

PRIMER NOTE

## DEVELOPMENT OF EST-SSR MARKERS FOR TAXILLUS NIGRANS (LORANTHACEAE) IN SOUTHWESTERN CHINA USING NEXT-GENERATION SEQUENCING<sup>1</sup>

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- 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

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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  $10 \ \mu g$  (400 ng/ $\mu L$ ) 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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TR114)F: accazameteracionante(T)16166NumberNumberRyndus apfunctionNumber<	Locus	Primer sequences $(5'-3')$	Repeat motif	Allele size (bp)	$T_{\rm a}$ (°C) <sup>a</sup>	Fluorescent dye	GenBank accession no.	r	Protein <sup>b</sup>	$Organism^c$	<i>E</i> -value <sup>d</sup>
	#TR7149	F: GGCAAAATCAACCGAGAAGA	(CT) <sub>1</sub> ,	164	09	6-FAM	KY412965	0.0156	NEN1-like	Populus euphratica	$3 \times 10^{-7}$
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	#TR11564		$(AAG)_{16}$	215	09	HEX	KY412966	0.0626	WD repeat-containing	Elaeis guineensis	$8 \times 10^{-8}$
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F:   Amochanistication   (M)	TR24412		$(CT)_{21}$	122	9	6-FAM	KY412967	0.0747	Predicted gene, 39330	Oryza sativa	4×10 <sup>-4</sup>
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B:   CTGGATCATTAGE   Muture   Muture   Muture   Muture   Muture   Technologie	#TR51334		$(AG)_{26}$	206	60	TAMRA	KY421969	0.0008	Transmembrane protein,	Medicago truncatula	$1 \times 10^{-21}$
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N:   01   0.1826   LOCI07268204   Capital circurs     7:   TGCGGATTGTTMGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTG	TR56117		$(TC)_{15}$	166	60	TAMRA	KY421970	0.1183	LOC107411880	Ziziphus jujuba	$2 \times 10^{-8}$
F:Tencocrametrocorr reconcretance(TO),157606-FAMKY4219710.1826LOC107568204Caphus cincusR:AGGAATCGAACGAGGGC(TD),24560HEXKY4219720.0705LOC107268204Caphus cincusR:TECTCTCTCCGCCCTTCTCCCCCCTTC(TD),24560HEXKY4219720.0705CAUB_v10002273mgCapella rubellaR:TCTCTCTCTCCGCCCTTGCCCCTTC(AG),219606-FAMKY4219740.1080DDB1- and CU14-Theohrana caccoR:TTACCCCTTTGCCCCTTGCCCCTTGCCCCTTGCCCCTTGCCCCTTGCCCCTTGCCCCCTTGCCCCCC					61						
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F:CCTCCGTCTTCCCTTCC(T)_124560HEXKY4219720.0748A13g02290 $O_{Y2a} sativaF:TCGTCCTTTCCACTTATCC(T)_121900HEXKY4219730.0705CARUB_V10002273mgO_{Y2a} sativaF:TCGTCGTTTCCACTTCGACTTAT(AG)_1219006-FAMKY4219740.1080DDB1- and CUL4-Theohrana cacaoF:TTGGAGGACTTATC(AG)_1216006-FAMKY4219740.1080DDB1- and CUL4-Theohrana cacaoF:TTGGAGGAATTGCACCTTC(AG)_1210006-FAMKY4219750.2512Restricted TevNiccinarF:AAGCGACTTATCGACTCC(TA)_321799HEXKY4219760.0708LOC10720001CapusF:AAGCGACTTATCGACTCC(AG)_121799HEXKY4219760.0708Loc10720001CapusF:AAGCGACTTATCGCACC(AG)_1217906-FAMKY4219760.1064LOC10720001CapusF:AAGCGACTCATTGCACCCACC(AG)_1217906-FAMKY4219760.1064LOC10750001CapusF:AAGCGACTCATTGCACCCACCACCACCACCA(AG)_1217906-FAMKY4219760.1064LOC10750001CapusF:CCCCAACTGACTGCACCACCA(AG)_12040.068HEXKY4219760.1068LOC1075001LocnoccacF:CCCCAACTGACTGCACCACCA(AG)_12040.068HEXKY4219760.1064LOC10950976$					60						
R:TCGTCCTCTCCCCTCCCCACCA CCCCTCTCTCCACTATT00HEXKY4219730.0705CARUB_V1000273mgCappella rubellaR:CCTCTCTCTCTCCCCCACCA CCCACCACCACCA(AG)_{14}216606-FAMKY4219740.1080DDB1- and CUL4-Theobrona caccoR:TTAACTCGTTTTAATTCCCACCACCA CCATTGCCACCTTCTCAATTCCCACCACCA(AG)_{14}216606-FAMKY4219740.1080DDB1- and CUL4-Theobrona caccoR:TTAACTCGTTTTAAATT TTAACTCGTTTCAATT(TXT)_{15}129606-FAMKY4219750.2512Restricted factor 13NicotimaR:TTGCAGGAACCTCAATCCCAACACA TC(GA)_{25}213606-FAMKY4219750.2512Restricted factor 13NicotimaR:TTCCAACTCAACTCCAACACC TC(GA)_{25}213606-FAMKY4219750.1064LOC107270001Cphus cinctusR:TCCAACTCAACTCAACTCCAACCTCAACTCAACCC TC(GA)_{25}213606-FAMKY4219750.1064LOC107270001Cphus cinctusR:TCCAACTCAACTCAACTCAACCTCAACTCAACTCCCCCC(GA)_{25}20460HEXKY4219750.1064LOC107270001Cphus cinctusR:TCCAACTCAACTCAACTCAACTCAACTCACCC(GA)_{25}20460HEXKY4219750.1064LOC107270001Cphus cinctusR:TCCAACTCAACTCAACTCAACTCAACTCAACTCAACTC	#TR83979		$(CT)_{22}$	245	09	HEX	KY421972	0.0748	At3g02290	Oryza sativa	$2 \times 10^{-15}$
F:TCCTCTCTCTCCTCCTC $(AG)_{13}$ $219$ $60$ HEX $KY421973$ $0.0705$ $CARUB_V10002273mg$ $Capsella rubella$ F:TCTTGGCAGCTCCTC $(AG)_{14}$ $216$ $60$ $6$ -FAM $KY421974$ $0.1080$ $DB1-$ and $CU14+$ $Theohrana cacaoF:TGGGGAGGGGGGTGCTTGGTAT(TAT)_{15}129606-FAMKY4219750.2512Rsvirted TevNicotianaF:TAACGGGCTATGGCTATGCCT(TAT)_{15}129606-FAMKY4219750.2512Rsvirted TevNicotianaR:TATGCAGGATAGGCT(TAT)_{15}21759HEXKY4219760.0708Loot107270011Cephus cinctusR:AAGGACTCATGCAACATGC(AG)_{15}21360HEXKY4219760.0683Tanscription factorNicotianaR:TCCCCACACATGCAACATGC(AG)_{15}21060HEXKY4219760.0683Tanscription factorNicotianaR:TCCCCACACATGCAACATGC(CT)_{15}23560HEXKY4219760.0633NicotianaR:TCCCCCACACATGCAACATGCAACATGC(CT)_{15}23560HEXKY4219760.0633NicotianaR:TCCCCCACATGCATGCATGCAACATGC(CT)_{15}23560HEXKY4219760.0633NicotianaR:TCCCCCCACATGCATGCATGCATGCAACATGC(CT)_{14}CT_{14}KY4219800.0738LOC10339601Pinus muneR$					60						
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F:AATGACGGTATTCGGAACGC(TA)3 $217$ $59$ HEX $KY421976$ $-0.0708$ LOC107270001 <i>Cephus cincus</i> F:AAGCGACTCATCGAACTC(GA)3 $59$ 606FAM $KY421977$ $0.1064$ LOC105638199 <i>Jatropha curcas</i> F:TCGAACTCACTCATCTGCCC(GA)3 $213$ 606FAM $KY421978$ $0.0685$ Transcription factor <i>Vigna angularis</i> F:TCGCACTCACTCATCTCACTCACTCACTCACTCACTCACT					60				Movement 1-like	tomento siform is	
R:AAGCGACCTCATCACATC59F:AAGCGACCTCAACATC(A)2,213606-FAMKY4219770.1064LOC105638199Jaropha curcasF:TCCAACTCACACTGCCCCA(A)3,20460HEXKY4219780.0685Transcription factorVigna angularisF:TCCAACTCACACTCATTCCCC(CT)1,25560HEXKY4219790.0685Tanscription factorVigna angularisF:TCCCCCCACTCATTCTCCC(CT)1,25560HEXKY4219790.0839LOC104597466Nelumbo nuciferaF:TGGCTAACGCTCTGGTCGGAG(GA)2,21760TAMRAKY4219800.0798LOC104597466Nelumbo nuciferaF:TGGCTAATGGTCGGTTGGTCGGAG(GA)1,16160TAMRAKY4219800.0798LOC104597466Nelumbo nuciferaF:TGGGAATGGTGTTGTTCT(GA)1,16160TAMRAKY4219800.1191LOC104727032Camelina sativaF:CTTGGAAGGGGTTGTTCTTCT(TC)s22860TAMRAKY421982NDEUTSA_V10007584mgEutrena salsugineumF:CCTTGGGAGGGGGTTCAACTT(GC)1,27160HEXKY4219830.4202LOC10396576Masa activaF:CCTTGGGAGGGGGTTCAACTTGC27160TAMRAKY4219830.4202LOC10398576Masa activaF:CCTTGGGAGGGGGTTCAACTTGC27160TAMRAKY4219830.4202LOC103986576Masa activaF:CCTTGGGAGGA	TR90181		$(TA)_{25}$	217	59	HEX	KY421976	-0.0708	LOC107270001	Cephus cinctus	$1 \times 10^{-22}$
F:AGAGGAATTGGCATCGTCAG $(\mathbf{GA})_x$ $213$ $60$ $6$ -FAM $\mathbf{KY421971}$ $0.1064$ $\mathbf{LOC105638199}$ $Jarropha curcas$ R:TCCCAACTCACACTTGCCTCA $(\mathbf{A})_1$ $204$ $60$ HEX $\mathbf{KY421978}$ $0.1064$ $\mathbf{LOC105638199}$ $Jarropha curcas$ R:TCCTCCTCACTCATTGCCTC $(\mathbf{CT})_1$ $204$ $60$ HEX $\mathbf{KY421978}$ $0.0685$ $\mathbf{Tanscription factorVigna angularisR:TCCTCCTCACTCATTGCTC(\mathbf{CT})_125560HEX\mathbf{KY421979}0.0839\mathbf{LOC104597466}Nelumbo mciferaR:AGACTCGAAGGCCTTGGTT(\mathbf{GA})_221760TAMRA\mathbf{KY421980}0.0798\mathbf{LOC104597466}Nelumbo mciferaR:TGGGAATGGTTGTTGTGTGTTGTT(\mathbf{GA})_116160TAMRA\mathbf{KY421980}0.0798\mathbf{LOC104597466Nelumbo mciferaR:TGGGAATGGTGTTGGTTGTTGTTGTTGGTTGTTGT(\mathbf{GA})_116160TAMRA\mathbf{KY421980}0.0798\mathbf{LOC104727032}\mathbf{Camelina sativa}R:CCGTCACTCTTCGT(\mathbf{TC})_522860TAMRA\mathbf{KY421982}\mathbf{ND}\mathbf{LUTSA_v10007584mg\mathbf{Eutrena salsugineum}R:ACCTTTGGGGACACTTTGGTCTCTTCGT(\mathbf{CC})_427160\mathbf{TAMRA}\mathbf{KY421982}\mathbf{ND}\mathbf{EUTSA_v10007584mg\mathbf{Eutrena salsugineum}R:CCCTTTGGGGACACTTTTAGTCTGCGT(\mathbf{CC})_427160\mathbf{TAMRA}\mathbf{KY421983}\mathbf{OL}_02\mathbf{LOC}03986576$					59						
R:TCCAACTCACTCA60HEX $KY421978$ 0.0685Transcription factor <i>Vigna angularis</i> R:TCTCTCCTCACTCATTGCCTC $(CT)_1s$ 20460HEX $KY421979$ 0.0685Transcription factor <i>Vigna angularis</i> R:TCTCCTCACTCATTGCCTC $(CT)_1s$ 25560HEX $KY421979$ 0.0839LOC104597466 <i>Nelumbo nucifera</i> R:TAGACTCGATGGTTGGTTG $(CT)_1s$ 25560HEX $KY421980$ 0.0798LOC104597466 <i>Nelumbo nucifera</i> R:TAGACTCGATGGTTGGTTG $(CT)_1s$ 21760TAMRA $KY421980$ 0.0798LOC103319601 <i>Prunus mune</i> R:TGGGAAATGGTTGCTTCTTC $(GA)_1a$ 16160TAMRA $KY421981$ 0.1191LOC104727032Canelina sativaR:TCGGTCATGGTCTCTTCTTC $(CG)_1a$ 21760TAMRA $KY421981$ 0.1191LOC104727032Canelina sativaR:TCGGTCATGGTCTCTTCTTC $(CG)_1a$ 218 $(CG)_1a$ 218 $(CG)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ R:ACTGGGACTTCTTCGT $(CT)_1a$ $(CG)_1a$ $(CG)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ R:ACTGGGACTTCTTCGT $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ R:ACTGGGACTTCTTCGT $(CG)_1a$ $(CG)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ $(CT)_1a$ R:ACTGGGACACTT	#TR91417		$(GA)_{26}$	213	09	6-FAM	KY421977	0.1064	LOC105638199	Jatropha curcas	$2 \times 10^{-21}$
F:CTGGAGTCGTAGTAGTCGA(AG)_520460HEXKY4219780.0685Transcription factorVigna angularisR:TCTCTCTCACTCATTGCTC(CT)_125560HEXKY4219790.0895DHLH35Nelumbo nuciferaR:TGGCTAAGGCTTGGTTGCTC(CT)_125560HEXKY4219790.0839LOC104597466Nelumbo nuciferaR:AGACTCGATGCTTGGTGGTT(GA)_921760TAMRAKY4219800.0798LOC103319601Prunus numeR:TGGGAAAGGCTTGGTGGTGGTT(GA)_416160TAMRAKY4219810.1191LOC104727032Canelina sativaR:TCTGGTCATGGTCGCTTCTTC(TC)_522860TAMRAKY421982NDEUTSA_V10007584mgEutrena salsugineumR:ACTGGGAAGGTTTAGTCTCGCTT(GG)_427160HEXKY4219830.4202LOC103396576Musa acuminataR:ACTGGGAAGTTTAGTCGC(GC)_427160HEXKY4219830.4202LOC103986576Musa acuminataR:TGGCGCAAGTTTAGTCGC60HEXKY4219830.4202LOC103986576Musa acuminataR:TGGCCGAAGTTTAGTCAGC60HEXKY4219830.4202LOC103986576Musa acuminata					09						
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F: TGGCTACCTCTATCTCC (CT) <sub>1</sub> 255 60 HEX KY421979 0.0839 LOC104597466 Nelumbo nucifera   R: AGACTCGAAGGCCTCTGGTT 60 TAMRA KY421980 0.0798 LOC104597466 Nelumbo nucifera   F: CAGCATGCATTGCTGGTG (GA) <sub>29</sub> 217 60 TAMRA KY421980 0.0798 LOC103319601 Prunus nume   R: CGGCAAGGCGTTGGTGGTT (GA) <sub>4</sub> 161 60 TAMRA KY421981 0.1191 LOC104727032 Camelina sativa   R: CCGTCACGGCTGCTCTCTTCT 60 TAMRA KY421982 ND EUTSA_V10007584mg Eutrena salsugineum   R: ACTGGGACGCTCTTCGT 60 HEX KY421983 0.4202 LOC103386576 Musa acuminata   R: ACTGGGACGCTTCTCGT 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata   R: ACTGGGACGCTCTTCGT 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata		•••			60				bHLH35		
R: AGACTCGAAGGCCTTGGT 60   F: CAGCATGCATTGCTAGGAG (GA) <sub>20</sub> 217 60 TAMRA KY421980 0.0798 LOC103319601 Prunus nume   R: TGGGAATGGCATTGCTGGGGTT (GA) <sub>14</sub> 161 60 TAMRA KY421981 0.1191 LOC104727032 Camelina sativa   R: CGGTCAGTGGCTTCTCGGTGCGTT (GA) <sub>14</sub> 161 60 TAMRA KY421981 0.1191 LOC104727032 Camelina sativa   R: CGGTCAGTGGTCTTCGGT (TC) <sub>5</sub> 228 60 TAMRA KY421982 ND EUTSA_V10007584mg Eutrena salsugineum   R: ACTGGGACACTTTGGG (TC) <sub>5</sub> 228 60 HEX KY421983 0.4202 LOC103386576 Musa acuminata   R: ACTGGGAGGGGTTCAACTT (GC) <sub>4</sub> 271 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata   R: TGGCCGAAGTTTAGTCAGC 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata	TR98683		(CT) <sub>15</sub>	255	60	HEX	KY421979	0.0839	LOC104597466	Nelumbo nucifera	$2 \times 10^{-9}$
F: CAGCATGCATTGCTAGGAGA (GA) <sub>2</sub> 217 60 TAMRA KY421980 0.0798 LOC103319601 Prunus mume   R: TGGGAAATGGACGTTGTTCT 60 TAMRA KY421980 0.0798 LOC103319601 Prunus mume   F: CTTGATCTTCTGGTGGGTT (GA) <sub>4</sub> 161 60 TAMRA KY421981 0.1191 LOC104727032 Camelina sativa   R: CGGTCACTGCTTCTGCTT 60 TAMRA KY421982 ND EUTSA_v10007584mg Eutrena salsugineum   R: ACTGGGGACACATTCTGGGT (TC) <sub>5</sub> 228 60 HEX KY421983 0.4202 LOC103768576 Musa acuminata   R: ACCGTGAGGGCTCATCTGCT 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata   R: TGGCGGAAGTTTAGTCAGC 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata					60						
R: TGGGAAATGGACGTTGTTC 60   F: CTTGATCTTCGGTGGGGT (GA) <sub>4</sub> F: CTTGATCTTCGGTCGGTTCAT (GA) <sub>4</sub> R: CCGTCATGGTCTCTTCAT 60   R: CCGTCATGGTCTTCGTT 60   R: CCGTCATGGTCTTCGTT 60   R: CCGTCATGGATTCCTTCGT (TC) <sub>5</sub> 228 60   R: ACTGGGACATTCCTGCAT (TC) <sub>5</sub> R: ACTGGGACATTCCTGCAT 0.0   R: CCTTTGGAGGGGTTCAACTT 0.0   R: ACTGGGACGATTCATCGCA 0.0   R: ACTGGGACGATTCATCGCAT 0.0   R: ACTGGGACGATTCAACTT 0.0   R: ACTGGGAGGTTCAACTT 0.0   R: ACTGGGAGGTTCAACTT 0.0   R: TGGCCGAAGTTTAGTCAGC 0.0   R: TGGCCGAAGTTTAGTCAGC 0.0	TR105177		$(GA)_{29}$	217	60	TAMRA	KY421980	0.0798	LOC103319601	Prunus mume	$1 \times 10^{-25}$
F: CTTGATCTTCGGTGCGGTT (GA) <sub>14</sub> 161 60 TAMRA KY421981 0.1191 LOC104727032 Camelina sativa   R: CCGTCATGGTCTTCATT 60 TAMRA KY421981 0.1191 LOC104727032 Camelina sativa   8 F: GTCGTCATGGTCTTCGCT (TC) <sub>5</sub> 228 60 TAMRA KY421982 ND EUTSA_v10007584mg <i>Eutrema salsugineum</i> 8 F: ACTGGGACACATTCCTGCAT (GCG) <sub>4</sub> 271 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata   8: TGGCCGAAGTTTAGTCAGC 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata					60						
R:CCGTCATGGTCTTCAT608F:GTCGTCATGGACTCTTCGCT(TC)s10007584mgEutrema salsugineum8F:CCTTTGGGACACATTCCTGCAT8ACTGGGACACATTCCTGCAT608ACTGGGAGGGTTCAACTT609HEXKY4219830.420210007584mgEutrema salsugineum10007584mgEutrema salsugineu	TR120023		$(GA)_{14}$	161	60	TAMRA	KY421981	0.1191	LOC104727032	Camelina sativa	$4 \times 10^{-9}$
3 F: GTCGTCATGGACTCTTCGCT (TC) <sub>5</sub> 228 60 TAMRA KY421982 ND EUTSA_v10007584mg Eutrema salsugineum R: ACTGGGACACATTCCTGCAT 60 TAMRA KY421983 0.4202 LOC103986576 Musa acuminata F: CCTTTGGAGGGGTTCAACTT (GCG) <sub>4</sub> 271 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata R: TGGCCGAAGTTTTAGTCAGC 60 HEX KY421983 0.4202 LOC103986576 Musa acuminata					60						
R: ACTGGGACACATTCCTGCAT 60 60 FIEX KY421983 0.4202 LOC103986576 Musa acuminata R: TGGCCGAAGTTTTAGTCAGC 60 FIEX 60 FIEX 60 FIEX 60 FIEX 60 FIEX FIEX FIEX FIEX FIEX FIEX FIEX FIEX	*#TR85478		(TC) <sub>5</sub>	228	60	TAMRA	KY421982	ą	EUTSA_v10007584mg	Eutrema salsugineum	$5 \times 10^{-4}$
F: CCTTTGGAGGGGTTCAACTT (GCG) <sub>4</sub> 271 60 HEX KY421983 0.4202 LOC103986576 <i>Musa acuminata</i> R: TGGCCGAAGTTTTAGTCAGC 60					60						
	*TR87192		$(GCG)_4$	271	60	HEX	KY421983	0.4202	LOC103986576	Musa acuminata	0.11
		R: TGGCCGAAGTTTTAGTCAGC			60						

Characteristics of 19 polymorphic microsatellite loci developed for Taxillus nigrans. TABLE 1.

*Note:* ND = not done; r = null allele frequency;  $T_a$  = annealing temperature, <sup>a</sup> 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. <sup>b</sup> Information from BLAST analysis on the protein most closely matching the EST. <sup>c</sup> Organism from which the BLAST match was obtained. <sup>d</sup> E-value associated with the BLAST match.

\* Null alleles (r > 0.4).

'Primers successfully amplified for Taxillus delavayi.

TABLE 2. Genetic properties of 17 newly developed polymorphic microsatellite loci in three populations of *Taxillus nigrans*. Loci exhibiting null alleles are not included.<sup>a</sup>

	Sicl	huan University (n	= 100)		Tazishan ( $n = 30$	))		Huanhuaxi ( $n = 3$	30)
Locus	A	$H_{\rm o}$	H <sub>e</sub>	A	H <sub>o</sub>	H <sub>e</sub>	A	$H_{\rm o}$	H <sub>e</sub>
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	8	0.931	0.829	10	0.828	0.757
TR85804	18	0.808	0.876	9	0.633	0.799	17	0.833	0.898
TR87965	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;  $H_e$  = expected heterozygosity;  $H_o$  = observed heterozygosity; n = number of individuals sampled. <sup>a</sup>Voucher and locality information are provided in Appendix 1.

low-quality ( $Q \le 3$ ) bases in a single read was restricted to less than 50%, and paired reads were discarded if the number of unknown nucleotide bases in either of the paired reads exceeded 3% following the sequencing company's protocol (Novogene). After removing the adapter sequences and ambiguous reads, the clean reads obtained were de novo assembled using Trinity (release 2013-02-25; Grabherr et al., 2011) with default settings. The final assembly was composed of 299,147 unigenes and had an N50 size of 1056 bp. Raw transcriptome read data

were deposited in the National Center for Biotechnology Information (NCBI) Short Read Archive (accession no. SRP105083).

SSRs were detected using the Perl script MISA (Thiel et al., 2003) with a motif size of one to six nucleotides and thresholds of eight, four, four, three, three, and three repeat units for mono-, di-, tri-, tetra-, penta-, and hexanucleotide SSRs, respectively. We selected 83,954 microsatellite loci and used the primer design software package Primer3 version 2.3.6 (Untergasser et al., 2012) to design primer sets. Following random browsing across the output files of these primer sets, 40 markers were selected based on length (19–20 bp), GC

TABLE 3. Fragment sizes detected in cross-amplification tests of the 19 newly developed microsatellite markers in *Taxillus delavayi* and *Scurrula parasitica*.<sup>a</sup>

Locus	Taxillus delavayi $(n = 2)$	Scurrula parasitica $(n = 5)$
TR7149	167	152-163
TR11564	192	193
TR24412	_	124
TR47466	_	272
TR51334	182	174–182
TR56117	_	155–185
TR59209	_	125–143
TR83979	244	177-211
TR85804	_	179–255
TR87965	_	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. <sup>a</sup>Voucher and locality information are provided in Appendix 1. content (40–65%) of the primers, and annealing temperatures (59–61°C) of the primer sets. Nineteen of the 40 tested markers were selected based on PCR success rate and degree of polymorphism (difference in band length), and these were used to genotype individual *Taxillus* plants (Table 1).

Genomic DNA was extracted from the silica-dried leaves of 160 individuals from three populations of T. nigrans, two individuals of T. delavayi, and five individuals of S. parasitica (Appendix 1) using the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987). PCR reactions were performed in 25-µL volumes containing 12.5 µL 2× PCR buffer, 300.0 µM each dNTP, 0.3 µM each primer, 1.25 unit Taq DNA polymerase (Vazyme Biotech, Nanjing, China), and ca. 50 ng of genomic DNA. The cycling conditions were as follows: 95°C for 5 min, followed by 35 cycles of 95°C for 45 s, 55°C for 30 s, and 72°C for 45 s; the reactions were completed by a final elongation step at 72°C for 10 min. The PCR products were checked on 1% agarose gels to confirm PCR success and then sent to TsingKe (Chengdu, China) for microsatellite genotyping. Primer pairs were synthesized with the forward primer of each pair 5' end-labeled with either 6-FAM, TAMRA, or HEX (Applied Biosystems, Foster City, California, USA), and amplicons were analyzed on an ABI PRISM 3100 genetic analyzer. The microsatellite genotype at each locus for each individual was determined using GeneMarker (SoftGenetics, State College, Pennsylvania, USA). Allele sizes at each locus were then scored and checked for possible genotyping errors, such as stuttering, large allele dropouts, or null alleles, using CERVUS (Dakin and Avise, 2004). In total, null alleles (null allele frequency [r] >0.4) were detected at two loci (Table 1). These loci were eliminated, and the remaining 17 microsatellite loci were subjected to further analyses (Table 2).

These 17 microsatellite loci were highly polymorphic, with two to 21 alleles per locus. We used GenAlEx version 6 (Peakall and Smouse, 2006) to calculate the number of alleles and the observed and expected heterozygosity at each locus (Table 2). When using GIMLET version 1.3.3 (Valière, 2002), a minimum of two loci and six loci are needed to estimate, respectively, the unbiased probability that a genotype is shared by two individuals ( $P_{ID}$ ) in a population, and the probability that a genotype is shared by two siblings ( $P_{ID}$ ).

In the cross-species transferability test, eight of the 19 loci were successfully genotyped in two individuals of *T. delavayi* taken from herbarium specimens (Table 3). In contrast, all polymorphic loci were successfully amplified in *S. parasitica* (Table 3). The difference in success between *T. delavayi* and *S. parasitica* may have been due to a higher proportion of degraded DNA from *T. delavayi* herbarium specimens.

## 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 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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Species	Ν	Population code	Locality	Geographic coordinates	Voucher specimen accession no. <sup>a</sup>
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,
T. nigrans	30	TZT	Tazishan, Sichuan	30°38'7"N, 104°7'15"E	SZ-00545044 SZ-00545045, SZ-00545046,
T. nigrans	30	НН	Huanhuaxi, Sichuan	30°39'28"N, 104°1'55"E	SZ-00545047 SZ-00545048, SZ-00545049, SZ-00545050
T. delavayi (Tiegh.) Danser T. delavayi Scurrula parasitica L.	1 1 5	Individual Individual TZS	Maerkang, Sichuan Muli, Sichuan Tazishan, Sichuan	31°54′46″N, 102°11′24″E 27°55′55″N, 101°16′43″E 30°38′7″N, 104°7′15″E	SZ-00343030 SZ-00280020 SZ-00280006 SZ-00545051

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

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

<sup>a</sup>All voucher specimens are deposited at the herbarium of Sichuan University (SZ), Sichuan, China.