DEVELOPMENT OF MICROSATELLITE MARKERS FOR Eurya acuminatissima (Theaceae)\(^1\)

Xianggang Shi\(^2\), Haidan Wu\(^2\), Hongxian Xie\(^2\), Yi Zheng\(^2\), Yelin Huang\(^2,3\), and Shixiao Yu\(^2,3\)

\(^2\)State Key Laboratory of Biocontrol and Guangdong Provincial Key Laboratory of Plant Resources, School of Life Sciences, Sun Yat-sen University, Guangzhou 510275, Guangdong, People’s Republic of China

- 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 cetyltrimethylammonium 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 non-redundant 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 Archive (accession no. SRR5512705). 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, 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 Skaletsky, 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).

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. 31300344, J1210074), the Natural Science Foundation of Guangdong Province (grant no. 31300344, J1210074), and the Fundamental Research Funds for the Central Universities (grant no. 14lgpy20).

2 Authors for correspondence: llssyx@mail.sysu.edu.cn (S. Y.), llsshy1@mail.sysu.edu.cn (Y. H.)

doi:10.3732/apps.1700037

Applications in Plant Sciences 2017 5(7): 1700037; http://www.bioone.org/loi/apps © 2017 Shi et al. Published by the Botanical Society of America. This is an open access article distributed under the terms of the Creative Commons Attribution License (CC-BY-NC-SA 4.0), which permits unrestricted noncommercial use and redistribution provided that the original author and source are credited and the new work is distributed under the same license as the original.
The 16 polymorphic primer pairs were tested for polymorphisms in 83 individuals from three populations of *E. acuminatissima*. In addition, 27 individuals from one population were used to detect the efficiency of these markers in cross-species amplification. PCR products were analyzed using the ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, California, USA), resolved with an internal size standard (GeneScan 500 LIZ, Applied Biosystems). The peaks of the loci were read by Peak Scanner Software version 1.0 on the ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, California, USA).

**Table 1.** Characteristics of 16 microsatellite loci developed in *Eurya acuminatissima*.

| Locus   | Primer sequences (5’–3’) | Repeat motif | Allele size (bp) | Fluorescent label | GenBank accession no. | Putative function                              | E-value |
|---------|--------------------------|--------------|------------------|-------------------|----------------------|-----------------------------------------------|---------|
| Ea-1374 | F: TGGTTTTCTCTGTTGCTTTACT | (CT) \(_{18}\) | 290              | JOE               | KY807054             | Homeobox-leucine zipper protein ATBH-6 [Vitis vinifera] | 7e-25   |
|         | R: CTTGTTCTCTGTTGCTTTGT   |              |                  |                   |                      |                                               |         |
| Ea-1519 | F: TCTGAAAAACCTACACGCTACC | (CA) \(_{17}\) | 261              | FAM               | KY807055             | Zinc finger and BTB domain-containing protein 21 [Poreicia mexicana] | 1e-11   |
|         | R: GTGAAACACTGATCCTTTCGAG |              |                  |                   |                      |                                               |         |
| Ea-1597 | F: GACACATTCTCTAAGGCGGATA | (TTCT) \(_{5}\) | 289              | FAM               | KY807056             | Probable nucleolar protein 5-2 [Vitis vinifera] | 0.000   |
|         | R: AGGAAAGAAGAATCTGAAACCC |              |                  |                   |                      |                                               |         |
| Ea-1853 | F: CACACATTCTCTGAGACACTCA | (TCCC) \(_{6}\) | 453              | JOE               | KY807057             | BZIP transcription factor [Camellia sinensis] | 2e-111  |
|         | R: GCACCTCTACTCTCCTCTCTT  |              |                  |                   |                      |                                               |         |
| Ea-46130| F: TTACGTTGAAAGACGAGATG    | (AT) \(_{3}\)(AG) \(_{14}\) | 360              | JOE               | KY807058             | Chromatin modification-related protein EAF7 [Vitis vinifera] | 0.000   |
|         | R: CCCCTGGAAACATTATTTTCTC  |              |                  |                   |                      |                                               |         |
| Ea-46976| F: AGCAGAACCCTGATAGCTACA   | (GA) \(_{3}\) | 267              | JOE               | KY807059             | E3 ubiquitin-protein ligase SIS3 isoform X3 [Vitis vinifera] | 0.000   |
|         | R: GATGTTCTCTGTTGAGACAAAA  |              |                  |                   |                      |                                               |         |
| Ea-47249| F: ATTTGCTCATGTTGTCGATT   | (TTTC) \(_{4}\) | 210              | JOE               | KY807060             | Uncharacterized protein [Juglans regia] | 4e-174  |
|         | R: CTCACACCTCCTCTGAACTT    |              |                  |                   |                      |                                               |         |
| Ea-47797| F: CTTCACACCCCTCTTATATT    | (ATCG) \(_{4}\) | 403              | JOE               | KY807061             | Hypothetical protein [Citrus clementina] | 0.000   |
|         | R: CAATGGACGGACAGATATTAG   |              |                  |                   |                      |                                               |         |
| Ea-35287| F: TTTTATCGGCTCTTTCGGA     | (TTTC) \(_{6}\) | 160              | JOE               | KY807062             | RalA-binding protein 1 [Aedes albopictus] | 0.026   |
|         | R: GGTAGCAGAATCAGGAGAAAA   |              |                  |                   |                      |                                               |         |
| Ea-31862| F: CATCTCGTGGCTACTCAAGAG   | (TTCC) \(_{3}\) | 100              | FAM               | KY807063             | 3-ketocoy-CoA synthase [Ricinus communis] | 6e-11   |
|         | R: CGAAGGGGGAGGCGGTCTTATT  |              |                  |                   |                      |                                               |         |
| Ea-33987| F: ACAATTGCTGAGTGTGAATCG   | (CTCAT) \(_{5}\) | 205              | JOE               | KY807064             | Uncharacterized protein LOC100246622 isoform X2 [Vitis vinifera] | 2e-152  |
|         | R: TCATTGCAAGGGTGTTGTTATTT |              |                  |                   |                      |                                               |         |
| Ea-804  | F: GGAGATTTGGAAATATCAGAGAG | (TGTC) \(_{3}\) | 267              | JOE               | KY807065             | Uncharacterized protein [Theobroma cacao] | 2e-15   |
|         | R: CTCTAATGACCTGCTAGGCTCTACCTT  |              |                  |                   |                      |                                               |         |
| Ea-27742| F: CACCTCATGTTTCTGACGAAACA | (TTTA) \(_{3}\) | 135              | FAM               | KY807066             | MD21-interacting receptor like kinase 2 [latropha curcas] | 3e-08   |
|         | R: CAGTACCTCGGTTGCTCTGAGGTTT |              |                  |                   |                      |                                               |         |
| Ea-24991| F: CAAAATGTTGTCACACGTTGTAGT | (TCTT) \(_{3}\) | 339              | JOE               | KY807067             | Oligosaccharide repeat unit polymerase Wzy [Streptococcus pneumoniae] | 0.26   |
|         | R: CTAGGAGACACCTCTGAGTACACA |              |                  |                   |                      |                                               |         |
| Ea-97085| F: CTGCTGTCTGTTGTTGCTT    | (TGC) \(_{3}\) | 215              | JOE               | KY829135             | Hypothetical protein [Monoraphidium neglectum] | 2e-14   |
|         | R: CTGCTGTTGCTTGGTTGCTT    |              |                  |                   |                      |                                               |         |
| Ea-98287| F: GAAACAGCTGCTCGGGAAGAAC | (CT) \(_{3}\) | 161              | JOE               | KY829136             | Uncharacterized protein, transcript variant X2 [Macaca nemestrina] | 2e-16   |
|         | R: CTGCTGCTGCTGCTGCTGCTGCT |              |                  |                   |                      |                                               |         |

**Note:** \( T_a \) = annealing temperature.

**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. auriformis* suggests that they may be useful in studies of other related species in *Eurya*.

**LITERATURE CITED**

CHUNG, M. G., and B. K. EPPERSON. 2000. Clonal and spatial genetic structure in *Eurya emarginata* (Theaceae). *Heredity* 84: 170–177.

FU, X., Y. HUANG, S. DENG, R. ZHOU, G. YANG, X. NI, W. LI, and S. SHI. 2005. Construction of a SSH library of *Aegiceras corniculatum* under salt stress and expression analysis of four transcripts. *Plant Science* 169: 147–154.

GRABIERRE, M. G., B. J. HAAS, M. YASSOUR, J. Z. LEVIN, D. A. THOMPSON, I. AMIT, X. ADONIS, et al. 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotechnology* 29: 644–652.

HUANG, X., AND A. MADAN. 1999. CAP3: A DNA sequence assembly program. *Genome Research* 9: 868–877.
### 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.*

| Locus     | Huizhou (N = 28) | Yingde (N = 25) | E. auriformis (N = 27) |
|-----------|------------------|-----------------|------------------------|
|           | A                | H<sub>e</sub>   | H<sub>b</sub>   | F<sup>b</sup>  | A                | H<sub>e</sub>   | H<sub>b</sub>   | F<sup>b</sup>  | A                | H<sub>e</sub>   | H<sub>b</sub>   | F<sup>b</sup>  |
| Ea-1374   | 10               | 0.733           | 0.863           | 0.150          | 9                | 0.600           | 0.802           | 0.252***       | 10               | 0.630           | 0.850           | 0.259**        |
| Ea-1519   | 14               | 0.900           | 0.884           | −0.018         | 7                | 0.600           | 0.792           | 0.177          | 4                | 0.074           | 0.173           | 0.571***       |
| Ea-1597   | 4                | 0.167           | 0.214           | 0.221          | 2                | 0.000           | 0.137           | 1.000***       | 3                | 0.077           | 0.500           | 0.846**        |
| Ea-1853   | 8                | 0.483           | 0.773           | 0.375***       | 5                | 0.000           | 0.621           | 1.000***       | 4                | 0.040           | 0.252           | 0.841          |
| Ea-46130  | 14               | 0.600           | 0.898           | 0.332***       | 8                | 0.036           | 0.842           | 0.958***       | 10               | 0.200           | 0.794           | 0.748          |
| Ea-46976  | 14               | 0.767           | 0.845           | 0.093***       | 10               | 0.571           | 0.767           | 0.255***       | 8                | 0.560           | 0.745           | 0.248          |
| Ea-47249  | 4                | 0.400           | 0.638           | 0.373**        | 3                | 0.036           | 0.409           | 0.913***       | 5                | 0.000           | 0.538           | 1.000***       |
| Ea-7797   | 4                | 0.276           | 0.582           | 0.373**        | 3                | 0.464           | 0.420           | −0.105         | 3                | 0.320           | 0.422           | 0.242          |
| Ea-35287  | 4                | 0.333           | 0.407           | 0.181          | 4                | 0.393           | 0.457           | 0.140          | 4                | 0.400           | 0.425           | 0.058          |
| Ea-38162  | 2                | 0.133           | 0.124           | −0.071         | 3                | 0.393           | 0.493           | 0.203**        | 2                | 0.320           | 0.269           | −0.190         |
| Ea-33987  | 3                | 0.107           | 0.427           | 0.749***       | 3                | 0.179           | 0.166           | −0.073         | 5                | 0.080           | 0.288           | 0.722***       |
| Ea-3804   | 5                | 0.600           | 0.554           | −0.083         | 4                | 0.286           | 0.559           | 0.489***       | 2                | 0.080           | 0.211           | 0.621**        |
| Ea-27742  | 1                | 0.000           | 0.000           | −             | 1                | 0.000           | 0.000           | −             | 2                | 0.080           | 0.077           | −0.042         |
| Ea-24991  | 3                | 0.333           | 0.335           | 0.005          | 4                | 0.214           | 0.197           | −0.087         | 3                | 0.320           | 0.368           | 0.130          |
| Ea-97085  | 2                | 1.000           | 0.500           | −1.000***      | 6                | 1.000           | 0.586           | −0.707***      | 2                | 1.000           | 0.500           | −1.000***      |
| Ea-98287  | 17               | 0.893           | 0.878           | −0.017         | 15               | 0.963           | 0.903           | −0.067*        | 14               | 0.952           | 0.880           | −0.082         |

**Note:** A = number of alleles; F = fixation index; H<sub>e</sub> = expected heterozygosity; H<sub>b</sub> = observed heterozygosity; N = number of individuals analyzed.

*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.

---

**References:**

LING, L. K. 1998. Theaceae (Ternstroemiaioideae). In L. K. Ling [ed.], Flora Reipublicae Popularis Sinicae, vol. 50(1), 70–176. Science Press, Beijing, China.

MING, T. L., and B. BARTHOLOMEW. 2007. Theaceae. In Z. Y. Wu and P. H. Raven [eds.], Flora of China. vol. 12. 447–478. Science Press, Beijing, China, and Missouri Botanical Garden Press, St. Louis, Missouri, USA.

MISHER, M., and N. KAWAKURA. 2015. Variations in leaf morpho-anatomy and photosynthetic traits between sun and shade populations of *Eurya japonica* (Pentaphylacaceae) whose seeds are dispersed by birds across habitats. *Plant Species Biology* 30: 147–158.

PEAKALL, R., and P. E. SMOUSE. 2012. GenAlEx 6.5: Genetic analysis in Excel. Population genetic software for teaching and research–An update. *Bioinformatics* (Oxford, England) 28: 2537–2539.

ROUSSET, F. 2008. *GENEPOP'007*: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8: 103–106.

---

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

| Species              | Population code | Voucher no.       | Collection locality                | Geographic coordinates | N |
|----------------------|-----------------|-------------------|-----------------------------------|------------------------|---|
| *Eurya acuminatissima* |                 |                   |                                   |                        |   |
| Merr. & Chun          |                 |                   |                                   |                        |   |
| Zhaoping              |                 | Shixg150801        | Heishiding Nature Reserve, Zhaoping, Guangdong, China | 23°27′37.39″N, 111°54′9.78″E | 30 |
| Huizhou               |                 | Shixg161203        | Nankunshan Nature Reserve, Huizhou, Guangdong, China | 23°38′17.60″N, 113°50′47.79″E | 28 |
| Yingde                |                 | Shixg161210        | Shimentai Nature Reserve, Yingde, Guangdong, China | 24°23′38.18″N, 113°09′4.66″E | 25 |
| *E. auriformis*       |                 |                   |                                   |                        |   |
| H. T. Chang           |                 | Shixg161205        | Nankunshan Nature Reserve, Huizhou, Guangdong, China | 23°38′17.60″N, 113°50′47.79″E | 27 |

**Note:** N = number of individuals sampled.

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