**Development of EST-SSR markers for *Taxillus nigrans* (Loranthaceae) in southwestern China using next-generation sequencing**

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**Primer Note**

*Premise of the study:* We developed transcriptome microsatellite markers (simple sequence repeats) for *Taxillus nigrans* (Loranthaceae) to survey the genetic diversity and population structure of this species.

*Methods and Results:* We used Illumina HiSeq data to reconstruct the transcriptome of *T. nigrans* by de novo assembly and used the transcriptome to develop a set of simple sequence repeat markers. Overall, 40 primer pairs were designed and tested; 19 of them amplified successfully and demonstrated polymorphisms. Two loci that detected null alleles were eliminated, and the remaining 17, which were subjected to further analyses, yielded two to 21 alleles per locus.

*Conclusions:* The markers will serve as a basis for studies to assess the extent and pattern of distribution of genetic variation in *T. nigrans*, and they may also be useful in conservation genetic, ecological, and evolutionary studies of the genus *Taxillus*, a group of plant species of importance in Chinese traditional medicine.

**Key words:** Chinese traditional medicine; conservation; Loranthaceae; microsatellite marker; next-generation sequencing; *Taxillus nigrans*; transcriptome.

*Taxillus nigrans* (Hance) Danser (Loranthaceae) is a mistletoe species that is found attached to many canopy tree species in low mountains, hills, and river basins in subtropical areas of southwestern China at elevations of 300–1300 m. Flowering can occur throughout the year, and the fruiting period is mainly in November. The entire plant of this species can be used as raw material for Chinese traditional medicine (Jiang, 1998). However, because the range of the species has undergone rapid expansion mediated by birds in the urban area of Chengdu (Sichuan Province, China), it forms large groves on garden tree species and is sometimes harmful to its host trees, so that individuals of this species are often removed by gardeners. To date, apart from some basic taxonomic data on the species (Gong et al., 2004) and genome studies on other species of *Taxillus* Tiegh. (Rist et al., 2011; Wei et al., 2017), nearly all published research has focused on aspects relating to its medicinal value, for example, the extraction and identification of medicinal components and the optimization of extraction methods (Li et al., 2006, 2009; Zhang et al., 2016; Zhao et al., 2016). There is little information on the genetic diversity and population structure of the species. We are also interested in developing genetic approaches for identification of individuals and assignment testing, which will help in understanding how this species expands its distribution and jumps from host to host in urban areas as well as in the field.

Simple sequence repeat (SSR) markers, also known as microsatellites or short tandem repeats, are highly polymorphic and are therefore useful as molecular markers in population genetic studies (Zhang et al., 2012; Jiang et al., 2015). Transcriptome sequencing has proven to be a powerful and cost-effective tool that has greatly accelerated the process of discovering molecular markers, including single nucleotide polymorphisms (SNPs) and SSRs (Ashrafi et al., 2012; Qi et al., 2016). In this study, we sequenced and assembled the transcriptome of *T. nigrans* and developed a set of expressed sequence tag (EST)–SSR markers for population genetic studies of *T. nigrans*. We also tested the transferability of these markers in herbarium samples of *T. delavayi* (Tiegh.) Danser and five individuals of *Scurrula parasitica* L. (collected from the field), another Loranthaceae parasite that co-occurs with *T. nigrans*.

**Methods and Results**

Approximately 10 μg (400 ng/μL) of total RNA was extracted from fresh leaf material of one individual of *T. nigrans* using TRizol Reagent (Invitrogen, Carlsbad, California, USA). Subsequently, mRNA was isolated using magnetic oligo (dT) beads (Illumina, San Diego, California, USA); it was then fragmented into short fragments using the Ambion RNA Fragmentation Kit (Ambion, Austin, Texas, USA) according to the manufacturer’s protocols. First-strand cDNA synthesis was performed using reverse transcriptase (Invitrogen) with random primers, and second-strand cDNA was synthesized by RNase H and DNA Polymerase I (Invitrogen). Finally, the transcriptome was sequenced on an Illumina HiSeq 2000 system at Novogene (Beijing, China). Prior to the assembly, a stringent filtering process of raw sequencing reads was conducted. The number of

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| Locus   | Primer sequences (5’–3’) | Repeat motif | Allele size (bp) | Fluorescent dye | GenBank accession no. | r       | Protein\(^b\)                          | Organism\(^c\)                  | E-value\(^d\) |
|---------|--------------------------|--------------|------------------|-----------------|----------------------|--------|------------------------------------------|---------------------------------|-------------|
| T\(^a\)R7149 | F: GGCAAAATCACACGGAAGA  | (CT)\(_{21}\) | 164              | 60              | 6-FAM                | 0.0156 | NEN1-like                               | *Populus euphratica*             | 3×10\(^{-7}\) |
|         | R: CTATATGACATCACACAC  |             |                  |                 |                      |        |                                          |                                 |             |
| T\(^a\)R11564 | F: CTTAAGACATTCACTCAAC  | (AGA)\(_{14}\) | 215              | 60              | HEX                  | 0.0626 | WD repeat-containing protein RUP2       | *Ekeis guineensis*               | 8×10\(^{-8}\) |
|         | R: CTCAGACAGCAGCCCTGGA  |             |                  |                 |                      |        |                                          |                                 |             |
| TR24412 | F: TTCTTCACACAGGGGCAA  | (CT)\(_{21}\) | 122              | 60              | 6-FAM                | 0.0747 | Predicted gene, 39330                    | *Oriza sativa*                   | 4×10\(^{-4}\) |
|         | R: AGCGAGTCTAGATCACGCT  |             |                  |                 |                      |        |                                          |                                 |             |
| TR47466 | F: AGTCCTCTGGCTCCGATACC | (AT)\(_{24}\) | 231              | 60              | TAMRA                | 0.3990 | Unknown                                  | *Vigna angularis*                | 2×10\(^{-21}\) |
|         | R: TCAGTGGCTATCCGCTGC  |             |                  |                 |                      |        |                                          |                                 |             |
| T\(^a\)R51334 | F: GTAAGATCCCAACACGAG  | (AG)\(_{26}\) | 206              | 60              | TAMRA                | 0.0008 | Transmembrane protein, putative         | *Medicago truncatula*            | 1×10\(^{-21}\) |
|         | R: CTCAGATCTACCCCGGTGT |             |                  |                 |                      |        |                                          |                                 |             |
| TR56117 | F: TTCTTCACACAGGGGCAA  | (TC)\(_{15}\) | 166              | 60              | TAMRA                | 0.1183 | LOC107411880                             | *Ziziphus jujuba*                | 2×10\(^{-4}\) |
|         | R: CTGATGTCACATCCGCTGC |             |                  |                 |                      |        |                                          |                                 |             |
| TR59209 | F: AGCGAGTCTAGATCACGCT | (TC)\(_{15}\) | 157              | 60              | 6-FAM                | 0.1826 | LOC107268204                             | *Cephus cinctus*                 | 2×10\(^{-8}\) |
|         | R: AGCAATGCCAAGGAGGTC  |             |                  |                 |                      |        |                                          |                                 |             |
| T\(^a\)R83979 | F: CTGAGGCTCTCCAGATGCC | (CT)\(_{22}\) | 245              | 60              | HEX                  | 0.0748 | At3g02290                                | *Oriza sativa*                   | 2×10\(^{-15}\) |
|         | R: CTGATGTCACATCCGCTGC |             |                  |                 |                      |        |                                          |                                 |             |
| TR85804 | F: TGCACTTCCTCCAGATGCC | (AG)\(_{33}\) | 219              | 60              | TAMRA                | 0.0705 | CARUB\(_{v1}\)002273mg                   | Capsella rubella                 | 1×10\(^{-26}\) |
|         | R: CTTGCTAAATTCACACACA |             |                  |                 |                      |        |                                          |                                 |             |
| TR87965 | F: TGAGAGCTTCTGGCTTCCGC| (AGA)\(_{14}\) | 216              | 60              | 6-FAM                | 0.1080 | DDB1- and CUL4-associated factor 13      | *Theobroma cacao*                | 2×10\(^{-12}\) |
|         | R: TAACCTGCTCCACACCTCC |             |                  |                 |                      |        |                                          |                                 |             |
| T\(^a\)R88317 | F: GAGGAGAGGAGGATCTGTA  | (TAT)\(_{15}\) | 129              | 60              | TAMRA                | 0.2512 | Restricted Tev Movement 1-like          | *Nicotiana tomentosiformis*      | 1×10\(^{-15}\) |
|         | R: TGGAGAGGAGGATCTGCTC |             |                  |                 |                      |        |                                          |                                 |             |
| TR90181 | F: AAAGACCTCTTCACAGCCTC| (TA)\(_{25}\) | 217              | 59               | TAMRA                | −0.0708 | LOC107270001                             | *Cephus cinctus*                 | 1×10\(^{-22}\) |
|         | R: AAGAGGCTCTACACACATC |             |                  |                 |                      |        |                                          |                                 |             |
| T\(^a\)R91417 | F: AGAGGATATTGGGATGCTC  | (GA)\(_{26}\) | 213              | 60              | 6-FAM                | 0.1064 | LOC105638199                             | Jatropha curcas                   | 2×10\(^{-21}\) |
|         | R: TCCAGCTCAACTGGGCTCA |             |                  |                 |                      |        |                                          |                                 |             |
| T\(^a\)R97121 | F: CTGAGGCTTCTGGATCCCGA| (AG)\(_{15}\) | 204              | 60              | HEX                  | 0.0685 | Transcription factor bHLH35             | *Vigna angularis*                | 3×10\(^{-13}\) |
|         | R: TCCAGCTCAACTGGGCTCA |             |                  |                 |                      |        |                                          |                                 |             |
| TR98683 | F: CTGGTACCCTTCTATCTCC | (CT)\(_{15}\) | 255              | 60              | TAMRA                | 0.0839 | LOC104597466                             | Nelumbo nucifera                 | 2×10\(^{-9}\) |
|         | R: AGACAGTACACACCTGCTG |             |                  |                 |                      |        |                                          |                                 |             |
| TR105177 | F: CAGGCTGCTTCTGATCCAGA| (GA)\(_{25}\) | 217              | 60              | TAMRA                | 0.0798 | LOC103319601                             | *Prunus mume*                    | 1×10\(^{-25}\) |
|         | R: TGGGAAATGGACGTCTGCTC|              |                  |                 |                      |        |                                          |                                 |             |
| TR120023 | F: CTGAGGCTTCTGGATCCCGA| (GA)\(_{14}\) | 161              | 60              | TAMRA                | 0.1191 | LOC104727032                             | *Camelina sativa*                | 4×10\(^{-9}\) |
|         | R: CCCTGACCTGCTTCTATCC |             |                  |                 |                      |        |                                          |                                 |             |
| **T\(^a\)R85478 | F: GTCCTGACATGGACTTCCCTGC | (TC)\(_{3}\) | 228              | 60              | TAMRA                | ND     | EUTSA\(_{v1}\)00075844mg                 | *Eutrema salsugineum*            | 5×10\(^{-3}\) |
|         | R: ACTGGGACATTGGCTGCTG |             |                  |                 |                      |        |                                          |                                 |             |
| **T\(^a\)R87192 | F: CCTTGGGAGGTTCCACTACCTT| (GGG)\(_{4}\) | 271              | 60              | HEX                  | 0.4202 | LOC103986576                             | *Musa acuminata*                 | 0.11          |
|         | R: TCGGCCAGTTTTAGTGGCA |             |                  |                 |                      |        |                                          |                                 |             |

Note: ND = not done; r = null allele frequency; \(T_a\) = annealing temperature.

\(^a\)The annealing temperature for each primer is listed, and the final annealing temperature for each PCR reaction is given as the average annealing temperature of the adopted primer pair.

\(^b\)Information from BLAST analysis on the protein most closely matching the EST.

\(^c\)Organism from which the BLAST match was obtained.

\(^d\)E-value associated with the BLAST match.

* Null alleles (r > 0.4).

** Primers successfully amplified for *Taxillus delavayi*. 

Table 1. Characteristics of 19 polymorphic microsatellite loci developed for *Taxillus nigrans*. 
Table 2. Genetic properties of 17 newly developed polymorphic microsatellite loci in three populations of *Taxillus nigrans*. Loci exhibiting null alleles are not included.

| Locus   | Sichuan University (n = 100) | Tazishan (n = 30) | Huanhuaxi (n = 30) |
|---------|-----------------------------|------------------|-------------------|
|         | A   | $H_o$ | $H_e$ | A   | $H_o$ | $H_e$ | A   | $H_o$ | $H_e$ |
| TR7149  | 7   | 0.717 | 0.815 | 5   | 0.900 | 0.728 | 10  | 0.967 | 0.844 |
| TR11564 | 5   | 0.667 | 0.781 | 4   | 0.767 | 0.672 | 5   | 0.667 | 0.727 |
| TR24412 | 6   | 0.551 | 0.628 | 4   | 0.633 | 0.691 | 7   | 0.621 | 0.722 |
| TR47466 | 6   | 0.333 | 0.453 | 2   | 0.034 | 0.034 | 4   | 0.367 | 0.476 |
| TR51334 | 11  | 0.525 | 0.776 | 2   | 0.966 | 0.499 | 5   | 0.967 | 0.577 |
| TR56117 | 9   | 0.583 | 0.745 | 7   | 0.724 | 0.737 | 8   | 0.567 | 0.787 |
| TR59209 | 10  | 0.626 | 0.789 | 6   | 0.310 | 0.596 | 6   | 0.643 | 0.786 |
| TR83979 | 17  | 0.737 | 0.859 | 9   | 0.633 | 0.799 | 10  | 0.828 | 0.757 |
| TR58804 | 18  | 0.808 | 0.876 | 9   | 0.633 | 0.799 | 17  | 0.833 | 0.898 |
| TR79695 | 7   | 0.646 | 0.786 | 5   | 0.586 | 0.703 | 6   | 0.667 | 0.764 |
| TR88317 | 11  | 0.347 | 0.714 | 5   | 0.607 | 0.702 | 4   | 0.517 | 0.644 |
| TR90181 | 14  | 1.000 | 0.786 | 7   | 0.963 | 0.747 | 5   | 1.000 | 0.621 |
| TR91417 | 10  | 0.717 | 0.809 | 6   | 0.400 | 0.665 | 7   | 0.700 | 0.749 |
| TR97121 | 2   | 0.380 | 0.476 | 2   | 0.500 | 0.408 | 2   | 0.400 | 0.464 |
| TR98683 | 14  | 0.690 | 0.860 | 10  | 0.833 | 0.815 | 15  | 0.933 | 0.813 |
| TR105177| 20  | 0.764 | 0.893 | 8   | 0.733 | 0.807 | 10  | 0.931 | 0.835 |
| TR120023| 21  | 0.802 | 0.885 | 6   | 0.633 | 0.776 | 6   | 0.552 | 0.797 |

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

Table 3. Fragment sizes detected in cross-amplification tests of the 19 newly developed microsatellite markers in *Taxillus delavayi* and *Scirrula parasitica*.

| Locus   | *Taxillus delavayi* (n = 2) | *Scirrula parasitica* (n = 5) |
|---------|-----------------------------|-------------------------------|
| TR7149  | 167                         | 152–163                       |
| TR11564 | 192                         | 193                           |
| TR24412 | —                           | 124                           |
| TR47466 | —                           | 272                           |
| TR51334 | 182                         | 174–182                       |
| TR56117 | 155–185                     | 155–185                       |
| TR59209 | 125–143                     | 125–143                       |
| TR83979 | 244                         | 177–211                       |
| TR58804 | 179–255                     | 179–255                       |
| TR79695 | —                           | 197                           |
| TR88317 | 100                         | 100–130                       |
| TR90181 | —                           | 205–207                       |
| TR91417 | 196                         | 196–204                       |
| TR97121 | 332                         | 353                           |
| TR98683 | —                           | 244–260                       |
| TR105177| —                           | 189                           |
| TR120023| —                           | 152                           |
| TR85478 | 229                         | 229                           |
| TR87192 | —                           | 269                           |

Note: — = amplification failed; $n$ = number of individuals sampled.

CONCLUSIONS

We developed and amplified a set of polymorphic EST-SSR markers for *T. nigrans*. These new SSR markers will serve as a basis for studies assessing the genetic diversity and population

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structure of *T. nigrans*. Our research will be useful for conservation genetic, ecological, and evolutionary studies of the genus *Taxillus*, a group of plant species of importance in Chinese traditional medicine. We plan to use these markers to explain the rapid demographic expansion and host specificity of *T. nigrans* in urban areas in southwestern China.

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APPENDIX 1. Voucher specimen information for Loranthaceae used in this study.

| Species                  | N  | Population code | Locality       | Geographic coordinates | Voucher specimen accession no.  |
|--------------------------|----|-----------------|-----------------|------------------------|---------------------------------|
| *Taxillus nigrans* (Hance) Danser | 100 | SCU             | Sichuan University, Sichuan | 30°37′48″N, 104°4′48″E | SZ-00545040, SZ-00545041, SZ-00545042, SZ-00545043, SZ-00545044 |
| *T. nigrans*             | 30  | TZT             | Tazishan, Sichuan | 30°38′7″N, 104°7′15″E | SZ-00545045, SZ-00545046, SZ-00545047 |
| *T. nigrans*             | 30  | HH              | Huanhuaxi, Sichuan | 30°39′28″N, 104°1′55″E | SZ-00545048, SZ-00545049, SZ-00545050 |
| *T. delavayi* (Tiegh.) Danser | 1   | Individual     | Maerkang, Sichuan | 31°54′46″N, 102°1′12″E | SZ-00280020 |
| *T. delavayi*            | 1   | Individual     | Muli, Sichuan    | 27°55′55″N, 101°16′43″E | SZ-00280006 |
| *Scurrula parasitica* L. | 5   | TZS             | Tazishan, Sichuan | 30°38′7″N, 104°7′15″E | SZ-00545051 |

Note: N = number of individuals sampled.

*All voucher specimens are deposited at the herbarium of Sichuan University (SZ), Sichuan, China.*