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Source: Applications in Plant Sciences, 5(7)
Published By: Botanical Society of America
URL: https://doi.org/10.3732/apps.1700037
**DEVELOPMENT OF MICROSATellite MARKERS FOR Eurya acuminatissima (Theaceae)**

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**Primer Note**

**Premise of the study:** Sixteen microsatellite markers were developed to study the fine-scale spatial genetic structure of *Eurya acuminatissima*, a dioecious tree species of Theaceae endemic to southern China.

**Methods and Results:** A total of 30 primer pairs were initially designed and tested on the basis of the transcriptome data of *E. acuminatissima*, of which 16 were successfully amplified and showed clear polymorphism. For these microsatellites, one to 17 alleles per locus were identified. The observed and expected heterozygosities ranged from 0 to 1.000 and 0 to 0.903, respectively.

**Conclusions:** The microsatellite markers described here can be used to study genetic diversity and spatial genetic structure of *E. acuminatissima*. Furthermore, all loci were successfully cross-amplified in a related species, *E. auriformis*.

**Key words:** *Eurya acuminatissima*; microsatellite marker; Theaceae; transcriptome.

_Eurya_ Thunb., a genus in the family Theaceae, is mainly distributed in tropical and subtropical Asia, including the southern and western Pacific Islands (Ling, 1998; Ming and Bartholomew, 2007). There are about 83 species in China, of which 63 are endemic (Ming and Bartholomew, 2007). *Eurya* species are dioecious, insect-pollinated, and bird-dispersed small trees that constitute an important component in forests from low to middle elevations. To date, little is known about the genetic diversity, spatial genetic structure, reproductive biology, and ecological adaptations of species in the genus (Chung and Epperson, 2000; Wang et al., 2014; Mishio and Kawakubo, 2015). In particular, microsatellite markers for genetic analysis in the genus *Eurya* are not available.

_Eurya acuminatissima_ Merr. & Chun, a species endemic to China, grows in forests on mountain slopes or in valleys from 200–1200 m and is a common component in the understory of old-growth and secondary evergreen broad-leaved forests in southern China. In this study, we developed 16 nuclear microsatellite markers for our ongoing research project regarding *E. acuminatissima*, in which we are investigating its genetic diversity and spatial genetic structure in a typical evergreen broad-leaved forest mountain area of southern China. We also tested the transferability of these markers in a congeneric species, *E. auriformis* H. T. Chang.

**METHODS AND RESULTS**

Total RNA of *E. acuminatissima* was extracted from fresh leaves of one seedling using an improved cetrimidethylammonium bromide (CTAB) method (Fu et al., 2005). The seedling was collected from Heishiding Nature Reserve, Guangdong Province, China (23°27′37.39″N, 111°54′9.78″E). Transcriptome sequencing of *E. acuminatissima* was conducted using the Illumina HiSeq 2500 system (Illumina, San Diego, California, USA). In total, 16,323,790 nucleotide paired-end reads were obtained and assembled into 143,640 nonredundant unigenes with an N50 length of 610 nucleotides using Trinity (Grabherr et al., 2011). The reads were then deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Accession (accession no. SRR512705). The redundant sequences were removed by CAP3 (Huang and Madan, 1999) with the criterion of a minimum identity of 99%.

All unigenes obtained in the study were used to screen for the presence of microsatellites using MISA (Thiel et al., 2003), with the criteria of a minimum of six, five, five, and five repeat units for di-, tri-, tetra-, penta-, and hexanucleotide motifs, respectively. Altogether, 23,872 simple sequence repeat (SSR) motifs were detected. Using Primer3 (Rozen and Skalesky, 1999), 30 primer pairs were designed on the basis of randomly selected SSR motifs with the optimum conditions set at a length of 22–25 bp and a product size range of 100–500 bp.

Genomic DNA was isolated from silica-dried leaves of 83 individuals from three populations of *E. acuminatissima* and 27 individuals from one population of its congener *E. auriformis* using the DNA Extraction Kit (Magen, Guangzhou, China) following the manufacturer’s protocol. All specimens are deposited at the Herbarium of Sun Yat-sen University (SYSU), Guangdong, China (Appendix 1). In the first PCR trial, five individuals were randomly selected from each population of *E. acuminatissima* to amplify the 30 primer pairs. PCR amplifications were performed according to Xie et al. (2015), except for the annealing temperatures as indicated in Table 1 for 45 s. PCR products were visualized in a 6% polyacrylamide gel with a 10-bp DNA ladder marker. Sixteen primer pairs produced PCR products with clear and polymorphic bands among the 15 individuals. The sequences of microsatellite loci were deposited into GenBank (Table 1).

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1 Manuscript received 16 April 2017; revision accepted 19 May 2017.

The authors thank Y. Li and W. Ye for their assistance in collecting plant materials. This work was supported by grants from the National Natural Science Foundation of China (grant no. 3130344, J1210074), the Natural Science Foundation of Guangdong Province (2015A030313136), and the Fundamental Research Funds for the Central Universities (grant no. 14lgpy20).

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doi:10.3732/app.1700037


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The 16 microsatellite loci showed successful transferability in its congeneric species. E. acuminatissima microsatellites were useful to investigate the genetic diversity and population structure of this species. We are currently using these markers to investigate fine-scale spatial genetic structure and to estimate gene flow among populations of E. acuminatissima in a 50-ha plot in Heishiding Nature Reserve, Guangdong Province, China. The successful transferability of these markers in its congeneric species E. acuminatissima suggests that they may be useful in studies of other related species in Eurya.

**LITERATURE CITED**

**CONCLUSIONS**

The 16 microsatellites of E. acuminatissima reported here are useful to investigate the genetic diversity and population structure of this species. We are currently using these markers to investigate fine-scale spatial genetic structure and to estimate gene flow among populations of E. acuminatissima in a 50-ha plot in Heishiding Nature Reserve, Guangdong Province, China. The successful transferability of these markers in its congeneric species E. acuminatissima suggests that they may be useful in studies of other related species in Eurya.

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Table 2. Results of initial primer screening of 16 microsatellite loci developed in Eurya acuminatissima in three populations of E. acuminatissima and one population of E. auriformis.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Zhaoming (N = 30)</th>
<th>Huizhou (N = 28)</th>
<th>Yingde (N = 25)</th>
<th>E. auriformis (N = 27)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>H₀</td>
<td>Hₑ</td>
<td>F₀</td>
</tr>
<tr>
<td>Ea-1374</td>
<td>10 0.733 0.863 0.150</td>
<td>14 0.571 0.893 0.360***</td>
<td>9 0.600 0.802 0.252***</td>
<td>10 0.630 0.850 0.259***</td>
</tr>
<tr>
<td>Ea-1519</td>
<td>14 0.900 0.884 −0.018</td>
<td>4 0.321 0.544 0.409***</td>
<td>7 0.600 0.729 0.177</td>
<td>4 0.074 0.173 0.571***</td>
</tr>
<tr>
<td>Ea-1597</td>
<td>4 0.167 0.214 0.221</td>
<td>2 0.000 0.137 1.000***</td>
<td>2 0.000 0.077 1.000***</td>
<td>3 0.077 0.500 0.846**</td>
</tr>
<tr>
<td>Ea-1885</td>
<td>8 0.483 0.773 0.375***</td>
<td>5 0.000 0.621 1.000***</td>
<td>4 0.040 0.252 0.841</td>
<td>7 0.038 0.752 0.949***</td>
</tr>
<tr>
<td>Ea-46130</td>
<td>14 0.600 0.898 0.332***</td>
<td>8 0.036 0.842 0.958***</td>
<td>10 0.200 0.794 0.748</td>
<td>10 0.296 0.842 0.648***</td>
</tr>
<tr>
<td>Ea-46976</td>
<td>14 0.767 0.845 0.093***</td>
<td>10 0.571 0.767 0.177</td>
<td>10 0.296 0.842 0.648***</td>
<td></td>
</tr>
<tr>
<td>Ea-47249</td>
<td>4 0.400 0.58 0.373**</td>
<td>3 0.036 0.49 0.913***</td>
<td>3 0.000 0.538 1.000***</td>
<td></td>
</tr>
<tr>
<td>Ea-47797</td>
<td>4 0.276 0.392 0.296</td>
<td>3 0.464 0.420 −0.105</td>
<td>3 0.320 0.422 0.242</td>
<td>5 0.148 0.267 0.445***</td>
</tr>
<tr>
<td>Ea-48527</td>
<td>14 0.333 0.407 0.181</td>
<td>4 0.393 0.457 0.140</td>
<td>4 0.400 0.425 0.058</td>
<td>4 0.296 0.680 0.564***</td>
</tr>
<tr>
<td>Ea-31862</td>
<td>2 0.133 0.124 −0.071</td>
<td>3 0.393 0.493 0.203**</td>
<td>2 0.320 0.269 −0.190</td>
<td>3 0.037 0.204 0.818***</td>
</tr>
<tr>
<td>Ea-33987</td>
<td>3 0.107 0.427 0.749***</td>
<td>3 0.179 0.166 −0.073</td>
<td>5 0.080 0.288 0.722***</td>
<td></td>
</tr>
</tbody>
</table>
| Ea-804    | 5 0.600 0.554 −0.083 | 4 0.286 0.559 0.489*** | 2 0.080 0.211 0.621** | 5 0.481 0.501 0.038
| Ea-27742  | 1 0.000 0.000 −0.000 | 1 0.000 0.000 −0.000 | 2 0.080 0.077 −0.042 | 2 0.037 0.036 −0.019
| Ea-24991  | 3 0.333 0.335 0.005 | 4 0.214 0.197 −0.087 | 4 0.320 0.388 0.130 | 2 0.259 0.266 −0.149
| Ea-97085  | 2 1.000 0.500 −1.000*** | 6 1.000 0.586 −0.707*** | 2 1.000 0.500 −1.000*** | 7 0.850 0.701 −0.263*** |
| Ea-98287  | 17 0.893 0.878 −0.017 | 15 0.963 0.903 −0.067* | 14 0.952 0.880 −0.082 | 12 0.778 0.807 0.037

Note: A = number of alleles; F = fixation index; Hₑ = expected heterozygosity; Hₑ = observed heterozygosity; N = number of individuals analyzed.

*Locality and voucher information are provided in Appendix 1.

**Significant deviations from Hardy–Weinberg equilibrium after sequential Bonferroni corrections: *** represents significance at the 0.1% nominal level; ** represents significance at the 1% nominal level; * represents significance at the 5% nominal level.

APPENDIX 1. Voucher and location information for the species and populations used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Population code</th>
<th>Voucher no.</th>
<th>Collection locality</th>
<th>Geographic coordinates</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eurya acuminatissima</td>
<td>Merr. &amp; Chun</td>
<td>Shaqing L58081</td>
<td>Shenhiding Nature Reserve, Shaqing, Guangdong, China</td>
<td>23°27′37.39″N, 111°54′9.78″E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shaqing L162103</td>
<td>Nankunsun Nature Reserve, Huizhou, Guangdong, China</td>
<td>23°38′17.60″N, 113°50′47.79″E</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Shaqing L162101</td>
<td>Shimentai Nature Reserve, Yingde, Guangdong, China</td>
<td>24°23′38.18″N, 113°09′4.66″E</td>
</tr>
<tr>
<td>E. auriformis H. T. Chang</td>
<td></td>
<td>Shaqing L162105</td>
<td>Nankunsun Nature Reserve, Huizhou, Guangdong, China</td>
<td>23°38′17.60″N, 113°50′47.79″E</td>
</tr>
</tbody>
</table>

Note: N = number of individuals sampled.

*Voucher specimens are deposited at the herbarium of Sun Yat-sen University (SYSU), Guangzhou, China.