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SHORT RESEARCH ARTICLE

Development and characterisation of EST-SSR-based markers in curry leaf (*Murraya koenigii*)

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Abstract

Crop improvement strategies depend on a better understanding of their genetic architecture. Murraya koenigii L. an important medicinal and aromatic value vegetable/spice tree crop, needs genetic improvement in yield and other agronomic traits. In the present study, EST-SSRs have been developed using the transcriptome sequence of curry leaf. A total of 12861 EST-SSRs were identified among the tested transcripts. The mononucleotide motif (58.8%) was more abundant than those with tri (20%), di (14.6%) and other higher order motifs (6.61%). About 24 EST-SSR primers were validated and all of them were polymorphic. The estimates of unbiased gene diversity ranged from 0.70 (for the marker MK1 locus) to 0.98 (at the MK8 locus), with an average of 0.88. The estimate of PIC values varied from 0.66 (at the MK1 locus) to 0.97 (at the MK8 locus) with a mean of 0.85, irrespective of the repeat motif-based markers. The 50 Murraya accessions could be classified into four clusters based on EST-SSR marker allelic data. Significant differences among the genotypes classified into different clusters for all three quantitative traits, namely, leaf yield, aroma intensity and essential oil content, suggested that EST-SSR marker alleles are efficient in discriminating the genotypes at loci controlling horticulturally important quantitative traits as well. To the best of our knowledge and belief, the present study is the first report on the development, validation and use of EST-SSR for assessing the genetic diversity of Murraya koenigii.

Keywords: Transcriptome, EST-SSR, polymorphism, PIC, clusters

Murraya koenigii, commonly known as used in culinary due to its aromatic quality (curry leaf or karipatta) in Indian dialects, belongs to the Family Rutaceae, which represents more than 150 genera and 1600 species. It is a perennial leafy vegetable tree species containing major phyto-chemicals like phenolic, carbazole alkaloids and phenols. The improvement of this crop through conventional breeding relies on phenotype-based selection, which is rather very slow and less effective for improving leaf yield and consumer/end-user evident traits, which are controlled by a large number of genes with crossover genotype-by-environment interaction. DNA markers have proved powerful surrogates of traits that are either difficult to score or those that express after a long juvenile period in perennial crops, including *Murraya*. Of the several types of DNA markers, those based on simple sequence repeats (SSR), owing to their abundance and distribution throughout the genome, co-dominant, multiallelic inheritance capture large genomic regions on a per-site basis (Hamblin et al. 2007), coupled with ease of assay in any basic molecular biology laboratory, have become breeder-preferred markers. Based on their origin, two types of SSR markers are recognized. These are (i) genomic SSR and (ii) expressed sequence tag-SSR (EST-SSRs)-based markers. As coding sequences are more conserved than non-coding sequences, the cross-genome comparison and evolutionary analysis would be more accurate with the use of EST-SSRs than genomic-SSRs (Eujayl et al. 2004).

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Identification of EST-SSRs has been more practical and cost-efficient with the emergence of less expensive sequencing platforms (Zalapa et al. 2012). In less-researched crops like *Murraya, the* development and validation of EST-SSR markers is seldom attempted. Therefore, the present study was conducted to develop and validate the EST-SSR-based markers in curry leaf to generate useful information for improving the medicinal traits.

Identification of EST-SSRs, primer design and validation

The transcriptome data of *Murraya* leaf were downloaded from NCBI (National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/sra/?term=SRR5590072). About 57,428 transcripts were analysed for identification of SSRs using 'Krait' software (Du et al. 2018). A total of 12861 sequences containing dinucleotide, trinucleotide, tetra, penta, hexa and other higher order SSR motifs were detected with a minimum of five, four, and three repeats, respectively. Primer pairs were designed to conserved sequences flanking randomly chosen 50 out of 300SSR targeting sequences using 'PRIMER3' software (http:// frodo. wi. mit. edu/primer3; Untergasser et al. 2012). Of these, 24 SSRs were validated.

A total of 50 representative genotypes maintained in the experimental plots of the Indian Council of Agricultural Research (ICAR) - Indian Institute of Horticultural Research (IIHR), Bangalore, India, were used as genetic material in the present study. Most of these genotypes were collected from Tamil Nadu, Karnataka and Odisha and a few wild species from Himachal Pradesh (Supplementary Table S1). High-quality DNA was isolated from young leaf tissues using the CTAB method as per the procedure described by Poornima et al. (2023); a 0.8% agarose gel was used for DNA quality check and a NanoDrop spectrophotometer ND 1000 (NanoDrop Products, USA) was used for measuring the quantity of DNA. DNA was normalized to 10 ng$\mu L^{-1}$for PCR amplification.

Genotyping assay

Fifty genotypes were genotyped using 24 EST-SSR primer pairs. PCR was performed in a 15 µL reaction volume in an Eppendorf tube with 50 ng genomic DNA, 10 pmol each of tagged forward and reverse primers, 10 pmol of "M13 tag" (FAM, VIC, NED and PET) and 2.5 mM of dNTPs, 0.3U of taq polymerase (Sigma USA) and 1.5 µL of 10X PCRbuffer (10 mM Tris pH 8.0, 50 mM KCl, 1.8 mM MgCl2 and 0.01 mg/ mL gelatin) using Thermal Cycler PCR (Applied Biosystems, Foster City, CA, USA). The PCR was performed using the program set for initial denaturation for 5 min at 95°C, followed by 35 cycles of denaturation for 1 min at 95°C, annealing for 30 s at 55–60°C (primer-specific) and extension for 1 min at 72°C, followed by final extension of 72°C for 10 min and hold at 4°C. Amplicons were confirmed on 1.5% agarose gel, post-PCR multiplex sets were constructed based on fluorescence-labelled primer dyes. The pooled PCR product was outsourced for capillary electrophoresis and analysed using GenScan (Bioserve biotechnologies, ltd, Hyderabad).

Statistical analysis

The population genetic parameters, such as total number of alleles (Na), effective number of alleles per SSR locus (Ne), observed heterozygosity (Ho) and expected heterozygosity (He) and polymorphic information content (PIC), were estimated as detailed below using "Cervus" software. Two other parameters, namely major allele frequency (MAF) and unbiased expected heterozygosity (Hue), were also estimated using "GenAlex" software (Peakall and Smouse, 2012). PowerMarker (Liu et al. 2005) was used to determine major allele frequency (MAF). The 50 accessions were classified into different clusters based on allelic data using the unweighted neighbour-joining method implemented in Darwin version. 6.0.14 software (Perrier et al. 2006).The historical data were used to estimate mean essential oil yield, leaf yield and intensity of the aroma of the genotypes classified into different clusters.

EST-SSRs identification, characterisation, validation and assessment of genetic diversity

A total of 12861 EST-SSRs were identified among the tested transcripts (Table 1). The mononucleotide motif (58.8%) was more abundant than those with tri (20%), di (14.6%) and other higher order motifs (6.61%) among these 12861 EST-SSRs. Similarly, the trinucleotide motif (33.33%) was more abundant than those with di (25%), tetra and penta (16.67%) among these 24 EST-SSRs used for validation.

All 24 EST-SSR primers amplified the genomic DNA and were polymorphic. The major allele frequency (MAF) varied between markers with different motifs (Supplementary Table S2). The frequencies of major alleles were higher at di- and tri-nucleotide repeat-based markers compared to those at other repeat motif-based markers. The number of alleles at these 24 polymorphic EST-SSR markers varied from 7 to 44, with a mean of 22 alleles per locus. The average number of alleles per locus differed with the repeat motif of the markers. While the average number of alleles was comparable between SSR markers with di-(25.16) and others-nucleotide repeat motifs (25.5), they were slightly lower in markers with tri-nucleotide repeat motifs (17.38). As expected, the range of the effective number of alleles per locus (3–29 with a mean of 12) was lower than that of the average number of alleles per locus (7–47 with a mean of 22.7). The average gene diversity of the 50 test genotypes at dinucleotide repeat-based markers(0.89) was slightly greater than that at tri-nucleotide repeat-based markers (0.83). The estimates of unbiased gene diversity ranged from 0.70 (for the marker MK1 locus) to 0.98 (at the MK8 locus),

Table 1. Details of ESTs and SSRs identified in M. koenigii

Parameters	Numbers
Total EST-sequences downloaded	57428
Total length of sequences	42379801
Total size of examined sequences (bp)	42379801
Number of SSR-containing sequences	12861
Number of sequences selected for EST- SSR primer development	50
Number of EST-SSR primers used for validation	24

Table 2. Estimates of population genetic parameters based on the frequency of the alleles at 24 EST–SSR marker loci in a set of 50 *M. koenigii* germplasm accessions

EST-SSR based marker locus	Alleles/locus (Na)	Effective alleles/ locus (Ne)	Major allele frequency (MAF)	Shannon's information Index (I)	Nei's gene diversity (He)	Heterozygo-sity (Ho)	Unbiased Expected Heterozygosity (uHe)	Polymorphic information content (PIC)
Mk1	9.00	3.25	0.46	1.58	0.69	0.31	0.70	0.67
Mk2	11.00	4.15	0.40	1.85	0.76	0.02	0.77	0.74
Mk3	19.00	8.57	0.22	2.46	0.88	0.65	0.89	0.87
Mk4	42.00	17.93	0.17	3.39	0.94	0.41	0.95	0.94
Mk5	10.00	5.29	0.29	1.94	0.81	0.16	0.82	0.79
Mk6	10.00	4.37	0.38	1.82	0.77	0.04	0.78	0.75
Mk7	13.00	3.48	0.44	1.69	0.71	0.37	0.72	0.68
Mk8	47.00	38.13	0.08	3.74	0.97	0.52	0.98	0.97
Mk9	18.00	6.44	0.30	2.35	0.84	0.02	0.85	0.83
Mk10	7.00	3.69	0.34	1.51	0.73	0.00	0.74	0.69
Mk11	28.00	16.14	0.12	3.04	0.94	0.42	0.95	0.94
Mk12	36.00	19.10	0.12	3.26	0.95	0.42	0.96	0.95
Mk13	20.00	10.22	0.24	2.68	0.90	0.00	0.91	0.90
Mk14	15.00	9.88	0.16	2.46	0.90	0.00	0.91	0.89
Mk15	33.00	17.34	0.17	3.22	0.94	0.16	0.95	0.94
Mk16	27.00	10.84	0.19	2.81	0.91	0.24	0.92	0.90
Mk17	12.00	5.84	0.34	2.09	0.83	0.00	0.84	0.81
Mk18	13.00	7.91	0.24	2.28	0.87	0.00	0.88	0.86
Mk19	30.00	20.49	0.11	3.22	0.95	0.32	0.96	0.95
Mk20	32.00	21.65	0.10	3.27	0.95	0.20	0.96	0.95
Mk21	15.00	7.70	0.22	2.31	0.87	0.02	0.88	0.86
Mk22	11.00	6.68	0.24	2.10	0.85	0.00	0.86	0.83
Mk23	44.00	29.24	0.07	3.57	0.97	0.48	0.98	0.97
Mk24	43.00	24.39	0.09	3.47	0.96	0.44	0.97	0.96
Total	545.00	302.73						
Average	22.71	12.61	0.23	2.59	0.87	0.22	0.88	0.86

with an average of 0.88. The difference in the estimates of absolute gene diversity (0.87) and those of unbiased gene diversity (0.88) was marginal. The estimate of PIC values varied from 0.66 (at MK1 locus) to 0.97 (at MK8 locus) with a mean of 0.85 irrespective of the repeat motif-based markers (Table 2). While the estimates of PIC value were comparable between di and other repeat motif-based markers, they were slightly lower at tri-nucleotide repeat motif-based markers (Supplementary Table S3).

Organisation of variability among the accessions

The 50 *Murraya koenigii* accessions could be classified into four clusters based on EST-SSR marker allelic data (Fig. 1). The number of accessions varied with the cluster. The first and third clusters had a greater number of accessions (18) compared to the other two clusters (7). The average essential oil yield, leaf yield and Aroma intensity of the accessions classified into clusters differed significantly (Table 3).

Developing genomic resources is very important for their use in genetic improvement of tree crops, with no

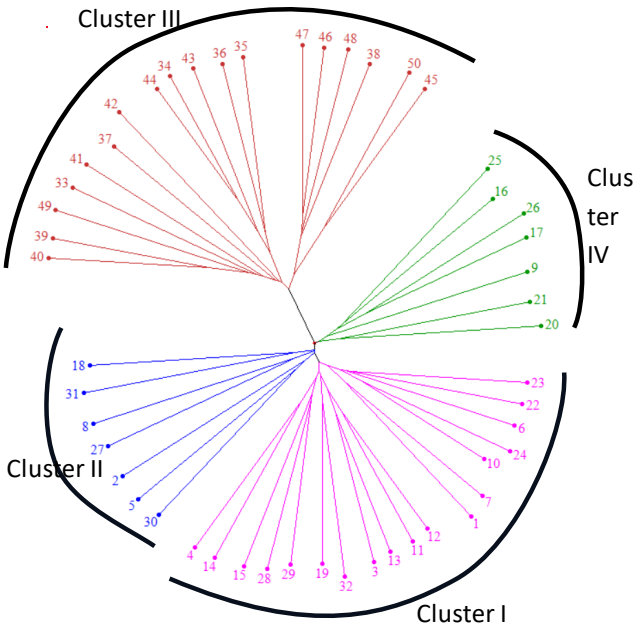

Fig. 1. EST-SSR marker assay-based grouping of *M. koenigii* germplasm accessions

for the marker assay. Our study reported a greater number of alleles with an average of 22 compared to those reported in other tree crops such as *J. mandshurica* (Wang et al. 2023). The major allele frequency estimates provide clues about the frequency of occurrence of specific alleles in the test genotypes. The markers with an intermediate frequency (0.5) would have a better ability to discriminate the test genotypes. In the present study, the markers MK1 and MK7 with intermediary frequency could be considered as ideal ones for polymorphism detection.

Genetic diversity at SSR loci measures the magnitude of heterozygosis and gene diversity is a measure of such a parameter (Laurentin 2009). The present study reports an average gene diversity of 0.88 which is comparatively higher than that reported (0.20) by Verma et al. (2013) in curry leaf. The two other alternative parameters of gene diversity are observed heterozygosis (Ho- proportion of genotypes that are heterozygous at a given SSR locus) and expected heterozygosis (He- heterozygosity expected at a locus under Hardy-Weinberg equilibrium). If Ho=0, then it means that the genotypes are homozygous at the tested marker loci. In the present study, Ho<He indicated that genotypes under test exhibit greater levels of inbreeding than is expected.

The estimates of PIC at SSR loci depend on the number

Table 3. Classification of 50 M. koenigii accessions into four clusters based on EST-SSR marker allelic data

Cluster number	Number of genotypes	Mean essential oil yield	Mean leaf yield	Mean aroma intensity
C1	18	0.3	21.9	2.7
C2	07	0.3	26.2	2.6
C3	18	0.3	25.5	2.2
C4	07	0.3	17.4	2.8
F statistics- 235.22				
F probability- 0.000467				

exception of *Murraya koeinigii*. In the present study, 24 EST-SSR markers were developed and validated, which are highly polymorphic with a number of alleles as high as 29, with an average of 12 alleles. The number of alleles of this magnitude indicated a greater genetic variation at the EST-SSR loci as earlier reported (Nei 1987). One hundred percent polymorphism of all the SSR-based markers in the present study could be attributed to two reasons. The genotypes used in the present study are of diverse origin, which are essentially subjected to natural selection pressure. This would have resulted in mutation followed by recombination of the mutant alleles, resulting in substantial variability at different loci at either coding and/or non-coding sequences. It is hypothesised that these loci might also include those at SSRs. Hence, the genotypes used in the present study differ at SSR loci as well. The estimates of Na give us an idea about the size and kind of population used of alleles and their frequencies (Hilderbrand et al. 1992). As reported by Guo and Elston (1999), the PIC>0.5 for a marker suggests that it is highly informative, while PIC <0.5 but more than 0.25 is moderately informative and PIC<0.25 represents that the marker considered is less informative. In the present study, all the EST-SSR markers were informative (PIC>0.25) with an average PIC value of 0.85. The estimates of average PIC value in the present study are comparatively higher than those reported in *Moringa* (0.78) by Poornima et al. (2023). To the best of our knowledge and belief, the present study on the development, validation and the use of EST-SSR for assessing the genetic diversity of *Murraya koenigii* is the first report.

Supplementary materials

The Supplementary Tables S1 to S3 are provided, which can be accessed at www.isgpb.org

Authors' contribution

Conceptualization of research (KNP, BRR, SR, KVR); Designing of the experiments (KNP, BRR, SR, KVR); Contribution of experimental materials (BRR, KVR); Execution of field/lab experiments and data collection (KNP); Analysis of data and interpretation (KNP); Preparation of the manuscript (KNP, BRR, SR, KVR).

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Supplementary Table S1. Details of *Murraya koenigii* germplasm accessions with geographical origin and their major phenotypic characteristic features

S. No.	Code	Genotype	District	State	Biological status	Essential oil yield (%)	Total fresh leaf yield/plant (kg)	Aroma intensity (1-3 scale), 1=low, 2=medium, 3=high
1	2	LSR/18/75	Bijapur	Karnataka	Landrace	0.2	18.1	3
2	4	BRR18/2	Selam	Tamil Nadu	Landrace	0.21	24.3	3
3	5	BRR/18/3	Selam	Tamil Nadu	Landrace	0.28	22.1	3
4	14	BRR/18/11	Selam	Tamil Nadu	Landrace	0.23	18.2	3
5	18	LSR/18/9	Gulbarga	Karnataka	Landrace	0.4	21.8	2
6	26	BRR/18/18	Kallakurichi	Tamil Nadu	Landrace	0.25	16.3	3
7	30	BRR/18/22	Kallakurichi	Tamil Nadu	Landrace	0.27	18.3	3
8	33	BRR/18/25	Namakkal	Tamil Nadu	Landrace	0.19	27.1	1
9	35	BRR/18/27	Namakkal	Tamil Nadu	Landrace	0.29	14.7	3
10	36	BRR/18/28	Namakkal	Tamil Nadu	Landrace	0.3	23.7	3
11	39	BRR/18/31	Namakkal	Tamil Nadu	Landrace	0.25	26.6	3
12	40	LSR/18/6-a	Gulbarga	Karnataka	Landrace	0.51	18.1	3
13	41	BRR/18/32	Namakkal	Tamil Nadu	Landrace	0.21	23.1	3
14	47	BRR/18/34	Namakkal	Tamil Nadu	Landrace	0.21	26.4	1
15	48	BRR/18/35	Namakkal	Tamil Nadu	Landrace	0.2	27.3	1
16	53	BRR/19/09	Bengaluru-U	Karnakata	Landrace	0.35	15.3	3
17	56	RPP/18/14	Malkangiri	Odhisa	Landrace	0.35	16.3	3
18	61	BRR/19/10	Bengaluru-U	Karnakata	Landrace	0.38	18.5	3
19	62	BRR/19/11	Bengaluru-U	Karnakata	Landrace	0.31	25.8	3
20	63	RRP/18/4	Malkangiri	Odhisa	Landrace	0.3	12.7	3
21	66	Suwasini-b	UHS-Bagalkote	Karnataka	Release variety	0.25	21.6	3
22	67	BRR/18/14	Kallakurichi	Tamil Nadu	Landrace	0.28	24.6	3
23	71	RRP/18/20-a	Malkangiri	Odhisa	Landrace	0.37	25.4	2
24	72	RRP/18/55	Malkangiri	Odhisa	Landrace	0.35	16.7	3
25	73	RRP/18/20-b	Malkangiri	Odhisa	Landrace	0.35	24.5	2
26	74	RRP/18/51	Malkangiri	Odhisa	Landrace	0.41	16.8	3
27	76	BRR/19/16	Bengaluru-U	Karnakata	Landrace	0.49	23.6	3
28	81	BRR/19/18	Bengaluru-U	Karnakata	Landrace	0.31	27.9	3
29	93	BRR/19/22	Bengaluru-U	Karnakata	Landrace	0.38	17.9	3
30	94	BRR/19/23	Bengaluru-U	Karnakata	Landrace	0.41	32.7	3
31	95	BRR/19/24	Bengaluru-U	Karnakata	Landrace	0.51	35.6	3
32	109	BRR/19/38	Bengaluru-U	Karnakata	Landrace	0.49	18.4	3
33	80	BRR/19/17	Bengaluru-U	Karnakata	Landrace	0.51	24.7	3
34	144	BRR/19/67	Bengaluru-U	Karnakata	Landrace	0.39	27.4	3
35	96	BRR/19/25	Bengaluru-U	Karnakata	Landrace	0.41	28.5	3
36	97	BRR/19/26	Bengaluru-U	Karnakata	Landrace	0.39	29.1	3
37	124	BRR/19/52	Bengaluru-U	Karnakata	Landrace	0.44	23.5	3
38	111	BRR/19/40	Bengaluru-U	Karnakata	Landrace	0.31	18.9	3
39	110	BRR/19/39	Bengaluru-U	Karnakata	Landrace	0.45	18.4	3
40	7	BRR/18/5	Selam	Tamil Nadu	Landrace	0.21	16.4	3
41	43	BRR/19/05	Bengaluru-U	Karnakata	Landrace	0.41	19.2	3

42	32	BRR/18/24	Kallakurichi	Tamil Nadu	Landrace	0.22	15.9	3
43	19	BRR/18/14	Kallakurichi	Tamil Nadu	Landrace	0.23	21.6	3
44	60	Suwasini-a	UAD-D	Karnataka	Release variety	0.23	18.6	1
45	1	BRR/19/01-a	Bengaluru-U	Karnataka	Farmers Variety	0.18	31.6	1
46	6	BR-NN-0-a	Solan	Himachal Pradesh	Wild	0.23	35.9	1
47	8	BR-NN-0-a	Bilaspur	Himachal Pradesh	Wild	0.18	32.8	1
48	23	BR-NN-1-b	Hamirpur	Himachal Pradesh	Wild	0.26	38.5	1
49	28	BR-NN-17-c	Hamirpur	Himachal Pradesh	Wild	0.19	28.7	1
50	31	BR-NN-18-b	Hamirpur	Himachal Pradesh	Wild	0.22	30.1	1

Supplementary Table S2. EST-SSR-based primer sequences designed from leaf transcriptome of Murraya koenigii

S. No.	Primer name*	Forward primer sequence (5′-3′)	Reverse primer sequence (3′-5′)	SSR motif	Length of SSR motif
1	MK1	GGGAGAAACACTGGATTGCAT	TGCTCTCCAGTTGCTACATTG	GCT	3
2	MK2	CACCACATAAGGGACAAATGG	CAGTGTCATTCCCCTGAGCTA	ATCC	4
3	MK3	TCATTGGCCAGTAGACGAGTT	CAAGGCTCCAGGTAATGATAGG	ATTT	4
4	MK4	ACAAGACCTTCCTCTGCATCA	AATTGGTGTCCGTGAAGTGAG	CAA	3
5	MK5	AACACAACCCTCTGCTGACTG	AAAAAGTGGGAGAGGGACAAG	TGAGT	5
6	MK6	CAATGGTGCTTGTAGGGTTGT	AGCAAGTTTTGCCAGCTACAG	GCT	3
7	MK7	GGCAGTCAATCCAATCATCTG	CTGCCTGGTTGTGAAAGAAAC	TGA	3
8	MK8	AGCTTCGCGACCAATGTAAAG	GATAAGAACCGTCCGTTGGAT	TGTTCT	6
9	MK9	CCCATTCGATACACTATCCTCA	CTCTCTCTCTCTCTTCTCTCTCC	AG	2
10	MK10	CGCACTTGTTCTGAACTGTCA	GGTTGGTGCAGTAAGTTGCAG	TC	2
11	MK11	GCATCCGGAGGACAATTTTAG	AAGTGAGGGCAAAGCATCAG	GATT	4
12	MK12	GCTTCACTTTCTTCCCTTTGG	ACGTTTCTGATGAAGCTGAGG	CTTCAC	6
13	MK13	TAGGACAAGAAGCCATTGTGC	ACCCCTCGAAACAGAAACATC	TC	2
14	MK14	ATTCTACGAGCATCAGCGTTC	CTGCTGGTCCTACTCTTGTGG	TCT	3
15	MK15	CCTCATCACCACCAAAACTCA	ATACATGCCACTGCTCTCACC	CA	2
16	MK16	TTTCAACCTTGAGCAGTCTGG	TGGATTCCTGTGATGACCACT	CTG	3
17	MK17	GAGCCGAGAATAGAGACGACA	GGACAATTATGGGAGCTTGTG	TTC	3
18	MK18	GTAAACGCTAGCCAACGACAA	GGTACCCACTATCACGACTGC	GTGA	4
19	MK19	ATGGCTCCCTTTTAACGTCTC	CTACCGCTCCCATTCATTCTT	AT	2
20	MK20	GCAGTTGCTCAGAATGACTCC	CCACCACTAACCCCACCTTAT	AATGC	5
21	MK21	ATGAAGCTGCTGCTGCTAGTC	ATGTCTCTCTGTGCTCGCAGT	TCTGT	5
22	MK22	CCCAGATCTGAAGGAAGATGA	CGCAAGTTTCTGTGAGAGACC	CAA	3
23	MK23	ACCACTCGCTCAATCAATCAG	AACTCACCGATCGAGTCAACA	ACCAA	5
24	MK24	GGGACCTAGAACTTGGACCAT	GGTGAGATTTCTGGTGGGTTT	TA	2
25	MK25	TGATGGATCTTGTTGGAGAGG	CTCTGCTGCAAGCAAAGCTAA	GA	2
26	MK26	TGAGCATGAGTAATGCGTCTG	CAGTCGTCCATAGGTTGATGA	ATAC	4
27	MK27	CATAACCCTGGCTTCTTGCTA	GCTGATATCATCAACCCCGTA	TTTATT	6
28	MK28	CCAAATGTGATCCTGCTGTCT	CCTCCCACCACCTAAAAAGAA	GAA	3
29	MK29	TTACTCTCCTTCGCTTCCACA	TCAGGATGAGAAGGTTGCTGT	CAT	3
30	MK30	ACGGAGGGATTTCTCAAAGAC	CAAGTCCCTCAAGGTTTATGC	ACAG	4
31	MK31	AGGGTCTTGCTAAACCCATTG	CCATGTCCCAAGTTAACCAGA	GGT	3
32	MK32	GAAGAGGAGCAACCACTCTCA	TCTAATAAGCCGGTGGTGTTG	ACC	3
33	MK33	GCTCTCCAATGTGAAAGCTGA	GACGACGATGTCGATGAGATT	AG	2
34	MK34	ACGATAGGGGAGATGAAGTGG	ATCTCCTCCACAACCCAGAAC	TGT	3

35	MK35	TGCCTCTATGCTTTCAACTGG	CGTACACATTTGACTGGCTCA	GAA	3
36	MK36	CGCCTTGCCTTCTACTAACTG	CGAGAGTTGAAGGAGCCATTA	TA	2
37	MK37	AGGCGCAGCTCCTCATATCT	GATTCACCACCTTCCTCTTCC	GGGCGA	6
38	MK38	CCCAGATCCTCTCCACAACTA	CTCAGCGGCTATACACAGCAT	AGAA	4
39	MK39	ACCCTTTCTGGCTCATCACTT	GAGGGTTCTTGTTTGGTGACA	ATC	3
40	MK40	GATGTTCCCAGTGCTGACATT	GCCCAACAGTTGTTGAAGAAG	TTTTTC	6
41	MK41	GTCCAAGGTTGGTTTGTCCTC	TTGGAGATAGGCTTTCCGACT	TTTC	4
42	MK42	ACATCTGCCCACAGATTGTTC	GTTTTCCTAATGGCTGGAGGA	ATC	3
43	MK43	TACTACTCCCCACTCCCCAGT	GGTCGGGTAAGGAGGAGATTA	AACA	4
44	MK44	CCAACCATCTTATCCACCACA	GTGGAAGGGACATCGTTGATA	TTTG	4
45	MK45	ACAATGAAGAACCCTCCTTGG	TCAATGATCAGAGGAGGCAAC	ATT	3
46	MK46	GCTTCCCATTTATCCACCATC	TTGAGGTCAAGGAATGCAGAC	GAA	3
47	MK47	CTCCTGAAACCCCAAAGTCTC	GCCTCTTTCTTGTGCTTCCTT	TTCT	4
48	MK48	TTGTAGCGGCTATGCTTCTTC	GTATGGTGGTGGTTCGGAGTA	TCA	3
49	MK49	CCGTTGTAGAAGTCCGACTCA	GCAGAGCAAAACTCACGTCTC	TCC	3
50	MK50	TAGAACCGTTACTGGTTGCAG	TCTGGTGATGATGTTGCTGAG	TTC	3

The first 24 EST-SSR based primers have been validated in the present study

SupplementaryTable S3. Estimates of population genetic parameters based on type of repeat motif at 24 EST–SSRs marker loci in a set of 50 M. koenigii germplasm accessions

Repeat motif type	Alleles/locus (Na)	Effective alleles/ locus (Ne)	Observed heterozygosity (Hobs)	Expected heterozygosity (Hexp)	Polymorphic information content (PIC)	Shannon's information Index (I)
Di	25.16	13.76	0.15	0.89	0.87	2.74
Tri	17.38	7.78	0.17	0.83	0.81	2.24
Others	25.5	15.79	0.29	0.91	0.88	2.77
	22.68	12.44	0.20	0.88	0.85	2.58