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ISOLATION AND CHARACTERIZATION OF 30 MICROSATELLITE LOCI FOR *CUNNINGHAMIA LANCEOLATA* (TAXODIACEAE)¹

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- **Premise of the study:** To quantify the population-level genetic characteristics of *Cunninghamia lanceolata* (Taxodiaceae), an important timber conifer, we developed 30 pairs of microsatellite primers based on the nuclear genome.
- **Methods and Results:** Using the streptavidin-biotin capture system, we developed 14 polymorphic and 16 monomorphic microsatellites. Polymorphisms were detected in 14 loci using 94 individual trees that were collected from three *C. lanceolata* populations in Hubei and Zhejiang provinces and in Chongqing Municipality, China. There were three to 30 alleles per locus, and the observed and expected heterozygosities ranged from 0.0313–0.8333 and from 0.0313–0.9246, respectively. Cross-species amplification showed that two to seven polymorphic loci were functional in three of the five related species that were collected.
- **Conclusions:** Our newly developed microsatellite primers provide neutral molecular markers that are beneficial to future studies of population genetics and germplasm conservation of *C. lanceolata*.

Key words: cross-amplification; *Cunninghamia lanceolata*; genetic diversity; microsatellite; Taxodiaceae.

Cunninghamia lanceolata (Lamb.) Hook. (Taxodiaceae), known as Chinese fir, is an evergreen, outcrossing, and long-lived conifer that is widely distributed in southern China and northern Vietnam. Because of its relatively low nutrient demands, fast rate of growth, and strong resistance to corrosion and insect attacks, *C. lanceolata* is an important timber source that has been cultivated for more than 2000 yr (Yeh et al., 1994). Its present plantations cover about 4 million hectares accounting for 20–25% of the total commercial production of timber in China (Bao and Jiang, 1998; Huang et al., 2005). Understanding its genetic background is therefore critical to selecting germplasm resources and managing forests.

Some codominant molecular markers have been reported for *C. lanceolata*, e.g., 10 pairs of polymorphic microsatellite primers based on the nuclear genome (Li et al., 2015), 28 polymorphic expressed sequence tag–simple sequence repeat (EST-SSR) markers (Wen et al., 2013), and 97 polymorphic SSR loci based on transcript data (Xu et al., 2016). Microsatellite loci located in noncoding regions are neutral and usually show higher mutation rates than those located in encoding regions of the genome (Charlesworth et al., 1994). Neutral markers can be used to study population genetic diversity that is not related to adaptive

traits, and therefore can better reveal spatial genetic structure, gene flow, and historical events (e.g., bottlenecks and founder effects) that contribute to conservation of germplasm. However, detecting gene flow patterns, especially in fine-scale analyses such as parentage analyses, requires genetic resolution high enough to distinguish every individual and is thus dependent on a large number of neutral markers with high polymorphism. It is therefore necessary to develop more informative neutral molecular markers for *C. lanceolata*. Here, 14 polymorphic and 16 monomorphic microsatellite loci were isolated and characterized in the nuclear genome of *C. lanceolata* to facilitate future studies on population genetics and germplasm conservation.

METHODS AND RESULTS

Total genomic DNA was extracted from dried leaves of one *C. lanceolata* individual (located in Tiantong in Zhejiang Province, China [Appendix 1]) using the Plant Genomic DNA Extraction Kit (Tiangen, Beijing, China). Approximately 250 ng of DNA were digested with the restriction enzyme *Mse*I (New England Biolabs, Beverly, Massachusetts, USA), and fragments of 200–800 bp were fractionated. The fragments were linked with an *Mse*I-adaptor pair (F: 5'-TACTCAGGACTCAT-3'; R: 5'-GACGATGAGTCCTGAG-3'). The diluted products were amplified by an *Mse*I-N primer (5'-GATGGTCCTGAGTAAN-3') under the following conditions: an initial step at 95°C for 3 min, followed by 20 cycles of 30 s at 94°C, 1 min annealing at 53°C, and 1 min at 72°C. The products were hybridized with 5'-biotinylated probes (AG)₁₅ in a 250-μL reaction system at 48°C for 2 h. The hybridization products were adhered by streptavidin-coated magnetic beads (Promega Corporation, Madison, Wisconsin, USA). The washed and eluted DNA fragments were further amplified with the *Mse*I-N primer using the conditions given above for 30 cycles. The products were purified using a multifunctional DNA Extraction Kit (BioTeke, Beijing, China) and were ligated to a pMD19-T vector (TaKaRa Biotechnology Co., Dalian, China). Then, the products were transformed into *Escherichia coli*

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TABLE 1. Characterization of 14 polymorphic and 16 monomorphic microsatellite loci developed in *Cunninghamia lanceolata*.^a

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | A | T _a (°C) | Fluorescent dye ^b | GenBank accession no. |
|--------|--|--|------------------------|----|---------------------|------------------------------|-----------------------|
| CL22 | F: TGGTAGTACTCGCAGGAAAT R: CAGAGAATGGACACAAACAG | (GA) ₅ | 170 | 1 | 64 | HEX | KY769227 |
| CL34* | F: TGTCAAAGACTCCTGAGAAG R: GCAGCAATACACACACATAG | (CT) ₅ | 176–190 | 5 | 61 | 6-FAM | KY769205 |
| CL90* | F: GCAAAAAGAGGTCACACATAC R: ACTGCAAAGGAGATACGCTG | (AG) ₈ | 214–222 | 5 | 65 | TAMRA | KY769206 |
| CL108* | F: GAGGTATAGACTTTTACGGTTTG R: TCCTATGTGTCTAGTCATGTCC | (GA) ₇ | 108–124 | 6 | 64 | HEX | KY769207 |
| CL164 | F: GCTGGATGAACCTGAGTTGATT R: GAAGTTTCTCTTTTGCTTTGGC | (CT) ₁₂ | 124 | 1 | 63 | 6-FAM | KY769226 |
| CL255 | F: CAACAGAGATATGCGACA R: TTCACCTGTAAGGGTTTTC | (AG) ₂₁ | 118 | 1 | 63 | ROX | KY769229 |
| CL256 | F: GGTTGCTCAGTTTGGA R: TGTCCCTCTACCTCTCA | (AG) ₁₅ | 107 | 1 | 64 | ROX | KY769230 |
| CL278 | F: GAGACTAAGCCTGGGAGAG R: GAGAGGGGATCGAAAGAGA | (TC) ₅ | 114 | 1 | 65 | ROX | KY769231 |
| CL287 | F: GCAGCAATACACATACACAGAAT R: ACAAGTGTCAAAGACTCCTAAGAAG | (AG) ₉ | 169 | 1 | 65 | 6-FAM | KY769220 |
| CL288 | F: GCAGCAATACACAAACATAAG R: TACCGAATTGTCTCCAGATA | (AG) ₉ | 176 | 1 | 62 | 6-FAM | KY769221 |
| CL291 | F: AATGGAGATATGCGAGC R: AATACATTGTGCGGGTT | (AG) ₂₄ | 153 | 1 | 61 | ROX | KY769232 |
| CL295* | F: ATCATCAACACAATTAGCACCTGGAG R: GCAGATCATCGAACGTGAGTTAGCTT | (CT) ₈ | 157–163 | 4 | 65 | ROX | KY769208 |
| CL343* | F: TGCAAGTGTGAATAGAACCC R: GTAAAAGGGGAAAGGGAGT | (GA) ₂₁ | 197–311 | 30 | 61 | HEX | KY769209 |
| CL346 | F: ACACACAGAATGTAGGCGAG R: ATTGTGCAGGTTTGGAGT | (AG) ₁₇ | 113 | 1 | 65 | ROX | KY769233 |
| CL389* | F: GGAGATTGTAAATGGACTCTAGCC R: CTTTTTCTTGTCTTTCCGGAGGAGC | (AG) ₁₅ | 147–171 | 10 | 63 | 6-FAM | KY769210 |
| CL396 | F: CCATGTGCCTCACTCTC R: GGTTAGGGGTTTCCAGGTT | (CT) ₅ | 111 | 1 | 65 | ROX | KY769234 |
| CL540* | F: GGGTAGTGATCATGGAAGA R: AGAAAGCAGATATCGGTTG | (TC) ₁₀ A(CA) ₁₀ | 118–160 | 19 | 63 | ROX | KY769211 |
| CL564* | F: TGAACCTGAACTTGTGACTTAGC R: TACAAAACCTGTGGGCTTGATGATG | (CT) ₁₂ | 204–238 | 15 | 65 | HEX | KY769212 |
| CL586* | F: CAGCAAAGAAACGGTTATGGT R: GTTTTGTGGTATCCAACCTAGG | (TC) ₉ | 208–224 | 6 | 63 | 6-FAM | KY769213 |
| CL631 | F: TGGTGAGGAAGGATTGAGCCGACAG R: TCAGTTCCGGTTAGGCTCAGTACAC | (TG) ₅ | 273 | 1 | 65 | ROX | KY769222 |
| CL653 | F: AATGGAGGATATGCAAC R: ACCTGTAAGGGTTTTC | (AG) ₂₀ | 114 | 1 | 59 | ROX | KY769223 |
| CL654* | F: TCTCTCTCCCTTTGCTTACG R: CCATGCGTTGAAGAAGTATCG | (TC) ₈ | 126–144 | 9 | 63 | HEX | KY769214 |
| CL723* | F: ATCTCTGTCTTTGCACTCTC R: GGAATTATTGTTGGGGTTAGG | (TC) ₆ | 211–227 | 5 | 63 | TAMRA | KY769215 |
| CL753 | F: TAGAATCAACGCACACAGAAAGGC R: ACTCAAAAACATGACTCGGTAGC | (GA) ₉ | 232 | 1 | 65 | HEX | KY769236 |
| CL761 | F: CCTCTTATGACACATGGT R: TTTTCAGATGACTCTCGGA | (CT) ₆ | 138 | 1 | 59 | HEX | KY769224 |
| CL776* | F: ACTGCAAAGGAGATACGCTGAAGG R: GACGCAAAAGAGGTCCACAATACA | (TC) ₈ | 221–225 | 3 | 65 | TAMRA | KY769216 |
| CL783 | F: CTAGATACGAGTGTGGAAGA R: GCAATACACATACACACAGA | (TC) ₆ | 183 | 1 | 65 | HEX | KY769237 |
| CL852* | F: CTAGTGTCCAAAAAGAGCA R: GAGATATGAGTAGAATGAGG | (CT) ₉ | 136–148 | 7 | 63 | ROX | KY769217 |
| CL871 | F: TGGTCCGCGTTACAAGTATACATG R: ACTCTGCCCTTTTCACTATTCTGC | (GT) ₅ | 228 | 1 | 65 | HEX | KY769225 |
| CL878* | F: CAGGGTAGCCTTTGAAACA R: GGCTCCATATAACAACATC | (AG) ₈ | 147–163 | 7 | 64 | ROX | KY769218 |

Note: A = number of alleles; T_a = annealing temperature.

^aAll values are based on samples representing three populations located in Lichuan in Hubei Province, Lin'an in Zhejiang Province, and Wanxian in Chongqing Municipality, China.

^bFluorescent dyes (i.e., HEX, ROX, 6-FAM, and TAMRA) used for fragment analysis.

*Polymorphic microsatellite loci.

TABLE 2. Genetic properties of 14 newly developed polymorphic microsatellites of *Cunninghamia lanceolata*.^a

| Locus | Lichuan population | | | | Lin'an population | | | | Wanxian population | | | |
|-------|--------------------|----------|----------------------|----------------------|-------------------|----------|----------------------|----------------------|--------------------|----------|----------------------|----------------------|
| | <i>n</i> | <i>A</i> | <i>H_o</i> | <i>H_e</i> | <i>n</i> | <i>A</i> | <i>H_o</i> | <i>H_e</i> | <i>n</i> | <i>A</i> | <i>H_o</i> | <i>H_e</i> |
| CL34 | 30 | 4 | 0.2000* | 0.5847 | 25 | 3 | 0.2000* | 0.5200 | 29 | 4 | 0.2069* | 0.5850 |
| CL90 | 32 | 5 | 0.6563 | 0.6880 | 32 | 4 | 0.3750 | 0.5709 | 30 | 4 | 0.5667 | 0.6356 |
| CL108 | 32 | 5 | 0.1875 | 0.2336 | 32 | 4 | 0.3125 | 0.3021 | 30 | 3 | 0.2333 | 0.3203 |
| CL295 | 32 | 4 | 0.5938 | 0.6047 | 32 | 4 | 0.6563 | 0.5843 | 30 | 4 | 0.5000 | 0.5701 |
| CL343 | 32 | 16 | 0.1875* | 0.8750 | 32 | 17 | 0.1563* | 0.9246 | 29 | 14 | 0.2759 | 0.8209 |
| CL389 | 30 | 7 | 0.2667 | 0.3819 | 32 | 5 | 0.1250 | 0.1220 | 30 | 5 | 0.2000 | 0.1904 |
| CL540 | 29 | 15 | 0.3793* | 0.9165 | 29 | 14 | 0.5172* | 0.9201 | 28 | 12 | 0.5000 | 0.8935 |
| CL564 | 32 | 12 | 0.8125 | 0.8705 | 32 | 11 | 0.6250 | 0.6999 | 30 | 10 | 0.8333 | 0.8621 |
| CL586 | 31 | 3 | 0.1613 | 0.2089 | 32 | 6 | 0.2813 | 0.3105 | 30 | 4 | 0.2667 | 0.2706 |
| CL654 | 32 | 7 | 0.5625 | 0.6002 | 32 | 6 | 0.6250 | 0.6969 | 30 | 6 | 0.6333 | 0.6418 |
| CL723 | 32 | 5 | 0.0938* | 0.4454 | 32 | 3 | 0.1563* | 0.4043 | 30 | 4 | 0.1000* | 0.5028 |
| CL776 | 32 | 3 | 0.3438 | 0.3031 | 32 | 2 | 0.0313 | 0.0313 | 30 | 2 | 0.3667 | 0.3045 |
| CL852 | 32 | 4 | 0.2500 | 0.2555 | 32 | 6 | 0.4688 | 0.3973 | 30 | 4 | 0.6000 | 0.4729 |
| CL878 | 32 | 6 | 0.1875 | 0.2073 | 32 | 5 | 0.2813 | 0.4772 | 30 | 3 | 0.2000 | 0.1859 |

Note: *A* = number of alleles; *H_e* = expected heterozygosity; *H_o* = observed heterozygosity; *n* = number of individuals genotyped.

^aLocality and voucher information for the populations are available in Appendix 1.

*Indicates significant deviation from Hardy–Weinberg equilibrium (*P* < 0.05).

strain JM109 (TaKaRa Biotechnology Co.) through transient thermal stimulation following the TaKaRa *E. coli* JM109 competent cell protocol.

A total of 1400 colonies were selected and tested by PCR with (AG)₁₀ and M13⁺/M13⁻ as primers, producing 469 positive sequences. The positive PCR products were sequenced on an ABI 3730 DNA Sequence Analyzer (Applied Biosystems, Foster City, California, USA). We designed 263 primer pairs using the software Primer Premier 5.0 (PREMIER Biosoft International, Palo Alto, California, USA). The criteria for primer design were: (1) primer length between 18–28 bp and amplicon length between 100–300 bp; (2) melting temperature (*T_m*) in the range of 45–65°C; (3) GC content of the sequence between 40–60%, with no more than three Gs or Cs in the last five bases at the 3' end of the primer without mismatch or secondary structures. Polymorphisms were detected using 24 individuals selected randomly from three populations from Lichuan (Hubei Province, China), Lin'an (Zhejiang Province, China), and Wanxian (Chongqing Municipality, China), according to the method proposed by Schuelke (2000). The nested PCR used three primers: a sequence-specific forward primer with an M13(–21) tail (5'-TGAAAACGACGGCCAGT-3') at its 5'-end, a universal M13(–21) primer labeled with a fluorescent dye, and a sequence-specific reverse primer. In the first 30 cycles, the forward primer was incorporated into the PCR products. Then these products were marked with the fluorescently labeled M13(–21) primer, which was incorporated during the following eight cycles at 53°C. Each reaction was run in a 20-μL system containing the following: 40 ng of DNA, 1× PCR buffer, 2.0 mM Mg²⁺, 0.2 mM of dNTPs, 0.1 μM M13(–21) primer labeled with HEX, ROX, or 6-FAM (Sangon Biotech, Shanghai, China), 0.025 μM forward primer with an M13(–21) tail, 0.1 μM reverse primer, and 2 units *Taq* DNA polymerase (Sangon Biotech). Thermocycling conditions were as follows: 5 min of denaturation at 94°C; 30 cycles of 30 s at 94°C, 45 s at 59–65°C (Table 1), and 45 s at 72°C; followed by eight cycles of 30 s at 94°C, 45 s at 53°C, and 45 s at 72°C; and a 10-min extension at 72°C. We genotyped the amplification products on an ABI 3730 automated sequencer using GeneScan 500 LIZ Size Standard (Applied Biosystems), and alleles were identified using GeneMapper 4.0 software (Applied Biosystems). Among the 263 primer pairs tested, 186 were not amplified in any samples, 32 did not produce clear and single-target bands, 12 could not be successfully amplified in the individuals used for testing, and three were

duplicates of previous markers in Li et al. (2015). Finally, 14 polymorphic and 16 monomorphic microsatellite loci were obtained (Table 1).

All polymorphic loci were further characterized using 94 *C. lanceolata* individuals from the same three populations mentioned above. One of four fluorescent dyes (HEX, ROX, 6-FAM, TAMRA) labeled the forward primers. PCRs were set up in 10-μL reaction volumes containing 40 ng of template DNA, 1× PCR buffer, 2.5 mM Mg²⁺, 0.2 mM of each dNTP, 0.1 μM forward and reverse primer, and 1 unit of *Taq* DNA polymerase. We used the following conditions: 5 min of denaturation at 94°C; 30 cycles of 30 s at 94°C, 45 s at 61–65°C (Table 1), and 1 min at 72°C; and a final extension at 72°C for 10 min. The amplification products were scanned on an ABI 3730 sequencer using GeneScan 500 LIZ Size Standard (Applied Biosystems), and alleles were called and binned using GeneMapper 4.0 software (Applied Biosystems).

The number of alleles varied from three to 30 with an average of 9.4 using FSTAT 2.9.3 software (Goudet, 1995) (Table 1). The observed and expected heterozygosities ranged from 0.0313–0.8333 and 0.0313–0.9246, respectively, analyzed by the software TFPGA version 1.3 (Miller, 1997) (Table 2). After the sequential Bonferroni adjustment (Rice, 1989), only two loci (CL34 and CL723) displayed significant deviations from Hardy–Weinberg equilibrium (*P* < 0.05) in all three populations, and two loci (CL343 and CL540) deviated significantly from Hardy–Weinberg equilibrium in the Lichuan and Lin'an populations. No loci exhibited significant linkage disequilibrium after sequential Bonferroni adjustment by GENEPOP version 4.0 (Rousset, 2008). Null alleles were likely to be present in the loci CF34, CF343, CF389, CF540, and CF723 using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004).

We also tested the performance of 14 polymorphic primer pairs in five related species belonging to the same family (Taxodiaceae), using one to 10 individuals from each species (Table 3; Appendix 1). In *Metasequoia glyptostroboides* Hu & W. C. Cheng, successful amplifications occurred in seven loci (CL90, CL295, CL343, CL723, CL776, CL852, and CL878), of which three (CL90, CL723, and CL776) could also be amplified in *Cryptomeria fortunei* Hooibr. ex Otto & A. Dietr. and two (CL852 and CL878) in *Taxodium ascendens* Brongn. No polymorphic loci amplified successfully in *Cryptomeria japonica* (Thunb. ex L. f.) D. Don or in *Sequoia sempervirens* (D. Don) Endl. (Table 3). Unfortunately,

TABLE 3. Allele size ranges tested in five additional taxa for cross-amplification trials of SSR loci isolated from *Cunninghamia lanceolata*.

| Locus | <i>Cryptomeria japonica</i> (<i>n</i> = 1) | <i>Cryptomeria fortunei</i> (<i>n</i> = 5) | <i>Metasequoia glyptostroboides</i> (<i>n</i> = 10) | <i>Sequoia sempervirens</i> (<i>n</i> = 2) | <i>Taxodium ascendens</i> (<i>n</i> = 2) |
|-------|--|--|---|--|--|
| CL90 | — | 268 | 218–220 | — | — |
| CL295 | — | — | 159–165 | — | — |
| CL343 | — | — | 193 | — | — |
| CL723 | — | 261 | 237–247 | — | — |
| CL776 | — | 225 | 217–225 | — | — |
| CL852 | — | — | 142 | — | 142 |
| CL878 | — | — | 145–155 | — | 145 |

Note: — = primers could not be amplified.

we were unable to sample the only congeneric species of *C. lanceolata* (*C. konishii* Hayata), and thus the cross-amplification capability of these primers is likely underestimated.

CONCLUSIONS

Of the 30 markers reported here for *C. lanceolata*, 14 microsatellite loci showed a high level of polymorphism. These loci will be used to study population genetic diversity, gene flow, and mating systems. Combined with the previously isolated loci, these markers will facilitate the further investigation of parentage analyses and kinships between the planted and natural populations of *C. lanceolata*, all of which are relevant to germplasm conservation and forest management of this timber species.

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APPENDIX 1. Locality information of *Cunninghamia lanceolata* and its related species used in this study. Voucher specimens were deposited in East China Normal University (HSNU), Shanghai, China.

| Species | Population | Collection locality | Geographic coordinates | <i>N</i> |
|---|------------|--|-------------------------|----------|
| <i>Cunninghamia lanceolata</i> (Lamb.) Hook. | Tiantong | Tiantong, Zhejiang, China | 29°48'19"N, 121°47'43"E | 1 |
| | Lichuan | Lichuan, Hubei, China | 30°10'37"N, 108°37'03"E | 32 |
| | Lin'an | Lin'an, Zhejiang, China | 30°19'14"N, 119°26'04"E | 32 |
| | Wanxian | Wanxian, Chongqing, China | 30°39'43"N, 108°45'05"E | 30 |
| <i>Cryptomeria japonica</i> (Thunb. ex L. f.) D. Don | Shanghai | Shanghai Botanic Garden, Shanghai, China | 31°08'48"N, 121°26'50"E | 1 |
| <i>Cryptomeria fortunei</i> Hooibr. ex Otto & A. Dietr. | Shanghai | Shanghai Botanic Garden, Shanghai, China | 31°08'48"N, 121°26'50"E | 5 |
| <i>Sequoia sempervirens</i> (D. Don) Endl. | Nanjing | Nanjing Botanic Garden, Nanjing, China | 32°04'15"N, 118°48'25"E | 1 |
| | Hangzhou | Hangzhou Botanic Garden, Hangzhou, China | 30°15'19"N, 120°07'22"E | 1 |
| <i>Metasequoia glyptostroboides</i> Hu & W. C. Cheng | Lichuan | Lichuan, Hubei, China | 30°10'22"N, 108°39'32"E | 10 |
| | Nanjing | Nanjing Botanic Garden, Nanjing, China | 32°04'15"N, 118°48'25"E | 2 |

Note: *N* = number of individuals.