Development of chloroplast microsatellite markers for *Glyptostrobus pensilis* (Cupressaceae)

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PREMISE: *Glyptostrobus pensilis* (Cupressaceae) is a critically endangered conifer native to China, Laos, and Vietnam, with only a few populations remaining in the wild.

METHODS AND RESULTS: Using a complete chloroplast genome sequence, we designed 70 cpSSR loci and tested them for amplification success and polymorphism in 16 samples. Ten loci were found to be polymorphic and their genetic diversity was characterized using a total of 83 individuals from three populations in China. A total of 43 haplotypes were present, the effective number of alleles per locus ranged from one to eight, and the effective number of alleles ranged from 8.04 to 16.00. Gene diversity ranged from 0.81 to 0.97 (average 0.89). The number of alleles per locus and population ranged from one to eight, and the effective number of alleles ranged from 1.00 to 3.90. All polymorphic loci were successfully amplified in the related species *Cryptomeria japonica* var. sinensis, *Taxodium distichum*, *T. ascendens*, and *Cunninghamia lanceolata*.

CONCLUSIONS: These newly developed chloroplast microsatellites will be useful for population genetic and phylogeographic analyses of *G. pensilis* and related species.

KEYWORDS: chloroplast microsatellite (cpSSR); Cupressaceae; *Glyptostrobus pensilis*; haplotypes.

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**Glyptostrobus pensilis** (Staunton ex D. Don) K. Koch, the only extant species in the genus *Glyptostrobus* Endl., is a relict conifer in the family Cupressaceae (Hao et al., 2016). In China, it is mainly distributed in the Pearl River delta region of Guangdong Province, the central region of Fujian Province, the lower reaches of the Minjiang River, and northeastern Jiangxi Province (Li and Xia, 2004). A few wild populations have recently been found in Laos and Vietnam, extending its latitudinal distribution from 28°N to 13°N (Averyanov et al., 2009; Thomas and LePage, 2011). The species preferred habitat of riverbanks and flood plains have been severely degraded by human activities (e.g., agriculture and rice cultivation) in many locations, which has led to a rapid decline of most *G. pensilis* populations (Li and Xia, 2004, 2005; Nguyen et al., 2013). Currently, the International Union for the Conservation of Nature (IUCN) Red List of Threatened Species has evaluated *G. pensilis* as Critically Endangered (CR) (Thomas et al., 2011).

Chloroplast microsatellites have been widely used to investigate the population genetic structure and phylogeographic history of a range of tree species (Ruhsam et al., 2016; Gryta et al., 2017). Previous molecular studies of *G. pensilis* have only used nuclear markers such as intersimple sequence repeats (Li and Xia, 2005; Wu, 2011), and recently Wang et al. (2019) developed 10 polymorphic nuclear microsatellite markers for this species. Compared with nuclear simple sequence repeats (SSRs), chloroplast SSRS (cpSSRs) are more likely to detect historical bottlenecks or genetic drift due to their uniparental inheritance, slower mutation rate, and lack of recombination (Ennos et al., 1999; Pleines et al., 2009; Li and Liu, 2012). Nguyen et al. (2013) analyzed *G. pensilis* populations from Vietnam using six cpSSRs; however, these loci were developed from *Pinus thunbergii* Parl. and designed for use in Pinaceae species (Vendramin et al., 1996). In this study, we developed new species-specific chloroplast microsatellite loci using the complete chloroplast genome of *G. pensilis* (Hao et al., 2016). Additionally, we tested the transferability of these loci in four related species: *Cryptomeria japonica* (Thunb. ex L. f.) D. Don var. *sinensis* Miq., *Taxodium distichum* (L.) Rich., *T. ascendens* Brongn., and *Cunninghamia lanceolata* (Lamb.) Hook.

**METHODS AND RESULTS**

We searched the complete chloroplast genome of *G. pensilis* (Hao et al., 2016; GenBank accession number KU_302768) for...
microsatellite loci exhibiting a minimum of eight repeats as these loci are likely to exhibit a higher level of polymorphism (Ueno et al., 2012). For loci with a minimum of eight repeats, primers were designed using the online software Primer3Plus (Untergasser et al., 2007) using default parameters. In total, 70 cpSSR loci were selected and evaluated for their amplification efficiency and level of polymorphism using 16 *G. pensilis* DNA samples from different populations (Appendix 1). DNA was extracted from *G. pensilis* leaves using a modification of the cetyltrimethylammonium bromide (CTAB) method (Tsumura et al., 1995).

PCR amplification was carried out in volumes of 15 μL using the following protocol: 7.5 μL of 2× Taq PCR Master Mix (Tiangen, Beijing, China), 0.75 μL of forward primer (10 μM), 0.75 μL of reverse primer (10 μM), 3 μL of 20–50 ng/μL DNA template, and 3 μL of ddH2O. The mixture was then cycled using the following profile: 94°C for 4 min; 34 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and 7 min for extension. The software GenAlEx6.5 (Peakall and Smouse, 2012) was used to calculate the following parameters: number of alleles (*N*), effective number of alleles (*N*<sub>e</sub>), Shannon’s information index (*I*), and diversity (*H*)

A total of 43 haplotypes were detected in the three assayed populations. The number of haplotypes per population ranged from 11 to 18, the number of private haplotypes ranged from nine to 16, the effective number of haplotypes ranged from 4.55 to 13.36, the haplotypic richness ranged from 8.04 to 16.00, and the gene diversity ranged from 0.81 to 0.97 (Table 2). The number of alleles per locus ranged from one to eight per population, the effective number of alleles ranged from 1.00 to 3.90, Shannon’s information index ranged from 0.00 to 1.52, and the diversity ranged from 0.00 to 0.74 (Table 3). The 10 polymorphic loci could also be successfully amplified in five individuals in each of the following four related species: *Cryptomeria japonica* var. *sinensis*, *Taxodium distichum*, *T. ascendens*, and *Cunninghamia lanceolata* (Table 4, Appendix 1).

### CONCLUSIONS

In this study, we developed 10 polymorphic cpSSRs (as well as 55 pairs of monomorphic primers) that can be used to assess the

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**TABLE 1.** Characterization of 10 polymorphic chloroplast microsatellite loci developed in *Glyptostrobus pensilis.*

| Locus      | Primer sequences (5′–3′) | Location | Repeat motif | Allele size range (bp) | GenBank accession no. |
|------------|--------------------------|----------|--------------|------------------------|-----------------------|
| Gp<sub>cp</sub>_1 | F: (ROX) TGACACACCAGGTCTGTATCA <br> R: GCTTGTGTGCTGCTGTGTGGTT | ycf4 to psa1 | (AT)<sub>10</sub> | 261–271 | MK386658 |
| Gp<sub>cp</sub>_1 | <br> Gp<sub>cp</sub>_2 | F: (FAM) GCCTGGCCCTTGAGCTCATAT | trnl to trnF | (AT)<sub>11</sub> | 195–203 | MK386659 |
| Gp<sub>cp</sub>_1 | <br> Gp<sub>cp</sub>_2 | F: (HEX) ACCTGCTACAATCGACTTCCC <br> R: CCTCCTCTCCAGACGAGAACA | ycf3 to psaA | (T)<sub>11</sub> | 149–167 | MK386660 |
| Gp<sub>cp</sub>_2 | <br> Gp<sub>cp</sub>_8 | F: (HEX) TGACCGATGATCTTACGCTT <br> R: AAATGATCCGCTTGGCTCC | psb to trnE | (TA)<sub>9</sub> | 267–279 | MK386661 |
| Gp<sub>cp</sub>_11 | F: (TAMRA) ACCTCTGAAAGTCACTGAGTTAG <br> R: GCTAGACCTATCTCCTGAATAGAATAG | chIB to rps16 | (AT)<sub>25</sub> | 363–414 | MK386662 |
| Gp<sub>cp</sub>_12 | F: (TAMRA) TTAAATGCGAGATGCTGATG <br> R: TGCCCATAGGATGCCAAGTG | accD to clpP | (T)<sub>11</sub> | 379–437 | MK386663 |
| Gp<sub>cp</sub>_13 | F: (TAMRA) TGGGGAATCAAAAATACAGCTA <br> R: GCTTACAAAGTGAATGAAAATACGA | rbcL to accD | (AT)<sub>11</sub> | 208–334 | MK386664 |
| Gp<sub>cp</sub>_14 | F: (FAM) TCCCCGCAAGCATTACTGTTT <br> R: AGGAAAGATGTGATACTTGGCT | ccsA to petA | (T)<sub>12</sub> | 208–214 | MK386665 |
| Gp<sub>cp</sub>_17 | F: (FAM) AATCCTGAAAGTCACTGAGTTAG <br> R: AGAATGCGGTGTGTCCTCTTC <br> R: AATCCTGAAAGTCACTGAGTTAG | trnD to psbM | (T)<sub>12</sub> | 116–119 | MK386666 |
| Gp<sub>cp</sub>_35 | F: (HEX) TTTTCTCTCTACCGCAGACC <br> R: AATCCTGAAAGTCACTGAGTTAG | psaI to rpl33 | (A)<sub>9</sub> | 115–117 | MK386667 |

<sup>a</sup>Optimal annealing temperature was 55°C for all loci.
TABLE 3. Characteristics of 10 polymorphic chloroplast microsatellite markers in 83 individuals of three Chinese Glyptostrobus pensilis populations.a

| Locus | DM (N = 33) | GZHN (N = 21) | PNSL (N = 29) |
|-------|-------------|---------------|---------------|
|       | N_a | N_e | I     | H        | N_a | N_e | I     | H        | N_a | N_e | I     | H        |
| Gp_cp_1 | 3   | 1.824 | 0.765 | 0.452 | 2   | 1.960 | 0.683 | 0.490 | 2   | 1.890 | 0.664 | 0.471 |
| Gp_cp_6 | 2   | 1.198 | 0.305 | 0.165 | 3   | 2.194 | 0.852 | 0.544 | 3   | 1.324 | 0.479 | 0.245 |
| Gp_cp_7 | 3   | 2.139 | 0.883 | 0.533 | 3   | 2.945 | 1.071 | 0.649 | 3   | 1.979 | 0.779 | 0.495 |
| Gp_cp_8 | 5   | 1.280 | 0.437 | 0.219 | 5   | 2.110 | 0.832 | 0.526 | 1   | 1.000 | 0.000 | 0.000 |
| Gp_cp_11 | 116–117 | 114–117 | 115–116 | 116–117 | 116–117 | 114–117 | 115–116 | 116–117 | 116–117 | 114–117 | 115–116 | 116–117 |
| Gp_cp_12 | 378 | 378 | 384 | 432 | 378 | 378 | 384 | 432 | 378 | 378 | 384 | 432 |
| Mean | 3,400 | 1,594 | 0.632 | 0.331 | 3,200 | 2,448 | 0.939 | 0.571 | 1,800 | 1,250 | 0.247 | 0.147 |

Note: N = number of individuals sampled; N_a = number of alleles; N_e = effective number of alleles; I = Shannon’s information index; H = diversity.

A = number of alleles; N_e = effective number of alleles; I = Shannon’s information index; H = diversity.

*Locality and voucher information are provided in Appendix 1.

TABLE 4. Results of cross-amplification of 10 polymorphic chloroplast microsatellite markers developed for Glyptostrobus pensilis in four closely related species.a,b

| Locus | Taxodium distichum (N = 5) | Taxodium ascendens (N = 5) | Cryptomeria japonica var. sinensis (N = 5) | Cunninghamia lanceolata (N = 5) |
|-------|---------------------------|---------------------------|----------------------------------------|-------------------------------|
|       | 254–256                   | 250–254                   | 258–262                                | 254–265                       |
|       | 179                       | 179                       | 197                                    | 171                           |
|       | 149                       | 149                       | 177                                    | 177                           |
|       | 276                       | 276                       | 284                                    | 286                           |
|       | 378–383                   | 381–383                   | 375                                    | 371                           |
|       | 303–305                   | 303                       | 384                                    | 432                           |
|       | 205                       | 205                       | 211                                    | 213                           |
|       | 117                       | 117                       | 117                                    | 117                           |
|       | 116–117                   | 114–117                   | 115–116                                | 115–116                       |

Note: N = number of individuals sampled.

*Locality and voucher information are provided in Appendix 1.

Numbers shown represent the size in base pairs of the amplified fragments.

population genetic and phylogeographic structure of G. pensilis populations. The high number of private haplotypes in the three assayed populations suggests geographically isolated populations. Additionally, the 10 loci can be successfully amplified in four related species of G. pensilis.

ACKNOWLEDGMENTS

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DATA ACCESSIBILITY

All polymorphic primer sequences were uploaded to the National Center for Biotechnology Information (accession number: MK386658–MK386667; Table 1).

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APPENDIX 1. Sampling information for species in this study. All voucher specimens are deposited at the herbarium of Central South University of Forestry and Technology, Changsha, Hunan, China.

| Species | Population code | Voucher no. | Collection locality | Geographic coordinates | Elevation (m) | N |
|---------|----------------|-------------|---------------------|------------------------|--------------|---|
| *Glyptostrobus pensilis* (Staunton ex D. Don) K. Koch | PNSL | Lin170804 | Pingnan, Fujian, China | 27°0’27.87"N, 118°5’1’59.75"E | 1260 | 29 |
| | GZHN | Lin170411 | Guangzhou, Guangdong, China | 23°11’24.6"N, 113°21’23.18"E | 40 | 21 |
| | DM | Lin170729 | Doumen, Guangdong, China | 22°23’42.5"N, 113°15’14.65"E | 20 | 33 |
| *Taxodium distichum* (L.) Rich. | Li180522 | Changsha, Hunan, China | 28°8’16.48"N, 112°59’28.36"E | 90 | 5 |
| *Taxodium ascendens* Brongn. | Li180522 | Changsha, Hunan, China | 28°8’16.48"N, 112°59’28.36"E | 90 | 5 |
| *Cryptomeria japonica* (Thunb. ex D. Don var. sinensis Miq. | Wang180720 | Jujiang, Jiangxi, China | 29°32’59.77"N, 115°58’03.32"E | 911 | 5 |
| *Cunninghamia lanceolata* (Lamb.) Hook. | Li180522 | Changsha, Hunan, China | 28°8’16.48"N, 112°59’28.36"E | 90 | 5 |

Note: N = number of individuals sampled.

APPENDIX 2. Characteristics of 55 monomorphic chloroplast microsatellite primers developed in *Glyptostrobus pensilis*.

| Locus | Primer sequences (5’–3’) | Repeat motif | Product size (bp) |
|-------|--------------------------|--------------|------------------|
| Gp_cp_2 | F: ACAATTGATTTCTAAAAAGAGGAGGTCA | (A)<sub>11</sub> | 211 |
| Gp_cp_3 | R: TCAGTCGAGAATTGTTGCTGA | (T)<sub>13</sub> | 369 |
| Gp_cp_4 | F: ATAGATTCGGAGCGGCTTG | (T)<sub>18</sub> | 292 |
| Gp_cp_5 | R: ACCGCTGAGTTATATCCCTTCC | (T)<sub>20</sub> | 230 |
| Gp_cp_6 | F: ATTTCTGCGGAAAGGTTCGCA | (T)<sub>20</sub> | 332 |
| Gp_cp_7 | R: CGAGGCAAGTCCATCTTCTTAC | (T)<sub>10</sub> | 280 |
| Gp_cp_8 | F: CGAACCCTCACCTGATGTC | (A)<sub>15</sub> | 280 |
| Gp_cp_9 | R: GTTTGTTCACCTGAAATAAAGAGGA | (A)<sub>12</sub> | 257 |
| Gp_cp_10 | F: TCAAGGAAAGTGAGATGTGAG | (A)<sub>12</sub> | 201 |
| Gp_cp_11 | R: TCTCAACCTTCCATTGGGAG | (A)<sub>12</sub> | 145 |
| Gp_cp_12 | F: TCAGATTCGGAGCGGCTTG | (T)<sub>12</sub> | 201 |
| Gp_cp_13 | R: TGGGACACAAAGGAAAGTTAAGTGCT | (A)<sub>11</sub> | 260 |
| Gp_cp_14 | F: GCAAAAAGCTGCGGAAACAT | (A)<sub>11</sub> | 190 |
| Gp_cp_15 | R: GCCCTTCTCTCCTCCGACAT | (A)<sub>11</sub> | 190 |

(Continues)
## APPENDIX 2. (Continued)

| Locus     | Primer sequences (5′–3′) | Repeat motif | Product size (bp) |
|-----------|--------------------------|--------------|-------------------|
| Gp_cp_22  | F: AGGGGCAGAGAACTCAGGGTT | (A)11        | 194               |
|           | R: CGGGTCATTTTCCACGTTGAC |             |                   |
| Gp_cp_23  | F: ATCCGGCTTGTATCCTCGTTT | (A)11        | 263               |
|           | R: ACAGGCAGCTTGGAAAGAT    |             |                   |
| Gp_cp_24  | F: TCTTCTTGGCTATCCCTCCC  | (T)11        | 231               |
|           | R: AAGAATTAGTTTCCGAATGGGT |             |                   |
| Gp_cp_25  | F: TCTTCGGGATTAATCTTATCTCT | (T)11    | 264               |
|           | R: AATCCGTAGACGCTAAAACC   |             |                   |
| Gp_cp_26  | F: TTGTAGCCTACGTGGCAC     | (AT)13       | 263               |
|           | R: AGCATAAAAACAAAACAGGCT  |             |                   |
| Gp_cp_27  | F: CGGGGGAATGATACCTGTCG   | (T)10        | 138               |
|           | R: ACGGAGACCTTATATGAGCTC  |             |                   |
| Gp_cp_28  | F: TCCTGAATTCTTTCCACAG    | (TA)10       | 213               |
|           | R: TCCACTCTACCTCAGCTCCT   |             |                   |
| Gp_cp_29  | F: GAGCTTACCTGGGATCTGAGC  | (A)10        | 126               |
|           | R: CATTCCGTCTCGAGCTAAGTG  |             |                   |
| Gp_cp_30  | F: TGAATTTTCCCTCTATATGTGT | (A)10    | 201               |
|           | R: TAAATTTCTCTTATAGTTGAGGTGT |         |                   |
| Gp_cp_31  | F: CGGGGAAGAACTTGAGATGCTC | (A)10        | 233               |
|           | R: GCATATGCTGAGATGAGCTC   |             |                   |
| Gp_cp_32  | F: CCGGACGACGCTCAAGAGGA  | (A)10        | 157               |
|           | R: GGGATGTACTGTGGGATTGGC  |             |                   |
| Gp_cp_33  | F: ATTAGCGGGGATATTCCACCC  | (A)10        | 226               |
|           | R: CCGACCTGTGATTTCTGAGCT  |             |                   |
| Gp_cp_34  | F: ACGGGCGCTACAATTGGATA   | (A)10        | 143               |
|           | R: CCTACAGACGGGTAGATCTCGC |             |                   |
| Gp_cp_36  | F: TCATTATTTTCCACGGAATGAAACAT | (A)10    | 156               |
|           | R: GATGGCTCTATTTATCTCAGATTG |         |                   |
| Gp_cp_37  | F: ACCCAAAAAAGGAGGAACAGG  | (A)10        | 165               |
|           | R: GAATGACCTCTGGGATGGGAG  |             |                   |
| Gp_cp_38  | F: ACTGGGAGAATCTCCCATTGGA | (AT)6     | 221               |
|           | R: CAGGGGAGTATGCTAGTGTG   |             |                   |
| Gp_cp_39  | F: TCTTATGTTCTCTAGTAACAGCCTT | (A)6    | 125               |
|           | R: TGGAGTGGAGAAACATCCGT    |             |                   |
| Gp_cp_40  | F: ATGCTCTGTATCCGGGACC    | (A)10        | 248               |
|           | R: TGACCTGTGTCCATCACCCGACC |         |                   |
| Gp_cp_41  | F: CTGCACATCTCCTCCCTCTGT  | (T)10        | 162               |
|           | R: TGCTTTGCTACCTCCGGCAAT  |             |                   |
| Gp_cp_42  | F: TCGCATGTAAGGAAATCCA    | (A)10        | 211               |
|           | R: TTTCCCTTGAGCCATTTGGG   |             |                   |
| Gp_cp_43  | F: GGTGATGCGCTCGTCTGTTGA  | (A)10        | 186               |
|           | R: TGTAACCTCTTGTGCTCGGTG   |             |                   |
| Gp_cp_44  | F: CCATTGCAATCTCCTCAGGTGC | (T)10      | 224               |
|           | R: CATCAACACCATCGGCAACTCT |             |                   |
| Gp_cp_45  | F: AGTAGGATGATTACGCCTAATCT | (A)10  | 222               |
|           | R: AGGCCAGTGTCTTATTTTGAAATATT |       |                   |
| Gp_cp_46  | F: CGGCTCGAAGCGCAAGTACA   | (A)10        | 132               |
|           | R: AATTTTTGCTGTTTCTGACTCATC |         |                   |
| Gp_cp_47  | F: GAAGCAGAGCCAATCTCTCA    | (T)6        | 190               |
|           | R: TGTCGCGGAGGAAAGGTGTT   |             |                   |
| Gp_cp_48  | F: TCTCTTCACATCTCTCGGAAAAAGGA | (T)6      | 228               |
|           | R: TGCTCGCTCTGTCCCAACTAT  |             |                   |
| Gp_cp_49  | F: AGCGAAGAAACTCCCTCTCGT  | (A)10        | 171               |
|           | R: ATCTGGGCGCCCCTCCCTAAT  |             |                   |
| Gp_cp_50  | F: CAGATACGCTCCGGGCTAGA   | (A)10        | 140               |
|           | R: CGTCCAGCCTCTCCTCCTAAG  |             |                   |
| Gp_cp_51  | F: CCGCAATCTTGGAGATGGGAG  | (T)10        | 233               |
|           | R: TGTGGCGGCTATAGTTCTTAGG |             |                   |
| Gp_cp_52  | F: CCGCCTTATAGTGGCATAGTG  | (A)10        | 298               |
|           | R: TGACTTAAATACCCGGCACTC   |             |                   |
| Gp_cp_53  | F: GCXACGAGACTTGGAGATCAG  | (A)10        | 164               |
|           | R: ATTGATTCATCGACCGCGGG    |             |                   |

(Continues)
### APPENDIX 2. (Continued)

| Locus   | Primer sequences (5′–3′) | Repeat motif | Product size (bp) |
|---------|-------------------------|--------------|-------------------|
| Gp_cp_54 | F: TGCATAAGAATGAGCCAACCTTGGA                     | (T)$_9$      | 187               |
|         | R: TCATAACGCTTTAACAAGAACAC                        |              |                   |
| Gp_cp_55 | F: CAGGCATTTACTTTTTTTTGGAGT                      | (T)$_9$      | 134               |
|         | R: TTTGGGATGGAATGGGATTG                            |              |                   |
| Gp_cp_56 | F: ATATTTCGCAAGAAATTTTTGGTT                      | (T)$_9$      | 202               |
|         | R: TGATTTGCTCAAACCTTTATCGAGA                     |              |                   |
| Gp_cp_57 | F: GCACGGCTTCATTAAGTAT                             | (T)$_9$      | 257               |
|         | R: ACCCTAAAGATGACATCGC                            |              |                   |
| Gp_cp_58 | F: TGTTATTTGCTTTTGGAAAGA                          | (T)$_9$      | 176               |
|         | R: TGCTTTGTGGCTCAATTTTTG                         |              |                   |
| Gp_cp_59 | F: TATTGGACCAGCGGTAGTAGG                        | (T)$_9$      | 144               |
|         | R: ATAAGCAGTCAGAGGGAGC                            |              |                   |
| Gp_cp_60 | F: ACAGATTATTCAGATGAGGCCTCCGA                    | (T)$_9$      | 201               |
|         | R: CCCCATTTACCTGATCATATAC                       |              |                   |
| Gp_cp_61 | F: GTTCAGCCAAATAGGGGAGG                         | (T)$_9$      | 159               |
|         | R: TAAAGCCAGCCAGCTCCGCA                        |              |                   |
| Gp_cp_62 | F: TGCTACTGATGCAAAAACCTCTTTTC                   | (T)$_9$      | 209               |
|         | R: ACCACGTCATCTCATGTCAC                         |              |                   |
| Gp_cp_63 | F: CCACCTATGCCCATAAGGGTC                         | (T)$_9$      | 127               |
|         | R: TCGATGGACCTGAGGACCCTT                        |              |                   |
| Gp_cp_64 | F: GGGATACGGGGTTCTATAGGAT                        | (A)$_9$      | 149               |
|         | R: TCGATCTATGCGCTCTACT                           |              |                   |
| Gp_cp_70 | F: TCGAGGAGATAGAAGATAAATACCT                    | (G)$_{11}$   | 137               |
|         | R: GCCTTTCCCTGCGCTCTGAG                          |              |                   |

*Optimal annealing temperature was 55°C for all loci.*