Twenty-Seven Low-Copy Nuclear Primers for Lindera obtusiloba (Lauraceae): A Tertiary Relict Species in East Asia

Authors: Ye, Jun-Wei, Li, Qin, Tian, Xiang-Yu, Bao, Lei, Wang, Hong-Fang, et al.

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Twenty-seven low-copy nuclear primers for Lindera obtusiloba (Lauraceae): A Tertiary relict species in East Asia

Jun-Wei Ye, Qin Li, Xiang-Yu Tian, Lei Bao, Hong-Fang Wang, and Jian-Ping Ge

2Natural History Research Center of Shanghai Natural History Museum, Shanghai Science and Technology Museum, Shanghai 200127, People’s Republic of China; 3State Key Laboratory of Earth Surface Processes and Resource Ecology and Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing 100875, People’s Republic of China; 4Center for Watershed Ecology, Nanchang University, Nanchang 330031, People’s Republic of China; and 5Institute of Biodiversity Science, Fudan University, Shanghai 200438, People’s Republic of China

Premise of the study: To investigate a more detailed evolutionary history of Lindera obtusiloba (Lauraceae) and other Lindera species, polymorphic low-copy nuclear primers were developed.

Methods and Results: Unigenes of the L. obtusiloba transcriptome greater than 800 bp in length were randomly chosen for initial design of 168 primers. Agarose gel electrophoresis and Sanger sequencing were used to select low-copy nuclear genes. Twenty-seven primers were obtained and were used to investigate genetic diversity in 90 individuals from 24 populations. The nucleotide diversity ranged from 2.11 × 10⁻³ to 8.99 × 10⁻³, and haplotype diversity ranged from 0.57 to 0.97. These primers were also cross-amplified in L. aggregata, L. chintii, L. erythrocarpa, and L. glauca; up to 15 primers were successfully amplified in these related species.

Conclusions: This methodology is effective for development of low-copy nuclear primers. The 27 primers developed here will be useful for evolutionary studies of L. obtusiloba and other Lindera species.

Key words: Lauraceae; Lindera; Lindera obtusiloba; low-copy nuclear gene; transcriptome.

Lindera obtusiloba Blume (Lauraceae) is a deciduous plant distributed in both northern and southern floral regions of the Tertiary relict flora in East Asia (Donoghue et al., 2001; Milne and Abbott, 2002). These two regions harbor two distinct L. obtusiloba genealogies that were probably triggered by the intermediate arid belt (Ye et al., 2017), providing a perfect system to investigate the floral subdivision of the East Asian Tertiary relict flora and the effect of the west-east–oriented arid belt. Only four chloroplast fragments and six nuclear microsatellites were used in Ye et al. (2017), limiting a detailed evolutionary history inference within each floral region. The nuclear microsatellites used in Ye et al. (2017) were designed for L. melissifolia (Walter) Blume (Echt et al., 2006) or L. benzoin (L.) Blume (Edwards and Niesenbaum, 2007); therefore, in this study, we aimed to design species-specific low-copy nuclear primers for L. obtusiloba.

Transcriptome sequences are widely used in studies of plant evolutionary history (e.g., Ai et al., 2015) and can be used for development of low-copy nuclear primers (Bai and Zhang, 2014). For example, Higashi et al. (2015) developed eight primers using 100 expressed sequence tag (EST) markers of Ericaceae, and the phylogeny of Shortia Raf. was inferred through these primers. In this study, the transcriptome data of L. obtusiloba were used to develop low-copy nuclear primers, and these primers were cross-amplified in other Lindera Thunb. species.

METHODS AND RESULTS

Two L. obtusiloba leaves were collected in the populations XRD and TMSH (Appendix 1) and used for transcriptome sequencing. Total RNA was extracted using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), and the NEBNext Ultra RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, Massachusetts, USA) was used to generate sequencing libraries. An index code was added to each sample. TruSeq PE Cluster Kit v3-cBot-HS (Illumina, San Diego, California, USA) on a cBot Cluster Generation System was used to cluster the index-coded samples. The Illumina HiSeq 2500 platform was used to sequence the libraries and generate paired-end reads.
## Table 1: Characteristics of the 27 *Lindera obtusiloba* low-copy nuclear loci.

| Locus | Primer sequences (5′-3′) | Length (bp) | Tm (°C) | GenBank accession no. | Exon (bp) | Intron (bp) | Putative function | Closest species | E-value |
|-------|-------------------------|-------------|----------|----------------------|-----------|-------------|------------------|-----------------|---------|
| 2AP   | ACTGGTACTTCTTGTGTTG     | 810         | 56       | MF152421             | 1–181; 778–810 | 182–777 | CSC1-like protein ERD1 (LOC104588785) | Nelumbo nucifera | 0       |
| 2DA   | CACAGACCTGGCTCTGCCAGTG  | 581         | 60       | MF152429             | 405–581   | 1–404 | Uncharacterized LOC103717698 (LOC103717698) | Phoenix dactylifera | 3E-35   |
| ACY   | GCTTCTGGTTCAAGAGTTGTT   | 944         | 52       | MF152435             | 1–451; 902–944 | 452–901 | ACO-coenzyme A oxidase 2, peroxisomal (LOC100090398) | Juglans regia | 0       |
| BAE   | TGACAGATTGACTGTTGA      | 629         | 60       | MF152452             | 141–155   | 1–140; 552–629 | Sucrose galactosyltransferase 2 (LOC104597400) | Nelumbo nucifera | 0       |
| COD1  | TGCGGGCTCATGAGAGAGT    | 154         | 56       | MF152461             | 1–154    | 1–154 | Flavonone-3-hydroxylase (F3H) gene | Persea americana | 0       |
| FASP  | CAGGCTGACTCTGGTGAC      | 626         | 56       | MF152466             | 71–225    | 1–70; 226–627 | Omega-6 fatty acid desaturase, chloroplastic-like (LOC104603036) | Nelumbo nucifera | 0       |
| GPN   | AAGGCAAGTCTGAATTACCA    | 516         | 48       | MF152474             | 1–23; 203–366; 457–516 | 24–202; 367–456 | Hsp70 nucleotide exchange factor FES1 (LOC100266149) | Vitis vinifera | 0       |
| HET   | GGACAGCCCCCTAGAAAT     | 612         | 56       | MF152477             | 580–612   | 1–579 | Heterogeneous nuclear ribonucleoprotein R (LOC109822129) | Asparagus officinalis | 2E-114  |
| HIST  | AATGACACCTCCTCACTAC     | 228         | 56       | MF152485             | 128–228   | 1–127 | Histone deacetylase 14 (LOC103720526) | Phoenix dactylifera | 0       |
| HPT   | CTACATGCTTCTCCTTTT     | 394         | 52       | MF152490             | 1–67     | 68–394 | Uncharacterized LOC104604799 (LOC104604799) | Nelumbo nucifera | 7E-126  |
| HYPO  | TCATCATGCTCTCACTACG    | 305         | 52       | MF152499             | 1–305    | 1–305 | Vesicle-associated protein 1-3-like (LOC103960124) | Pyrus bretscheri | 4E-103  |
| INTE  | TGCAAGAACACAGGGAGAG    | 324         | 48       | MF152505             | 1–47; 169–324 | 48–168 | Proton pump-interactor 1-like (LOC109013106) | Juglans regia | 4E-85   |
| ISOM  | AAAGGCCCTAAACCTCGTT    | 341         | 48       | MF152511             | 313–341   | 1–312 | Protein disulphide-isomerase A6 (LOC105032579) | Elaeis guineensis | 0       |
| LEP2  | GCATGGATATCCTGGTAC     | 397         | 56       | MF152517             | 1–53; 144–275; 369–391 | 54–143; 276–368 | F-box family protein 14 (LOC102437954) | Vitis vinifera | 0       |
| LG3   | GGGTGTGGTGAGGGTTTGA    | 472         | 56       | MF152522             | 191–462   | 1–190; 463–472 | Transcinnamate 4-monooxygenase (LOC104593756) | Nelumbo nucifera | 0       |
| LPD   | CGCGGACGTTGGTTTAAGG    | 187         | 56       | MF152530             | 1–187    | 1–187 | N-succinylaminopimelate aminotransferase DapC (LOC104882468) | Nelumbo nucifera | 0       |
| MALA  | GTGCAGGATCACTGAAGG     | 328         | 56       | MF152532             | 93–172; 296–328 | 1–92; 173–295 | Malate dehydrogenase [NADP], chloroplastic-like (LOC104587331) | Nelumbo nucifera | 0       |
| MPD   | CACAGACCTAACGAGAAGCT   | 622         | 48       | MF152537             | 471–621   | 1–470; 622 | Ankyrin repeat domain-containing protein 2A-like (LOC104592662) | Nelumbo nucifera | 0       |
| PENT  | TCAGGACATTACGGCACTCG   | 456         | 56       | MF152540             | 1–37; 288–456 | 38–287 | Tetrahexapeptide repeat-like superfamily protein | Cinnamonomum camphora | 0       |
| POR1  | ATGACACCTCTCTGTTCAAG   | 417         | 56       | MF152544             | 1–102; 215–417 | 103–219 | Mitochondrial outer membrane protein porin of 34 kDa (LOC108992813) | Juglans regia | 3E-159  |
| PRUP  | GCCAGACCTGCTGGTGGCT    | 586         | 60       | MF152547             | 1–64; 154–184; 65–153;185–530 | 53–186 | Peroxiredoxin-2, mitochondrial (LOC108792220) | Prunus persica | 5E-96   |
| SPT2  | CTGGCAGAGTATCTTGTGGCT  | 497         | 56       | MF152552             | 1–62; 460–497 | 63–459 | F-box protein SKIP31-like (LOC104610293) | Nelumbo nucifera | 3E-175  |
| STOP  | GCAGCTCAGGCGGGAGTCT    | 600         | 60       | MF152560             | 569–600   | 1–568 | Phosphatidylinositol decarboxylase proenzyme m-2-like (LOC104599579) | Nelumbo nucifera | 0       |
| STP   | GCCGCGGTCATATCAGAGA    | 386         | 56       | MF152569             | 1–386    | 1–386 | Serine/threonine-protein kinase HT1 (LOC104592094) | Nelumbo nucifera | 0       |
| TDM   | ATCTCGTTGCTCGCTCCT    | 686         | 56       | MF159113             | 643–677   | 1–642; 678–686 | F-box protein PP2-A15 (LOC104612503) | Nelumbo nucifera | 1E-168  |
reads. The raw reads were cleaned by removing reads containing adapters, reads including more than 10% unknown base information, and reads with low quality. All clean reads were assembled by Trinity (v2012-10-05) (Grabherr et al., 2011). The transcriptome data can be accessed in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) (NCBI Resource Coordinators, 2017) under accession numbers SRR5888830 and SRR5892454. In total, 191,545 unigenes were obtained, and unigenes greater than 800 bp in length were randomly chosen for initial design of 168 primers. We BLASTed these unigenes in nucleotide collection (nr/nt) database using MEGABLAST (optimized for highly similar sequences) in the NCBI database. The exon position, intron length, and putative function were justified by the gene information of the closest gene in the NCBI database. Primer pairs were designed in separate exon regions using Primer Premier 5 (PREMIER Biosoft International, Palo Alto, California, USA). The loci with all nucleotide sites that exhibit fewer than two types of nucleotide variants were treated as low-copy nuclear loci. Low-copy nuclear loci were tested in 90 individuals sampled from 24 populations of L. obtusiloba (Appendix 2). After reading in CodonCode Aligner 3.6.1, PHASE function in DnaSP 5.10.01 (Rozas et al., 2003) was used to determine heterozygous and polymorphic sites, determine haplotypes, and to calculate genetic diversities, including nucleotide diversity (π) and haplotype diversity (H_s), of each locus. SPADS 1.0 (Dellicour and Mardulyn, 2014) was used to calculate haplotype π, and allele richness in 24 populations. Genotypic disequilibrium was assessed using all locus pairs in all populations by randomization using FSTAT 2.9.3 with Bonferroni correction (Goudet, 2001). Local BLAST function in BioEdit 7.1.9 (Hall, 1999) was used to determine the intron and exon positions of all low-copy nuclear loci, and the unigenes for primer design were used as database (Appendix S1). Low-copy nuclear genes were cross-amplified in two individuals of four other Lindera species, including L. aggregata (Sims) Kosterm., L. chunii Makino, and L. erythrocarpa, and 14 primers were successfully amplified in L. aggregata and L. obtusiloba (Appendix 2). No significant genotypic disequilibrium was observed among 351 locus pairs. Fifteen primers were successfully amplified in L. aggregata and L. erythrocarpa, and 14 primers were successfully amplified in L. chunii and L. glauca (Table 2).

CONCLUSIONS

Given that information regarding exon position and intron sequence are not included in transcriptome sequencing, the success rate of primer development using transcriptome data would be expected to be low (Bai and Zhang, 2014). In this study, we developed 27 polymorphic primers out of a set of 168 primers, with a ratio of approximately 16%. The success rate is increased twofold compared with that of Higashi et al. (2015). This methodology provides an effective approach for the development of new low-copy nuclear primers.

Twenty-seven novel polymorphic low-copy nuclear primers were developed using transcriptome data from L. obtusiloba. These primers can be used to investigate the evolutionary history of L. obtusiloba and other Lindera species.
Table 2. Genetic diversity and cross-amplification of the 27 *Lindera obtusiloba* low-copy nuclear loci.

| Locus | n  | V  | S  | P  | H  | \(H_d\) | \(\pi\) \((\times 10^{-3})\) | *Lindera aggregata* | *Lindera erythrocarpa* | *Lindera chunii* | *Lindera glauca* |
|-------|----|----|----|----|----|--------|-----------------|------------------|------------------|----------------|----------------|
| 2AP   | 82 | 41 | 11 | 30 | 31 | 0.88   | 8.04            | —                | —                | —              | —              |
| 2DA   | 84 | 44 | 4  | 40 | 19 | 0.88   | 8.99            | +                | +                | +              | +              |
| ACY   | 85 | 71 | 14 | 57 | 49 | 0.97   | 8.53            | +                | —                | +              | +              |
| BAE   | 86 | 36 | 11 | 25 | 28 | 0.84   | 8.86            | —                | +                | +              | —              |
| COD1  | 87 | 10 | 2  | 8  | 13 | 0.73   | 8.55            | +                | +                | —              | —              |
| FASP  | 88 | 44 | 18 | 26 | 23 | 0.80   | 6.72            | +                | +                | +              | +              |
| GPN   | 88 | 28 | 11 | 17 | 17 | 0.73   | 4.79            | —                | +                | +              | —              |
| HET   | 88 | 22 | 4  | 18 | 15 | 0.77   | 6.27            | +                | —                | —              | +              |
| HIST  | 85 | 20 | 7  | 13 | 14 | 0.79   | 7.39            | —                | +                | —              | —              |
| HPT   | 86 | 32 | 9  | 23 | 24 | 0.84   | 5.46            | +                | —                | +              | —              |
| HYPO  | 87 | 16 | 4  | 12 | 15 | 0.72   | 6.65            | —                | —                | —              | —              |
| INTE  | 88 | 23 | 8  | 15 | 15 | 0.71   | 8.78            | +                | +                | +              | +              |
| ISOM  | 87 | 16 | 5  | 11 | 13 | 0.75   | 5.39            | +                | —                | +              | +              |
| LEP2  | 81 | 20 | 10 | 10 | 18 | 0.68   | 5.16            | +                | —                | +              | —              |
| LG3   | 86 | 16 | 4  | 12 | 16 | 0.84   | 3.55            | +                | —                | —              | —              |
| LPD   | 89 | 6  | 0  | 6  | 5  | 0.57   | 3.71            | —                | +                | —              | —              |
| MALA  | 88 | 16 | 0  | 16 | 12 | 0.71   | 6.16            | +                | +                | —              | —              |
| MPD   | 86 | 20 | 2  | 18 | 16 | 0.82   | 6.27            | +                | —                | +              | —              |
| PENT  | 88 | 20 | 2  | 18 | 13 | 0.57   | 2.11            | —                | —                | —              | —              |
| PORI  | 88 | 27 | 11 | 19 | 12 | 0.68   | 6.75            | +                | +                | —              | —              |
| PRUP  | 88 | 22 | 4  | 18 | 21 | 0.85   | 4.75            | +                | —                | —              | —              |
| SPT2  | 87 | 30 | 8  | 22 | 22 | 0.81   | 5.24            | —                | +                | +              | —              |
| STOP  | 89 | 30 | 9  | 21 | 23 | 0.80   | 8.56            | —                | —                | —              | —              |
| STP   | 89 | 12 | 2  | 10 | 15 | 0.73   | 3.31            | —                | +                | +              | —              |
| TDM   | 87 | 40 | 11 | 29 | 21 | 0.80   | 5.29            | —                | +                | —              | —              |
| TPP   | 88 | 35 | 12 | 23 | 21 | 0.76   | 6.03            | —                | —                | —              | —              |
| VEST  | 88 | 28 | 12 | 16 | 28 | 0.82   | 4.22            | +                | —                | —              | —              |

_Note:_ — = unsuccessful amplification; + = successful amplification; \(H\) = haplotypes; \(H_d\) = haplotype diversity; \(\pi\) = nucleotide diversity; \(P\) = parsimony informative sites; \(S\) = singleton variable sites; \(V\) = variable sites.

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APPENDIX 1. Location and voucher information for Lindera species used in this study.

| Species                     | Population | Location                  | Latitude | Longitude | Voucher no.* |
|-----------------------------|------------|---------------------------|----------|-----------|--------------|
| Lindera obtusiloba Blume    | ANZH       | Anzihe Nature Reserve, Sichuan, China | 30.81    | 103.13    | SHM23259     |
|                            | BDGS       | Mt. Badagong, Hunan, China | 29.69    | 109.79    | SHM23260     |
|                            | BHSN       | Bukhansan National Park, Seoul City, Korea | 37.65    | 126.99    | SHM23261     |
|                            | BM         | Bomi, Xizang, China       | 29.87    | 95.73     | SHM23262     |
|                            | DAL        | Dalian, Liaoning, China   | 38.90    | 121.46    | SHM23263     |
|                            | DBSH       | Mt. Daba, Anhui, China    | 31.01    | 116.11    | SHM23264     |
|                            | JAP        | Tokyo, Japan              | 35.95    | 139.30    | SHM23074     |
|                            | JWS        | Gariwangsan, Gangwon Province, Korea | 37.43    | 128.56    | SHM23265     |
|                            | KI         | Mt. Iizumai, Japan        | 36.72    | 138.15    | SHM23266     |
|                            | KYSH       | Mt. Kunyu, Shandong, China | 37.26    | 121.73    | SHM23267     |
|                            | LAJ        | Lajing, Yunnan, China     | 26.49    | 99.28     | SHM23073     |
|                            | LISH       | Mt. Li, Shanxi, China     | 35.43    | 111.98    | SHM23268     |
|                            | MCSS       | Mt. Micang, Shannxi, China | 32.69    | 107.53    | SHM23269     |
|                            | NI         | Nikko, Japan              | 36.75    | 139.42    | SHM23270     |
|                            | PMA        | Pianma, Yunnan, China     | 25.99    | 98.66     | SHM23271     |
|                            | TMSS       | Mt. Tianmu, Zhejiang, China | 30.42    | 119.41    | SHM23070     |
|                            | UH         | Masada, Japan             | 34.55    | 132.04    | SHM23272     |
|                            | WEIX       | Weixi, Yunnan, China      | 27.18    | 99.29     | SHM23273     |
|                            | WYSH       | Mt. Wuyi, Jiangxi, China  | 27.93    | 117.69    | SHM23274     |
|                            | XRD        | Zhuanghe, Liaoning, China | 40.02    | 122.96    | SHM23071     |
|                            | XYSH       | Seoraksan National Park, Gangwon Province, Korea | 38.17    | 128.49    | SHM23275     |
|                            | XZD        | Xiaozhongdian, Yunnan, China | 27.34    | 99.84     | SHM23276     |
|                            | YTSN       | Mt. Yuntai, Jiangsu, China | 34.72    | 119.44    | SHM23277     |
|                            | ZYSH       | Mt. Jiri, South Gyeongsang Province, Korea | 35.29    | 127.49    | SHM23278     |
| Lindera aggregata (Sims) Kosterm. | TMSS | Mt. Tianmu, Zhejiang, China | 30.42    | 119.41    | SHM22266     |
| Lindera chunii Merr.       | DPSN       | Mt. Dinghu, Guangdong, China | 23.17    | 112.55    | SHM23280     |
| Lindera erythrocarpa Makino | KYSN       | Mt. Kunyu, Shandong, China | 37.26    | 121.73    | SHM23279     |
| Lindera glauca (Siebold & Zucc.) Blume | TMSS | Mt. Tianmu, Zhejiang, China | 30.42    | 119.41    | SHM23281     |

*Voucher specimens were deposited in Shanghai Natural History Museum (SHM), Shanghai, China.

APPENDIX 2. Genetic diversity in 24 populations of the 27 low-copy nuclear loci in Lindera obtusiloba.

| Population | n | No. of haplotypes | π (×10−3) | Allelic richness |
|------------|---|------------------|-----------|-----------------|
| ANZH       | 5 | 55               | 0.77      | 1.58            |
| BDGS       | 3 | 61               | 1.50      | 1.94            |
| BHSN       | 3 | 56               | 1.23      | 1.79            |
| BM         | 3 | 51               | 1.08      | 1.64            |
| DAL        | 3 | 41               | 1.14      | 1.43            |
| DBSH       | 5 | 60               | 1.25      | 1.71            |
| JAP        | 6 | 76               | 1.80      | 1.89            |
| JWS        | 5 | 53               | 1.09      | 1.51            |
| KI         | 3 | 59               | 1.80      | 1.92            |
| KYSH       | 3 | 52               | 1.39      | 1.70            |
| LAJ        | 5 | 70               | 1.48      | 1.83            |
| LISH       | 3 | 50               | 0.87      | 1.64            |
| MCSS       | 3 | 65               | 1.44      | 1.90            |
| NI         | 2 | 39               | 0.76      | 1.44            |
| PMA        | 4 | 56               | 1.29      | 1.69            |
| TMSS       | 5 | 55               | 1.25      | 1.72            |
| UH         | 6 | 68               | 1.10      | 1.68            |
| WEIX       | 3 | 59               | 1.60      | 1.85            |
| WYSH       | 3 | 49               | 1.13      | 1.63            |
| XRD        | 5 | 55               | 1.49      | 1.66            |
| XYSH       | 3 | 47               | 1.47      | 1.57            |
| XZD        | 3 | 55               | 1.26      | 1.73            |
| YTSN       | 3 | 47               | 1.00      | 1.58            |
| ZYSH       | 3 | 51               | 1.49      | 1.71            |

Note: n = number of individuals; π = nucleotide diversity.