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Microsatellite primers in the native perennial cycad *Cycas taitungensis* (Cycadaceae)[1]

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- *Premise of the study:* Microsatellite primers were developed for the native perennial cycad *Cycas taitungensis* to evaluate the genetic variation of this endangered insular species.
- *Methods and Results:* Using a magnetic bead enrichment method and EST data, 16 primer sets were developed and identified for the native Taiwan cycad *C. taitungensis*. The primers amplified dinucleotide, trinucleotide, and complex repeats with 1–9 alleles per locus. Most primers also amplified DNA from *C. revoluta* and *C. debaoensis*.
- *Conclusions:* These results indicate the utility of primers for future studies of the genetic structure of *C. taitungensis*. In addition, the primers are useful for further phylogeographic studies between *C. taitungensis* and *C. revoluta*, which is a closely related species.

Key words: cycad; *Cycas taitungensis*; genetic structure; reintroduction.

The perennial cycad *Cycas taitungensis* Shen, Hill, Tsou & Chen is an endemic species in Taiwan. There are only two remaining populations, and these have little genetic differentiation (Huang et al., 2001). In the past decade, these populations have been in extreme decline because of individual deaths caused by *Aulacaspis yasumatsui*, an invasive species that infests cycad plants. The Forestry Bureau and the Conservation Management Office in Taiwan have considered *C. taitungensis* for *ex situ* conservation strategies such as "seed storage" to allow its reintroduction if the wild population becomes extinct. To evaluate population genetic variation, identify distinct genetic units, and select individuals for seed storage, primers to detect microsatellite loci were developed.

Cycas revoluta Thunb. and *C. taitungensis* belong to section *Asiorientales* and are paraphyletic in phylogenetic analyses (Chiang et al., 2009). To investigate the transferability of loci between these species, newly developed microsatellite primers must also be able to amplify microsatellites in *C. revoluta*. Furthermore, the amplification of another species from section *Stangerioides*, *C. debaoensis* Zhong & Chen, can be used to evaluate the utility of the primers in additional *Cycas* species.

METHODS AND RESULTS

Twenty individuals were collected from one population of *Cycas taitungensis*, *C. revoluta*, and *C. debaoensis* from Taitung (Taiwan; 22°51′17″N, 120°57′13″E), Amami-O-Shima (Japan; 28°09′04″N, 129°21′14″E), and Guangxi (China; 23°13′44″N, 106°22′12″E), respectively. Genomic DNA was extracted from silica-dried leaves following a CTAB procedure. Two strategies were used to develop microsatellite loci: a direct design of the microsatellite loci that was based on EST data, and a selective capture and enrichment of microsatellite loci using magnetic beads. First, a total of 21 999 ESTs from *C. rumphii* Miq. (Brenner et al., 2003) were scanned for microsatellites using Tandem Repeats Finder version 4.04 (Benson, 1999). Second, microsatellite loci were isolated using the method from Liao et al. (Liao et al., 2009) and modified as in Zane et al. (Zane et al., 2002). Genomic DNA was digested using the restriction enzyme MseI (Promega, Madison, Wisconsin, USA) and ligated to a phosphorylated double stranded adaptor (complementary oligo A: 5′-TACTCAGGACTCAT-3′, 5′phosphorylated oligo B: 5′-GACGATGAGTCCTGAG-3′). Fragments in the range of 400–1 000 bp were excised from agarose gels and purified using the MinElute Gel Extraction Kit (Qiagen, Hilden, Germany). The partial genomic library was enriched through 15 cycles of prehybridization polymerase chain reaction (PCR) using adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′, hereafter referred to as MseI-N). The enriched DNA fragments were denatured and hybridized to two biotinylated probes (B-$(AG)_{15}$, B-$(AC)_{15}$) at 48°C for 1 h followed by incubation with Streptavidin MagneSphere Paramagnetic Particles (Promega). After washes of increasing stringency, the enriched DNA fragments were used as templates for 25 cycles of PCR amplification using MseI-N. The purified PCR products were cloned and screened using PCR (primer pairs: $(AG)_{10}$ or $(AC)_{10}$ and SP6 or T7). The selected plasmids were subsequently sequenced using an ABI 377 sequencer. Sequence data were examined for microsatellites by Tandem Repeats Finder version 4.04 (Benson, 1999). Using both the magnetic bead enrichment method and EST data, primers were designed to recognize the flanking sequences of microsatellite loci using FastPCR software version 5.4 (Kalendar, 2009).

Twenty-five primer pairs were screened using a gradient PCR protocol with a Labnet MultiGene 96-well Gradient Thermal Cycler (Labnet, Edison, New Jersey, USA). PCR conditions were as follows: 94°C for 5 min; 30 cycles of 94°C for 40 s; 50–63°C for 60 s; 72°C for 60 s; and a final extension of 72°C for 10 min. PCR products were separated on 1% agarose gels to evaluate for the optimal annealing temperature (*Ta*) (Table 1). Sixteen of 25 microsatellite loci were successful for stable target DNA bands. To screen for polymorphisms, 16 primer pairs were labeled with 6-FAM, HEX, NED, PET, or VIC fluorescent dyes (Applied Biosystems, Carlsbad, California, USA), and 20 samples were examined using the designed primers. PCR was performed using the GeneAmp PCR System 9700 (Applied Biosystems). Microsatellite fragments labeled with fluorescent dyes were detected by an ABI 3730 for genotyping, and GeneMapper

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Table 1. Characteristics of 16 microsatellite loci designed from an EST library or directly isolated from *C. taitungensis* (Cycadaceae).

Locus	Primer sequence (5′–3′)	Repeat motif	Allelic size (bp)	*Ta* (°C)	GenBank Accession No.
Cy-Tai EST-SSR01	F: CGAGAAGTAATTTGCAAATGC R: TGTGAAGCTAAATAGTTGGG	$(TC)_{24}(TG)_5$	98	55	EX921265
Cy-Tai EST-SSR02	F: ATGTACACATTCCATCCATC R: AAAAATCAGTGTGAATGGCC	$(CT)_{15}N(TG)_9(TC)_4(TA)_4N(TG)_{12}(TC)_8(TA)_5$	370–378	55	EX928734
Cy-Tai EST-SSR03	F: TGGCTCAAGAACAAATACAC R: GAACTCGGAGGGACACAACC	$(CT)_{20}(CA)_5(TA)_4$	171–177	60	EX929469
Cy-Tai EST-SSR04	F: ATTTCTTGGTGTTGAGAGTG R: GATGGCTAACCTCATTCTCC	$(CT)_8(AT)_{22}(GTAT)_4$	105–108	55	EX929539
Cy-Tai EST-SSR05	F: AACAGACCATGAGGACCAGG R: GCTGGTATTCCTTAATGCAC	$(TGG)_7N(ATA)_5(ACA)_3$	232–235	55	EX929834
Cy-Tai EST-SSR06	F: CGTCATCAAATTCTGTGCCC R: GCTGAATAGATGTTGATTTG	$(TA)_{17}$	83–89	57	CB091383
Cy-Tai EST-SSR07	F: AGCTATTCAGAATGCTGGGG R: GCCCTTTCTCTTTGTGTATG	$(GAA)_{10}$	114–120	54	DR063232
Cy-Tai EST-SSR08	F: GAAAATGCTTTGATGTTCCC R: TGGGCCAACTTTAAGCACAC	$(ATGT)_4(TA)_{10}(CA)_9$	170–177	60	DR063107
Cy-Tai EST-SSR09	F: AGTTGTCACTTCTATGCACC R: GGAGGTGACTGTTATTTTTGC	$(ATAAT)_3(ATATT)_{19}N(CT)_{11}(TG)_{13}$	234–236	58	DR063002
Cy-Tai EST-SSR10	F: CTGTGAATTTGAATTGCCCT R: TATCGGAACAAAAGATGCTG	$(TC)_{12}(TA)_9$	183–191	50	DR061996
Cy-Tai EST-SSR11	F: GATATTAAAGGCACGGGAG R: TGAAGCTGCTGCATTTGCAT	$(CAG)_{34}$	168–174	56	DR062467
Cy-Tai EST-SSR12	F: ATCGAAATCACGCATGCTTG R: GGCACGAGGCTCCTCCTCCT	$(AGG)_{13}$	119–123	57	CB094252
Cy-Tai EST-SSR13	F: CACCATCTGGCAGTCATGAT R: CCCCTGAACTGTCAAACAGG	$(TA)_{26}(TTTTC)_3$	193–230	60	CB091079
Cy-Tai Genomic-SSR1	F: TGGTCTTCCAACAACGGTG R: GGGACTGCTAGTAAGGAAGCT	$(AGGAA)_5$	219	56	FR744449
Cy-Tai Genomic-SSR2	F: AGCTTACAGCACCACCGCCAA R: TCAAGCTATGCATCCAACG	$(GAG)_{11}$	144–153	54	FR744450
Cy-Tai Genomic-SSR3	F: GCAGCTTACAGCACCACAATC R: TCACTAGTGATTGGCACGAG	(AGG)12N(AGG)11	138–150	55	FR744451

Ta, optimized annealing temperature.

3.7 software (Applied Biosystems) was used for fragment analysis. Diversity indices and Hardy–Weinberg equilibriums (HWE) were calculated in GenAlEx 6.1 (Peakall and Smouse, 2006). Fourteen polymorphic and two monomorphic microsatellite loci were obtained from *C. taitungensis*.

In this study, we present 16 novel primer pairs for *C. taitungensis* microsatellite loci and expand these primer pairs for use in *C. revoluta* and *C. debaoensis*, including 13 from an EST library and 3 isolated directly (Table 1). Genotypic data for 16 microsatellite loci (Table 2) were obtained for one population each of *C. taitungensis*, *C. revoluta,* and *C. debaoensis*. From the 16 assayed microsatellite loci, all primer pairs were successfully amplified in *C. taitungensis* and *C. revoluta*, and 11 primer pairs were amplified in *C. debaoensis.* There were 14, 15, and 10 polymorphic loci in *C. taitungensis*, *C. revoluta*, and *C. debaoensis*, respectively. The range for the number of alleles per locus was from 1 to 9 in *C. taitungensis* and from 1 to 11 and 1 to 15 in *C. revoluta* and *C. debaoensis*, respectively. The number of effective alleles (*Ne*) varied from 1 to 4.908, 1 to 6.504, and 1 to 11.111 in the three cycad species, respectively. As shown in Table 2, the observed and expected heterozygosity (H_O and H_E) ranged from 0 to 0.750 and 0 to 0.796 in *C. taitungensis*, from 0 to 0.750 and 0 to 0.749 in *C. revoluta*, and 0 to 0.650 and 0 to 0.910 in *C. debaoensis*. Tests for linkage disequilibrium between loci were calculated with Arlequin Version 3.11 (Excoffier et al., 2005), and no loci pairs revealed significant linkage disequilibrium. Three, five, and one of the 16 loci deviated significantly from the Hardy–Weinberg equilibrium as a result of heterozygote deficiency (Table 2) for the three cycads surveyed.

CONCLUSIONS

The 16 novel primer sets for microsatellite loci are adequate to investigate genetic variation in *C. taitungensis.* The genetic information collected for *C. taitungensis* will allow implementation of a more efficient conservation strategy for the rare and endangered taxa. The primers also amplified microsatellites successfully in *C. revoluta*, a species with high genetic similarity to *C. taitungensis* (Chiang et al., 2009). In addition, most of the primers can amplify microsatellites in *C. debaoensis*. These data suggest the potential utility of these primers for a variety of population genetics studies across species. These new molecular resources will enhance our genetic knowledge and improve the management of the threatened taxa such as the *Cycas* species.

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TABLE 2. Average genetic diversity for *Cycas taitungensis*, *C. revoluta*, and *C. debaoensis* using the 16 newly developed polymorphic microsatellite markers.

Locus	*Na*	*Ne*	H_O	H_E	*p*-value (HWE)
C. taitungensis (*N* = 20)					
Cy-Tai EST-SSR01	1	1.000	0.000	0.000	—
Cy-Tai EST-SSR02	5	3.687	0.550	0.729	0.484
Cy-Tai EST-SSR03	4	3.433	0.600	0.709	0.708
Cy-Tai EST-SSR04	2	1.280	0.150	0.219	0.160
Cy-Tai EST-SSR05	2	1.663	0.350	0.399	0.585
Cy-Tai EST-SSR06	4	3.556	0.450	0.719	0.135
Cy-Tai EST-SSR07	4	3.137	0.350	0.681	0.006*
Cy-Tai EST-SSR08	7	4.848	0.400	0.794	0.000*
Cy-Tai EST-SSR09	2	1.724	0.400	0.420	0.831
Cy-Tai EST-SSR10	5	3.320	0.600	0.699	0.433
Cy-Tai EST-SSR11	3	2.867	0.400	0.651	0.062
Cy-Tai EST-SSR12	5	2.817	0.500	0.645	0.000*
Cy-Tai EST-SSR13	9	4.908	0.550	0.796	0.247
Cy-Tai Genomic-SSR1	1	1.000	0.000	0.000	—
Cy-Tai Genomic-SSR2	3	2.100	0.450	0.524	0.207
Cy-Tai Genomic-SSR3	4	2.640	0.750	0.621	−0.347
C. revoluta (*N* = 20)					
Cy-Tai EST-SSR01	7	1.852	0.400	0.460	0.075
Cy-Tai EST-SSR02	3	2.930	0.450	0.659	0.210
Cy-Tai EST-SSR03	7	2.867	0.750	0.651	0.976
Cy-Tai EST-SSR04	3	2.847	0.400	0.649	0.120
Cy-Tai EST-SSR05	1	1.000	0.000	0.000	—
Cy-Tai EST-SSR06	6	3.077	0.650	0.675	0.803
Cy-Tai EST-SSR07	3	1.766	0.200	0.434	0.017*
Cy-Tai EST-SSR08	7	3.980	0.600	0.749	0.001*
Cy-Tai EST-SSR09	3	1.597	0.200	0.374	0.049*
Cy-Tai EST-SSR10	5	2.888	0.500	0.654	0.371
Cy-Tai EST-SSR11	4	1.699	0.400	0.411	0.438
Cy-Tai EST-SSR12	4	1.441	0.250	0.306	0.002*
Cy-Tai EST-SSR13	11	6.504	0.650	0.846	0.000*
Cy-Tai Genomic-SSR1	3	1.831	0.350	0.454	0.593
Cy-Tai Genomic-SSR2	3	2.572	0.650	0.611	0.005*
Cy-Tai Genomic-SSR3	6	3.419	0.550	0.708	0.709
C. debaoensis (*N* = 20)					
Cy-Tai EST-SSR01	1	1.000	0.000	0.000	—
Cy-Tai EST-SSR02	—	—	—	—	—
Cy-Tai EST-SSR03	6	4.420	0.650	0.774	0.946
Cy-Tai EST-SSR04	5	4.520	0.600	0.779	0.609
Cy-Tai EST-SSR05	—	—	—	—	—
Cy-Tai EST-SSR06	—	—	—	—	—
Cy-Tai EST-SSR07	3	2.100	0.400	0.524	0.207
Cy-Tai EST-SSR08	12	7.339	0.350	0.864	0.000*
Cy-Tai EST-SSR09	6	5.333	0.650	0.813	0.180
Cy-Tai EST-SSR10	5	3.902	0.400	0.744	0.012*
Cy-Tai EST-SSR11	4	3.556	0.600	0.719	0.914
Cy-Tai EST-SSR12	9	4.145	0.500	0.759	0.082
Cy-Tai EST-SSR13	15	11.111	0.250	0.910	0.000*
Cy-Tai Genomic-SSR1	4	3.213	0.650	0.689	0.208
Cy-Tai Genomic-SSR2	—	—	—	—	—
Cy-Tai Genomic-SSR3	—	—	—	—	—

Na, number of alleles; *Ne*, *No*. of Effective Alleles; H_O, observed heterozygosity; H_E, expected heterozygosity; *P* values indicate a test for a significant deficit of heterozygotes from that expected under Hardy–Weinberg equilibrium (HWE). * $P < 0.05$.

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