



ORIGINAL ARTICLE

Development and characterisation of nine polymorphic microsatellite markers for *Tephrosia calophylla* Bedd. (Fabaceae)



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Abstract *Tephrosia calophylla* Bedd. (Fabaceae) is an endangered tropical plant endemic to south-western Ghats, India. The objective of this study was to contribute to the characterisation of the diversity of this rare species, which is necessary for its future conservation. Accordingly, microsatellite markers were designed, and their ability to detect polymorphisms was determined. Nine microsatellite markers were developed using genomic libraries, and all of the markers were successfully amplified in 42 individuals. Three to nine alleles per locus were observed, and the heterozygosity of the loci ranged from 0.381 to 0.905. The nine newly developed polymorphic markers recognise a sufficient number of varying loci to perform further studies on the conservation and breeding of this medicinal cultivar.

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1. Introduction

There are approximately 400 species of *Tephrosia* (Fabaceae), a complex pantropical genus, which are mainly distributed in Asia, Africa, Australia and America (Willis, 1973; Geesink, 1981). *Tephrosia* belongs to the tribe Millettieae (formerly Tephrosieae) of the family Fabaceae (Leguminosae) and is represented by 24 species in India and 13 in Andhra Pradesh (Saldanha and Singh, 1984; Pullaiah and Chennaiah, 1997). *Tephrosia* is well known for its richness in presenting a wide variety of unusual flavonoids that have been isolated and exploited by the medicinal and pharmaceutical industries

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(Dewick, 1993). *Tephrosia calophylla*, a perennial woody undershrub endemic to south India, is one of 13 rare or threatened *Tephrosia* species (Thammanna et al., 1994; Lakshmi et al., 2008; Sasikala et al., 2013).

Unlike other species of the genus *Tephrosia* that exhibit normal taproots, compound leaves, pink flowers and compressed pods, this species shows variation in morphological characteristics in terms of the presence of tuberous roots, simple oblanceolate leaves with winged petioles, red flowers in terminal racemes and 8–10 seeded glabrous pods. It is included in the subgenus *Reineria* together with *Tephrosia strigosa*, which displays simple leaves with pink flowers (Baker, 1876). The chemical profile of *T. calophylla* includes flavonoids, such as tephcalostan, coumestans, 7-O methyl glabranins and many secondary metabolites (Hari Kishore et al., 2003; Ganapaty et al., 2009), which represent lead molecules for the development of novel drugs. Ethanobotanical studies of this species have also revealed the utility of its tuberous roots for treating diabetes and jaundice and of its leaf extracts for curing ulcers, inflammation and microbial infections.

Due to the effects of adverse climatic changes, mining and other disruptive events, such as overgrazing and human exploitation, this species is presently on the verge of extinction and has been listed as endangered by the Botanical Survey of India (Nayar and Sastry, 1987, 1988, 1990, 1997 and Botanical Survey of India, 2011). The populations of *T. calophylla* in the Talakona forest have declined rapidly in the last two decades, possibly due to changes in forest management and excessive harvesting by collectors of rare medicinal plants. Because information on genetic variation is a prerequisite for breeding programmes for the improvement of any plant species, detailed knowledge of the genetic structure of these populations is

required for the remaining wild germplasm to be developed (Naomab, 2004). However, there are no available reports on the population genetics, phylogeographics or evolution of the species *T. calophylla*, and no microsatellite DNA markers have been developed for investigation of the *Tephrosia* genus. Thus, in view of the medicinal importance of this species, the present investigation was initiated to develop microsatellite markers to study the extent of variability among *T. calophylla*.

2. Material and methods

Fresh leaves of *T. calophylla* were collected from the interior of the Talakona forest in the Chittoor district, Andhra Pradesh, and stored at -80°C . Genomic DNA was extracted from 80 to 100 mg of leaf material using the DNeasy Plant Mini Kit (Qiagen). Extracted genomic DNA was digested with *Bsa*AI and *Hinc*II and ligated to SNX linkers using T4 DNA ligase (Hamilton et al., 1999). The ligated fragments were enriched through hybridisation with biotinylated dimeric and trimeric nucleotide repeats (CT_8 , TC_8 , TA_8 , TG_8 , GC_8 , CTT_8 , ATA_8 and GAA_8) in three reactions, with incubation at 97°C for 10 min, followed by 56°C for 30 min. Streptavidin-coated magnetic beads (New England Biolabs) were used for recovering single-stranded DNA.

Double-stranded DNA was generated from the enriched DNA fragments via polymerase chain reaction (PCR) using SNX primers. The PCR products were then digested with the restriction enzyme *Nhe*I (New Engl and Biolabs) and ligated into the pUC19 plasmid (digested with *Xba*I and dephosphorylated). These ligates were transformed into *Escherichia coli* DH5- α cells and cultured on Luria–Bertani (LB) agar/ampicillin plates. The resultant colonies were transferred

Table 1 Characteristics of the nine polymorphic microsatellite loci developed for *Tephrosia calophylla*.

Locus name (GenBank accession no.)	Primer sequence 5'–3'	Dye (forward primer)	Repeated motif	T_A ($^{\circ}\text{C}$)	Allele size range (bp)	N_A	H_O	H_E	PIC	P
TPM02 q	F:CCTCCTCTTCCTCCAAATCC R:AGAAGCAGGAAGGCAAACAA	FAM	(CTT) ₁₀	58	212–236	2	0.381	0.477	0.360	0.2810 ^Y
TPM03 JF262785	F:AGCCATAGACAGAGCGAGGA R:TGTCCATTGCTCAAAACAGC	FAM	(AG) ₁₅	56	100–115	4	0.619	0.517	0.422	0.1990 ^Y
TPM05 JF262786	F:GCTTAATGCCCTTCCCTTTT R:CCAGCCTTCTATCCTCCAAC	HEX	(AG) ₁₀	59	90–102	4	0.571	0.523	0.425	0.7986 ^Y
TPM06 JF262790	F:GCGTTTAAAGGACGGCAATA R:TGCCATCTTAGGTCCTTGC	FAM	(TAT) ₁₁	60	116–128	5	0.571	0.506	0.439	0.3021 ^Y
TPM09 JF262787	F:TCTTCCTTCCAAAGCAAAAA R:GCAACCCTAGGGCTCTTCTT	HEX	(AC) ₈ (AT) ₄ AA (AGGA) ₉	56	160–180	5	0.738	0.567	0.468	0.0128 ^Y
TPM11 JF715421	F:TCCTTGGGAATTCAGTGCC R:TTGCTTAAAATCAATGGAGTGAA	FAM	(AT) ₉	59	154–180	6	0.857	0.675	0.608	0.0079 ^Y
TPM14 JF262788	F:TGAGCATTTTGGAAAGGACAA R:AAAACGCCTGTTTTGGGTTA	HEX	(ACT) ₁₆	56	168–176	3	0.905	0.549	0.446	*** < 0.005
TPM17 JF715422	F:TGATTGTTGAAGAGCTTTGTGA R:GCCTATGCAGCCAAATCTTC	FAM	(AT) ₁₀	60	82–100	4	0.619	0.566	0.467	0.5246 ^Y
TPM18 JF262789	F:TCTTTAGATCTAGGAGAATGTAAT R:ATATCAAAGTTTGTAGTGAACAAACAGC	FAM	(AGA) ₁₁	58	104–120	3	0.524	0.575	0.496	0.8770 ^Y

The annealing temperature (T_A); size range of alleles (base pairs); number of alleles (N_A); observed heterozygosity (H_O); expected heterozygosity (H_E) and probability of deviation from Hardy–Weinberg proportions (P) are reported. Each locus was genotyped in a minimum of 48 plants (range 38–42).

^Y Not significant following Bonferroni correction.

*** Significant following Bonferroni correction.

to nylon membranes and then hybridised with ^{33}P radiolabeled probes with the same repeat motifs used in the enrichment. To identify the positive DNA fragments of interest, the membranes were again hybridised with the probes, and the positive clones were sequenced on an ABI 3730xl DNA Analyzer (Applied Biosystems, CA). Repetitive elements were located using the Msatcommander programme (Faircloth, 2008), and flanking primers were designed with OligoPerfect™ Designer (Invitrogen) software.

PCR amplification was carried out in a Mastercycler® ep Eppendorf thermocycler (Eppendorf, Hamburg, Germany). The reaction mixture consisted of 1 μl of DNA (50 ng/ μl), 2.5 μl of 10 \times PCR buffer with 15 mM MgCl_2 , 0.3 μl of 5 U Taq polymerase (AmpliTaq Gold), 2.5 μl 2 mM dNTPs, 1 μl (10 pM) each of the forward and reverse primers and sterile double distilled water to a final volume of 25 μl . PCR amplifications with all primer pairs were performed using the following programme: an initial denaturation at 95 °C for 10 min, followed by 35 cycles of denaturation at 95 °C for 50 s, primer annealing at the temperatures given in Table 1 for 50 s and extension at 72 °C for 55 s, with a final extension at 72 °C for 10 min. The forward primer in each pair was labelled with a fluorescent tag (FAM, HEX), and the obtained fluorescent PCR products were diluted 1:15 in water and mixed with formamide and the Genescan LIZ-500 size standard (Applied Biosystems). The allele sizes were determined using an ABI PRISM 3730 DNA analyser and GeneMapper version 3.5 software (Applied Biosystems). Individuals were genotyped by assessing their allele sizes using an ABI 3130xl Genetic Analyser (Applied Biosystems) with forward primers labelled with FAM and HEX (Applied Biosystems), together with the LIZ 500 size standard (Applied Biosystems).

The expected (H_E) and observed (H_O) heterozygosities as well as the polymorphic information content (PIC), departure from the expected Hardy–Weinberg proportions and gametic disequilibrium were calculated using the CERVUS v3.0 (Kalinowski et al., 2007) and Arlequin version 3.5.1 programmes (Excoffier and Lischer, 2010). The programme Micro-Checker version 2.2.3 (vanOosterhout et al., 2004) was used to test for null alleles, large allele dropout and scoring errors due to stutter peaks. The significance values for all diversity tests were corrected through the sequential Bonferroni procedure (Rice, 1989).

3. Results and discussion

A total of 46 different microsatellite-containing clones were identified in *T. calophylla*. However, only 18 of the clones were suitable for designing PCR primer pairs. Nine out of the 18 primer pairs yielded products of various sizes within the expected size range from ten different *T. calophylla* samples and were selected for fluorescent labelling.

These nine primer pairs were used to assess the associated loci in 42 *T. calophylla* individuals from the Talakona forest. Out of the nine loci, three (TPM09, TPM11 and TPM 14) deviated significantly from Hardy–Weinberg proportions (Table 1). The number of alleles detected at each locus ranged from two to six. The expected heterozygosity ranged from 0.477 to 0.675, while the observed heterozygosity ranged from 0.381 to 0.905. Analysis performed with Micro-Checker indicated an increased percentage of homozygosity at loci, with a departure

from Hardy–Weinberg proportions that can be attributed to the presence of null alleles.

Genomic library microsatellite enrichment is an efficient procedure for marker development in *T. calophylla*. This is the first reported attempt to use enriched libraries for microsatellite marker development in this species. A sample of the tested microsatellite markers proved efficient for genetic analysis of *T. calophylla*. These nine newly developed polymorphic markers recognise a sufficient number of varying loci to perform further studies. This information will be useful for conservation and restoration efforts aimed at this species.

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References

- Baker, J.G., 1876. Leguminosae. In: Hooker, J.D. (Ed.), Flora of British India. Reeve and co., London, pp. 110–114.
- Botanical survey of India, 2011. Threatened plants of India. <http://www.bsienviis.nic.in/threatened_plants_of_india.htm> .
- Dewick, P.M., 1993. Isoflavonoids. In: Harbone, J.B. (Ed.), The Flavonoids: Advances in Research Since 1986. Chapman and Hall, London.
- Excoffier, L., Lischer, H.E.L., 2010. Arlequin suite ver 3.5: a new series of programs to perform population genetics analyses under linux and windows. Mol. Ecol. Res. 10, 564–567.
- Faircloth, B.C., 2008. Msatcommander: detection of microsatellite repeat arrays and automated, locus-specific primer design. Mol. Ecol. Res. 8, 92–94.
- Ganapathy, S., Srilakshmi, G.V., Pannakal, S.T., Rahman, H., Laatsch, H., Brun, R., 2009. Cytotoxic benzil and coumestan derivatives from *Tephrosia calophylla*. Phytochemistry 70, 95–99.
- Geesink, R., 1981. Tephrosiaceae. In: Ravan, P.H., Polhill, M. (Eds.), Advances in Legume Systematics. Royal Botanic Gardens, Kew, pp. 245–260.
- Hari Kishore, P., Vijaya Bhaskar Reddy, M., Gunasekar, D., Marthanda Murthy, M., Caux, C., Bodo, B., 2003. A new coumestan from *Tephrosia calophylla*. Chem. Pharmacol. Bull. 51, 194–196.
- Hamilton, M.B., Pincus, E.L., Di Fiore, A., Fleischer, R.C., 1999. Universal linker and ligation procedures for construction of genomic DNA libraries enriched for microsatellites. Biotechniques 27 (3), 500–502.
- Kalinowski, S.T., Taper, M.L., Marshall, T.C., 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol. Ecol. 16 (5), 1099–1106.
- Lakshmi, P., Akbar Ali Khan, P., Narasimha Reddy, P., Lakshminarayana, K., 2008. Genetic relationship among *Tephrosia* species as revealed by RAPD analysis. Asian J. Biol. Sci. 1, 1–5.
- Naomab, E., 2004. Assessment of Genetic Variation in Natural Populations of Marama bean (*Tylosema esculentum*) Using Molecular Markers (Dissertation). University of Namibia.
- Nayar, M.P., Sastry, A.R.K., 1987. In: Red Data Book of Indian Plants, vol. 1. BSI, Calcutta, pp. 217–218.
- Nayar, M.P., Sastry, A.R.K., 1988. In: Red Data Book of Indian Plants, vol. 2. BSI, Calcutta, pp. 214–218.
- Nayar, M.P., Sastry, A.R.K., 1990. In: Red Data Book of Indian Plants, vol. 3. BSI, Calcutta, pp. 243–244.
- Nayar, M.P., Sastry, A.R.K., 1997. Red data book plants of India. Bot. Surv. India, 1987–1988.

- Pullaiah, T., Chennaiah, E., 1997. In: Flora of Andhra Pradesh, vol. I. Scientific Publishers, Jodhpur, India, pp. 185–194.
- Rice, W.R., 1989. Analyzing tables of statistical tests. *Evolution*, 223–225.
- Saldanha, C.J., Singh, B.G., 1984. Leguminosae. In: Saldanha, C.J. (Ed.), . In: Flora of Karnataka, vol. I. Oxford and IBH, pp. 495–499.
- Sasikala, T., Rao, S.V., Kamakshamma, J., 2013. Intrapopulation genetic diversity assessed in *Tephrosia calophylla* bedd. A rare medicinal plant using RAPD markers. *Inter. J. Pharmacol. Biosci.* 4 (3).
- Thammanna, R., Narayana, K., Chetty, M.K., 1994. In: Angiospermic Wealth of Tirumala, vol. I. TTD Press, Tirupati, pp. 49.
- vanOosterhout, C., Hutchinson, W.F., Wills, D.P.M., Shipley, P., 2004. Micro-Checker: software for identifying and correcting genotyping errors in microsatellite data. *Mol. Ecol. Notes* 4, 535–538.
- Willis, J.C., 1973. *Dictionary of Flowering Plant and Ferns*. University Press, Cambridge, p. 1135.