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Species Specific AFLP Markers for authentication of *Zanthoxylum acanthopodium & Zanthoxylum oxyphyllum*

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Zanthoxylum acanthopodium and Zanthoxylum oxyphyllum, found in North East India, are commercially important due to its edible fruits and leaves containing essential oils used in cosmetics and perfume industries. These species have also been used by local population as ethno medicine. We used amplified fragment length polymorphism (AFLP) to produce DNA fingerprints for two *Zanthoxylum* species viz. *Z. acanthopodium*, and. *Z. oxyphyllum*. Twelve collections (eight of *Z. acanthopodium*, four of *Z. oxyphyllum*) were used in the study. Six selective primer pairs were found to detect polymorphism. A total of 483 alleles were produced. Species-specific markers were identified in the two *Zanthoxylum* species (23 for *Z .acanthopodium.* and 13 for *Z. oxyphyllum*). The dendogram showed species specific clustering of two species. The AFLP markers developed in this study could be used to authenticate two *Zanthoxylum* species to resolve adulteration-related problems faced by pharmaceutical industries to supplement conventional drug assessment protocols.

Keyword: DNA Fingerprinting; *Z. acanthopodium*; *Z. oxyphylum*; AFLP; Species Specific Marker.

1. Introduction

The genus *Zanthoxylum,* under the family Rutaceae, growing in tropical and temperate regions [28], consists of about 250 species [2] that are represented by thorny, dioecious shrubs or small trees with dense foliage and prickly trunk and branches bearing edible fruits and leaves with a strong and pungent taste resembling the flavour of lemon, anise or mint [7]. Commonly found in the Himalayan region, plants of this genus are also found in Central, South, Southeast and East Asia [22], some species are also reported from America and Africa. Many of the species are recorded as traditional medicines of Africa [1], Asia [36] and America [4] for treatment of a number of diseases in humans and animals [1], [5], [10], [26]. Some of the Asian species, in addition to being used for medicinal purpose, are also known for their culinary use as spices [30], [31] unlike the African and American species [29]. Hooker [13] described eleven species from India. viz *Z. budrunga, Z. oxyphyllum, Z. ovalifolium, Z. acanthopodium, Z. planispinum, Z. armatum, Z. nitidium, Z. rhesta, Z. simulans, Z. avicennae* and *Z. limonella.* Out of these *Z. armatum DC, Z. acanthopodium DC., Z. oxyphyllum Edgew,* and *Z. budrunga* have been reported from Uttarakhand [15] and *Z. hamiltonianum, Z. rhesta, Z. oxyphylum, Z. alatum, Z. ovalifolium* and *Z.acanthopodium* have been reported from North East India [12]. All these species are commonly used by local population for ethnomedicinal purpose. Seeds of most *Zanthoxylum* fruits are rich in oil containing large amounts of alkaloids and unsaturated fatty acids possessing notable antioxidant activity [40]. This accounts for several types of biological activities of the *Zanthoxylum* genus such as anti-inflammatory, analgesic,

antinociceptive, antioxidant, antibiotic, hepatoprotective, antiplasmodial, cytotoxic, antiproliferative, anthelminthic, larvicidal, antiviral and antifungal. [24]. Other commercial importance for the edible fruits [15] and leaves containing essential oils constitute their use in cosmetics and perfume industries [29].

Zanthoxylum is a difficult genus with many different and not well-researched species [2]; many of the species exhibit apparent similarity with related and unrelated plant groups. An interesting example is the plant species *Rhus hookeri* that has striking similarity with the leaves and fruits of *Zanthoxylum.* This posses the risk of adulteration in vendor-collected drug plant material that constitute the starting material in pharmaceutical/nutraceutical industry. It is thus important for pharmaceutical industries to supplement conventional (morphological, anatomical, biochemical) drug assessment protocols with use of species specific DNA markers for selection of the prescribed drug plants. It is pertinent to mention here that while studies on chemical constituents of *Zanthoxylum* of medicinal importance viz alkaloid [3], [16], [25]; and antioxidant, anti-inflammatory compounds found in stem bark of some species [32], [41] are often found in the literature, studies on molecular authentication of the medicinally important species have seldom been reported [20], [35], [43]. Considering the urgency of molecular documentation of important plant bioresource we are undertaking studies on DNA Fingerprinting based authentication of plant species.

We present here AFLP analysis of 2 important species of *Zanthoxylum* that are widely used as folk medicine in North East India. Such studies, in addition to helping pharmaceutical industry for using precisely identified drug plants, would also help in establishing ownership rights and resolve associated IPR conflicts.

Zanthoxylum oxyphyllum: In North East India the plant has been used as traditional medicine where tender shoots of this plant are taken as vegetable, which are useful against stomach trouble, as a blood purifier and reducing the incidence of leucoderma [3]. Fruits are used as spice and help in digestion. *Z. oxyphyllum* bark is commonly applied in skin diseases, rheumatism, varicose ulcers and varicose veins, leg pains. Additionally, it can be applied to relieve inflammations, fevers, and hypotension [2]. Extracts from the bark and root of *Z. oxyphyllum* has recently been shown to have antiproliferative activity against the growth of human keratinocytes [18]. Besides it has stimulant, stringent and digestive properties and is used in dyspepsia and diarrhea [20].

Zanthoxylum acanthopodium: The fruits are used in carminative and anthelmintic treatments. Fruits, leaves and seeds are eaten as vegetable [17]. Besides this the leaves are also used as insecticides and an insect repellent [39]. The fruit and seeds are also prescribed in the treatment of rheumatism, dysentery and stomach ache [12]. Seeds and barks are also used as an aromatic, tonic in fever, dyspepsia and in cholera [6]. Of particular concern is the report that these plants are now decreasing in numbers [23]. The wide use of this plant in traditional folk medicine [42] and the threat of it's drastic reduction in number calls for precise molecular studies for documentation, evaluation and conservation of this species.

The present study was conducted for development of species specific markers of Z. acanthopodium and *Z. oxyphyllum*, using plants collected from Sikkim, Meghalaya & Arunachal Pradesh.

2. Material and Methods

2.1 Plant materials

The plant materials used in this study were collected from different parts of Eastern and North Eastern India, viz. Arunachal Pradesh, Sikkim, and Meghalaya (Table 1). The samples consisted of 8 collections of *Z. acanthopodium* and 4 collections of *Z. oxyphyllum.*

2.2 DNA extraction

Genomic DNA from all plant samples was isolated from young leaves from each genotype. Total genomic DNA was extracted using) DNA extraction protocol [9] where an extraction buffer [120 mM Tris HCl (pH 8.0), 80 mM EDTA (pH 8.0), 0.5% Triton X (v/v) and 0.5% β mercaptoethanol (v/v)] was primarily used to reduce the secondary metabolite levels followed

by a separate lysis buffer [120mM Tris HCl (pH 8.0), 10 mM EDTA (pH 8.0), 2% (w/v) N-lauryl sarcosine sodium salt (sarkosyl), 0.8 M NaCl, 2% Poly Vinyl Pyrrolidone (PVP) and 0.2% β mercaptoethanol (v/v)] comprising of sarcosyl detergent (N-lauryl sarcosine sodium salt), two different reducing agents and higher concentration of chelating agents

Table 1: Name, family, locality, GPS data, elevation, date of collection and accession number of the experimental *Zanthoxylum* germplasm.

S. No	Name of the plant	Family	Locality	Latitude & Longitude	Elevation (m)	Collection No.
1	*Zanthoxylum acanthopodium* DC.	Rutaceae	Meghalaya: East Khasi Hills, Barapani	N 25°39'11.19"; E 91°53'58.51"	910	52517 52518
			Meghalaya: East Khasi Hills, on the way to Cherrapunjee	N 25°23.193 E 91°45.559	1512	52520 52521 52522
			Meghalaya: East Khasi Hills, near Shillong Peak	N 25°32'50.24"; E 91°52'28.22"	1908	52523 52524
			Meghalaya: East Khasi Hills, near Shillong Peak	N 25°32'51.32"; E 91°52'32.50"	1900	52526
2	*Zanthoxylum oxyphyllum* Edgew	Rutaceae	Sikkim, North District, Lachung	N 27°41.478' E 88°44.681'	2761	52513
			Near, Mayudia, Lower Dibang Valley	N 28°14.021 E 95°54.758	2434	52568
			Near Tiwari Gaon, Lower Dibang Valley	N 28°12.768 E 95°51.121	2490	52569
			4.5 km from Mayudia towards Hunli, Lower Dibang Valley	N 8°14.543 E 95°54.138	2359	52578

The concentration of DNA in the samples was determined by the 260/280 O.D. value as observed in nanophotobiometer. The DNA samples were subjected to 0.8% agarose gel

electrophoresis; genomic λ DNA (25 ng/µL) was used as standard. All DNA samples taken for AFLP study showed a 260/280 O.D. value of 1.75-2.00.

2.3 AFLP fingerprinting

AFLP electropherograms were produced for each variety using the ABI prism fluorescent dye labeling and detection technology (Perkin-Elmer). AFLP analysis was performed using the kit supplied by Applied Biosystems (USA) and was used according to manufacturer instructions.

High-quality genomic DNA (500 ng) was digested with 1 U *Mse*I and 5 U *Eco*RI restriction endonucleases. *Eco*RI and *Mse*I adaptors were ligated with 1 U T4 DNA ligase (all enzymes were from New England Biolabs, Beverly, MA, USA). Restriction and ligation were done simultaneously [37] in a single step by incubating at 37 °C for 2 h in a thermocycler (Applied Biosystems).

Polymerase chain reaction (PCR) amplification and selective amplification were carried out according to instructions provided in the kit. Pre-amplifications were evaluated running pre-amplified samples on a 1.5% agarose gel. A smear of product from (100-1500) bp was clearly visible.

Selective amplification was carried out using six primer pairs for two species of the *Zanthoxylum* genus, viz., *Z. acanthopodium and Z. oxyphyllum*. The amplified products were mixed with Size Standard Gene Scan 500 ROX, and the samples were then analyzed on an automated DNA sequencer (ABI Model 3130 XL genetic analyzer, Applied Biosystems).

2.4 Scoring and Data Analysis

Fragment analysis was carried out for bands in the range of 35-500 bp. For diversity analysis, bands were scored as presence (1) or absence (0) to form a raw data matrix. A square symmetric matrix of similarity was then obtained with the Jaccard's similarity coefficient [14]. The average similarity matrix was used to generate a tree for cluster analyses by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using the NTSys v 2.1 software.

3. Results and Discussion

AFLP fingerprinting patterns of collected landraces of *Z. acanthopodium*, and *Z. oxyphyllum* were generated in a DNA sequencer using the six pairs of primers for both *Z. acanthopodium* and *Z. oxyphyllum*. Of total of 483 alleles, only 2 alleles were monomorphic and 835 were polymorphic. A polymorphism of 83.9% was detected among the species. Representative AFLP patterns of *Z. acanthopodium* and *Z. oxyphyllum* are given in Figure 1 & 2. The study could identify species-specific AFLP markers for the two *Zanthoxylum* species, where all landraces within a single species showed similarity in fingerprinting pattern. The AFLP alleles common to all the landraces of *Z. oxyphyllum*, using six primer pairs and their respective allele size (in bp) are shown in Table 2. Similarly, alleles, specific for *Z. acanthopodium* with their respective size (in bp) are shown in Table 3. Such data on DNA characterization reveal variation between the two species, landraces of each species being similar regardless of their place of collection.

The dendogram (Figure 3) obtained after analysis of data using the NTSys v 2.1software indicated two major clusters, cluster I representing landraces of *Z. acanthopodium* and cluster II representing landraces of *Z. oxyphyllum*. Cluster I representing 8 landraces is divided into 2 sub clusters viz cluster Ia and cluster 1b. Cluster Ia contains 4 landraces (52517, 52520, 52522, 52523) and cluster Ib contains the other 4 landraces (52518, 52521, 52526, 52524) of *Z. acanthopodium*. Thus the eight landraces of *Z. acanthopodium* collected from Meghalaya remained together in cluster I, showing 49% similarity among the genotypes. The second cluster (cluster II) consisted of four landraces of the *Z. oxyphyllum* separated into 2 subclusters correlated to the site of collection. From the cluster II it appears that the landrace collected from Sikkim (52513) shows 10% phylogenetic

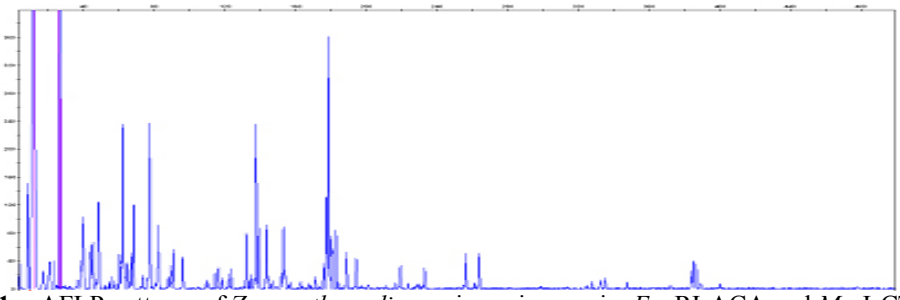

Fig 1a: AFLP patterns of *Z. acanthopodium* using primer pairs *Eco*RI-ACA and *Mse*I-CTA.
X-axis = base pair of alleles; Y-axis = intensity of alleles.

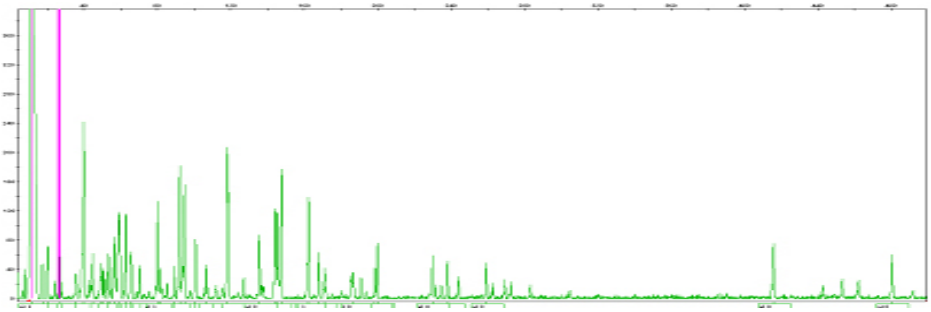

Fig 1b: AFLP patterns of *Z. acanthopodium* using primer pairs *Eco*RI-AAG and *Mse*I-CTC.
X-axis = base pair of alleles; Y-axis = intensity of alleles.

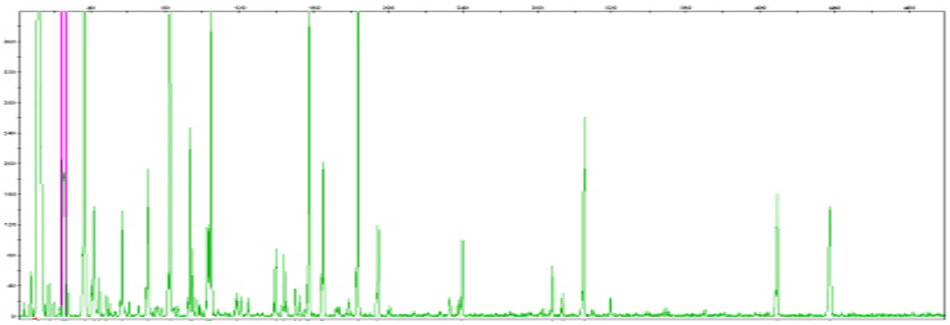

Fig 1c: AFLP patterns of *Z. acanthopodium* using primer pairs *Eco*RI-ACG and *Mse*I-CTG.
X-axis = base pair of alleles; Y-axis = intensity of alleles.

dissimilarity with the other three landraces of *Z. oxyphyllum* (52568, 52569 and 52578) collected from Arunachal Pradesh. Overall *Z. oxyphyllum* landraces showed 22% similarity among the landraces represented within the cluster II. The distribution of landraces belonging to one particular species within one cluster is due to infraspecific genetic variation which was precisely depicted in the dendogram using sophisticated AFLP Fingerprinting analysis.

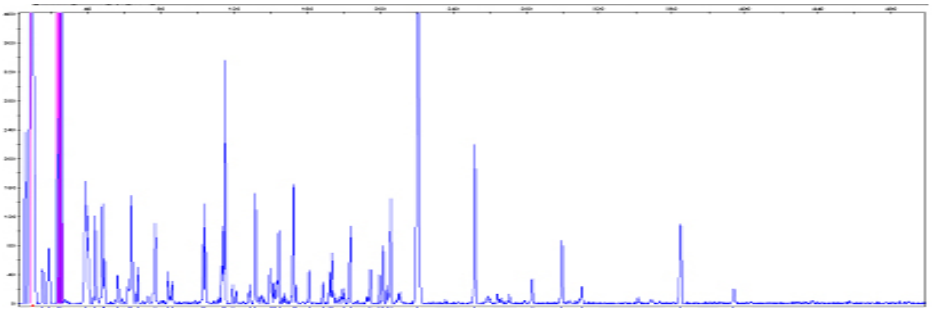

Fig 2a: AFLP patterns of *Z. oxyphyllum* using primer pairs *Eco*RI-ACA and *Mse*I-CTA.
X-axis = base pair of alleles; Y-axis = intensity of alleles.

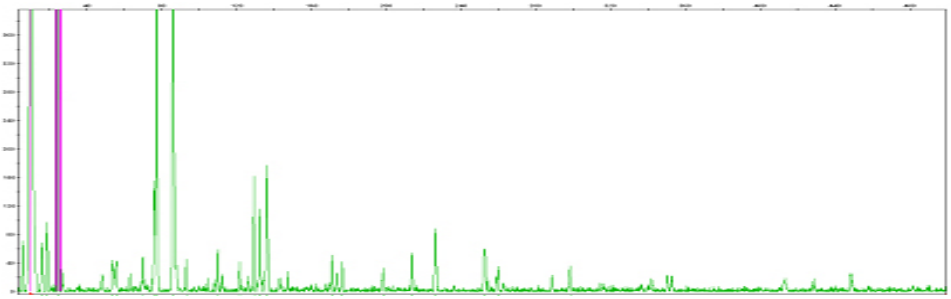

Fig 2b: AFLP patterns of *Z. oxyphyllum* using primer pairs *Eco*RI-AAG and *Mse*I-CTC. X-axis = base pair of alleles; Y-axis = intensity of alleles.

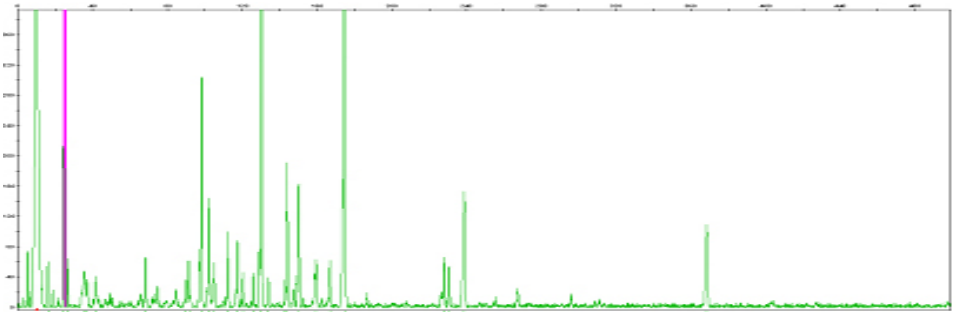

Fig 2c: AFLP patterns of *Z. oxyphyllum* using primer pairs *Eco*RI-ACG and *Mse*I-CTG. X-axis = base pair of alleles; Y-axis = intensity of alleles.

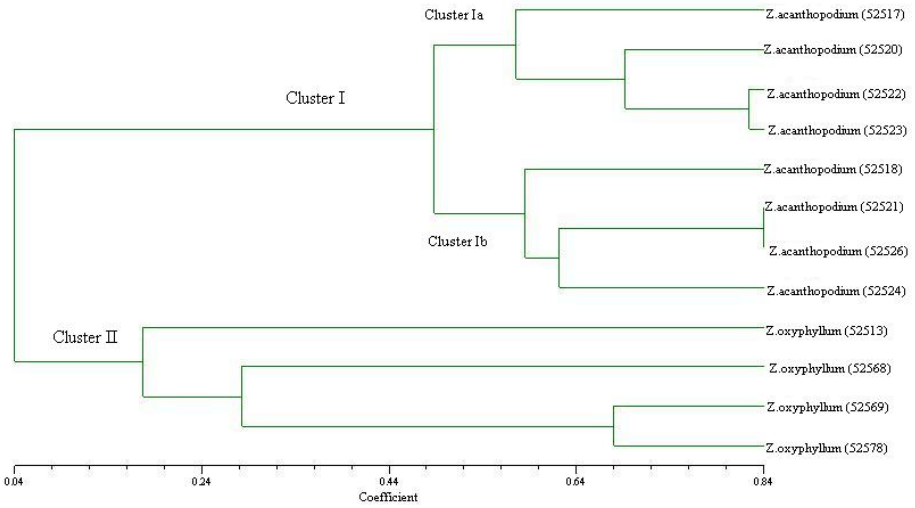

Fig 3: Cluster diagram showing relationship among two *Zanthoxylum* species.

Table 2: Number and size (in bp) of common alleles (indicating species specificity), of *Z.oxyphyllum* land races collected from widely different elevations.

EcoRI ACA+ MSeI CTA		EcoRI AAG +MSeI CTC		EcoRI ACG+ MSeI CTG		EcoRI AGC+ MSeI CAA		EcoRI ACA+ MSeI CTC		EcoRI AAG+ MSeI CTA	
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)
5	31	25	76	57	130	32	64	43	74	29	84
60	115	26	77			65	135	78	145	37	98
135	252	30	86			68	139	96	175		
		44	129			71	142				
		49	136								

Table 3: Number and size (in bp) of common alleles (indicating species specificity), of *Z. acanthopodiuum* land races collected from widely different elevations. Eco RI – AAG MSe I – CTC did not reveal any Species Specific DNA marker in these land races.

EcoRI ACA+ MSeI CTA		EcoRI AAG +MSeI CTC	EcoRI ACG+ MSeI CTG		EcoRI AGC+ MSeI CAA		EcoRI ACA+ MSeI CTC		EcoRI AAG+ MSeI CTA	
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
32	63		27	71	33	67	48	70	28	49
45	83		31	83	46	93	77	105	41	89
79	168		37	93	67	138	95	140	49	150
104	178		38	95	81	180	98	158	56	213
105	179		45	104			109	210	106	418
			46	105			117	249		
		No Specific allele found for this primer pair	59	140			121	301		
			72	165						
			80	184						
			82	185						
			91	240						
			98	305						
			111	409						
			113	437						

AFLP is a useful method for precise identification of genotype within a short period of time. This method also proves to be very important in plant taxonomy for species-specific identification [8, 11, 19, 21, 27, 33, 34, 38]. As per molecular works of *Zanthoxylum* only few relevant work of *Zanthoxylum* species has been done comprising development and characterization of microsatellite markers in *Zanthoxylum ailanthoides* [43] molecular identification of medicinally important *Zanthoxylum schinifolium* using ribosomal DNA internal transcribed spacers [35] and Genetic diversity of *Z. hamiltonianum* of North- East India through RAPD analysis [20] which itself depicts the need of more molecular works as per as this plant is concerned. So it is quite evident that molecular documentation of this particular genus has not been thoroughly explored.

We found 13 species specific alleles/markers for *Z. oxyphyllum* and 23 species specific alleles/markers for *Z. acanthopodium*. The identification of species specific DNA markers for the two species of *Zanthoxylum* genus viz. *Z. acanthopodium* and *Z. oxyphyllum* regardless of their site of collection should provide useful reference tool for species identification that circumvents problems associated with morphological or biochemical markers that is often used as a dependable identifying parameter for species identification even if collected from different locations.

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