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Microsatellite Markers: Cross Species Amplification in Myristicaceae

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Abstract: Western Ghats of India one of the mega-biodiversity hotspot harbours several important and endemic plant taxa and Myristicaceae is one such example. It is also referred as "nutmeg" family one of the ancient families. Five species of Myristicaceae occur in Western Ghats, among them *Gymnacranthera canarica* and *Myristica fatua* var. *magnifica* are exclusively associated with *Myristica* swamps (India's most threatened ecosystem). Other three species namely *Myristica malabarica M. dactyloides* and *Knema attenuate* are non-swampy but are endemic to Western Ghats. Most of these species are listed in RET data book. In this study, an attempt was made to identify microsatellite markers through cross species amplification in Myristicaceae. Totally, 21 microsatellite (SSR) primers that are developed for *Virola sebifera* and *Myristica malabarica* were examined against three different genera. The study indicated possibility of cross species amplification and use of marker for genetic studies in new species. Microsatellite priming sites are more likely to be conserved and thus successful in closely related species (e.g., within subgenera) compared to those that are distantly related (e.g., between genera). Close to 90 percent of species/primer combinations tested within subgenera were successful, a much higher success rate than between genera and geographically distant species.

Keywords: Microsatellite, SSR, Cross amplification, Myristicaceae, Swamps

Myristicaceae is one among the ancient angiosperm families, popularly known as nutmeg family (i.e. source of spice such as nutmeg and mace). Myristicaceae are a medium-sized family of angiosperm trees with a wide pan tropical distribution, mostly confined to lowland rainforest habitats (Sauquet 2003) and consist of about 21 genera, with about 520 species (Christenhusz and Byng 2016). The Western Ghats of India, one of the 36 hot spots of Biodiversity in the world (Myers et al., 2000) harbours several species of family Myristicaceae. These Myristicaceae are being used as timber, medicines, food and other purposes (Tambat et al., 2014). Most of the species area are endemic (Ramesh and Pascal 1997) and also listed under endangered a threatened category (FRLHT 2000). Several Myristicaceae members endemic to Western Ghats, show poor regeneration, seed abnormality, discontinuous girth class distribution, etc (Tambat et al., 2007; Tambat et al., 2014). Most geneticists suggested this could be the result of inbreeding and poor genetic structure in endemic species (Savolainen and Kuittien 2000, Gitzendanner and Soltis 2000).

However, to assess the genetic structure, there are no specific and precise molecular markers for Myristicaceae (Hemmila et al., 2010). Among the available molecular marker, microsatellite (or) simple sequence repeats (SSRs) are the genetic markers of choice for plant and animal systems due to their abundance, co-dominant inheritance, hyper-variability, multi-allelic nature, reproducibility and transportability between species (Wang et al., 2009) and extensive genome coverage. In forestry, tree species microsatellite (SSR) are found to be power full markers for genetic mapping, diversity analysis, genotyping and SSR markers are capable of detecting large number of alleles, and DNA fingerprinting applications and are used to investigate questions related to effective population size, structure, migration, colonization rates and mating system. The ability of these hyper-variable regions to reveal high allelic diversity and delimit fine genetic structure has resulted in an increase in elaborate population studies (Rossetto et al., 1999, Singh et al., 2014).

The widespread adoption of species-specific SSR (Simple Sequence Repeat) loci in plant research is hindered by their availability for only a limited number of taxa. While many laboratories possess the resources and expertise to conduct SSR - based studies, the characterization of new loci remains a costly and labor-intensive process (Hemmila et al., 2010). An alternative approach involves using microsatellite primers developed for one species to detect polymorphisms in homologous regions of related species. This method significantly reduces resource requirements and broadens the applicability of SSR markers. The success of this heterologous PCR amplification largely depends on the evolutionary proximity between the source and target species. Greater genomic homology often results in higher conservation of SSR-flanking regions, improving primer

transferability (Rossetto 2001). Research has demonstrated that SSR markers can be successfully transferred across species within the same genus and, in some cases, even to species in other genera. This study focuses on examining the cross-species amplification of microsatellite markers originally developed for *Virola sebefera* and *Myristica malabarica* and testing their applicability to other species within the Myristicaceae family of the Western Ghats, India.

MATERIAL AND METHODS

In this study six species belonging to 3 genera were used. Species such as *Gymnacranthera canarica* (King) Warb. and *Myristica fatua* Houtt. Var *magnifica* (bedd) are known to associate with swampy habitats in Western Ghats. Species namely as *Knema attenuate* (J.Hk and Thw.) Warb) and *Myristica dactyloides* Gaerten, occurs in non-swampy habitat (Chandran et al., 1999, Chandran and Mesta 2001, Tambat et al., 2004, Banik and Bora 2016). However, a lone species known as *Myristica malabarica* Lam. occurs under both swampy and non-swampy conditions (Chandran Mesta 2001) (Table 1). *Myristica fragrance* Menities, Sinclair, (Mollucas island) a domesticated species in India was also used in this study.

DNA extraction and PCR: Genomic DNA (g-DNA) was extracted from washed leaf samples using a modified cTAB method (Doyle and Doyle, 1990). The leaf tissue was digested at 65°C for 1 hour in an extraction buffer containing 100 mM Tris-HCl (pH 8), 1 mM EDTA (pH 8), 5 M NaCl, 2% CTAB, 1% PVP-40, and β-mercaptoethanol (2%). The concentration of β-mercaptoethanol was increased to 2% due to the high tannin content in the leaf samples, and SDS was excluded from the buffer to optimize DNA extraction. To enhance DNA purity, chloroform and isoamyl alcohol (24:1) were added, followed by precipitation of g-DNA with chilled isopropanol. The resulting DNA pellets were washed twice with 70% ethanol and suspended in 10 mM Tris-HCl (pH 8). The extracted DNA was dissolved in TE buffer, and its concentration was measured spectrophotometrically at 260/280 nm. The samples were treated with RNase to remove RNA contamination and analyzed using 0.8% agarose gel electrophoresis. Finally, DNA samples were stored at -20°C for further use.

Microsatellite primer sequences were obtained from published sources (Hemmila et al., 2010; Wei et al., 2013) and synthesized by Bangalore Genie Private Limited, Bangalore. The study utilized 11 primers developed for *Virola sebifera Aubl.* and 10 primers for *Myristica malabarica Lam* (Table 2).

The PCR amplification was carried out in 10µl volume reaction mixture containing 50ng of template DNA, 2.5 mM dNTPs, 4mM each forward and reverse primers, 1.5mM taq buffer and 0.1U taq polymerase. PCR reaction consisted of an initial denaturation at 94^0 C for 2min followed by 35 cycles of 40 sec denaturation at 94^0C, 1min annealing at 50^0C and 2min elongation at 72^0 C. Final elongation was carried out at 72^0 C for 10min. DNA fragments were separated on 2.5 % agarose gel at 40mA. At each of the PCR amplified microsatellite locus, the frequency of each allele was computed and compared (Wickneswari and Boyle 1999).

RESULTS AND DISCUSSION

Totally, 21 different primer sets were used (Table 3). Three primers namely Mlr10, M2r6 and M4s14 showed cross amplification with all the five species of Myristicaceae.

Table 1. Distribution, habitat and leaf characteristics of the Myristicaceae species of the Western Ghats, India

Species	Habitat	Reference	Roots	Reference	Tree height (in feet)	Leaf characters	Forest types	Distribution	Reference
Gymnacranthera canarica (King) Warb.	Swampy	Tambat et al., 2004	Knee roots	Chandran and Mesta (2001)	Large (Up to 80)	Thin and smooth	Occasional in evergreen forest on windward side of Western Ghats	Endemic to WG	Ramesh and Pascal (1997)
Myristica fatua Houtt. Var. *magnifica* (Bedd.) Sinclair	Swampy	Gamble (1935)	Prominent stilt root	Chandran and Mesta (2001)	Lofty (Up to 100)	Thick and smooth	In swampy areas of evergreen forest of Western Ghats	Endemic to WG	Ramesh and Pascal (1997)
Myristica malabarica Lam.	Both	Tambat et al., 2004	Stilt root	Chandran and Mesta (2001)	Moderate (Up to 50)	Thin and smooth	Occasional in evergreen forest of Western Ghats	Endemic to WG	Ramesh and Pascal (1997)
Myristica dactyloides Gaertn.	Non-swampy	Tambat et al., 2004	No prominent stilt root	Chandran and Mesta (2001)	Large (Up to 90)	Thick and smooth	Frequent in evergreen forest of Western Ghats	Widespread	
Knema attenuata (J. Hk. & Thw.) Warb.	Non-swampy	Tambat et al., 2004	No prominent stilt root	Chandran and Mesta (2001)	Moderate (Up to 50)	Thin and rough	Frequent in semi-evergreen forest of Western Ghats	Endemic to WG	Ramesh and Pascal (1997)

Table 2. Details of the primer sequences, base pairs (bp), annealing temperature and PCR amplification product size range

Primers	Primer sequence	bp	Temp (^{0}C)	PCR product size range (bp)
Mlr6-L	CCTGACAACTGGCGAAGATGG	21	59.5	141-159
Mlr6-R	TGATTTCAAACCCAGTCAAGG	21		
Mlr10-L	GATCTTGGTGTAAGCTTCTTCTTC	20	58	145-169
Mlr10-R	CATGCCCCAAAATCACTTC	19		
Mls20-L	GACACTGGCCAATATTGATACG	22	63	185-188
Mls20-R	GCAGCAGCAGCAGGAGTTA	19		
Mls75-L	AAGAAAATGGGGCAGACGTT	20	60	155-179
Mls75-R	TGCTGCTAATTCTTCTTTCTCTGAT	25		
M2r6-L	GGTTCACTTGCCCTTTTGT	20	59.5	141-159
M2r6-R	GATGCACTTTAGTAAGGTTTCAAGC	25		
M2r9-L	CTCTCCACATGTCTGAGGAACTT	23	59	208-217
M2r9-R	AGGTGATATGGCCCATTTTG	20		
M2r31-L	AGCTTGGGATCAGGTGATATG	21	59	151-159
M2r31-R	CGGCCCAATTGAGCTACTAA	20		
M4S14-L	GTTTACCAGATTGGCACACG	20	63	181-227
M4S14-R	GCACATTGAGTGGACAGCA	19		
M4S73-L	AAGGTCTGGTTTGCTGATGA	20	63	244-268
M4S73-R	AAGGTCTGGTTTGCTGATGA	20		
M4S90-L	CAGCACGGTGACTACAGCAG	20	65	203-239
M4S90-R	TTTTTGGTGTCGTGTCCTTG	20		
M5r33-L	TCAAACAAAACCACCCATACC	21	62	141-156
M5r33-R	GGTTTAAAAGAGGCCATGATTC	22		
VSE02-L	CGGTAGTCCATTGATTGGCA	20	55	266-296
VSE02-R	GCTGTCATTGTCATCTTCCT	20		
VSE11-L	TATAGATGCCTGCCATTGGA	20	55	237-267
VSE11-R	TCGTGCGAAATTCCCTTCTA	20		
VSE30-L	CATGCATGCTGGTCCATA	18	55	159-186
VSE30-R	TTCAGCATATTCTCATGTTCCA	22		
VSE31-L	AACTAGGGCTCTCGCAGCTT	20	55	183-210
VSE31-R	CCAAAGAAGTGCTCCTCAGC	20		
VSE32-L	TGCCCAAGTGGGTTTCTCTA	20	55	197-221
VSE32-R	CCAGTGTTTCTTCTCTTGCATC	22		
VSE36-L	AGACGGATTGAGGAGAAGCC	20	55	222-243
VSE36-R	CGGAGCACAGGAATGAAATC	20		
VSE38-L	CCATTTGCTCTAAGCAATTCATC	23	55	214-253
VSE38-R	TCACATGCGAATTGTTCACAC	21		
VSE42-L	CACCGCTACTGTTTCCTGGT	20	55	283-306
VSE42-R	GTGGGATGTGCGATAGAAGC	20		
VSE45-L	TGAAATTTGTTCCCTTCTGAGG	22	55	132-163
VSE45-R	TGATCCATTATTCAGATGAGGC	22		
VSE55-L	GTTGGAGACTGTCCTCGGTG	20	55	162-192
VSE55-R	TGCTTAACAGCATGGAATGG	20		

1-10 M-series primers and 12-21 VSE series primers Source: Hemmila, et al., 2010; Wei, et al., 2013

Primers like Mls20, M2r9 and VSE30 exhibited cross amplification probability with four species. Four primers namely Mlr6, Mls75, M2r31 VSE02 showed cross amplification with three species. Remaining primers showed cross amplification less than two species. The two primers Viz., VSE31 and VSE45 showed no cross amplification with Myristicaceae members.

The mean cross amplification across the species observed as 2.04 species per primer. The primers developed for *M. malabarica* (M-series) indicated on an average 2.27 species per primer whereas the primers developed for *Virola sebifera* (VSE series) showed 1.64 species per primer. Number of alleles per locus ranged from 0 to 4 across the species. The primer M2r9 exhibited maximum diversity with an average value 2.00 allele per locus, whereas most other alleles exhibited less than 0.50 alleles per locus with an overall mean of 0.79 alleles per locus.

Cross amplification of *M. Malabarica* primers with the native species *G canarica* showed 45 % success. Similarly with other native species *Knema attenuate*, *Myristica dactyloides Myristica fatua* were 18, 64 and 73 percent respectively whereas its was 27 percent with domesticated species *Myristica fragrance*. The cross amplification of *V sebifera* primers with the Western Ghats species namely *G. canarica, Myristica fatua Knema attenuate* and *Myristica dactyloides* 10, 20, 40 and 50 percent respectively whereas its was 30 percent with domesticated species *Myristica fragrance* (Table 4). The results suggest that within genera cross amplification is high compared to across the genera. Further, Western Ghats species show more cross amplification with native species than with the distant species *V. sebifera*.

These results are in accordance with many previous studies (Zucchi et al., 2002, Yasodha et al., 2005, Williamson et al., 2002, Aldrich, et al., 2003, Wilson et al., 2004, Gutierrez et al., 2005, Magdalena et al., 2007, Barbara et al., 2007, Ravishanker et al., 2011, Wei et al., 2013). They have shown intergeneric transferability of SSR, for instance tall fescue to several grasses (57%), *Litch chinensis* to *Blighia sapida* (58%) and *Setaria italic* to six grass species (74%).

Table 3. Primers used and amplification quality, alleles numbersper locus and number of species showed cross amplification

Primer	Gc	Ka	Mm	Mfr	Md	Mfa	Alleles/ locus	# species amplified
Mlr6	*	*	+	*	+	++	1.5	3
Mlr10	+	++	+	*	+	+	1.5	5
Mls20	*	++	+	*	+	+	1	4
Mls75	*	*	+	+	++	_	1	3
M2r6	+	_	+	+	++	++	1.5	5
M2r9	+	_	+	*	+	++	2	4
M2r31	+	_	+	_	_	+	0.5	3
M4s14	+	*	+	+	+	+	1	5
M4s73	*	*	+	*	_	_	0.5	1
M4s90	*	*	+	*	_	_	0.5	1
M5r33	*	*	+	*	*	+	1	2
VSE02	*	+	+	+	_	_	0.5	3
VSE11	*	*	+	+	_	_	0.5	2
VSE30	*	+	+	*	+	+	0.5	4
VSE31	*	*	*	_	_	_	0.5	0
VSE32	+	*	*	*	+	_	1	2
VSE36	*	++	+	*	*	_	0.5	2
VSE38	*	*	*	+	_	+	0.5	2
VSE42	*	*	+	*	_	_	0.5	1
VSE45	*	*	*	*	_	_	0.5	0
VSE55	*	+	*	*	+	_	0.5	2
Mean							0.79	2.04

Amplification quality: ++ very good, + good, * very poor and – No amplification) Gc-*Gymnacranthera canarica,* Ka-*Knema attenuate,* Mm-*Myristica malabarica* , Mfr -*Myristica fragrance,* Md-*Myristica dactyloides* and Mfa *Myristicafatua*

Table 4. Percentage cross amplifications of SSR markers across the species

Species	M-series primer	VSE series primer
Gymnacranthera canarica	45%	10%
Knema attenuate	18 %	40%
Myristica malabarica	100%	50%
Myristica dactyloides	64%	30%
Myristica fatua	73%	20%
Myristica fragrance	27%	30%
Average	55%	30%

M-series – SSR of *Myristica malabarica* VSE series – SSR of *Virola sebifera*

CONCLUSION

This study demonstrated the utility of *Virola sebifera* and *Myristica malabarica* SSRs markers for the genetic diversity and evolutionary studies of Myristicaceae member of Western Ghats. These results also indicate high levels of cross genera transferability possibility and demonstrated that distantly related species (*Virola sebifera* primers) show less amplification possibility than closely related native species (*Myristica malabarica*). Further, close to 90 percent of species/primer combinations tested within subgenera were successful, a much higher success rate than between genera attempts.

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