*#	F: CCAAGCAAGTAGTATTTG	$(AG)_{14}$	114–124	52	JN712071
	R: ATCCCACCTATCGTTATCTA				
CLE011	F: CGATGGCAAAATCAAATCTT	$(TG)_8$	159	52	JN712072
	R: ATCTTAGTTTGTTGGTTCAC				
CLE012*#	F: TCAAATGTGCGAGGTCCAAA	$(TG)_7$	208–220	50	JN712073
	R: TTCAAGAGTTTTTATCCACC				
CLE015*	F: CTCTTATCAGCAGCATCAAT	$(TC)_9(AC)_{12}$	113–159	51	JN712074
	R: GTAAATAGGTTCAAGTGTGT				
CLE018*#	F: CGAATCAGAATCCTCCGAAG	$(AG)_{15}$	246–268	60	JN712075
	R: GCTTTGCCTACATCTCCATC				
CLE019*	F: CCGTTTCTGGGTTGTCATTT	$(AG)_{10}$	179–213	56	JN712076
	R: ATTCCCACATTGTTTTCGTA				
CLE020*	F: TTGACTGGAATGAGATAA	$(TG)_8$	165–183	52	JN712077
	R: CATCACCCAAAACCCACATA				
CLE022*	F: GCTTTATTGAGGGAGACAGA	$(AG)_{15}$	158–184	60	JN712078
	R: CCCACGTTCCTTATTATCCT				
CLE025*#	F: GGAACCACCACTACCACAAT	$(AG)_{15}$	182–196	60	JN712079
	R: GCTGCTTGGTCTCTTGACAA				
CLE026*	F: GTACAAAAAGCTGAGTGCAA	$(AG)_{14}$	169–193	54	JN712080
	R: ATTAGACAAAACACCTACAT				
CLE031*	F: ATATGGAAGAGGCTAGGAGG	$(AG)_{15}$	153–177	51	JN712081
	R: TGGATTTATCAACAAAGATT				
CLE034*#	F: CTCAATGCGTTCTTTTATCA	$(TC)_9$	169–187	57	JN712082
	R: CAATACATACCGAGTCCAGG				
CLE036*	F: TCCCAAAGCCAGCCACCTCT	$(TC)_{13}(AC)_8$	156–184	57	JN712083
	R: CCAAAACTACATAAACCCTA				
CLE043*	F: ATGTGATGTGTGTGTTCTTG	$(AG)_{12}$	97–111	52	JN712084
	R: TTTCTTCCTCTTTAGCTTTT				
CLE049*	F: TGGAATACTGTGGGTGAGAT	$(TC)_{19}$	112–146	52	JN712085
	R: ATTCAGGGGATTCGCTCATA				
CLE050*	F: CGCCATTTGAGAGATTGTGT	$(AG)_9$	162–178	59	JN712086
	R: CACTTCAGTTCCCGTACATC				
CLE053*	F: TGCTGAATCTGTTGGCTAAG	$(TC)_{10}$	164–172	50	JN712087
	R: ATTTTTTCCTTTGTTTTCTG				
CLE054*	F: CGCCGCCGACCATCACCAGC	$(TC)_{13}$	135–167	62	JN712088
	R: GGGGGAGCGAGATACCATTG				
CLE056*	F: GCATCAAGAACCAGAGCAAC	$(AG)_{16}$	185–225	56	JN712089
	R: CTTCTCCTCCTCTGTAAACG				
CLE058*	F: TTATCTCAGGGACTCAAAAC	$(TC)_{10}$	133–167	54	JN712090
	R: GCAAACAACAACTAGCAAGA				
CLE059*	F: TGTTTTCCTGTTTTGCTGAT	$(TC)_{14}$	132–140	49	JN712091
	R: ATTATGGCAGACAACCAAGA				
CLE062*	F: GGAAATCAGTAAGTGGTAT	$(AT)_{11}AGAT(AG)_{16}$	132–188	48	JN712092
	R: CACAAAGTCATAATTCATAG				
CLE063*	F: CTGTCACCAGAGAGGTTACA	$(TC)_{14}(AC)_{15}$	136–148	56	JN712093
	R: GGAAGCCACCTGTCTCATAA				
CLE066*	F: AAGAAACGGAAGAACTAACC	$(AG)_8\ldots(AG)_{11}$	159–191	53	JN712094
	R: AAGAAATACAGGAACACCAA				
CLE068*	F: GGCTAATGAGTTTCGCTTTC	$(TG)_{16}(AG)_6$	176–200	57	JN712095
	R: TGTCTTTCTCCCGCAGTTAC				
CLE069*#	F: TTGTTGATAGATTAGAGGCT	$(AG)_{15}$	265–273	55	JN712096
	R: ATGGCTTATATTCGGCATTC				
CLE071	F: CCCCAGAGACTTGAGTGATT	$(AG)_8$	145	56	JN712097
	R: GGACGAGGTTTGTATGTTAT				
CLE076	F: AGAAGCCTCAAGTTATGGAT	$(AG)_{14}$	262	57	JN712098
	R: GGTCAGTCGGTCATGGGT				
CLE080*	F: CACTTCTATCTGTTGACCCT	$(TC)_{11}$	148–156	55	JN712099
	R: AGTATGCTGGTTCTAAGGTT				
CLE081	F: GTTGGGTCTACTGAGGATTC	$(TC)_{19}TGTC(AC)_8$	186	56	JN712100
	R: CATCAAACACTTCTACTTCC				
CLE085	F: AGAGGGGTGACGGAATAATG	$(TC)_{14}$	252	58	JN712101
	R: TGGGAGTTCAATCGGTGGTG				
CLE087*	F: GTTGTGTTTTGAAGTGGGAG	$(AG)_{16}$	133–155	59	JN712102
	R: CCCTTTCCTTTCTCTCACCA				
CLE094*	F: AAAGGTTGTCCATTGAGGCA	$(TC)_{13}$	161–179	60	JN712103
	R: CTTCGGATATGACATGCTG				
CLE095*	F: CTCTTCTTCCAAGTCCGATT	$(TCCCTC)_4\ldots(TC)_8$	160–168	55	JN712104
	R: GAGTGTGAGATAAGCCCCAA				

TABLE 1. Continued.

Locus	Primer sequences (5′–3′)	Repeat motif	Size range (bp)	T_a (°C)	GenBank accession no.
CLE097	F: AGTATTTTTCTTTCTGTGGT	$(TC)_{13}$	133	52	JN712105
	R: AACCAGATGATTACTAACCG				
CLE102*	F: GGCAAGATAAGCAACGACAT	$(AG)_{10}$	186–212	59	JN712106
	R: AGCCACTCTCCATTCCATAG				

Note: T_a = annealing temperature.
*Displayed polymorphisms in *C. scandens*.
#Loci deviating from Hardy–Weinberg equilibrium (HWE).

KCl, 3 mM $MgCl_2$) (Tiangen, Beijing, China). PCR amplifications were conducted under the following conditions: 95°C for 3 min followed by 32–35 cycles at 94°C for 30 s, at the annealing temperature for each specific primer (optimized for each locus, Table 1) for 45 s, 72°C for 1 min, and a final extension step at 72°C for 7 min. The amplification products were separated and visualized using the QIAxcel system for capillary gel electrophoresis (QIAGEN, Irvine, California, USA).

The DNA data were analyzed by GENEPOP 4.0 (Raymond and Rousset, 1995), which included statistics for observed heterozygosity (H_o) and expected heterozygosity (H_e) for the 30 polymorphic microsatellite loci. The same program was used to test the Hardy–Weinberg equilibrium (HWE).

A total of 287 positive clones were sequenced, and 195 of these clones contained microsatellites. One hundred four sequences containing microsatellites were selected for primer design. A total of 36 microsatellite loci were successfully amplified in *C. scandens* with amplicon sizes approximately matching the expected sizes. This species is diploid with $2n = 2x = 48$ chromosomes (He et al., 2005). As expected, all the loci had clear single-locus amplification products. Of these amplification products, 30 loci displayed polymorphisms and six showed similar genetic diversity (Table 1). The number of alleles ranged from two to eight in 24 individuals of the species sampled from the two natural populations. Values for H_o and H_e ranged from 0.000 to 0.500 and from 0.042 to 0.444, respectively (Table 2).

CONCLUSIONS

In summary, 36 microsatellite markers were developed for *C. scandens*. The high discriminatory power of 30 polymorphic loci suggests that they should be suitable for surveys of population genetics and speciation process. These developed and characterized microsatellite markers should also be useful for studies on breeding system and patterns of gene flow.

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Table 2. Result of 30 polymorphic primers screening in two populations of *Clematoclethra scandens*.

	Population SNJ (N = 12)[a]			Population EMS (N = 12)[a]		
Locus	N_a	H_e	H_o	N_a	H_e	H_o
CLE007	2	0.080	0.000	3	0.082	0.083
CLE012	2	0.042	0.042	2	0.226	0.167
CLE015	4	0.183	0.208	4	0.351	0.125
CLE018	6	0.331	0.250	5	0.274	0.250
CLE019	2	0.143	0.000	3	0.183	0.208
CLE020	4	0.245	0.208	4	0.257	0.167
CLE022	6	0.329	0.250	5	0.351	0.333
CLE025	4	0.333	0.083	4	0.281	0.167
CLE026	4	0.283	0.333	4	0.371	0.208
CLE031	5	0.375	0.292	3	0.355	0.375
CLE034	3	0.259	0.167	2	0.042	0.042
CLE036	5	0.277	0.167	4	0.375	0.292
CLE043	4	0.304	0.333	3	0.324	0.500
CLE049	8	0.413	0.417	8	0.444	0.417
CLE050	4	0.154	0.083	4	0.328	0.292
CLE053	3	0.212	0.167	3	0.150	0.167
CLE054	5	0.368	0.333	5	0.400	0.500
CLE056	4	0.361	0.417	5	0.395	0.375
CLE058	5	0.275	0.333	3	0.302	0.250
CLE059	4	0.282	0.292	4	0.303	0.083
CLE062	7	0.377	0.375	5	0.370	0.375
CLE063	3	0.248	0.250	3	0.150	0.042
CLE066	3	0.310	0.250	4	0.324	0.250
CLE068	5	0.349	0.333	4	0.339	0.250
CLE069	2	0.042	0.042	2	0.172	0.125
CLE080	2	0.219	0.167	2	0.208	0.292
CLE087	4	0.351	0.250	4	0.292	0.292
CLE094	3	0.319	0.375	3	0.299	0.292
CLE095	2	0.042	0.042	3	0.324	0.333
CLE102	3	0.210	0.250	3	0.292	0.125
Mean	3.9	0.257	0.224	3.7	0.285	0.246

Note: H_e = expected heterozygosity; H_o = observed heterozygosity; N = population sample size; N_a = number of alleles observed.

[a] SNJ = Shennongjia, Hubei (31°33′12.51″N, 110°21′28.91″E; elevation 1747 m); EMS = Emeishan, Sichuan (29°32′38.81″N, 103°19′27.29″E; elevation 2328 m).