Chapter I

INTRODUCTION
CHAPTER-I

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1.1. Introduction of the genus Zanthoxylum

The genus Zanthoxylum (Rutaceae) is distributed worldwide from the tropics to temperate zones. The genus comprises of over 250 species ranging from small shrubs to large trees (Engler, 1896; 1931). Morphologically, it is the only truly choricarpous genus in the family Rutaceae, with fully free and stalked carpels (Gut, 1966). The much unspecialized flower morphology and vascular supply suggest a primitive position of Zanthoxylum within the family Rutaceae (Moore, 1936; Das et al., 1988). It is economically important in different parts of the world because of their alimentary, industrial and medicinal applications (Seidemann, 2005; Chase et al., 1999). Zanthoxylum comes from the Greek word "xanthon xylon" that means "yellow wood", hence the use of the terms Xanthoxylum or Zanthoxylum by some authors. The genus Zanthoxylum was created by Linné in 1757 and since its inception it has been confused with the genus Fagara. But in 1896, Engler made a clear distinction between the two genera by the characteristics of the perianth. The species of the genus Zanthoxylum have a simple perianth, while in species of the genus Fagara have twofold. Literatures have contradictory information regarding the species available in India. In "Flora of British India" by Hooker (1875) reports 11 species of Zanthoxylum while in "Wealth of India" (Anonymous, 2003) mentions about 13 species of India. In Assam, Kanjilal (1992) in his book "Flora of Assam" reported only 9 species that are available in Assam. This genus includes trees and shrubs, usually dioecious. The trees have leafy crown, with few branches and reach up to 20 meters. The species of this genus are characterized by the presence of recurved spines on its
trunk and branches. The leaves are varied, may be alternate or opposite, simple or composed, imparipanadas or parimpanadas with up to 15 pairs of leaflets. The inflorescences are usually in form of panicles or umbels compound, axillary or terminal of small flowers. The flowers are actinomorphic, hermaphrodite and unisexual, rarely bisexual, usually white or green. The fruits are follicles or esquizocarp contains from one to five carpels usually aromatic, and are ordinarily bivalve with a single red or black, shiny seeds (Melo and Zickel, 2004; Silva and Paoli, 2004).

The genus *Zanthoxylum* has great importance due to its ethnobotanicals, phytochemicals and biological activity, and it is a promising source of various secondary metabolites including benzophenanthridine alkaloids.

Assam state located on foothills of the eastern Himalayas comprises the Brahmaputra and the Barak river valleys and the Karbi Anglong and the North Cachar Hills. Such a diverse situation has favoured the rich occurrence of floral diversity in the state. The characteristics of three commonly available *Zanthoxylum* species under study in Assam are reported contradictorily by various authors.


A large, climbing, evergreen and scandent shrub armed with short, recovered and scandent prickles (Fig 1). The species is occurring in south-east Asian countries and in Australia (Hu et al., 2006).

Fairly common throughout the province in low level forests except in Khasi and Jaintia hills, mainly distributed in Assam, Sikkim, Nagaland and Burma (Kanjilal, 1992; Bhattacharya and Zaman 2009).
Fig 1: *Z. nitidum*: 1) the whole plant, 2) Flower, 3) Seed, 4) Prickles, and 5) arrangement of leaves
In Wealth of India (Anonymous, 2003), it was described as a scanted, erect, foetid shrub, with retrose prickles, fairly common throughout the low level forest from Bihar onwards to Sikkim and Assam and Naga hills.

The stem bark and root (wood as well as the bark) contain Nitidine, a benzophenanthridine alkaloid. Nitidine is unstable and gets converted to dihydronitidine and oxynitidine by disproportionation. The fruit is considered to be an aromatic stimulant; it is prescribed in stomachache. It is also used as a condiment. The seeds yield 3.5-5.0% of an essential oil with a pleasant persistant odour resembling that of bergamot and geranium.

The different morphological traits of the species were described differently by various authors. In Flora of Assam (Kanjilal, 1992), it was mentioned that the plant of the species is generally known as Tezmoi or Tezmaibih or Tezmuri in Assam. Leaves are imperipinnate, 5-40 cm long with leaflets of 2-4 pairs, opposite, ovate to elliptic; both male and female flowers dull white to pale yellow or rarely reddish, in axillary or terminal fascicles. Follicles: subglobose, seeds pitted, brownish black, glossy. The bark is dark grey with white lenticels, armed with short recurved prickles, old stems occasionally covered with thick corky spine up to 2 cm long. Wood is pale yellow and compact, pores orbicular, rather small for a climber; medullary rays nearly equidistant and somewhat wavy.

Fruits are tubercle and medicinally used for its stimulant and aromatic properties. Fruits have piscicidal component (Kirtikar and Basu, 1993; Kanjilal, 1992).

1.1.2 Zanthoxylum rhetsa Syn. Z. budrunga Syn. Z. limonella:

A deciduous tree up to 75 ft in height and 4-5 ft in girth. The plant is commonly known as brajamani and bajarnali in Assam (Fig 2). In Wealth of India (Anonymous, 2003), the species was described as a lofty, deciduous tree, upto 35 m tall, with a spreading crown and a bole of 4-6 m, commonly found in the evergreen monsoon...
forest of the foothills of Assam and Meghalaya and in the eastern and western ghats in peninsular India. The main stem is generally armed with broad conical spines 2-3 cm long. Branchlets are usually sparsely armed with straight or ascending prickles, often swollen and hollow, apparently housing ant; bark cream coloured or yellowish grey, studded with conical spines, thick, deeply and finely reticulate-fissure. Leaves are paripinnate or imparpinnate, 30-45 cm long, glabrous, clustered at the end of branches. Leaflets are 5-8 pairs, opposite or sub-opposite, ovate to elliptic, chataceous, occasionally with scattered pellucid dots, oblique, entire to glandular-crenate. Flower is white to yellowish white; in terminal peniculate, cymes which may sometimes be located in the upper leaf axils; follicles globose, aromatic, red, rugose, 6-7 mm in diameter; seed blue black, subglobose, shining. The tree is essentially of spice value. The bark is bitter and aromatic. It has the taste of the rind of orange and is prickled. The fruits are digestive and appetizing and the tender leaves are cooked and eaten in Assam. In Western and Southern India, they are used as a condiment in both sweet and sovoury preparation, especially in preparation of fish curries. The pericarp of the unripe fruit is pleasantly aromatic and tastes like the rind of a fresh orange; the seeds are pungent in taste, more or less like black pepper. The fruits are prescribed in atrabiliary dyspepsia. They are also used in asthma and bronchitis, heart troubles, toothache, and rheumatism. The pericarp is credited with astringent, stimulant and digestive properties. A tincture of the seeds is used in cholera. The fruits yield an essential oil called Mullilum oil, which is obtained by steam distillation of the dried ripe fruits. The oil has pleasant odour resembling that of sweet orange and tangerine. It is used in the indigenous system of medicine for the treatment of cholera. The oil is used as an antiseptic and disinfectant. It show anti-inflammatory activity against the exuddative phase of inflammation in formalin an carragenin induced rat's hind- paw oedema. This property has been proved clinically by topical application of the drug in cases of inflammatory dermatosis. The rind on extraction with ether yielded 7.2% of an oil possessing anti-bacterial activity. The tree is well
known in Ayurvedic system of medicine. The bark is credited with tonic and aromatic properties and is used with advantage in rheumatism and otonic dyspepsia, the root-bark is preferred. It is of a reddish brown colour and covered with a light yellow suber that separates easily in the form of flat and slightly recurved pieces 7 cm long, 5 cm wide and 1 cm thick. It is reputed in Western India as a diuretic. Aqueous and alcoholic extracts of the bark have exhibited cholinergic, hypoglycaemic and spasmylytic activity in preliminary trials. The bark is aromatic and contains an essential oil, resin and alkaloids. Peputine hydrochloride, isolated from the stem bark from Mudigera (Kornataka) had a stimulating action on the mammalian heart and a spasmylytic effect on smooth muscles. The wood is canary yellow, light greyish yellow to yellowish grey, rather dull, and with a smooth feel. It is straight often wavy or sometimes interlocked-grained, medium and fairly even textured, hard, strong, tough and rather light to moderately heavy.

The wood is easy to saw; works to a smooth finish by hand or machine, and takes a good polish or paint. However, it requires mild presematric treatment. The wood is recommended as a substitute for sal and teak.

In Flora of Assam (Kanjilal, 1992), the species was described as large prickles found over every aerial parts of the plant. Bark corky and pale yellow outside and light brown inside so finally turns dark chocolate on exposure. Leaves are peripinnate or imperipinnate. Small flowers represent light green or pale yellow in color. The tree is essentially of spice value. Bark is bitter and aromatic. Fruit is used as an appetizer, analgesic, antiasthmatic, antidiarrhoerial and antirheumatic and to cure bronchitis, diseases of heart, mouth and teeth and dyspepsia. Root bark is used as purgative.

Essential oil of fruits is anesthetic, antagonistic and anti-inflammatory. Wood is suitable for furniture (Drury H, 1982; Kanjilal, 1992).
Fig 2: *Z. rhesta*: 1) Whole tree, 2) Flower, 3) Seeds, 4) arrangement of the leaves and 5) prickles
1.1.3. *Zanthoxylum oxyphyllum*

The *Z. oxyphyllum* is found to be distributed in the temperate and subtropical Himalayas from Kumaun eastwards to Bhutan and Sikkim at altitudes of 1,800 - 2,700 m and in Assam and the Khasi hills at 1,200 - 1,800 m (Chopra *et al.*, 1956; Anonymous, 2003). In Wealth of India (Anonymous, 2003), described it as an aromatic, evergreen, sarmentose shrub, up to 5 m tall clothed with hooked prickles, commonly found in the shady forests of the Himalayas from Kumaun, Eastwards to Bhutan and Sikkim at altitudes of 1,800 - 2,700 m and in Assam and the Khasi Hills at 1,200 - 1,800 m. The bark is greenish brown. Leaves are variable in size, imparipinnate. Leaflets are aromatic, 3-10 pairs, alternate and opposite, ovate-lanceolate, acuminate, and crenate-serrate. Flowers are white to light yellow, in terminal, axillary panicles. Fruits are globose follicle of 1-5 carpels, dull red; seeds solitary, shining black. The tender shoots are cooked and eaten as a vegetable in Assam. The fruits are employed as a condiment in curries. The bark is considered as stimulates stomachic and digestive and is used in the Philippines for colic. It is also administered in fevers as a sudorific. The stem bark yielded indoquinazoline alkaloid.

Kanjilal (1992) described the species as an evergreen shrub with weak rambling stems usually supported by the surrounding trees and shrubs, known as Mezenga in Assam (Fig 3). Stems and branches armed with straight or hooked prickles. Bark stimulant, stomachic and sudorific. Leaves are imparipinnate. Flowers are globose, glabrous and dull red in color. Seeds are solitary and shining black. Fruits are sweetish bitter, hot and digestible, appetizer, anthelmintic and remove pain, tumors, useful in gastric problem, headache, body ache, fever, cold and cough and also used for the purification of blood (Kirtikar and Basu, 1993; Kanjilal, 1992).
1.2. PCR Based Molecular Markers:

*Zanthoxylum* plant is the source of many phytotoxic chemical compounds. Different types of alkaloids are commonly present in *Zanthoxylum* species. Previous chemical studies on the genus have shown it to be a rich source of secondary metabolites (Waterman and Grundon, 1983). The root, bark and berries are used medicinally in the United States Pharmacopoeia. Throughout the world, *Zanthoxylum* is recognized as an important medicinal plant for its qualities such as...
treating stomach ache, toothache, intestinal worms, rheumatism, scabies, snakebites, fever, cholera, cancer treatment, antioxidant, anticoagulant and antibacterial agents (Chen et al. 1994b; Hisatomi et al. 2000; Islam and Ahsan, 1997; Xiong et al., 1995).

Apart from the ethno medicinal use of the various species available in India, it has got tremendous commercial credence for their well valued essential oil, medicinal and timber quality. Linalool extracted from Zanthoxylum has been used commercially in soaps, detergents, insecticides, and as a precursor for the production of vitamin E (Jain et al., 2001); and therefore may have commercial potential beyond spice production. Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies. This has been brought about by the acknowledgement of the value of medicinal plants as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development. The secondary metabolites, especially the benzophenanthridine alkaloids are considered to be very important in the world of medicine. Zanthoxylums species eliminates fly maggots in wastewater and latrines (A Barefoot Doctor's Manual, 1978). In addition to food and medicinal qualities, Zanthoxylum has also been identified as a potential plant for reforesting degraded hillsides in Hong Kong (Hau and Corlett, 2003) and in Central America (Condit et al., 1993).

Zanthoxylum is a difficult genus with varied degree of morphological variation to differentiate the species. The active constituents of various Zanthoxylum species vary greatly (Farnsworth et al., 1968; Chhabra et al., 1982) and the taxonomy in certain cases is somewhat obscure, both scientifically and by local nomenclature. In spite of having immense use, Zanthoxylum species are not commercially much important in Assam due to lack of awareness about the plant regarding their chemical compositions, medicinal values or their other uses. Although, little studies on chemical composition and their uses have been done (Nath et al., 1993; Jain et al. 2001; Tiwary et al. 2007) for a few Indian Zanthoxylum species, but it includes one or
two components only. Lack of knowledge also leads to destroying the natural population creating a threatening point to the *Zanthoxylum* population. Genus *Zanthoxylum* is typically characterized by sharp thorns on either the stem or foliage, and leaves that are ash-like in appearance. People cut away these plants as they are Thorny in nature. Moreover, the genus is dioecious, and therefore male and female trees must be in close proximity in order for pollination to take place and seed setting. Seed production can be particularly low in shaded situations (Popp and Reinartz, 1988). Seed of numerous *Zanthoxylum* species have been found to have low germination rates (Rodriguez, 1995; Francis, 2000; Frances, 2004; WAC, 2005). The species are now seriously threatened by increasing stress of population, fluctuating climate and deforestation etc. Moreover, *Zanthoxylum* is a difficult genus with many different, similar and not well-researched species. Characterization and extent of genetic variation is essential to identify various genotypes, which will help the breeder, geneticist and conservationist for their effective utilization (Orhan et al., 2007). Analysis of genetic diversity among the species of *Zanthoxylum* in NE India will reveal an efficient and authentic way to measure the genetic variation and phenetic relationship between and within the species. Various PCR (Polymerase chain reaction) based molecular markers have been used frequently for evaluating genetic distances among different cultivars.

PCR is a novel technique for the amplification of selected regions of DNA. It is an *in vitro* method for enzymatic amplification of specific DNA segment from the genomic DNA or from RNA following reverse transcription.

1.2.1. Random Amplified Polymorphic DNA (RAPD):

Williams *et al.* (1990) proposed the use of single short random primers (usually 10-base primers) as a method of generating polymorphic markers (RAPDs). This method is based on the use of polymerase chain reaction (PCR) to
amplify specific genes or DNA sequence to such an extent that they can be visualized after ethidium bromide staining and thus there is no need of Southern blotting or hybridization with labelled probes as required in RFLP technique. Differences in banding patterns represent differences between DNA samples, and hence diversity (Ford-Lloyd et al., 1997). In this technique primers are designed. In general, a single short oligonucleotide (10 bases in length) of random chosen DNA sequence with at least 50% GC content is preferred which hybridized with the homologous genomic DNA. No knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. This makes the method popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared as it is not suitable for forming a DNA databank. Due to the fact that it relies on a large, intact DNA template sequence, it has some limitations in the use of degraded DNA samples. Its resolving power is much lower than targeted, species specific DNA comparison methods, such as short tandem repeats. In recent years, RAPD is used to characterize, and trace, the phylogeny of diverse plant and animal species. RAPD has been successfully used to analyze diversity and genetic structure of wild plant species (Xu et al., 2003; Zhang et al., 2005; Vyas et al. 2009; Bewal et al. 2009).

1.2.2. Inter-Simple Sequence Repeats (ISSR):

A marker system called Inter-Simple Sequence Repeats (ISSRs) [Zietkiewicz et al., (1994)], has RAPDs-like approach that accesses variation in the numerous microsatellite regions dispersed throughout the various genomes (particularly the nuclear genome) and circumvents the challenge of characterizing individual loci that other molecular approaches require. Microsatellites are very short, usually 10-20 base-pair stretches of DNA that are "hypervariable", expressed as different variants within
populations and among different species. They are characterized by mono-, di- or trinucleotide repeats, e.g., AA..., or AG..., CAG..., that have 4-10 repeat isunits' side-by-side. In ISSRs, it specifically targets the di- and trinucleotide repeats types of microsatellite, because these are characteristic of the nuclear genome. Mononucleotide types are found in the chloroplast genome and these are not required.

The ISSR primers are used to generate the variation in a given DNA sample that includes one of these highly variable microsatellite sequences and an arbitrary pair of bases at the 3’ (rear) end. One samples for variation among DNA samples in small PCR (polymerase chain reaction) reactions using one primer at a time. Where the primer successfully locates two microsatellite regions within an amplifiable distance away on the DNA strands of the DNA sample (the "template DNA"), the PCR reaction will generate a band of a particular size molecular weight for that "locus" representing the intervening stretch of DNA between the microsatellites. Usually several to many such "paired" microsatellite areas exist in a particular DNA sample, so one gets many bands generated in the reaction, for that sample.

ISSR markers have been successfully applied various plant species, including many rare or endemic plants, Neopicrorhiza scrophulariiflora (Lui et al., 2011), Swertia tetraptera (Yang et al., 2011), Michelia coriacea (Zhao et al., 2012), etc.

1.2.3. Amplified Fragment Length Polymorphism (AFLP):

AFLP is an ingenious combination of RFLP and PCR and is extremely useful in detection of polymorphism between closely related genotypes (Saiki et al., 1988; Ehrlich et al., 1991). The technique was originally described by Zabeau and Vos (1993). AFLP uses restriction enzymes to cut genomic DNA as template for PCR amplification. The genomic DNA is digested with two restriction enzymes; one enzyme, e.g. pstI has a 6 bp recognition sequence, while the other, e.g. Msel has a 4
bp recognition site, followed by ligation of complementary double stranded adaptors to the ends of the restriction fragments. The fragments are now amplified using two AFLP primers; one primer has the sequence of the adapter for one restriction enzyme, while the other is specific to the adapter for the second enzyme used for digestion of the genomic DNA. The fragments are visualized on denaturing polyacrylamide gels either through autoradiography or fluorescence methodologies. AFLP is a highly sensitive method for detecting polymorphisms in DNA. The AFLP technology has the capability to detect various polymorphisms in different genomic regions simultaneously. It is also highly sensitive and reproducible. As a result, AFLP has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals, and bacteria. The AFLP technology has been used in criminal and paternity tests, in population genetics to determine slight differences within populations, and in linkage studies to generate maps for QTL analysis.

AFLP have been used in several plants such as *Jatropha curcas* (Tatikonda et al., 2009), *Rhodiola rosea* (Elameen et al., 2008), wild populations of *Agave angustifolia* (Teyer et al., 2009), *Croton alabamensis* (Van et al., 2006), *Pulsatilla vernalis* (Ronikier et al., 2002) etc.

1.3. Morphological Characterization:

Traditionally, the classification of any genus was based on morphological traits only. Now, with the advancement of comparative genomics, the reconstructing phylogeny with morphological data seems to be very unnecessary. But understanding the evolution of morphological traits remains essential for a comprehensive view of the evolution of a group.

RAPD, ISSR and AFLP segregate as dominant markers and must be treated as phenotypic characters, i.e., presence or absence data. But, these methods are
differing in principle, in application, in the type and amount of polymorphism detected, and in cost and time requirements. The choice of marker type depends upon the plant used in the study. Therefore, a Marker comparison study is essential to establish the suitable marker for characterization of the plant (Powell et al., 1996; Milbourne et al., 1997; Russell et al., 1997, Mignouna et al., 2003).

*Zanthoxylum* genus has proven to be a very valuable genus to the discovery and utilization of medicinal and agrochemical natural products. The collected information provides a means to understand the latest developments in the biological activity and phytochemistry of the genus. The potential for development of leads from *Zanthoxylum* continues to grow, particularly in the development of new antiparasitary, antitumor and antimicrobial agents.

*Zanthoxylum* is not a fast-growing species and has low population sizes in Assam. Some of the mechanisms of collection adopted by the local people are harmful to existing populations. However, the main question is whether *Zanthoxylum* can be conserved when the race for commercial tapping of its fruit and different parts is escalating, and when maximising income is the chief concern of local harvesters. Recognising the current demand, *Zanthoxylum* plantations can be developed as a viable source of income for resource-poor villagers.

Information on the *Zanthoxylum* species composition of a forest is essential for its wise management in terms of economic value, regeneration potential and ultimately may be leading to conservation of biological diversity. Nevertheless, very scanty or almost no information on *Zanthoxylum* genetic diversity in India and especially Assam state is available on the composition, distribution and status of natural regeneration. Literature often gives contradicting information about the local species used as spice. Therefore, rapid and accurate identification of the genus *Zanthoxylum* is required. The levels and patterns of genetic diversity in genomes are shaped by demographic processes and natural selection. Previous isozyme studies of
genetic variation reveal that life history and breeding systems, in addition to demography, considerably affect the pattern of genetic polymorphism and structure in natural populations (Hamrick and Godt, 1989, 1996). However, it is still uncertain to what extent those findings from the isozyme studies hold true for DNA polymorphisms. Furthermore, Zanthoxylum is a complicated genus with many different, similar and not well-researched species.

The present study was carried out with the following objectives:

1. To Survey and collect germplasm of different Zanthoxylum species of Assam, India.
2. DNA fingerprinting of Zanthoxylum species by using different PCR based RAPD, ISSR, AFLP markers.
3. Morphological characterization of the species.
4. To identify the genetically diverse species.
5. To determine Geographical variation among different species.