Research Note

Development and characterization of microsatellite markers for *Phyllanthus emblica* Linn., an important Non-timber Forest Product species

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Abstract

Phyllanthus emblica and *P. indofischeri,* commonly called as the Indian Gooseberry are important non-timber forest products (NTFP) species widely distributed across the Indian sub-continent. The fruits of these species are rich in Vitamin C and are used in the preparation of number of herbal medicines for treating wide range of disorders. Due to increased demand for these fruits, they are harvested extensively and form a major source of income for the forest-dwelling communities living in Southern India. There have been 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 allele per locus ranged from 2-9 (*P. emblica*) and 2-11 (*P. indofischeri*). The observed and expected heterozygosity for *P. emblica* ranged from 0-1 and 0.401-0.825 respectively. Similarly, the observed and expected heterozygosity of *P. indofischeri* ranged from 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: *Phyllanthus indofisheri*; SSR markers; heterozygosity; population Genetics

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, and 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 also cultivated in southern India (Ravikanth *et al* 2011, Srirama *et al*. 2010).

Phyllanthus emblica L. and *P. indofischeri* Bennet, commonly known as the Indian Gooseberries, form an important Non-timber 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). The fruits of these species are also used in preparing pickles, jams, juices and are also used in the cosmetic, hair dye and shampoo industries (Ganesan and Setty 2004; Siva 2003). Both the species are medium-sized trees widely distributed in the forests in south India. While *P. emblica* is commonly found in the evergreen to moist deciduous forests, *P. indofischeri* is often seen in the dry deciduous forests in 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 industrial consumption of dry Amla by the herbal industries is 17,000 MT that 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 an enormous dependence on the 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 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 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 assist in designing germplasm collections (Ravikanth et al 2001).

Pandey and Changtragoon (2012), have reported the characterization of six microsatellite markers for *P. emblica* of which five are polymorphic. These five microsatellite markers however are far too few to address the impacts of harvesting on the genetic structure of these two important NTFP species or to study the population genetic variability or assess the gene flow across the populations. In this study, we have additionally developed 15 polymorphic

85 microsatellite markers of *P. emblica* in order to understand the population genetic diversity of
86 both *P. emblica* and *P. indofischeri*. We also show the cross-amplification of these markers with
87 other related species of *Phyllanthus*. These primers can be useful in assessing the genetic
88 diversity, gene flow and spatial genetic structure both *P. emblica* and *P. indofischeri* and related
89 species.

90 **Material and Methods**

91 Young leaves from twenty individuals each of *P. emblica* and *P. indofischeri* were collected
92 from Biligiri Rangaswamy Temple Wild Life Sanctuary (BRT-WLS) (11°59′38″N: 77°8′26″E),
93 India. Two to three individuals each of the related species were also collected from School of
94 Ecology and Conservation Garden, GKVK, Bangalore. The genomic DNA was extracted from
95 the leaves using the DNeasyPlant Mini kit (Qiagen). The purified genomic DNA of *P. emblica*
96 was used for the microsatellite development using the hybridization capture approach described
97 by Glenn and Schable (2005) with minor modifications. The genomic DNA (20µg) of *P. emblica*
98 was initially digested by the *Rsa1* and *Xmn1* restriction digestion enzymes (New England
99 Biolabs) for 1 h at 37 °C. The digested DNA was ligated to double-stranded Super SNX linkers
100 (SNX-F- 5'-GTTTAAGGCCTAGCTAGCAGAATC and SNX-R- 5'-
101 ATTCTGCTAGCTAGGCCTTAAACAAAA) and the ligated DNA was amplified with the SNX
102 forward primer. The amplified products were hybridized with the Biotin labelled oligonucleotide
103 probes containing the microsatellite repeats (AC, GA, AA, CTT, AGG and ACAG). The
104 hybridized microsatellite rich products were captured magnetically using streptavidin-coated
105 dyna beads (Invitrogen, Oslo, Norway). These enriched fragments were amplified using the SNX
106 linker primer and cloned to pTZ57R/T vector using the Thermo Scientific TA cloning kit. The
107 cloned vector was transformed into the competent *E. coli* cells (CB5α, Chromos Biotech,

Bangalore). The recombinant or the positive clones were identified as the 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 150bp were selected. Based on this criterion, 132 colonies were selected and sequenced using ABI PRISM 3100 Genetic Analyzer Applied Biosystems (Chromos). The sequences were screened for the presence of microsatellite repeat motifs using the web-based SSR Finder program (Martins *et al*. 2009). Of these 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 forty-one primers were initially screened with five individuals of *P. emblica*. Of these 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, Table 1). Twenty individuals each of *P. emblica* and *P. indofischeri* from the BRT-WLS population were amplified with the labeled primers. The PCR reaction for the amplification was carried out using 5-10 ng DNA template, 1X polymerase buffer, 1mM dNTPs, 5 pmoles of each 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 labeled products were genotyped on an Applied Biosystems 3730 Genetic Analyzer with a GeneScan 500 LIZ (-250) Size Standard (SciGenome). The genotype results were evaluated and scored for the allele sizes using Peak Scanner version 2 (Applied Biosystems).

The data was analysed for the genetic diversity indices such as observed heterozygosity (Ho), expected heterozygosity (He), number of alleles per locus (Na), effective number of alleles (Ne); Fixation index (F) and deviation from Hardy Weinberg's equilibrium (HWE). The analysis was carried out in Cervus 3.0 (Kalinowski *et al*. 2007) and Genealex 6.5 (Peakall and Smouse 2006). The presence of null alleles and allele dropouts were assessed using Microchecker 2.2.3 (Van *et al*. 2004).

All the fifteen primers were also analyzed for cross-amplification with seven related *Phyllanthus* species namely *P. amarus*, *P. debilis*, *P. tenellus*, *P. kozhikodianus*, *P. polyphyllus*, *P. baillonianus* and *P. acidus* (Table 3).

Results

Twenty individuals each of both the species *P. emblica* and *P. indofischeri* were assessed for their genetic variability with 15 microsatellite markers. Of these fifteen primers, two were mononucleotide, seven di-nucleotides and six tri-nucleotide repeat motifs (Table 1). All the microsatellite markers were found to be polymorphic and the number of allele per locus ranged from 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 ranged from 1.63-5.02 (*P. emblica*) and 1.69-5.59 (*P. indofischeri*) with an overall mean of 2.70 and 2.53 respectively. The observed and expected heterozygosity for *P. emblica* ranged from 0-1 and 0.401-0.825, respectively. Similarly, the observed and expected heterozygosity of *P. indofischeri* ranged from 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 confidence level of 95% based on Oosterhout and Chakraborty null allele estimates for this population.. 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 out of the fifteen primers showed significant deviation from HWE ($P < 0.05$) in the case of *P. emblica* and six of the fifteen 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,* eleven primers were found to cross amplify (Table 3); however, only four primers in *P. amarus,* five in *P. baillonianus* and six in *P. acidus* cross amplified (Table 3).

Discussion

Only few studies have investigated the genetic diversity of *Phyllanthus* species (Padmini *et al* 2001; Uma Shaanker and Ganeshaiah 1997). However, these studies using isozymes provided limited information on the spatial genetic structure as well as 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* show 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

174 years, there has been increased mortality and reduced fruit set of both the species due to the
175 infestation of mistletoe, *Taxillus tomemtosus* (Rist et al 2011). Spread of invasive species such
176 as *Lantana camara* in recent decades, mistletoe infection and harvesting, have led to poor
177 regeneration causing significant reduction in population size of these species. Reduced
178 population size could significantly increase inbreeding in the population.

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

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

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Table Legend

Table 1: Microsatellite primers developed for *Phyllanthus emblica* with their repeat motifs, primer sequence, GenBank numbers, allele size and annealing temperature

†**Table 1:** List of fifteen polymorphic microsatellite primers developed for *Phyllanthus emblica* with their GenBank numbers

Locus	Repeat Motif	Primer sequence (5'-3')	GenBank Acc No.	Label dye	Allele size range (bp)	Annealing Temperature (°C)
PE3	(T)19	F : CTGCAACTTCCAATTGGGTT	MF487804	FAM	130-200	51
		R : AGATTTCATGCAGGCCTTTG				
PE6	(CT)7	F : CACGGCTCTTGATTACGGAT	MF487805	PET	200-310	52.8
		R : GTACCTCCTGTCACCCCAAA				
PE8	(CT)12	F : ACTTCCCACACACCAACCTC	MF487806	FAM	250-330	51
		R : ACCATGATTACGCCAAGCTC				
PE9	(TGT)4	F : GCTAGCAGTAATCACCTCGC	MF487807	PET	180-215	48.4
		R : TCGGAGTATAAGGAGAAGGAGG				
PE10	(GGT)5	F : TGAGCTGCAGTGGTGTATGA	MF487808	FAM	150-190	52.8
		R : GCGTCATGATCAAAGCAGTG				
PE11	(CAA)6	F : TGAGTACGCGTTCACCTGT	MF487809	HEX	210-240	51
		R : CAGTTACTTTCGTCTTTTGCGC				
PE12	(GAA)10	F : GACGACGACGACTACGAAGA	MF487810	NED	150-208	52.8
		R : CTAGCAGAATCGCTTCACCC				
PE15	(AAG)6	F: CAGCAGAAGCAGAAGAGAATATG	MF487811	PET	190-230	52.6
		R: CCGGGTTACTGAAATGCTTG				
PE16	(ACT)6	F: TGTTTAAGGCCTAGCTAGCAGAA	MF487812	HEX	200-250	46.3
		R: CCATGATTACGCCAAGCTCT				
PE27	(CTT)8	F: CTCGGGTTATATTCACTTGGCTA	MF487813	FAM	190-240	47.7
		R: TGTCACAGTTTGGAGGGACA				
PE34	(TC)18	F: TCCTCTCCTCTTCATCTCACTTC	MF487814	FAM	210-260	47.7
		R: GATATGTCCATGATTACGCCAA				
PE35	(TG)8	F : TTGTTGATGGAAGAAGTTGGC	MF48715	FAM	180-220	48.8
		R : CAGGAAACAGCTATGACCATGA				
PE36	(TC)9	F : GGTGAAGGCTCATCTTCTCAAT	MF487816	NED	125-150	52.8
		R : ATTTGGTGCTAGTGGCTGATTT				
PE37	(CA)8	F : CCACTTTTCCACTCTCTCCTCT	MF487817	PET	175-190	51.2

		R :TGGGCAAGATTACCTACACAAA				
PE40	(GT)17	F : GCAGAATCACAATGCTGGTAGA	MF487818	HEX	150-168	48.4
		R : GACCCTTCCAATGCTAGATGAG				

†F: Forward primer; R: Reverse primer

Table 2: Characterization of the fifteen polymorphic loci for *Phyllanthus emblica* and *P. indofischeri*

Table 2: Characterization of the fifteen polymorphic loci for *Phyllanthus emblica* and *P. indofischeri*

	Phyllanthus emblica							***Phyllanthus indofischeri***			
Primer	**N**	**Na**	**Ne**	**Ho**	**He**	**F**	**HWE**	**N**	**Na**	**Ne**	**Ho**
PE3	17	5	3.18	1	0.706	-0.460	NS	19	3	2.31	
PE6	17	9	5.03	0.941	0.825	-0.175	NS	20	5	2.26	0
PE8	18	2	1.98	0	0.508	1.000	***	19	5	3.18	0.9
PE9	18	3	2.45	0.944	0.608	-0.598	*	20	4	2.51	0.
PE10	20	5	3.52	1	0.735	-0.396	NS	20	4	2.49	0
PE11	20	2	1.98	0.9	0.508	-0.818	*	20	3	1.70	0
PE12	14	2	1.99	0.929	0.516	-0.867	NS	20	3	2.10	
PE15	18	2	2.00	1	0.514	-1.000	**	9	2	1.53	0.4
PE16	14	4	3.73	0.714	0.759	0.024	NS	20	3	2.12	0
PE27	18	2	1.98	0.889	0.508	-0.800	NS	20	3	2.36	0
PE34	17	4	1.89	0.588	0.485	-0.250	NS	19	3	2.11	
PE35	18	3	2.31	0.889	0.584	-0.565	NS	20	5	2.57	0
PE36	19	8	3.86	0.947	0.761	-0.279	NS	20	11	5.59	0.
PE37	18	6	3.00	0.889	0.686	-0.333	NS	20	5	3.33	0
PE40	16	3	1.64	0.438	0.401	-0.126	NS	20	4	1.81	0

Overall mean	**17.467**	**4**	**2.70**	**0.805**	**0.607**	**-0.376**	**19.067**	**4.2**	**2.53**	**0.81**

†N: number of individuals ; Na: Number of alleles; Ne: Number of effective alleles; Ho: observed heterozygosity; He: expected heterozygosity (He); F: Fixation Index; HWE: Hardy-Weinberg equilibrium

Table 3: Cross amplification of *Phyllanthus emblica* microsatellite markers with closely related *Phyllanthus* species

Table 3: Cross amplification of *Phyllanthus 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