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## Altitudinal effect in active principle content in *Murraya koenigii* (L) correlated with DNA fingerprinting study

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### Abstract

*Murraya koenigii*, commonly growing in eastern Asia, is a herb that is highly valued for its medicinal potential; leaves of this plant, commonly known as Curry Patta, are also used widely for culinary purpose, particularly in India. Medicinal value of the plant include antioxidant, antibacterial, anticancer, hypoglycemic, hypolipidemic and antidiabetic activity. The active principle conferring such effect consists of carbazole alkaloids viz. Mahanine, Mahanimbine and Coumarins; the most potent being Mahanimbine. AFLP based Species Specific DNA Marker(s), identified in accessions of *Murraya koenigii* collected from different altitudinal regions established that all the genotypes used in this study belong to the same species viz. *Murraya koenigii*. Comparative HPLC analysis of the most potent active principle i.e. Mahanimbine revealed that content of this compound varies among plants growing in different altitudes: plants collected from high altitude (viz. Nagaland and Arunachal Pradesh) exhibited high content of mahanimbine in comparison to plants collected from low altitude places (viz. Kolkata and West Midnapur). This study indicates a correlation between altitude (related UV fluence) and mahanimbine content.

The phylogenetic tree, obtained through analysis of AFLP derived binary matrix, exhibits clustering of *Murraya* plants on the basis of the altitude of collection site; plants collected from high, intermediate and low altitude formed separate clusters. Our study indicated that altitude related variation in content of active principle correlate with variation in genome as reflected in the dendrogram. Multiple Regression Analysis (MRA) correlating active principle content and associated DNA fingerprinting data identified one AFLP allele (156 base pair allele generated by primer pair EcoRI-AAG / MseI-CTG) as Trait (Mahanimbine) Related AFLP Marker(s) in *Murraya koenigii*.

**Keywords:** *Murraya koenigii* (L), medicinal potential, Mahanimbine, altitudinal variation, species specific marker, trait related marker

### 1. Introduction

*Murraya Koenigii*, commonly known as curry-leaf tree, is a medicinal plant belonging to the family Rutaceae. This plant is found almost everywhere in the Indian subcontinent (Mhaskar *et al.*, 2000) [13] and is native to tropical Asia from Himalaya foothill's of India to Srilanka eastward through Myanmar, Indonesia, Southern China and Hainan. This plant has several important uses in the traditional system of medicine in Eastern Asia (Ningappa and Srinivas., 2008) [17]. *Murraya koenigii* is also used in ethanomedicine / folk medicine as a stimulant, antidysentric, anticancer, hypoglycemic, hypolipodermic and in the management of diabetes mellitus (Xie *et al.*, 2006) [29]. The leaves of this plant are highly valued as an important ingredient in Indian cuisine, known to promote appetite and digestion. The leaves, root and bark are used as tonic, stomachic and carminative. The aromatic leaves, that retain their flavor and other qualities even after drying, are slightly bitter, acrid, cooling, weakly acidic in taste and exhibit effect as a tonic, anthelmintic, analgesic, digestive and appetizing agent ; leaves are also useful for curing dysentery and vomiting (Parrota, 2001, Mhaskar *et al.* 2000) [20, 13]. Leaves of this plant are widely used in Indian cookery for flavoring food stuffs (Adebajo *et al.*, 2005; Mandal *et al.*, 2010) [2, 11]. Ppastes of leaves are applied externally to treat the bites of poisonous animals (Parrota, 2001) [20] and also to cure bruises and eruption (Kumar *et al* 1999) [8]. Steam distillate of the leaves can be used as stomachic, purgative, febrifuge and antianemic (Parrota, 2001) [20]. The juice of the root is good for pain associated with kidney. Fruits are considered as astringent in Indo-China and are known to have very high nutritional value together with many medicinal properties such as hepatoprotective activity against ethanol-induced hepatotoxicity.

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The phytoconstituents isolated so far from the leaves of *Murraya koenigii* are mainly alkaloids viz. Mahanine, Mahanimbine, Koenine, Koenigine, Koenidine, Girinimbiol, Girinimibine, Koenimbine, O-methyl Murrayamine A, Omethyl Mahanine, Iisomahanine, Bismahanine, Bispyrayafoline (Narasimhan *et al.*, 1970; Narasimhan *et al.*, 1975; Adebajo *et al.*, 2006; Tachibana *et al.*, 2003; Adebajo and Reisch, 2000) [16, 15, 1, 23, 3]. Among all these chemicals Mahanine and Mahanimbine constitute the most potent medicinal compounds. These two compounds are well known for their various pharmacological activities, including anti-HIV, anticancer, antibacterial and antifungal activities.

In ancient days, the activity of herb procurement, storage, preparation of drugs and its distribution remained mainly the responsibility of local physicians (Vaidyas and Hakims) who were very well versed with the identity of medicinal plants through their experience inherited through generation. In the present age of studies on use of medicinal plant for drug preparation, the first step is to ensure selection of authentic plant material by checking the presence of Species specific DNA marker(s). Although studies on DNA fingerprinting revealing genetic diversity in populations of *Murraya koenigii* have been performed by various DNA fingerprinting methods such as RAPD, ISSR (Verma and Rana, 2013) [24], identification of Species Specific DNA Marker(s) by AFLP is not available in the literature till date. DNA Fingerprinting data correlated with active principle content in plant helps to identify Trait Related DNA marker(s) through Multiple Regression analysis (Virk *et al.*, 1996; Kar *et al.*, 2008). Such DNA Trait Related DNA marker(s) may be used for screening wild germplasms for selection of elite genotype containing high amount of the desired medicinal compound.

In this study we have.

- Developed AFLP based Species Specific DNA markers for authentication of experimental *Murraya koenigii* plants collected from different altitude.
- Assayed Mahanimbine content by HPLC in *Murraya* plants collected from different altitude to enquire effect of UV radiation in the biosynthesis of this carbazole alkaloid.
- Developed medicinal potential related trait related DNA marker by statistical correlation analysis between DNA fingerprinting and chemical analysis data.

## 2. Material

*Murraya koenigii* plants were collected from different altitudinal regions of Eastern and NE India viz. West Bengal (Kolkata (9m), West Midnapur(16m), Doors(32m)), Nagaland (1400m), Arunachal Pradesh (700m) in the same month viz. in June (i.e during summer solstice) within almost the same latitude (~27°N).

## 3. Methods

### 3.1 DNA extraction

DNA was extracted from fresh leaves by the 2% CTAB method (Bousquet *et al.*, 1990) [4]. The extraction buffer consisted of 2% (w/v) CTAB (cetyltrimethyl ammonium bromide, Sigma), 1.4M NaCl, 20mM EDTA, 100mM Tris-HCl pH 8.0, and 0.2% (v/v)  $\beta$ -mercaptoethanol. The concentration of DNA in the samples was determined by calculation from 260/280 O.D value. The DNA samples are subjected to electrophoresis on 0.8% agarose gel; genomic  $\lambda$  DNA (25ng/ $\mu$ l) was used as molecular weight standard. All DNA samples taken for AFLP study showed 260 O.D/280 O.D value between 1.75-2.00.

### 3.2 DNA fingerprinting study

AFLP analysis of genome in landraces (collected from different altitudes) was done with the help of ABI prism fluorescent dye labeling with detection technology (Perkin-Elmer) that uses the kit supplied by Applied Biosystems (USA). The basic procedure of AFLP was according to Vos *et al.*, 1995 [26]. High-quality genomic DNA (500 ng) extracted from experimental plants was digested with restriction endonucleases (1 U MseI and 5 U EcoRI). EcoRI and MseI adaptors were ligated with 1 U T4 DNA ligase (all enzymes were from New England Biolabs, Beverly, MA, USA). Restriction and ligation are done simultaneously (Vos *et al.*, 1995) [26] in a single step by incubating extracted DNA at 37 °C for 2h in a thermocycler (Applied Biosystems). Pre-amplification and selective amplification are carried out according to instructions provided in the kit. Pre-amplifications were checked by running pre-amplified samples on a 1.5% agarose gel. A smear of DNA fragments ranging from 100-1500 bp was visible after separation in 1.5% agarose gels. Selective amplification was carried out using six primer pairs for all the landraces of *Murraya koenigii* studied. The amplified products are mixed with Size Standard Gene Scan 500 ROX, and the samples are then analyzed on an automated DNA sequencer (ABI Model 3130 XL genetic analyzer, Applied Biosystems).

AFLP analysis was carried out for alleles in the range of 35-500 bp. For diversity analysis, alleles were scored as present (1) or absent (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained with Jaccard's similarity coefficient (Jaccard, 1908) [6]. This similarity matrix were then used for cluster analysis using the unweighted pair group method of arithmetic averages (UPGMA) technique which led to development of a phylogenetic tree by using the software NTSys v 2.1.

### 3.3 Active Principle analysis

Mahanimbine is a carbazole alkaloid found in *Murraya koenigii* leaves. Extraction of plant material was done using 90% methanol and complete removal of the solvent from extract was done under vacuum evaporator under reduced pressure to get a sticky mass which was finally lyophilized to obtain a dry powder (Kumar *et al.*, 2010) [9]. Isocratic HPLC assays were performed using the instrument HPLC 1100, Shimadzu, Japan, equipped with a reversed phase column C18 (Hypersil ODS 250 mm x 4.0 mm i.d., 5 micron). Mobile phase composition was optimized to methanol and 0.5% acetic acid in water (90:10 v/v) which was degassed and filtered through membrane prior to run in the column. Temperature of the column was kept at 25 °C, and each injection volume was 20 mL. Flow rate was set at 1.0mL/min. Detection of the compounds was performed at 254 nm (Pandit *et al.*, 2011). Peak identification was achieved by comparison of the retention time of Mahanimbine standards (Sigma-Aldrich) with the extract.

### 3.4 Statistical Analysis

Correlations between parameters are examined using Karl Pearson correlation. The association between AFLP markers and the quantitative traits is estimated through stepwise multiple regression analysis (MRA), where each quantitative trait is treated as dependent variable, while the AFLP markers treated as independent variables. The analysis is based on the model:  $Y = a + b_1m_1 + b_2m_2 + \dots + b_jm_j + \dots + b_nm_n + d + e$  which related the variation in the dependent variable (Y= accession means for a quantitative trait) to a linear function of the set of

independent variables mj, representing AFLP markers. All analysis is performed using the same SPSS software package.

#### 4. Results

AFLP analysis of collected *Murraya koenigii* genotypes by six pairs of primers revealed Species Specific DNA Marker(s) that are present in all the genotypes of this species regardless of their place of collection (Table-1); in addition to Species Specific AFLP alleles, there are other AFLP alleles which reveal genetic variability among the genotypes collected from different altitudinal locations. Representative AFLP chromatogram of *Murraya koenigii* plants from different locations are shown in Figure-1. As evident from phylogenetic tree, *Murraya* genotypes from Nagaland and Arunachal Pradesh (both states situated at high altitude) clustered together whereas plants from West Midnapur and Kolkata (both places of low altitude) clustered separately. *Murraya* plants collected from Doors (location of intermediate altitude) appeared in a cluster separated from the other two groups (Figure-2).

Comparative HPLC analysis reveals highest Mahanimbine content (21.09 µg/g) in plants of Nagaland (1400m) and lowest content (9.71 µg/g) in plants of Kolkata (9m). Plants of Arunachal Pradesh (700m) have also high mahanimbine content (16.76 µg/g) (Table-2) in comparison to lower altitudinal plants but lower than plants collected from Nagaland (1400m). The Mahanimbine content of Plants collected from West Midnapur (11m) is comparable to Kolkata (9.89 µg/g). Mahanimbine content of *Murraya* plants collected from Doors (32m), West Bengal found to be 10.01 µg/g. Representative HPLC chromatograms showing variable content of Mahanimbine from different altitudes are shown in Figure-3. Mahanimbine content is found to be higher in plants collected from high altitude (where UV fluence is high) evidently due to upregulation of its biosynthetic pathway by UV radiation. Multiple Regression Analysis (MRA) correlating active principle content (Table-2) and associated DNA fingerprinting data (Figure-1) identified an allele (156 base pair allele generated by primer pair EcoRI-AAG / MseI-CTG) as Trait (Mahanimbine) Related AFLP Marker(s) in *Murraya koenigii* (Table-3).

**Table 1:** Species specific alleles with their size (in bp) for *Murraya koenigii*

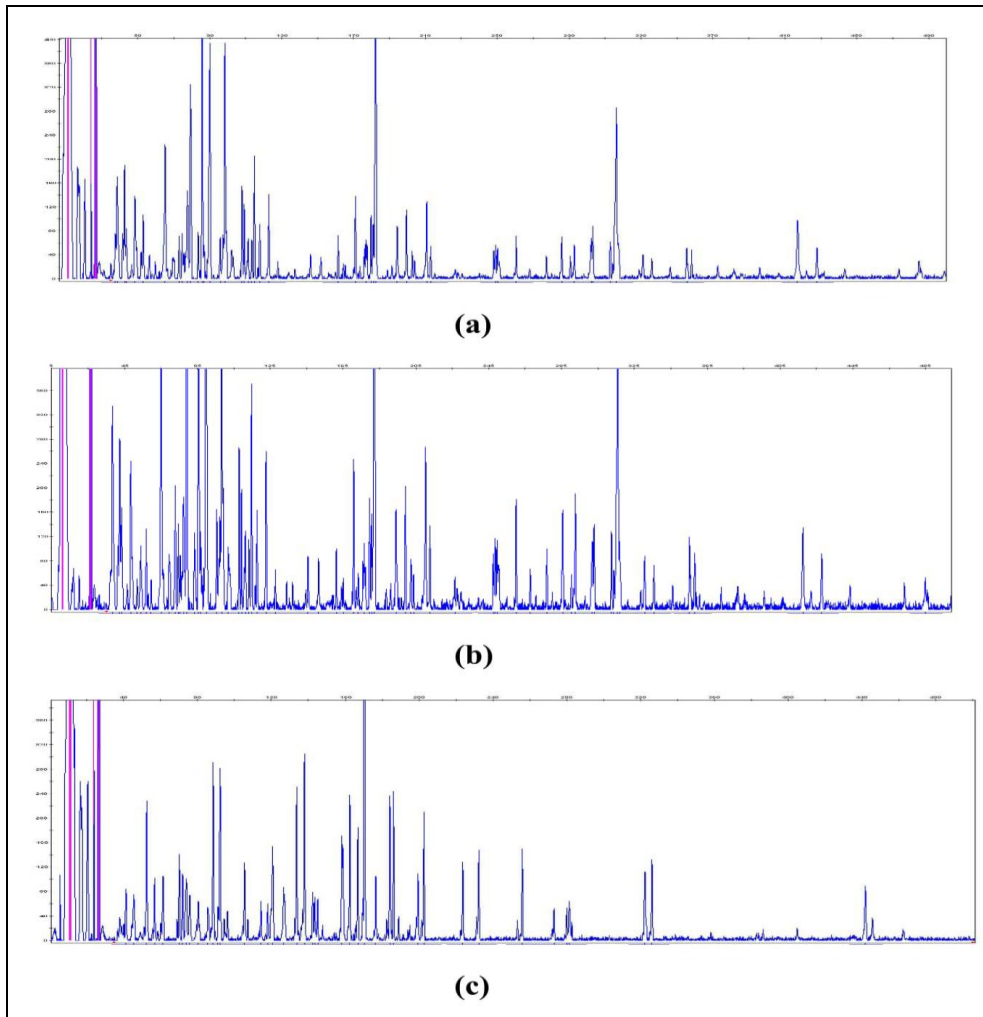
Primer combination MseI-CAG/ EcoRI-ACG		Primer combination MSeI-CTT/ EcoRI-AAG		Primer combination MseI-CTG/ EcoRI-ACA		Primer combination MseI-CTG/ EcoRI-AAG		Primer combination MseI-CTG/ EcoRI-ACT		Primer combination MseI-CAG/ EcoRI-ACA	
Allele no	Allele size (bp)	Allele no	Allele size (bp)	Allele no	Allele size (bp)	Allele no	Allele size (bp)	Allele no	Allele size (bp)	Allele no	Allele size (bp)
16	54	2	38	4	45	10	55	5	46	10	45
35	91	42	106	23	72	22	68	19	76	11	46
40	100	72	186	31	86	23	70	24	84	37	77
52	130	73	187	37	95	32	94	36	124	38	78
56	132	95	264	62	161	37	118	37	126	127	292
57	134	86	235	96	247	41	126	40	128	136	328
58	144	56	132	61	159	45	139	46	141	142	344
61	157	58	135	29	82	50	159	49	145	143	346
62	158	84	227	91	237	51	165	54	169	150	381
63	159	92	253	102	293	56	180	59	180		
64	160			105	304	73	295	61	181		
65	170			106	312	74	303	64	188		
66	171			107	314	76	315	71	203		
92	432			110	350	85	391	76	228		
				117	437	91	450	87	256		
								102	312		
								108	374		

**Table 2:** Content of Mahanimbine in *Murraya koenigii* landraces collected from different locations of variable altitudes

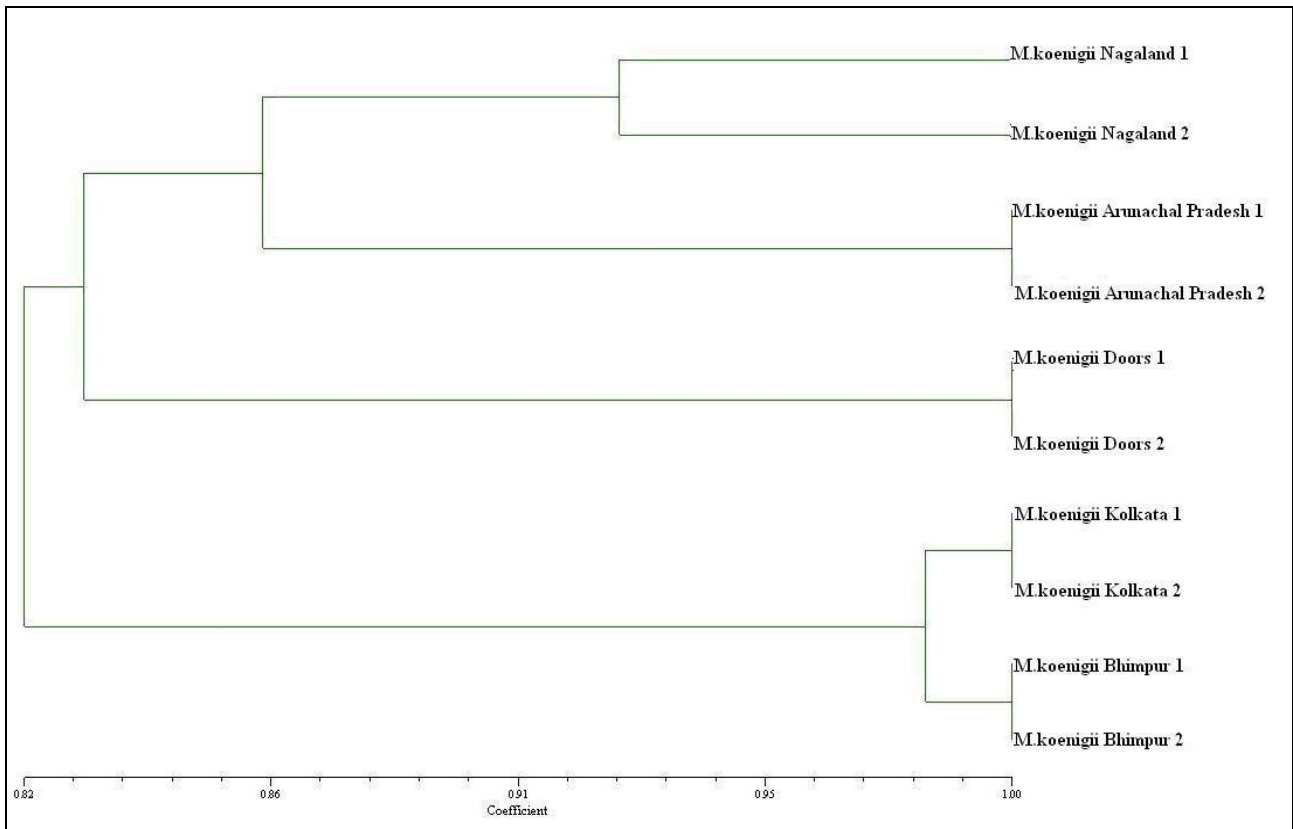
Location	Altitude(m)	Mahanimbine content
Nagaland	1400m	21.09 µg/g
Arunachal Pradesh	700m	16.76 µg/g
Kolkata, West Bengal	9m	9.71 µg/g
West Midnapur, West Bengal	16m	9.89 µg/g
Doors, West Bengal	32m	10.01 µg/g

**Table 3:** Trait Related Marker for Mahanimbine in *Murraya koenigii*

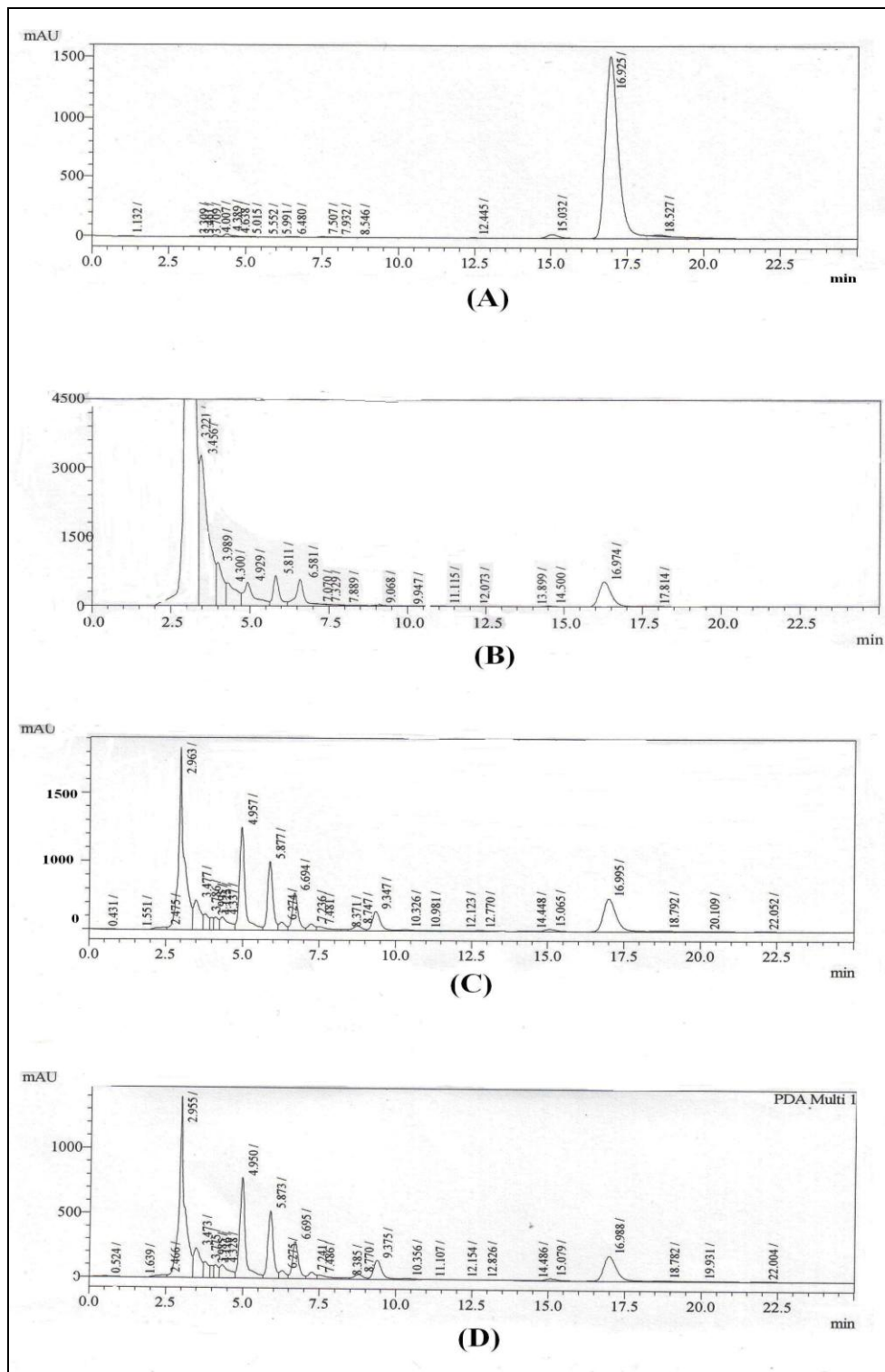
Biochemical parameter	Primer pair used	AFLP marker	r	R <sup>2</sup>	Standardized coefficient β	t value	Standard Error (SE)	P Value
Mahanimbine content	EcoRI-AAG / MseI-CTG	156bp	0.9848	0.970	0.903	10.567	1.987	<0.01



**Fig 1:** Representative AFLP patterns of *Murraya koenigii* landraces collected from (a) Kolkata (b) Nagaland and (c) Arunachal Pradesh using primer pairs EcoRI-ACA and MseI-CTA. X-axis = base pair of alleles; Y-axis = intensity of alleles.



**Fig 2:** AFLP analysis based phylogenetic tree showing clustering of *Murraya koenigii* genotypes correlated with place of origin.



**Fig 3:** Representative HPLC chromatogram showing variation in Mahanimbine content in *Murraya koenigii* genotypes collected from different altitudinal region.

(A) - HPLC Standard (Sigma-Aldrich), (B) – HPLC chromatogram of Mahanimbine from genotype collected from Nagaland (1400m) showing high content, (C) - HPLC Chromatogram of Mahanimbine from genotype collected from Arunachal Pradesh (700m) showing intermediate content and (D) HPLC Chromatogram of Mahanimbine from genotype collected from Kolkata (9m) showing low content (X-axis – Time, Y-axis – mAU)

## 5. Discussion

For ensuring use of the same species of plants collected from different locations (including variation in altitude) AFLP analysis of genomes was undertaken to identify Species Specific markers. AFLP analysis by six pairs of primers revealed Species Specific alleles which are present in all the landraces of *Murraya koenigii*, used in the present study, irrespective of their collection from different altitudes (Table - 1). Such authentication demonstrated by presence of species

specific alleles in experimental material may also be useful for pharmaceutical companies for production of medicines from *Murraya* with uniform efficacy in each batch. In addition to Species Specific AFLP markers, several other peaks are also evident in the experimental material collected from different altitudes (Figure 1). The DNA fingerprinting data indicates that while all the studied *Murraya* plants belong to the same species, some genetic variability at infraspecific level exists between them. This genetic variability is reflected

in the phylogenetic tree (Figure-2). Plants collected from high (viz. Nagaland and Arunachal Pradesh), intermediate (Doors) and low (Kolkata and Bhimpur) altitudes clustered separately in the phylogenetic tree (Figure-2). Thus it appears that clustering of *Murraya* genotype is correlated with altitude of their origin; indicating a trend towards phylogenetic variation due to ecological (altitude related) variation such as UV fluence.

Mahanimbine content was found to be correlated with respective altitude of the collected plant (Table-2). Among the plants studied, Mahanimbine content is highest (17.09 µg/g) in plants collected from Nagaland (alt-1400m) and lowest (10.89µg/g) in plants collected from Kolkata (alt-9m) (Figure-3). Mahanimbine content in plants from other low altitude regions viz. Bhimpur (alt 32m) is comparable to content found in Kolkata growing plants. Content of this compound is comparatively higher (13.76 µg/g) in Arunachal Pradesh (alt-700m) growing plants in comparison to plants of Kolkata but lesser than Nagaland growing plants (Table-2). This altitudinal difference in Mahanimbine content is evidently due to the fact that being an (carbazole) alkaloid, this compound is UV upregulated Lyddon *et al.*, (1987) <sup>[10]</sup> also reported UV related enhancement in tetrahydrocannabinoid in Cannabis. This study indicates that altitude related variation in content of active principle correlate with variation in genome as reflected in the AFLP derived dendrogram. Similar observation has been reported by Ghosh and SenMandi (2013) in *Zingiber officinale*.

Multiple Regression Analysis (MRA) correlating DNA fingerprinting data with active principle (i.e Mahanimbine) content identified one AFLP allele (156 bp allele generated by primer pair EcoRI-AAG / MseI-CTG) to be associated with high Mahanimbine content (Table-3). During the last two decades, DNA-based molecular markers associated with traits of importance have been extensively studied in *Camellia sinensis* for drought tolerance- in *Cocos nucifera* for mite resistance (Shalini *et al.*, 2007) <sup>[22]</sup>, in *Morus* sp. for protein content (Kar *et al.*, 2008) <sup>[7]</sup>, in *Medicago sativa* for forage yield in *Betula platyphylla* for fibre length (Wang, 2007), in *Medicago sativa* for resistance against downy mildew (Obert *et al.*, 2000) <sup>[18]</sup>, in *Dendrocalamus hamiltonii* for cyanogens and antioxidant potential (Waikhom *et al.*, 2012) <sup>[27]</sup>.

This is the first report correlating AFLP allele with high Mahanimbine content, representing Trait Related marker; this could be used for selecting medicinally superior landraces of *Murraya koenigii*, collected from different altitudinal regions.

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