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RESEARCH NOTE

Development and characterization of microsatellite markers for *Phyllanthus emblica* Linn., important nontimber forest product species

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Abstract. *Phyllanthus emblica* and *P. indofischeri*, commonly known as the Indian gooseberry, are important nontimber forest product (NTFP) species widely distributed across the Indian subcontinent. The fruits of these species are rich in vitamin C and are used in the preparation of a number of herbal medicines for treating a wide range of disorders. Due to the increased demand, they have been harvested extensively and form a major source of income for the forest-dwelling communities living in southern India. There are limited studies to understand the impact of harvesting on the genetic structure of these species. In this study, 15 polymorphic microsatellite markers have been developed for *P. emblica* and were characterized by screening 20 individuals each of *P. emblica* and *P. indofischeri*. The number of alleles per locus ranged 2–9 for *P. emblica* and 2–11 for *P. indofischeri*. The observed and expected heterozygosity of *P. emblica* ranged 0–1 and 0.401–0.825, respectively. Similarly, the observed and expected heterozygosity of *P. indofischeri* ranged 0.5–1 and 0.366–0.842, respectively. Cross-amplification of the designed primers was assessed with seven related *Phyllanthus* species. The microsatellite markers developed can be used for studying the population genetic structure, gene flow and genetic diversity of *P. emblica* and *P. indofischeri*.

Keywords. simple-sequence repeat markers; heterozygosity; population genetics; *Phyllanthus indofisheri*.

Introduction

Phyllanthus is one of the most species-rich genera of the family Phyllanthaceae, comprising over 800 species worldwide and over 50 species in India (Ravikanth *et al.* 2011). They are characterized by diverse growth forms, including shrubs, trees, annual or biennial herbs, and are distributed throughout the tropical and subtropical regions of both hemispheres. Among the species found in India, *P. amarus*, *P. debilis*, *P. fraternus*, *P. urinaria*, *P. kozhikodianus*, *P. maderaspatensis*, *P. acidus*, *P. emblica* and *P. indofischeri* are widely used in preparation of herbal medicines, and some of these species are cultivated in southern India (Srirama *et al.* 2010; Ravikanth *et al.* 2011).

P. emblica L. and *P. indofischeri* Bennet, commonly known as the Indian gooseberries, form an important nontimber product species (NTFP) in southern India. The fruits of these species are rich in vitamin C and used for treating digestive disorders, constipation, fever, cough, asthma and to stimulate hair growth (Ravikanth *et al.* 2011). The fruits of both the species are traded as '*Amla*' in India, and the extracts of fruits have been reported to have antioxidant, analgesic, anti-inflammatory and chemoprotective properties (Khopde *et al.* 2001; Srirama *et al.* 2012). They are also used in the preparation of pickles, jams, juices and also used in cosmetics, hair dye and shampoo industries (Siva 2003; Ganesan and Setty 2004). Both the species are medium-sized trees widely distributed in the forests of south India, where, *P. emblica* is commonly found in the evergreen to moist deciduous forests, and *P. indofischeri* is often seen in the dry deciduous forests of southern India.

The fruits of these species form a major source of livelihood for many forest-dwelling communities in India (Ganesan and Setty 2004). Besides these species, a number of *Phyllanthus* species are used in the herbal industries as a source for many Ayurvedic preparations (Ravikanth *et al.* 2011; Srirama *et al.* 2012). The predicted annual

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industrial consumption of dry Amla by the herbal industries is 17,000 MT, which correlates to 85,000 MT of green fruits (Babu 2010). It is also estimated that the forest-dwelling communities obtain 65% of the fruits from the wild (Ganesan and Setty 2004; Ved and Goraya 2008). Such enormous usage of NTFP can have a detrimental effect on the wild populations of both *P. emblica* and *P. indofischeri* leading to the lower regeneration and reduction in population size (Ravikanth and Siddappa Setty 2017). Reduced population size and fragmentation could result in mating among closely related individuals, leading to inbreeding and alteration of the genetic structure of the populations and finally to local extinction of populations (Ravikanth and Siddappa Setty 2017). Understanding how harvesting of fruits of these species modifies the genetic structure could provide important information in developing sustainable harvesting practices. Secondly, for effective utilization and conservation of genetic resources of *Phyllanthus* species, critical information on the spatial distribution of genetic variability of the species is crucial. Mapping the distribution of genetic variability could aid in the identification of genetic hot spots for *in situ* conservation, as well as to assist in designing germplasm collections (Ravikanth *et al.* 2001).

Pandey and Changtragoon (2012) reported the characterization of six microsatellite markers of *P. emblica*, of which five were polymorphic. These five microsatellite markers are however extremely low to address the impacts of harvesting on the genetic structure of these two important NTFP species, to study the population genetic variability or to assess the gene flow across the populations. In this study, we additionally developed 15 polymorphic microsatellite markers of *P. emblica* to understand the population genetic diversity of both *P. emblica* and *P. indofischeri*. We also show the cross-amplification of these markers with other related species of *Phyllanthus*. These primers can be useful in assessing the genetic diversity, gene flow and spatial genetic structure of *P. emblica*, *P. indofischeri* and related species.

Materials and methods

Young leaves from 20 individuals each of *P. emblica* and *P. indofischeri* were collected from Biligiri Rangaswamy Temple Wildlife Sanctuary (BRT WLS) (11°59′38″N: 77°8′26″E), India. Two to three individuals each of the related species were also collected from School of Ecology and Conservation Garden, GKVK, Bengaluru. Genomic DNA was extracted from the leaves using the DNeasyPlant Mini kit (Qiagen, Hilden, Germany). Purified genomic DNA of *P. emblica* was used for microsatellite development using the hybridization capture approach, described by Glenn and Schable (2005) with minor modifications. The genomic DNA (20 μg) of *P. emblica* was initially digested by the *Rsa*I and *Xmn*I restriction digestion enzymes (New England Biolabs, Massachusetts, USA) for 1 h at 37°C. The digested DNA was ligated to double-stranded Super SNX linkers (SNX-F- 5′-GTTTAAGGCCTAGCTAGCA GAATC and SNX-R- 5′-ATTCTGCTAGCTAGGCCTT AAACAAAA) and the ligated DNA was amplified with the SNX forward primer. The amplified products were hybridized with the Biotin labelled oligonucleotide probes containing the microsatellite repeats (AC, GA, AA, CTT, AGG and ACAG). The hybridized microsatellite rich products were captured magnetically using streptavidin-coated Dynabeads (Invitrogen, Oslo, Norway). These enriched fragments were amplified using the SNX linker primer and cloned to pTZ57R/T vector using the Thermo Scientific TA cloning kit. The cloned vector was transformed into the competent *E. coli* cells (CB5 α, Chromous Biotech, Bengaluru). The recombinant or the positive clones were identified as white colonies by the blue/white screening procedure on LB Agar plates containing Ampicillin and X-gal. These positive colonies were further amplified from the plasmid DNA using the M13 primers and inserted length that was more than 150 bp were selected. Based on this criterion, 132 colonies were selected and sequenced using ABI PRISM 3100 Genetic Analyzer Applied Biosystems (Chromous Biotech, Bengaluru). The sequences were screened for the presence of microsatellite repeat motifs using the web-based SSR Finder program (Martins *et al.* 2009). Of the 132 colonies, 41 had sufficient SSRs and the forward and reverse primers were designed using a web-based Primer3 program (http://primer3.wi.mit.edu/; Rozen and Skaletsky 2000).

These 41 primers were initially screened with five individuals of *P. emblica*. Of the 41 primers, 15 primer pairs were selected based on the length variations in the agarose gel (table 1). The selected primers were labelled using different fluorescent label dyes (6-FAM, HEX, NED, PET) at the 5′ end of the forward primers (Invitrogen, California, USA; table 1). Twenty individuals each of *P. emblica* and *P. indofischeri* from the BRT WLS population were amplified with the labelled primers. The PCR reaction for the amplification was carried out using 5–10 ng DNA template, 1X polymerase buffer, 1 mM dNTPs, 5 pmol each of forward and reverse primer and 1 unit of *Taq* polymerase (Bangalore Genei, India). The PCR's cycling conditions were 5 min at 95°C, followed by 38 cycles of 30 s at 94°C, 45 s at the annealing temperature of designed specific primer (47.7–52.8°C) and 45 s at 72°C, with an extension of 10 min at 72°C in the final cycle. The labelled products were genotyped on an Applied Biosystems 3730 Genetic Analyzer with a GeneScan 500 LIZ (-250) Size Standard (SciGenom, Cochin, India). The genotype results were evaluated and scored for the allele sizes using Peak Scanner ver. 2 (Applied Biosystems).

The data was analysed for the genetic diversity indices such as observed heterozygosity (H_o), expected heterozygosity (H_e), number of alleles per locus (N_a), effective number of alleles (N_e); fixation index (F) and deviation

Table 1. List of 15 polymorphic microsatellite primers developed for *P. emblica* with their GenBank numbers.

Locus	Repeat motif	Primer sequence (5′–3′)	GenBank accession no.	Label dye	Allele size range (bp)	Annealing temperature (°C)
PE3	$(T)_{19}$	F: CTGCAACTTCCAATTGGGTT R: AGATTTCATGCAGGCCTTTG	MF487804	FAM	130–200	51
PE6	$(CT)_7$	F: CACGGCTCTTGATTACGGAT R: GTACCTCCTGTCACCCCAAA	MF487805	PET	200–310	52.8
PE8	$(CT)_{12}$	F: ACTTCCCACACACCAACCTC R: ACCATGATTACGCCAAGCTC	MF487806	FAM	250–330	51
PE9	$(TGT)_4$	F: GCTAGCAGTAATCACCTCGC R: TCGGAGTATAAGGAGAAGGAGG	MF487807	PET	180–215	48.4
PE10	$(GGT)_5$	F: TGAGCTGCAGTGGTGTATGA R: GCGTCATGATCAAAGCAGTG	MF487808	FAM	150–190	52.8
PE11	$(CAA)_6$	F: TGAGTACGCGTTCACCTGT R: CAGTTACTTTCGTCTTTTGCGC	MF487809	HEX	210–240	51
PE12	$(GAA)_{10}$	F: GACGACGACGACTACGAAGA R: CTAGCAGAATCGCTTCACCC	MF487810	NED	150–208	52.8
PE15	$(AAG)_6$	F: CAGCAGAAGCAGAAGAGAATATG R: CCGGGTTACTGAAATGCTTG	MF487811	PET	190–230	52.6
PE16	$(ACT)_6$	F: TGTTTAAGGCCTAGCTAGCAGAA R: CCATGATTACGCCAAGCTCT	MF487812	HEX	200–250	46.3
PE27	$(CTT)_8$	F: CTCGGGTTATATTCACTTGGCTA R: TGTCACAGTTTGGAGGGACA	MF487813	FAM	190–240	47.7
PE34	$(TC)_{18}$	F: TCCTCTCCTCTTCATCTCACTTC R: GATATGTCCATGATTACGCCAA	MF487814	FAM	210–260	47.7
PE35	$(TG)_8$	F: TTGTTGATGGAAGAAGTTGGC R: CAGGAAACAGCTATGACCATGA	MF48715	FAM	180–220	48.8
PE36	$(TC)_9$	F: GGTGAAGGCTCATCTTCTCAAT R: ATTTGGTGCTAGTGGCTGATTT	MF487816	NED	125–150	52.8
PE37	$(CA)_8$	F: CCACTTTTCCACTCTCTCCTCT R: TGGGCAAGATTACCTACACAAA	MF487817	PET	175–190	51.2
PE40	$(GT)_{17}$	F: GCAGAATCACAATGCTGGTAGA R: GACCCTTCCAATGCTAGATGAG	MF487818	HEX	150–168	48.4

F, forward primer; R, reverse primer.

Table 2. Characterization of 15 polymorphic loci for *P. emblica* and *P. indofischeri*.

	P. emblica							*P. indofischeri*						
Primer	N	N_a	N_e	H_o	H_e	F	HWE	N	N_a	N_e	H_o	H_e	F	HWE
PE3	17	5	3.18	1	0.706	−0.460	NS	19	3	2.31	1	0.582	−0.765	**
PE6	17	9	5.03	0.941	0.825	−0.175	NS	20	5	2.26	0.6	0.572	−0.076	NS
PE8	18	2	1.98	0	0.508	1.000	***	19	5	3.18	0.947	0.704	−0.382	NS
PE9	18	3	2.45	0.944	0.608	−0.598	*	20	4	2.51	0.95	0.617	−0.580	*
PE10	20	5	3.52	1	0.735	−0.396	NS	20	4	2.49	0.9	0.614	−0.503	NS
PE11	20	2	1.98	0.9	0.508	−0.818	*	20	3	1.70	0.5	0.422	−0.216	NS
PE12	14	2	1.99	0.929	0.516	−0.867	NS	20	3	2.10	1	0.537	−0.909	***
PE15	18	2	2.00	1	0.514	−1.000	**	9	2	1.53	0.444	0.366	−0.286	NS
PE16	14	4	3.73	0.714	0.759	0.024	NS	20	3	2.12	0.8	0.542	−0.513	NS
PE27	18	2	1.98	0.889	0.508	−0.800	NS	20	3	2.36	0.9	0.591	−0.562	*
PE34	17	4	1.89	0.588	0.485	−0.250	NS	19	3	2.11	1	0.539	−0.905	**
PE35	18	3	2.31	0.889	0.584	−0.565	NS	20	5	2.57	0.9	0.627	−0.472	*
PE36	19	8	3.86	0.947	0.761	−0.279	NS	20	11	5.59	0.75	0.842	0.087	NS
PE37	18	6	3.00	0.889	0.686	−0.333	NS	20	5	3.33	0.9	0.718	−0.286	NS
PE40	16	3	1.64	0.438	0.401	−0.126	NS	20	4	1.81	0.6	0.458	−0.345	NS
Overall mean		4	2.70	0.805	0.607	−0.376			4.2	2.53	0.813	0.582	−0.448	

N, number of individuals; N_a, number of alleles; N_e, number of effective alleles; H_o, observed heterozygosity; H_e, expected heterozygosity; F, fixation index; HWE, Hardy–Weinberg equilibrium. NS, not significant; *significant at 0.05; **significance at 0.01; ***significant at 0.001 based on P values.

Table 3. Cross-amplification of *P. emblica* microsatellite markers with closely related *Phyllanthus* species.

Locus	*P. tenellus*	*P. debilis*	*P. amarus*	*P. kozhikodianus*	*P. polyphyllus*	*P. baillonianus*	*P. acidus*
PE3	+	+	−	+	+	+	+
PE6	+	+	−	−	+	−	−
PE7	+	+	+	+	+	+	+
PE8	+	+	+	+	+	+	+
PE9	+	−	−	+	−	−	−
PE10	+	+	+	+	+	+	+
PE11	+	+	−	+	−	−	−
PE12	−	+	−	+	+	−	−
PE15	+	+	−	+	−	−	−
PE35	+	+	+	−	+	−	+
PE36	+	+	−	+	+	+	+
PE37	+	−	−	+	+	−	−
PE40	−	+	−	+	+	−	−

(+) Successful amplification; (–) no amplification.

from Hardy–Weinberg equilibrium (HWE). The analysis was carried out in Cervus 3.0 (Kalinowski *et al.* 2007) and Genealex 6.5 (Peakall and Smouse 2012). The presence of null alleles and allele dropouts were assessed using Microchecker 2.2.3 (Van Oosterhout *et al.* 2004). All the 15 primers were also analysed for cross-amplification with seven related *Phyllanthus* species, namely *P. amarus*, *P. debilis*, *P. tenellus*, *P. kozhikodianus*, *P. polyphyllus*, *P. baillonianus* and *P. acidus*.

Results

Twenty individuals each of both the species *P. emblica* and *P. indofischeri* were assessed for their genetic variability using 15 microsatellite markers. Of these 15 primers, two were mononucleotide, seven dinucleotides and six trinucleotide repeat motifs (table 1). All the microsatellite markers were found to be polymorphic and the number of alleles per locus was in the range of 2–9 in *P. emblica* and 2–11 in the case of *P. indofischeri* with an average of 4 and 4.2, respectively. The effective number of alleles was in the range of 1.63–5.02 for *P. emblica* and 1.69–5.59 for *P. indofischeri* with an overall mean of 2.70 and 2.53, respectively. The observed and expected heterozygosity for *P. emblica* was in the range of 0–1 and 0.401–0.825, respectively. Similarly, the observed and expected heterozygosity of *P. indofischeri* was in the range of 0.5–1 and 0.366–0.842, respectively. The overall mean for observed and expected heterozygosity for both the species were 0.805 and 0.607 (*P. emblica*) and 0.813 and 0.582 (*P. indofischeri*), respectively (table 2). The presence of null alleles

and allele dropouts in all the loci was not detected using Microchecker at the confidence level of 95%, based on Van Oosterhout *et al.* (2004). The fixation index for *P. emblica* ranged from 1 to −1 with an overall mean of −0.376. Similarly, the fixation index for *P. indofischeri* ranged from 0.087 to −0.909 with an overall mean of −0.448. Four of the 15 primers showed significant deviation from HWE ($P<0.05$) in the case of *P. emblica* and six of the 15 primers showed significant deviation from HWE for *P. indofischeri*.

The primers developed also cross-amplified with all the seven related *Phyllanthus* species. However, not all the primers successfully amplified in all the seven species. In case of *P. debilis*, *P. tenellus*, *P. kozhikodianus* and *P. polyphyllus*, 11 primers were found to cross amplify (table 3); however, only four primers in *P. amarus*, five in *P. bailloni-anus* and six in *P. acidus* cross-amplified (table 3).

Discussion

Only a few studies have investigated the genetic diversity of *Phyllanthus* species (Uma Shaanker and Ganeshaiah 1997; Padmini *et al.* 2001). However, these studies using isozymes provided limited information on the spatial genetic structure as well as on gene flow across the populations. Our initial study with the markers in one population for each of the two species revealed a mean observed and expected heterozygosity of 0.805 and 0.607 (*P. emblica*); and 0.813 and 0.582 (*P. indofischeri*). The results indicate that the primers PE8, 9, 11 and 15 in case of *P. emblica* and PE 3, 9, 12, 27, 34 and 35 in case of *P. indofischeri* showed significant deviation from HWE (table 2). One of the possible reasons for this deviation from HWE could be due to higher levels of inbreeding in the sampled population of both the species. There has been a long history of extraction of fruits of these species in sites sampled (Ganesan and Setty 2004). Besides, over the years, there has been increased mortality and reduced fruit set of both the species due to the infestation of mistletoe, *Taxillus tomentosus* (Rist *et al.* 2011). The spread of invasive species such as *Lantana camara* in recent decades, mistletoe infection and harvesting, have led to poor regeneration causing a significant reduction in population size of these species. Reduced population size could significantly increase inbreeding in the population.

In an earlier study, of the six microsatellite loci five showed polymorphism with the number of alleles ranging from four to seven and the observed and expected heterozygosity ranging from 0.360 to 0.760 and 0.499 to 0.806 similar to the observed and expected heterozygosity found in our study (Pandey and Changtragoon 2012).

In addition to the markers reported by Pandey and Changtragoon (2012), the microsatellite markers developed in this study could be used to study the population genetic structure, gene flow and genetic diversity of *P. emblica*, *P. indofischeri* and other *Phyllanthus* species. Besides *P. emblica* and *P. indofischeri*, our study has also shown cross-amplification of these microsatellite markers in a number of *Phyllanthus* species. Thus, these markers could also aid in assessing the impact of harvesting and other anthropogenic pressures on the genetic structure of other *Phyllanthus* species, which are also subject to harvesting pressures.

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