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TECHNICAL NOTE

Development of EST-microsatellites from the cycad *Cycas rumphii*, and their use in the recently endangered *Cycas micronesica*

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Abstract We developed a set of 12 EST-microsatellite markers (EST-STRs) from *Cycas rumphii* Miq. and tested them on an ex situ collection of the endangered *Cycas micronesica* K.D. Hill. The number of alleles per locus in both species ranged from 1 to 15. Observed and expected heterozygosities ranged from 0.000 to 0.842, and 0.000 to 0.883, respectively. All primers amplified in four additional *Cycas* species. These markers are the first genome-enabled tools for cycad population level studies, and are now available to inform conservation efforts and disentangle the biogeographic history of the genus.

Keywords Cycad · *Cycas micronesica* · EST-STRs · Invasives

Many of the ca. 300 cycad species distributed worldwide are currently threatened by human-induced habitat change, poaching, and invasive alien pests (Hill and Stevenson 2006). *Cycas* is the oldest (*c.a.* 280My) and most widespread cycad genus. The roughly 100 species are divided into several species complexes including the *Cycas rumphii* complex, whose distribution extends from Madagascar to Micronesia (Mamay 1969; Norstog and Nicholls 1997). One species within the *C. rumphii* complex is of critical conservation importance. *Cycas micronesica* is endemic to the Mariana and western Caroline Islands, and is the only native gymnosperm from the Mariana Islands (Hill and Stevenson 2006). Up to 12 m tall and previously locally abundant (Fig. 1), this species is an important ecological component of tropical forests (Hill et al. 2004). Consumption of its seeds has been associated with a high historical incidence of ALS-PDC in Guam's Chamorro residents (Borenstein et al. 2007) making it a medically important species that may one day help understand environmental causes of neurological disorders (Cox et al. 2006; Ince and Codd 2005). Wild *C. micronesic*a populations in Guam are currently threatened by an invasive, armored scale (*Aulacaspis yasumatsui* Takagi) accidentally introduced in 2003 and rapidly spreading within Guam (Fig. 2). In the past few months, we have detected it in *C. micronesica* populations in the neighboring island of Rota and it is likely to continue moving through Micronesia due to frequent inter-island human travel. These developments thrust the species into endangered status on the IUCN Red List in less than 3 years after the invasion (Marler et al. 2006).

Our goal here is to develop highly transferable, cost and time effective microsatellite markers, or simple tandem repeats (STRs), using an expressed sequence tag (EST) database from *C. rumphii*. EST-STRs will facilitate population level studies, identify populations that maintain overall genetic connectivity across the endemic range and that harbor unique genotypes that may be sources for ex situ nurseries. Population studies using EST-STRs within genes of ecological relevance will also enable

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Fig. 1 Healthy adult plant of *Cycas micronesica* in wild habitat in Guam

research on genetic patterns driven by less understood factors, such as selection (Stinchcombe and Hoekstra 2007).

Simple Sequence Repeat Identification Tool (Temnykh et al. 2001) was used to scan for microsatellites with a minimum of six and a maximum of ten repeats, from 9,510 assembled contigs from *C. rumphii* EST libraries (Brenner et al. 2003). A total of 112 microsatellite loci were identified, and 30 were used to design primers using PRIMER3 (Rozen and Skaletsky 2000). Genomic leaf DNA was prepared with a QIAGEN DNeasy Plant MiniKit from eight leaf samples of *C. rumphii* from Jawa Timur Indonesia, 27 from *C. micronesica* from three localities within northern Guam, and to test transferability across species, one sample each from *C. curranii* (J. Schust) K.D. Hill (Philippines), *C. edentata* de Laub. (Philippines), *C. hainanensis* C.J. Chen (China) and *C. wadei* Merrill (Philippines). To protect the endangered or threatened species, exact localities may be provided only upon request.

PCR amplification was carried out in 25 µl reactions of 12.5 µl of Lucigen EconoTaq™ Plus Green 2X Master Mix, 1 pmol/µl of each forward and reverse primer, 1 µl DNA (20–50 ng). Cycling conditions were: 1 × (95°C-3′), 28 × (95°C-45″, 53-1′, 72°C-45″, 1 × (72°C-3′) in a GeneAmp PCR System 9700 (Applied Biosystems).

All 30 primers yielded the predicted size ranges (150–300 bp) in all six species. Only 12 loci that consistently amplified in both *C. rumphii* and *C. micronesica* were fluorescently labeled with 6-carboxy-fluorescine (FAM) and M13 tags (Schuelke 2000).

For their PCR amplification we used 12.5 µl of Lucigen EconoTaq™ Plus Green 2X Master Mix, 1 pmol/µl of each M13 reverse and FAM-M13(21) primer, 0.5 1 pmol/µl of forward primer, 1 µl DNA. Amplification was also successful following the PCR amplification protocol as provided by Schuelke (2000). PCR cycling conditions followed Schuelke (2000) with 28 and then 8 PCR cycles, and annealing temperatures as shown in Table 1 for *C. rumphii* and *C. micronesica*, and of 53–51°C for all other species. We used an ABI 3730 × l for genotyping and GENEMAPPER 3.5 software (Applied Biosystems) for fragment analysis.

A blastx search against the Genbank NR peptide sequence database (E-20) showed that 7 of the 12 match genes with known functions including metabolic genes and transcription factors (Table 1).

Population statistics were implemented only for *C. rumphii* and *C. micronesica* in ARLEQUIN 3.11 (EXCOFFIER 2005) (Table 1). Locus 248 and 232 were monomorphic for *C. rumphii* and *C. micronesica*, respectively, and were excluded from analyses within each species. Departure from Hardy-Weinberg equilibrium (HWE) in *C. rumphii* was detected only in locus 226 for one locality ($P < 0.01$). In *C. micronesica*, loci 226, 238, 282 deviated significantly from HWE in one or maximum two localities ($P < 0.01$). There was no evidence for scoring error due to stuttering and no evidence for large allele dropout according to MICROCHECKER 2.2.3 (Oosterhout et al. 2004). The comparison of observed genotypes with the distribution of randomized genotypes was significant for some loci ($P < 0.01$), which could be explained by null alleles. Linkage disequilibrium was not observed on either species based on Fisher's test implemented in GENEPOP ON THE WEB (Raymond and Rousset 1995). The number of alleles for *C. rumphii* and *C. micronesica* ranged from 2 to 7, and from 5 to 15, respectively. Observed and expected heterozygosities ranged from 0.000 to 0.800 and 0.000 to 0.805 in *C. rumphii*, and 0.000 to 0.842 and 0.000 to 0.883 in *C. micronesica* (Fig. 2).

The levels of polymorphism of the four congeners and the population genetic patterns of wild populations of *C. micronesica* are currently investigated to enlighten the evolutionary and biogeographic history of this genus. The fact that three of the tested species reside in the *C. rumphii* complex, and three in the *C. pectinata* complex, indicates that the utility of our markers is potentially far-reaching. We are also characterizing current ex situ collections to select potential seeds for re-introduction in areas where *C. micronesica* will likely go locally extinct.

Table 1 Characteristics of EST-microsatellite loci in *Cycas rumphii* and *Cycas micronesica*

Locus	Primer sequence (5′-3′)	Repeat motif	T (°C)	Allele range	*Na* (*C. rumphii*/*C. micronesica*)	*Ho* (*C. rumphii*/*C. micronesica*)	*He* (*C. rumphii*/*C. micronesica*)	Protein
CY226*,**	F:ACAGGGCATCGGAACACTAC R:CTACTCTTCGGCTTCCAACG	(TA)9	55–53	228–265	5/15	0.125/0.231	0.664/0.858	Putative membrane transporter
CY232	F:TCTTGCTTACCCGTTTGCTT R:CTCCTCGACGTTCAATCACA	(GT)9(GCGT)3	56–54	230–240	3/1	0.600/0.000	0.460/0.000	None
CY238*,**	F:TGCCCATTGATTTTGGTTTT R:AAATTTGCTGATTCGGCTTC	(GA)24	55–53	240–270	6/9	0.286/0.130	0.786/0.626	None
CY240**	F:ATTGCGGAACGAATATCGAC R:TATCGCGAGGCCATAGGTAG	(AT)9	56–53	185–220	2/13	0.000/0.313	0.320/0.883	Ubiquitin/Zinc finger, C3HC4 type
CY248**	F:TCACAATGCCTTCCAGATCA R:TGTGAAGGAAGTTGGCTGTG	(AT)10(GA)14	53–51	200–220	1/5	0.000/0.100	0.000/0.545	MYB transcription factor MYB172
CY250	F:ATGAACAAGCGGCTGAGTCT R:CCCACCCTCTTTCTCTCTCC	(AT)8	53–51	200–250	6/10	0.400/0.545	0.800/0.717	NADH dehydrogenase subunit 5
CY266**	F:AAATGCTTTGATGTTCCCAAA R:ATGCAATGCTCAACAAGCTG	(AT)9	56–54	210–270	6/8	0.667/0.400	0.750/0.828	None
CY270	F:CGGATTTGGAGGTTCAAAGA R:CAGTTTGTATAGCTGAACAAGAATAGA	(TG)11	55–53	170–180	5/7	0.571/0.611	0.551/0.772	None
CY272**	F:TGGTGTGTATTTTGCATTTTCA R:TGGGCATGGAAAACAAGTTAG	(TC)11	55–53	260–280	7/6	0.750/0.200	0.805/0.534	NADH dehydrogenase subunit 6
CY280	F:CAGAGACTATTCGGGCCAAG R:TCAAACCCTTCCACACATCA	(TA)25	56–54	170–190	3/7	0.800/0.842	0.640/0.735	Sex hormone-binding globulin
CY282*	F:TTCCTGGAGTGAATAGAGACAGG R:TGGCATTGAGTTTGAATGAA	(TG)3(AT)11(GA)14	56–54	200–210	7/9	0.625/0.778	0.773/0.785	Catalytic enzyme
CY284**	F:TTTGGTCCACGTTACCATGA R:TCAACGGCGTCTAGTTGTTG	(TA)16	53–51	200–210	3/8	0.200/0.100	0.340/0.663	Allene oxide synthase

*Significant departure from Hardy-Weinberg Equilibrium ($P < 0.01$); **Null alleles may be present ($P < 0.01$); T (°C), Annealing temperature for the first and second cycle; *Na*, number of different alleles; *Ho*, observed heterozygosity, *He*, expected heterozygosity; Protein—blastx hit using default parameters in the nr peptide sequence database

Fig. 2 Female sporophylls and seeds (left) and male reproductive cones (right) covered in insect scale (white) as they are typically found in infected populations

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References

Borenstein A, Mortimer JA, Schofield E et al (2007) Cycad exposure and risk of dementia, MCI, and PDC in the Chamorro population of Guam. Neurology 68:1759–1761

Brenner ED, Stevenson DW, McCombie RW et al (2003) Expressed sequence tag analysis in *Cycas*, the most primitive living seed plant. Genome Biol 4:R78

Cox PA, Banack S, Murch S, Sacks O (2006) Commentary on: return of the cycad hypothesis-does the amyotrophic lateral sclerosis/Parkinsonism dementia complex (ALS/PDC) of Guam have new implications for global health? Neuropathol App Neuropathol 32:679–682

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. Evol Biol Online 1:47–50

Hill K, Stevenson D (2006) The cycad pages http://plantnet.rbgsyd.nsw.gov.au/PlantNet/cycad/index.html08/15/2007. Accessed 11 April 2007

Hill KD, Stevenson DW, Osborne R (2004) The world list of cycads. In: Waters T (ed) Cycad classification: concepts and recommendations. CABI publishing, Wallingford, Oxon

Ince P, Codd GA (2005) Return of the cycad hypothesis-does the amyotrophic lateral sclerosis/parkinsonism dementia complex (ALS/PDC) of Guam have new implications for global health? Neuropathol App Neuropathol 31:345–353

Mamay S (1969) Cycads: fossil evidence of late paleozoic origin. Science 164:295–296

Marler T, Haynes J, Lindström A (2006) *Cycas micronesica*. In: IUCN 2006. 2006 IUCN red list of threatened species. http://www.iucnredlist.org. Accessed 11 April 2007

Norstog K, Nicholls TJ (1997) The biology of the cycads. Comstock Pub. Associates, Ithaca, New York

Oosterhout C, Hutchinson WF, Wills DPM, Shipley P (2004) MicroChecker: software for identifying and correcting genotyping errors in microsatellite data. Mol Ecol Notes 4:535–538

Raymond M, Rousset F (1995) Genepop: population genetic software for exact tests and ecumenicism. J Heredity 86:248–249

Rozen S, Skaletsky H (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) Bioinformatics methods and protocols: methods in molecular biology. Humana Press, New Jersey

Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. Nat Biotech 18:233–234

Stinchcombe JR, Hoekstra HE (2007) Combining population genomics and quantitative genetics: finding the genes underlying ecologically important traits. Heredity 1–13. doi: 10.1038/sj.hdy.6800937

Temnykh S, DeClerck G, Lukashova A et al (2001) Computational and experimental analysis of microsatellites in rice (Oryza sativa L.): frequency, length variation, transposon associations, and genetic marker potential. Cold Spring Harbor Laboratory Press, New York