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厚壳桂新微卫星体分子标记的开发与应用

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摘 要:生境破碎化是导致全球生物多样性危机的主要原因,即使常见物种也会受到很大影响。厚壳桂(*Cryptocarya chinensis*)是我国南亚热带季风常绿阔叶林群落演替顶极物种,由于这一森林植被的破坏而呈片断化分布。在以往的研究中,我们利用筛选到的一些微卫星体对其遗传多样性研究后发现其可能以无性生殖为主。为进一步证实这一发现,该文又报道了 11 个新微卫星体,这些新微卫星体和以往报道的微卫星体将有助于更好分辨厚壳桂的有性和无性生殖,特别是在空间小尺度(Fine spatial scale)。选取鼎湖山 21 个厚壳桂个体,对这 11 个新微卫星体进行多态性检测。结果表明,这些微卫星体位点所包含的等位基因数目为 2~3 个,观察杂合度(Observed heterozygosities)和期望杂合度(Expected heterozygosities)分别为:0.048~1.000,0.048~0.535。11 个位点中有 8 个偏离哈迪—温伯格平衡,FIS 在 8 个位点表现为负值,并极显著。所有两两位点在 0.05 水平上均表现出连锁不平衡,但这一不平衡在使用 Bonferroni 校正(Bonferroni correction)后不显著。研究同时发现 21 个厚壳桂个体中 20 个有相同的多位点基因型(multilocus genotype)。上述遗传多样性结果符合无性生殖物种的遗传特征,表明厚壳桂以无性生殖为主。

关键词:无性生殖;保育;森林片断化;遗传标记

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Isolation and characterization of new set of microsatellite loci in *Cryptocarya chinensis*

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Abstract: Habitat fragmentation is the main threat to global biodiversity. Common species could also greatly suffer from habitat fragmentation. As a result of the destruction of the monsoon evergreen broad-leaved forest in South China, *Cryptocarya chinensis*, one of the climax species, is now patchily distributed. Because a few microsatellite loci reported previously might cause low resolution to detect asexual reproduction in this species, we reported a new set of microsatellite which will assist to detect the extensiveness of sexual and asexual reproduction at fine spatial scale in

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the future. Eleven microsatellite markers from repetitive DNA enriched libraries for *C. chinensis* were developed. Twenty-one individuals from Dinghu Mountain in South China were used to characterize their polymorphism. The number of alleles of 11 loci ranged from two to three, observed and expected heterozygosities ranged from 0.048 to 1.000, and 0.048 to 0.535, respectively. Deviations from Hardy-Weinberg equilibrium were detected in 8 loci due to heterozygote excess (highly significant negative Fis). All locus pairs showed significant linkage disequilibrium at the 0.05 significant level, but such significance disappeared after Bonferroni correction. 20 of 21 detected individuals showed identical multilocus genotype. All these results conformed to the genetic characteristic of clonal species, confirming extensive clonal growth in *C. chinensis*.

Key words: asexual reproduction; conservation; forest fragmentation; genetic marker

Habitat fragmentation is the main threat to global biodiversity. Alike to rare and endemic species, common species could also greatly suffer from habitat fragmentation (Hooftman *et al.*, 2003; Jump & Peñuelas, 2006). As a successional climax species, *Cryptocarya chinensis* (Lauranceae) is the main component of typical monsoon evergreen broad-leaved forest in lower subtropical China (Peng, 1996). As a result of the destruction of the monsoon evergreen broad-leaved forest, it is now patchily distributed and an ideal system to study the influence of forest fragmentation on the population genetics of forest species. Surprisingly, our previous study revealed unexpected clonal growth in *C. chinensis* in fragmented forest (Wang *et al.*, 2007, 2008). Because only eight previous microsatellite loci might cause low resolution to detect sexual growth in this species, we reported here new set of microsatellite which will assist to detect the extensiveness of sexual and asexual growth in *C. chinensis* at fine spatial scale in the future.

C. chinensis is a forest-dwelling species and occupies the second or third level of the tree layer. Its hermaphroditic flowers form paniculate inflorescences and are pollinated by insects. Seeds of *C. chinensis* are dispersed by vertebrates (e. g. birds and small mammals) and gravity (Wang *et al.*, 2003).

Genomic DNA was extracted from one dry leaf tissue by using CTAB method (Doyle, 1991). Approximately 250 ng of the total genomic DNA was digested by a restriction enzyme MseI (NEB) and the resulting fragments ligated with MseI adaptor (5′-TACTCAGGACTCAT-3′/5′-GACGATGAGTCCTGAG-3′) with T4 ligase (NEB) overnight at 16 ℃. The digestion-ligation mixture was subsequently diluted 10 times, and 2 μL was used for PCR amplification using adaptor-specific primers (5′-GATGAGTCCTGAGTAAN-3′, i. e. MseI-N). PCR products hybridized to a 5′ biotin-labeled oligonucleotide probe (GA) 15. Subsequent probe-bound DNA fragments were enriched for GA repeats using streptavidin-coated magnetic beads (NEB). Enriched fragments were recovered with PCR amplification using MseI-N as primer. PCR products were then ligated into the pGEM-T plasmid vector (Promega), and transformed into the *Escherichia coli* DH5α competent cells (Takara). The PCR-based method described by Lunt *et al.* (1999) was used to screen the recombinant clones. Identified positive clones were sequenced by United Gene Holdings, LTD (Shanghai, China) with M13R or M13F as primer. Primers were designed using OLIGO 6.54 software (MBI) for the sequences contain microsatellite repeats.

Polymorphisms of these micosatellite loci were assessed by 21 *C. chinensis* individuals, newly collected from Dinghu Mountain, Guangdong Province, China. PCR amplification were performed in 10 μL reaction mixtures, consisting of approximately 5 ng of template DNA, 50 mmol/L KCl, 20 mmol/L Tris-HCl (pH 8.0), 1.5 mmol/L $MgCl_2$, 0.5 μmol/L of each primer, 0.2 mmol/L of each dNTP, and 1U of Taq DNA polymerase (Takara). The reaction mixture was subjected to PCR amplification in a PTC-100 (MJ) using a PCR program, 4 min at 95°, followed by 35 cycles of 94 ℃ for 30 s, 52-60 ℃ (depending on locus) annealing tempera-

ture for 30 s, and 72 ℃ for 45 s, followed by 10 min at 72 ℃. PCR products were then resolved on 6% denaturing polyacrylamide gels and visualized by silver staining.

Observed heterozygosity (*HO*), the unbiased expected heterozygosity (*HE*) and fixation index (F_{IS}) were calculated using GDA 1. 1 (Lewis & Zaykin, 2001). Deviations from Hardy-Weinberg equilibrium (*HWE*) for each locus and genotypic linkage disequilibrium (*LD*) between all pairs of loci were tested using FSTAT 2. 9. 3 (Goudet, 1995).

The number of alleles per locus varied from 2 to 3, observed and expected heterozygosities ranged from 0. 048 to 1. 000, and 0. 048 to 0. 535, respectively (Table 1). Deviations from *HWE* were detected in 8 loci due to highly significant negative F_{IS}. All locus pairs showed significant *LD* ($P < 0.05$) without Bonferroni correction.

Table 1　Details of microsatellite loci in *Cryptocarya chinensis* including locus name, forward and reverse primer sequences, repeat motif, annealing temperature (*Ta*), numbers of alleles (*A*), observed/expected heterozygosities (*HO*/*HE*), fixation index (F_{IS}) by Weir & Cockerham's (1984), significant value of *HWE* (P-value of HWE), GenBank accession number

Locus	Primer sequence (5′-3′)	Repeat	*Ta* (℃)	*A*	*HO*	*HE*	F_{IS}	P-value of HWE	GenBank accession number
256	F:GTTCCCAAGAAACCGATAGAA R:GTAGTAGTGAATGGCGTAG	(AG)20	58	2	0.048	0.048	0.000	0.911	GU117095
185	F:CCAAATGGCAATATGCAA R:CTAACACTATCCACGCATCTC	(CT)21	52	3	1.000	0.535	-0.909**	0.000	GU117096
225-2	F:GGGTTTGTTTGGGCGGTGAG R:GAAGGTGGGCCATCGCATCAG	(GA)16	60	2	0.048	0.048	0.000	0.911	GU117097
155	F:CTGTTTATTCATTCATAAGGG R:CCAGTTGCTGCAAATCCAA	(TC)20	54	3	1.000	0.535	-0.909**	0.000	GU117098
212	F:GAACATGCGGAATGAG R:GTGTCTCCAACTGCTCGTTCA	(CT)22	56	2	0.952	0.511	-0.905**	0.000	GU117099
104	F:GGGTCACCATCCATTGCATCT R:GGACGCATGTCGAATCTATCT	(TC)22(AC)2	60	2	0.952	0.511	-0.905**	0.000	GU117100
170	F:TGCGCTTCTCTATCTT R:CTCACTTCTGCCATCAATCTC	(TC)19	52	3	1.000	0.535	-0.909**	0.000	GU117102
353	F:GCTTCTCTGCCTACTATGCCT R:CCATTTCACTGCTCCAACCAT	(TC)16	60	2	0.048	0.048	0.000	0.911	GU117103
268	F:GGAAATATGGGAAATAGTCCT R:GCGCAGACGTGCTAAC	T10GT(TC)19	60	2	0.952	0.511	-0.905**	0.000	GU117104
172	F:GGCCATGAATGCAATAA R:TCTGCAACGAAACCCTAGAAG	(AG)6AT(AG)4	52	2	0.952	0.511	-0.905**	0.000	GU117105
174	F:GGGCGAATCTTATATT R:GGACTTGGTAACTGGCTAACA	Complex[a]	52	3	1.000	0.535	-0.909**	0.000	GU117106

[a]: $T_{23}C(CTT)_4CCTT(CCT)_8(CT)_{16}$; ** $P<0.01$ after Bonferroni correction

Unlike its congener *Cryptocarya concinna*, which distributes in the same Dinghu Mountain, showed no clonal growth by using microsatellite markers (Wang *et al.*, 2009), *C. chinensis* showed unexpected clonal growth in our previous study (Wang *et al.*, 2007, 2008). The new set of microsatellite confirmed this. Although no linkage disequilibrium occurred between any locus pairs after Bonferroni correction, eight of total eleven loci showed highly significant negative F_{IS} value. Genotypic diversity of G/N (where G was number of observed multilocus genotypes, N was number of sample size) was 0. 048, much lower than previous reported G/N value in clonal species (see references in Wang *et al.* 2008). In fact, total twenty individuals showed the same identical mutiloci genotype. Strong negative F_{IS} values (meaning excess of heterozygote), high occurrence of identical genotypes (G/N values) confirmed growth mode of *Cryptocarya chinensis* is predominantly clonal (Balloux *et al.*, 2003; Halkett *et al.*, 2005).

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