High gene flow and genetic diversity in three economically important Zanthoxylum Spp. of Upper Brahmaputra Valley Zone of NE India using molecular markers

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Abstract
The genetic diversity in Zanthoxylum species viz. Zanthoxylum nitidum, Zanthoxylum oxyphyllum and Zanthoxylum rhesta collected from the Upper Brahmaputra Valley Zone of Assam (NE India) was amplified using 13 random amplified polymorphic DNA (RAPD) markers and 9 inter-simple sequence repeat (ISSR) markers. RAPD markers were able to detect 81.82% polymorphism whereas ISSR detected 98.02% polymorphism. The genetic similarities were analyzed from the dendrogram constructed by RAPD and ISSR fingerprinting methods which divided the 3 species of Zanthoxylum into 3 clear different clusters. The principle component analysis (PCA) was carried out to confirm the clustering pattern of RAPD and ISSR analysis. Analysis of molecular variance (AMOVA) revealed the presence of significant variability between different Zanthoxylum species and within the species by both RAPD and ISSR markers. Z. nitidum was found to be sharing a high degree of variation with the other two Zanthoxylum species under study. The Nei’s gene diversity (h), Shannon’s information index (I), observed number of alleles (nO) and effective number of alleles (ne) were also found to be higher in ISSR markers (0.3526, 0.5230, 1.9802 and 1.6145) than in RAPD markers (0.3144, 0.4610, 1.8182 and 1.5571). The values for total genotype diversity for among population (HT), within population diversity (HS) and gene flow (Nm) were more in ISSR (0.3491, 0.2644 and 1.5610) than RAPD (0.3144, 0.4610, 1.8182 and 1.5571). The mean coefficient of gene differentiation (GST) was more in RAPD (0.2764) than ISSR (0.2426). A comparison of these two fingerprinting methods was done by calculating MR, EMI and MI. The correlation coefficient between data matrices of RAPD and ISSR based on Mantel test was found to be significant (r = 0.65612).

Keywords:
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Introduction

The genus *Zanthoxylum* L. (Rutaceae) includes some 250 species of trees and shrubs, with a worldwide, but predominantly tropical distribution (Arun and Paridhavi, 2012; Engler, 1896, 1931). Morphologically, it is the only truly choricarpous genus in the Rutaceae, with fully free and stalked carpel's (Gut, 1966). The much unspecialized flower morphology and vascular supply of the genus *Zanthoxylum* L. suggest a primitive position within the family (Das Gracas et al., 1988; Moore, 1936). Different species of *Zanthoxylum* L. are extensively used and found in the Himalayan belt, America and Africa Central, South, Southeast and East Asia (Medhi et al., 2009, 2013; Negi et al., 2011). Upper Brahmaputra Valley of Northeast India is a treasure house of rare and endangered flora with abundant rainforests. These forests include a number of tree species, which have different uses like in medicine, timber and many others (Ahmad et al., 1980, 1993, 2003; Arun and Paridhavi, 2012). *Zanthoxylum* is one of such tree genus, typically characterized by sharp thorns on either the stem or the foliage, and leaves that are ash-like in appearance (Medhi et al., 2009, 2013). Various species of the genus are used as medicine for different elements like in stomach ache, toothache, intestinal worms, rheumatism, scabies, snake-bites, fever, cholera, cancer treatment, antioxidant, anticoagulant, and antibacterial (Ahmad et al., 1980, 1993, 2003; Arun and Paridhavi, 2012; Medhi et al., 2013). Moreover *Zanthoxylum* seed oil is used for making soaps, detergents, insecticides, as a precursor of Vitamin E etc. (Medhi et al., 2013; Singh and Singh, 2011). *Zanthoxylum* has also been identified as a potential plant for reforesting of degraded hillsides in Hong Kong (Hau and Corlett, 2003) and in Central America (Condit et al., 1993). The problem with the genus is in the identification of the species due to the varied degree of morphological variation resulting in a contradictory classification of the species (Appelhans et al., 2013). Wealth of India (Anonymous, 2003) reported 13 different *Zanthoxylum* species available in India whereas Hooker (1875) in his book “Flora of British India” documented 11 species of *Zanthoxylum* in India, whereas “Flora of Assam” (Kanjilal, 1992) reported 9 species in India. Moreover, the population of the *Zanthoxylum* genus has been reduced and fragmented due to unscientific collection for ethno-medicinal use, cutting the plants from their close vicinity due to their thorny nature and lack of knowledge among the local people (Gupta et al., 2011; Medhi et al., 2009, 2013). The dioecious nature and seed dormancy of *Zanthoxylum* also adds in making them rare in nature (Frances, 2004; Francis, 2000; Gupta et al., 2011; Rodriguez, 1995; WAC, 2005). The extensive and uncontrolled illegal cutting and use of different *Zanthoxylum* species for various purposes and the threat of it’s drastic reduction in number call for a detailed assessment of genetic diversity studies for documentation, evaluation and conservation of this species. Three species viz. *Zanthoxylum nitidum*, *Zanthoxylum rhesta* and *Zanthoxylum oxyphyllum* were selected for the study based on their economical importance and morphological traits. All the three species under study have been used in many countries for medicinal purpose and as important spices (Medhi et al., 2009, 2013). Traditionally all the parts of *Z. nitidum* were used in folklore medicines in different places of India according to its availability. The roots of *Z. nitidum* was reported in the treatments of toothache, stomachache, fever, rheumatism, paresis, boils and as an insecticide and piscicide whereas fruits of the plant were reported for stomachache, cough, colic vomiting, diarrhea, and paresis and as an aromatic, stimulant and piscicide. The small branches, seed and stem bark were used for the treatment of fever, diarrhea and cholera (Anonymous, 2003; Hu et al., 2007; Kanjilal, 1992; Kirtikar and Basu, 1993). Nitidine is the specific active component in the dried root version of *Z. nitidum* DC (Xiao, 2002) and nitidine chloride is reported to be beneficial for both killing and constraining the growth of cancerous cells (Hua et al., 2009; Li et al., 2009; Liu et al., 2009; Qin et al., 2007).

Similarly, all the parts of the plant *Z. rhesta* were reported to be used by the local people of India in their traditional medicines or in others. Fruit is used as an appetizer, analgesic, antiasthmatic, antidiarrheal and antirheumatic and to cure bronchitis, diseases of heart, mouth and teeth and dyspepsia. Essential oil of fruits is anesthetic, antagonistic and anti-inflammatory. Tender leaves are cooked and eaten in Assam. Wood is
suitable for furniture (Anonymous, 2003; Drury H, 1982; Kanjilal, 1992). The species is reported to have a considerable level of antioxidant activity of hydrophilic extraction of leaves (Tukun et al., 2014). Z. oxyphyllum has also been reported as stimulant, stomachic and sudorific, appetizer, and anthelmintic and removes pain, tumors, useful in gastric problem, headache, body ache, fever, cold and cough and also used for the purification of blood (Anonymous, 2003; Kanjilal, 1992; Kirtikar and Basu, 1993). Extracts from the bark and root of Z. oxyphyllum have been reported to have antiproliferative activity against the growth of human keratinocytes (Kumar and Muller, 1999).

DNA based molecular markers have been proved to be valuable tools in the characterization and evaluation of genetic diversity within and between species and populations. Different molecular markers might reveal different classes of variation (Powell et al., 1996; Russell et al., 1997) and they is correlated with the genome fraction surveyed by each kind of marker, their distribution throughout the genome and the extent of the DNA target which is analyzed by each specific assay [Davila et al., 1999]. RAPD (random amplified polymorphic DNA) and ISSR (Inter-simple sequence repeat) were used for evaluating the genetic diversity of the genus Zanthoxylum as they are also very simple, fast, cost effective, highly discriminative, and abundant throughout the genome, require small quality of template. Moreover, several researchers have reported the use of RAPD and ISSR markers for genetic diversity study in different species of Zanthoxylum individually like RAPD for species like Zanthoxylum bungeanum (Li et al., 2009; Song and Liu, 2012; Meng et al., 2013) whereas ISSR for Zanthoxylum dissitum (Li et al., 2013) and Z. bungeanum (Han et al., 2011). These markers have a high potentiality to classify closely related accessions.

Materials and methods

Study sites and sample collection

Fresh and young leaf samples of different Zanthoxylum species (Z. nitidum, Z. rhesta & Z. oxyphyllum) from natural populations in different parts of Upper Brahmaputra Valley Zone (NE India) were collected (Table 1). Young leaves of 46 genotypes including 18 nos. of Z. nitidum, 16 nos. of Z. rhesta and 12 nos. of Z. oxyphyllum were collected for the evaluation of genetic diversity existing in the genus as well as between the species (Table 1). Leaf material was kept in airtight plastic bags containing self-indicating silica gel (coarse), then transported to the lab, and stored at −45 ºC until DNA extraction. Fresh leaves were also collected from nearby areas for DNA extraction.

DNA isolation

Genomic DNA was extracted from lyophilized as well as fresh leaves using modified CTAB method (Doyle and Doyle, 1990). Quantification was done against a known quantity of unrestricted Lambda (λ) DNA by running it in a 1.5% agarose gel electrophoresis. To optimize the PCR amplification condition, experiments were carried out with the varying concentration of DNA template, primer, Taq polymerase, MgCl₂ as well as dNTPs.

RAPD and ISSR reactions

A total of 13 RAPD (Williams et al., 1990) and 9 ISSR (Zietkiewicz et al., 1994) primers were used for this study. Amplification was performed on ATC 401 (4.8 Ver) thermo cycler with 15 μl reaction mixtures containing 0.05 μg of template DNA, 0.4 mM of each dNTPs (dATP, dTTP, dCTP & dGTP), 0.25 μl of Taq DNA polymerase, 10 pmol of each primer, 2 μl of 1% BSA and 2.4 mM of MgCl₂ (all from Fermentas, MBI) for both the reactions.

The RAPD and ISSR amplification regimes were performed at 94 ºC for 3 min followed by 35 cycles at 94 ºC for min, 35 ºC/37 ºC and 55 ºC/57 ºC respectively for 2 min, 72 ºC for 2 min and a final extension at 72 ºC for 10 min. A slight modification to the reaction condition especially the annealing temperature was done depending upon the exact nature of the primer in both RAPD as well as ISSR.

Amplified PCR products (15 μl) were performed onto an agarose (1.5%; w/v) gel electrophoresis in 1× TBE buffer at 70 V for 150 min. The gel was visualized after Ethidium bromide staining and photographed under UV light by a gel Doc System (G Box HR, Syngene, UK).
Table 1
Different Zanthoxylum accessions collected from different places of Upper Assam.

| Sample No. | Place of collection                          | Species name | Latitude/Longitude |
|------------|---------------------------------------------|--------------|--------------------|
| 1          | Gibbon wild life sanctuary, Mariani, Jorhat  | Z. nitidum   | 26°41 N/94°20 E    |
| 2          | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 3          | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 4          | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 5          | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 6          | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 7          | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 8          | Bamungaon, Jorhat                           | Z. nitidum   | 26°46 N/94°14 E    |
| 9          | Hatimuriah, Sibsagar                        | Z. nitidum   | 26°50 N/94°34 E    |
| 10         | Hatimuriah, Sibsagar                        | Z. nitidum   | 26°50 N/94°34 E    |
| 11         | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 12         | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 13         | Bamungaon, Jorhat                           | Z. nitidum   | 26°46 N/94°14 E    |
| 14         | Baghjan Gharfaliagaon, Jorhat               | Z. nitidum   | 26°48 N/94°17 E    |
| 15         | Rongjan, Golaghat                           | Z. nitidum   | 26°28 N/93°54 E    |
| 16         | Gulongso gaon, Golaghat                     | Z. nitidum   | 26°28 N/93°54 E    |
| 17         | Gulongso gaon, Golaghat                     | Z. nitidum   | 26°28 N/93°54 E    |
| 18         | Rongjan, Golaghat                           | Z. nitidum   | 26°28 N/93°54 E    |
| 19         | Ratneswar Chutia Tea Estate, Makum          | Z. rhesta    | 27°30 N/95°23 E    |
| 20         | Rangman Chawrok Tea Estate, Makum           | Z. rhesta    | 27°30 N/95°23 E    |
| 21         | Ratneswar Chutia Tea Estate, Makum          | Z. rhesta    | 27°30 N/95°23 E    |
| 22         | Rangman Chawrok Tea Estate, Makum           | Z. rhesta    | 27°30 N/95°23 E    |
| 23         | Rangman Chawrok Tea Estate, Makum           | Z. rhesta    | 27°30 N/95°23 E    |
| 24         | Rangman Chawrok Tea Estate, Makum           | Z. rhesta    | 27°30 N/95°23 E    |
| 25         | Rangman Chawrok Tea Estate, Makum           | Z. rhesta    | 27°30 N/95°23 E    |
| 26         | Rangman Chawrok Tea Estate, Makum           | Z. rhesta    | 27°30 N/95°23 E    |
| 27         | Manoj Chutia Tea Estate, Makum              | Z. rhesta    | 27°30 N/95°23 E    |
| 28         | Manoj Chutia Tea Estate, Makum              | Z. rhesta    | 27°30 N/95°23 E    |
| 29         | Manoj Chutia Tea Estate, Makum              | Z. rhesta    | 27°30 N/95°23 E    |
| 30         | Manoj Chutia Tea Estate, Makum              | Z. rhesta    | 27°30 N/95°23 E    |
| 31         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 32         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 33         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 34         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 35         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 36         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 37         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 38         | Padumoni Researve Forest, Tinsukia          | Z. oxyphyllum| 27°32 N/95°18 E    |
| 39         | Upper Dehing Rain Forest, Tinsukia          | Z. oxyphyllum| 27°22 N/95°35 E    |
| 40         | Upper Dehing Rain Forest, Tinsukia          | Z. oxyphyllum| 27°22 N/95°35 E    |
| 41         | Rawriah, Jorhat                             | Z. rhesta    | 26°43 N/94°10 E    |
| 42         | Rawriah, Jorhat                             | Z. rhesta    | 26°43 N/94°10 E    |
| 43         | Rawriah, Jorhat                             | Z. rhesta    | 26°43 N/94°10 E    |
| 44         | Toklai (Tea Research Institute), Jorhat     | Z. rhesta    | 26°45 N/94°13 E    |
| 45         | Toklai (Tea Research Institute), Jorhat     | Z. rhesta    | 26°45 N/94°13 E    |
| 46         | Toklai (Tea Research Institute), Jorhat     | Z. rhesta    | 26°45 N/94°13 E    |

Data analysis

Each gel was analyzed by scoring the present (1) or absent (0) bands. The data matrices were entered into the NTSYS version 2.02 K and a pairwise comparison of populations was made and genetic diversity parameters were determined. Genetic similarities based on Jaccard’s coefficient were calculated among all possible pairs using the SIMQUAL option and ordered in a similarity matrix. The two-dimensional fold lines each for RAPD and ISSR were obtained using similarity matrices generated by Excel 2007 (Microsoft Corporation, Washington, DC). Dendrogram was constructed by employing UPGMA (Unweighted Pair Group Method with Arithmetic Average) to group individual into discrete clusters (Sokal and Sneath, 1963). Principal component analysis (PCA) was carried out to obtain a 3D image, in order to represent the genetic diversity among the accessions graphically. It was generated by using EIGEN programme of NTSYS-pc software 2.02.
Bootstrapping (500 replicates) was done by using the DARwin 6.0.0 software program (Perrier and Jacquemoud-Collet, 2006) based on Jaccard’s coefficient matrix and UPGMA (Unweighted Pair Group Method) in the in neighbor-joining module.

Polymorphism information content (PIC) was calculated for each assay as follows:

\[
\text{PIC} = 1 - \sum_{i=1}^{k} p_i^2,
\]

where, \(k\) is the total number of alleles detected for a given marker locus and \(p_i\) is the frequency of the \(i^{th}\) allele in the set of genotypes investigated. Multiplex ratio (MR) for each assay was estimated by dividing the total number of bands (monomorphic \(- m\) and polymorphic \(- p\)) amplified by the total number of assays (primer combination employed \(- n\)) as per Powell et al. (1996). The average number of DNA fragments amplified or detected per genotype using a marker system is considered as multiplex ratio \((n)\). The number of loci polymorphic in the germplasm set of interest, analyzed per experiment is called effective multiplex ratio \((E)\). It is estimated as

\[
E = n \beta,
\]

where \(\beta\) is the fraction of polymorphic markers and is estimated after considering the polymorphic loci \((np)\) and non-polymorphic loci \((nnp)\) as \(\beta = np / (np + nnp)\).

The utility of a given marker system is a balance between the level of polymorphism detected and the extent to which an assay can identify multiple polymorphisms. A product of information content, as measured by PIC, and effective multiplex ratio, known as marker index (MI) may provide a convenient estimate of marker utility.

\[
MI = \text{PIC} \times E \quad \text{or} \quad MI = n \times \beta \times \text{PIC}
\]

Mantel matrix-corresponding test (Mantel, 1967) was also carried out to compare the RAPD and ISSR data matrices.

AMOVA was performed to analyze the genetic diversity of the *Zanthoxylum* using the software Arlequin ver. 3.11 (Excoffier et al., 2005) to estimate the variance components of RAPD and ISSR profiles. POPGENE software version 1.32 (Yeh et al., 1997) was used to estimate the intra- and inter-genetic diversity parameters such as % polymorphism, Nei’s gene diversity \((h)\), Shannon’s information index \((I)\), observed number of alleles \((Na)\) and effective numbers of alleles \((Ne)\). The genetic structure was investigated using Nei’s gene diversity statistics, including the diversity within-population \((H_s)\), total gene diversity \((H_T)\) and coefficient of gene differentiation \((G_{ST})\) calculated on the basis of Nei’s method (Nei and Li, 1973) in POPGENE software. The \(H_s\) was defined in terms of gene diversities. The estimate of gene flow from \(G_{ST}\) was calculated as

\[
N_m = 0.5(1 - G_{ST}) / G_{ST}.
\]

## Results

**RAPD and ISSR banding patterns**

Genomic DNA was successfully isolated from the young tender leaves and lyophilized leaves of all the 3 *Zanthoxylum* species under study (Table 1). The average DNA concentration varied considerably among the different samples. The Doyle and Doyle (1990) method provided intact DNA with average concentration of 5.42 ng/μl. A total of ninety-seven RAPD primers (Operon Technologies, US) were screened for satisfactory application and thirteen primers were finally selected for this study. A total number of 132 distinct bands were amplified of which 108 (81.82%) were polymorphic in nature (Table 2). The number of fragments amplified by each primer ranged from 8 (OPBH-10, OPBH-07, OPC-01, OPC-11) to 20 (OPA-09) with an average of 10.15 (Table 2).

Among the 25 primers tested, 9 proved useful to characterize the samples (Table 3), whereas 16 were excluded due to the absence of amplification or to the amplification of the same single fragment in all samples (10 primers). The 9 selected ISSR primers produced 101 bands of which were 98.02% polymorphic and with an average of 11.22 bands per primer (Table 3). The number of band amplified by each of the 9 ISSR primers ranged from 8 (825) to 16 (868 and 841) with an average 11.22 (Table 3).
Genetic similarity, cluster analysis, principal component analysis (PCA) and PIC

The PIC values, a reflection of allele diversity and frequency among the Zanthoxylum species under study, were not uniformly higher for all the RAPD loci tested. In RAPD, the Jaccard's coefficients of similarity varied from 0.37 to 0.89 and the dendrogram derived from UPGMA cluster analysis indicates 2 broad groups at 48% similarity including all the accessions of Z. nitidum and Z. oxyphyllum in group A and Z. rhesta in group B (Fig. 1). The values of bootstrap analysis also support the classification of three species into 3 distinct clusters by RAPD markers with bootstrap values greater than 50. The average PIC value (Table 2) of the RAPD primers was 0.648 ranging from 0.344 (OPBH-03) to 0.884 (OPA-02).

The Jaccard’s similarity coefficient varied from 0.16 to 0.85 in ISSR. UPGMA cluster analysis reveals 3 groups at a similarity coefficient of 37% where group 1 can be further divided into 4 sub-groups — 1a, 1b and 1c have the Z. nitidum genotypes and 1d includes the Z. oxyphyllum genotypes. Groups 2 and 3 include Z. rhesta from Tinsukia and Jorhat district of Assam state of India respectively (Fig. 2). The bootstrap values were also lower than 50 for Z. nitidum and Z. oxyphyllum. The reason behind the clustering does not correspond to the species may be due to the excessive use of A and G rich repeat primers. The average PIC value of the ISSR primers (Table 3) was ranging from 0.578 (primer 842) to 0.984 (primer 880).

The principle component analysis was carried out to confirm the clustering pattern of RAPD and ISSR analysis. The first three Eigen vector accounted for 68.67% of the total variance in RAPD and 53.26% of the total variance in ISSR whereas the first component accounted 57.80% and 40.93% variance respectively

Table 2
Characteristics of different RAPD primers in Zanthoxylum.

| SL. No. | RAPD primers | Total bands | Monomorphic bands | Polymorphic bands | % polymorphic bands | PIC  |
|---------|---------------|-------------|-------------------|-------------------|---------------------|-----|
| 1.      | OPA-04        | 10          | 1                 | 9                 | 90%                 | 0.688|
| 2.      | OPA-03        | 10          | 1                 | 9                 | 90%                 | 0.563|
| 3.      | OPBH-03       | 9           | 2                 | 7                 | 78%                 | 0.394|
| 4.      | OPBH-07       | 8           | 0                 | 8                 | 100%                | 0.755|
| 5.      | OPBH-20       | 9           | 0                 | 9                 | 100%                | 0.778|
| 6.      | OPBH-18       | 10          | 5                 | 5                 | 50%                 | 0.531|
| 7.      | OPE-01        | 8           | 0                 | 8                 | 100%                | 0.771|
| 8.      | OPAA-11       | 12          | 1                 | 11                | 91%                 | 0.396|
| 9.      | OPAA-15       | 10          | 0                 | 10                | 100%                | 0.786|
| 10.     | OPA-02        | 10          | 0                 | 10                | 100%                | 0.884|
| 11.     | OPE-11        | 8           | 0                 | 8                 | 100%                | 0.869|
| 12.     | OPBH-10       | 8           | 4                 | 4                 | 50%                 | 0.466|
| 13.     | OPA-09        | 20          | 10                | 10                | 50%                 | 0.546|
| Total   | 132           | 24          | 108               |                   | 81.82%              | 8.427|

Here, Y = (C, T); B = (C, G, T); H = (A, C, T).

Table 3
Characteristics of ISSR primers in Zanthoxylum.

| SL no. | Primer name | Repeat Motifs | Anchor | Total bands | Monomorphic bands | Polymorphic bands | % Polymorphism | PIC  |
|--------|-------------|---------------|--------|-------------|-------------------|-------------------|---------------|-----|
| 1.     | 868         | (GAA)6        | -      | 16          | 0                 | 16                | 100%          | 0.895|
| 2.     | 818         | (CA)8         | G      | 9           | 0                 | 9                 | 100%          | 0.928|
| 3.     | 811         | (GA)8         | C      | 10          | 0                 | 10                | 100%          | 0.862|
| 4.     | 825         | (AC)8         | T      | 8           | 0                 | 8                 | 100%          | 0.643|
| 5.     | 841         | (GA)8         | Y'C    | 16          | 0                 | 16                | 100%          | 0.832|
| 6.     | 842         | (GA)8         | YG     | 10          | 0                 | 10                | 100%          | 0.578|
| 7.     | 885         | (GA)7         | B'H'B  | 11          | 2                 | 9                 | 82%           | 0.632|
| 8.     | 880         | (GA)8         | G      | 9           | 0                 | 9                 | 100%          | 0.984|
| 9.     | 807         | (AC)8         | T      | 12          | 0                 | 12                | 100%          | 0.853|
| Total  |             |               |        | 101         | 2                 | 99                | 98.02%        | 78.20|

Average = 0.8
Three dimensional (3D) distribution of the *Zanthoxylum* species based on PCA values in both RAPD and ISSR analysis gave a clear distinction of the three species used in the study (Fig. 4a and b). PCA plot of RAPD clearly shows the grouping of the 3 different species of *Zanthoxylum* (Fig. 4a), whereas in PCA plot of ISSR, *Z. oxyphyllum* and *Z. nitidum* groups are overlapping but are distinct (Fig. 4b).

The cluster analysis based on the combined RAPD–ISSR data matrices resulted into 4 groups where groups 3 & 4 include all the genotypes of the species *Z. rhesta* and showing the maximum differences with other two species under study (Fig. 3). Like in RAPD, *Z. nitidum* and *Z. oxyphyllum* were forming separate groups i.e. group 1 and group 2 respectively. The bootstrap values were more than 90 with 500 replicates for all the three species grouped into three clusters.

**Analysis of molecular variance (AMOVA)**

The analysis of molecular variance based on RAPD, 68.03% diversity was attributed to within species diversity, whereas 31.97% was among the species (Table 5). ISSR analysis of the total diversity in *Zanthoxylum* species reveals that 72.83% diversity can be attributed to diversity within species, whereas 27.17% was attributed to among species diversity.

AMOVA analysis between every combination of the *Zanthoxylum* species under study was performed to test the relationship among various species (Table 5). RAPD analysis showed that the variance between the *Z. nitidum* and *Z. rhesta* was highest (33.80) among between *Z. nitidum* and *Z. oxyphyllum* (32.60) as well as between *Z. rhesta* and *Z. oxyphyllum* (28.67). But the ISSR analysis showed the highest variation between *Z. nitidum* and *Z. oxyphyllum* (33.54%) followed by *Z. nitidum* and *Z. rhesta* (27.06) and between *Z. rhesta* and *Z. oxyphyllum* (23.91%). $F_{ST}$ value for “among all the three species of *Zanthoxylum*”, between *Z. nitidum* and *Z. rhesta*, between
Z. nitidum and Z. oxyphyllum and between Z. rhesta and Z. oxyphyllum were found to be high in RAPD (0.31969, 0.33796, 0.32599 and 0.28670) than ISSR (0.27173, 0.27063, 0.30539 and 0.23908) respectively. Significant P-values estimated for these combinations with 1023 permutations with random value less than or equal to observed value for FST and it was 0.00000 for all these combinations (Table 5).

**Table 4**
Eigen value, explained variance and cumulative variance in the PCA using characters to classify 46 Zanthoxylum accessions by RAPD & ISSR.

| Markers | Principal coordinate | Eigen value  | Explained variance (%) | Cumulative variance (%) |
|---------|----------------------|--------------|------------------------|-------------------------|
| RAPD    | 1.                   | 26.59120991  | 57.8070                 | 57.8070                 |
|         | 2.                   | 2.94610187   | 6.4046                  | 64.2115                 |
|         | 3.                   | 2.05346492   | 4.4641                  | 68.6756                 |
| ISSR    | 1.                   | 18.83185240  | 40.9388                 | 40.9388                 |
|         | 2.                   | 3.25986713   | 7.0867                  | 48.0255                 |
|         | 3.                   | 2.40916207   | 5.2373                  | 53.2628                 |
Gene diversity in Zanthoxylum

The genetic parameters like percent polymorphic bands (PPB), Nei’s gene diversity (h), Shannon’s information index (I), observed number of alleles (Na), and effective number of alleles generated with the Pop gene software version 1.32 are given in Table 6.

In the analysis of both the markers (RAPD and ISSR), the highest PPB was estimated in Z. nitidum (71.21% & 87.13%) followed by Z. rhesta (62.88% & 85.15) and Z. oxyphyllum (58.33% & 69.31%) respectively (Table 6). But comparatively, ISSR was able to produce a high range of PPB in all the three species of Zanthoxylum than by RAPD. The Nei’s gene diversity (h) among all the Zanthoxylum species was detected by the ISSR markers (0.3526) which was higher than the gene diversity detected by the RAPD (0.3144). Similarly the estimated values of Shannon’s information index (I), observed number of alleles (na) and effective number of alleles (ne) were also found to be higher in ISSR (0.5230, 1.9802 and 1.6145) than in RAPD (0.4610, 1.8182 and 1.5571).

The values for total species diversity for among population (Ht), within population diversity (Hs) and Gene flow (Nm) were more in ISSR (0.3491, 0.2644 and 1.5610) than RAPD (0.3128, 0.2264 and 1.3087) but the mean coefficient of gene differentiation (GST) was more in RAPD (0.2764) than ISSR (0.2426) (Table 7).

Comparison of RAPD and ISSR in evaluating genetic diversity of Zanthoxylum

Both the marker systems (RAPD and ISSR) were compared on the basis of different criteria (Table 8). The polymorphism detected and average PIC values were high in ISSR but RAPD reveals a higher MR, EMR and MI.
Fig. 4. 3D distribution of *Zanthoxylum* accessions revealed by PCA analysis based on RAPD (a) and ISSR (b) data.
value. The correlation coefficient between data matrices of RAPD and ISSR based on Mantel test was significant ($r = 0.65612$).

**Discussions**

In the past the analysis of genetic diversity in *Zanthoxylum* has been done comprising development and characterization of microsatellite markers in *Zanthoxylum ailanthoides* (Yoshida et al., 2010) molecular identification of medicinally important *Zanthoxylum schinifolium* using ribosomal DNA internal transcribed spacers (Sun et al., 2010a,b) and genetic diversity of *Zanthoxylum hamiltonianum* of north-east India through RAPD analysis (Medhi et al., 2013) which itself depicts the need of more molecular works as per as this plant is concerned. *Zanthoxylum* is a complicated genus with many different, similar and not well-researched species. Literature often gives contradicting information about the local species used. Considering the urgency of molecular characterization of this important plant bioresource, we undertook studies on DNA fingerprinting. The results indicated that the percentage of ISSR polymorphic bands (88.8%) was higher than that of RAPD (46.15%). The mean number of amplification ISSR bands (11.22) was more than that of RAPD (10.15). Moreover, the total number of polymorphic bands (108) detected by RAPD primers was higher than that of the ISSR primers (99), which suggested that the ISSR markers were superior to RAPD markers in the capacity

| Marker Source of variance | df | Sum of squares | Variance components | Percentage of variation | $F_{ST}$ |
|---------------------------|----|----------------|---------------------|------------------------|---------|
| **Between *Z. nitidum* and *Z. rhesta*** | | | | | |
| RAPD Among the species | 1 | 158.11 | 8.37 | 33.80 | 0.33796 |
| RAPD Within the species | 33 | 524.42 | 16.39 | 66.20 | |
| ISSR Among the species | 1 | 112.50 | 5.729 | 27.06 | 0.27063 |
| ISSR Within the species | 33 | 494.09 | 15.44 | 72.94 | |
| **Between *Z. nitidum* and *Z. oxyphyllum*** | | | | | |
| RAPD Among the species | 1 | 118.70 | 7.521 | 32.60 | 0.32599 |
| RAPD Within the species | 26 | 404.33 | 15.55 | 67.40 | |
| ISSR Among the species | 1 | 100.84 | 6.307 | 30.54 | 0.30539 |
| ISSR Within the species | 26 | 372.98 | 14.35 | 69.46 | |
| **Between *Z. rhesta* and *Z. oxyphyllum*** | | | | | |
| RAPD Among the species | 1 | 110.85 | 6.564 | 28.67 | 0.28670 |
| RAPD Within the species | 28 | 457.25 | 16.33 | 71.33 | |
| ISSR Among the species | 1 | 75.66 | 4.30 | 23.91 | 0.23908 |
| ISSR Within the species | 28 | 383.44 | 13.69 | 76.09 | |
| **Among all the three species of *Zanthoxylum*** | | | | | |
| RAPD Among the species | 2 | 261.41 | 7.57 | 31.97 | 0.31969 |
| RAPD Within the species | 43 | 693.00 | 16.12 | 68.03 | |
| ISSR Among the species | 2 | 193.49 | 5.43 | 27.17 | 0.27173 |
| ISSR Within the species | 43 | 626.01 | 14.56 | 72.83 | |

Table 5

Summary of analysis of molecular variance (AMOVA) based on RAPD and ISSR analysis of *Zanthoxylum* species.

| Marker | Species | Sample size | P% | Mean $n_a$ | Mean $n_e$ | Mean $h$ | Mean I |
|--------|---------|-------------|-----|------------|------------|----------|--------|
| RAPD   | *Z. nitidum* | 18 | 71.21 | 1.7121 | 1.4168 | 0.2430 | 0.3649 |
| RAPD   | *Z. rhesta*  | 16 | 62.88 | 1.6288 | 1.3854 | 0.2232 | 0.3335 |
| RAPD   | *Z. oxyphyllum* | 12 | 58.33 | 1.5833 | 1.3658 | 0.2192 | 0.3170 |
| ISSR   | All accessions | 46 | 81.82 | 1.8182 | 1.5571 | 0.3144 | 0.4610 |
| RAPD   | *Z. nitidum* | 18 | 87.13 | 1.8713 | 1.4617 | 0.2775 | 0.4232 |
| RAPD   | *Z. rhesta*  | 16 | 85.15 | 1.8515 | 1.5100 | 0.2993 | 0.4486 |
| RAPD   | *Z. oxyphyllum* | 12 | 69.31 | 1.6931 | 1.3586 | 0.2164 | 0.3323 |

Table 6

Mean genetic parameters based on RAPD, ISSR and combined RAPD–ISSR analysis of the three species of *Zanthoxylum*: P% = percent polymorphism; $n_a$ = observed number of allele; $n_e$ = effective number of alleles; $h$ = Nei’s gene diversity; and I = Shannon’s Information index.
of revealing more informative bands in a single amplification. The similar results were observed by in *Cicer arietinum* and *Cajanus cajan* L. (Lal et al., 2010), *Oryza sativa* L. (Parsons et al., 1997), *Picea* sp. (Nkongolo et al., 2005) and *Catharanthus roseus* (Shaw et al., 2009). They concluded that both the marker systems (RAPD and ISSR) are equally potential to differentiate the closely related plant cultivars. In general, the efficiency of a molecular marker technique depends on the amount of polymorphism that could be detected among the set of plant genotypes under study. The high reproducibility of ISSR markers may be efficient because of using longer primers and higher annealing temperature than those used for RAPD (Zietkiewicz et al., 1994). Results of ISSR and RAPD marker analyses both showed that genetic variation of *Zanthoxylum* species under study from northeast India was mainly internal and less between populations. Findings for total gene diversity, gene diversity within populations, and gene diversity among populations were similar in RAPD and ISSR approaches.

In the present investigation, polymorphism information content (PIC) was calculated for RAPD and ISSR primers. PIC analysis provides an estimate of the discriminating power of a marker. The suitability of molecular marker techniques based on the polymorphism detected, range of Jaccard’s similarity coefficients and average PIC value were found to be higher in ISSR than RAPD. PIC values are dependent on the genetic diversity of the genotypes chosen (Manimekalai and Nagarajan, 2006). The cluster analysis based on RAPD of the three species of *Zanthoxylum* was more or less similar with the phenotypic classification of the species. These three species were clustered into 2 distinct groups where as all the accessions of *Z. nitidum* and *Z. oxyphyllum* were in one single group and *Z. rhesta* accessions were in another group. An interesting observation was that group B divided the *Z. rhesta* accessions into two subgroups, separating genotypes collected from Jorhat from genotypes collected from Tinsukia. But ISSR marker analysis was slightly deviating from phenotypic classification of the species. Grouping of different accessions of *Z. nitidum* and *Z. oxyphyllum* in dendograms with high bootstrap was high in RAPD (77 & 60) and combined RAPD–ISSR (95 & 90) but low in ISSR. The accessions of *Z. rhesta* were found to be grouped together in all the three dendrograms based on RAPD (Fig. 1), ISSR (Fig. 2) and combined RAPD-ISSR (Fig. 3) with high bootstrap values (60, 73 and 99). High bootstrap values at most of the nodes supported the stability of the dendrograms. This may be due to the amplification of non-coding region and also there is little information to indicate that ISSR markers are functionally important (Esselman et al., 1999). RAPD markers are distributed throughout the genome and may be associated with functionally important loci (Penner, 1996). However, some researchers considered RAPD markers to represent a portion of DNA with noncoding regions and to be selectively neutral (Bachmann, 1997; Landerhoff et al., 2001).

In the present study, RAPD markers were found to be superior in PCA analyses and revealing variation among the population with AMOVA. In contrast to this, the ISSR markers attributed a high percentage of polymorphic bands (PPB), expected heterozygosity, Nei’s gene diversity and Shannon’s index for the three

### Table 7
Genetic structure of *Zanthoxylum* and estimate of gene flow within the genus: $H_T$ = Total diversity; $H_S$ = Diversity within population; $G_{ST}$ = coefficient of gene differentiation and $N_m$ = gene flow based on $G_{ST}$.

| Marker  | $H_T$     | $H_S$     | $G_{ST}$ | $N_m$  |
|---------|-----------|-----------|----------|--------|
| RAPD    | 0.3128 (0.0342) | 0.2264 (0.0216) | 0.2764 | 1.3087 |
| ISSR    | 0.3491 (0.0206) | 0.2644 (0.0147) | 0.2426 | 1.5610 |

### Table 8
Comparison of RAPD and ISSR Marker in evaluating genetic diversity of *Zanthoxylum*.

| Parameter                          | RAPD        | ISSR        |
|------------------------------------|-------------|-------------|
| Number of assay units              | 13          | 9           |
| Number of polymorphic band         | 108         | 99          |
| Number of loci                     | 132         | 101         |
| Fraction of polymorphic marker ($\beta$) | 0.82        | 0.98        |
| Average PIC                        | 0.648       | 0.80        |
| Multiplex ratio ($n$)               | 2.87        | 2.2         |
| Effective multiplex ratio ($E = \beta \times n$) | 2.35        | 2.16        |
| Marker index ($MI = E \times PIC$) | 1.52        | 1.73        |
species as well as for population as a whole. The evolutionary rate of change within microsatellites is considerably higher than most other types of DNA (Reddy et al., 2002). So, there are chances of having some changes in the non-coding regions of genus that leads to the variation in the species. AMOVA based on RAPD markers had resulted about 32% variation among the Zanthoxylum species while 68% variation was within the species. Similarly, AMOVA based on ISSR represents about 27% variation among the species while 73% variation was within the species. Both the marker techniques, RAPD and ISSR suggested a significant differentiation with the very large $F_{ST}$ values 0.31960 and 0.27173 respectively. While comparing the variations between the species, AMOVA based on RAPD reveals the highest variation among the species $Z.\ nitidum$ and $Z.\ rhesta$ (33.8%) and ISSR reveals the highest variation between $Z.\ nitidum$ and $Z.\ oxyphyllum$ (30.54%). Similarly, the percentage of polymorphic bands (PPB), expected heterozygosity, Nei’s gene diversity and Shannon’s index had the highest value in the species $Z.\ nitidum$ as shown by RAPD while these parameters were found to be highest in $Z.\ rhesta$ with ISSR. Here, both RAPD and ISSR analyses reveal a high amount of variation of $Z.\ nitidum$ with the $Z.\ rhesta$ and $Z.\ oxyphyllum$ while the lowest variation was between the later two species. The reason might be due to the evolution of $Z.\ oxyphyllum$ as intermediate of $Z.\ rhesta$ and $Z.\ nitidum$. Genetic diversity within the species is usually related with geographic range, mode of reproduction, mating system, and seed dispersal and fecundity (Gupta et al., 2008). Mehra and Khosla (1973) reported different ploidy levels of Zanthoxylum species. The haploid chromosomes were $n = 34$ in both $Z.\ nitidum$ and $Zanthoxylum\ limonella$ or $Z.\ rhesta$ whereas $n = 36$ in $Z.\ oxyphyllum$.

Genetic structure of the Zanthoxylum as discovered by the mean of the parameters like percent polymorphism (P%), observed number of alleles ($n_o$), effective number of alleles ($n_e$), Nei’s genetic diversity ($h$) and Shannon’s information index ($I$), were found to be highest in ISSR with the high gene flow and coefficient of gene differentiation than RAPD (Table 6). In population genetics, a value of gene flow (Nm) $< 1.0$ (less than one migrant per generation into a population) or equivalently, a value of gene differentiation ($G_{ST}$) $> 0.25$ is generally regarded as the threshold quantity, beyond which significant population differentiation occurs (Slatkin, 1987). Considering these criteria, the Zanthoxylum population was just above the threshold level and RAPD was superior in evaluating gene flow and gene differentiation (RAPD ($Nm = 1.3087, G_{ST} = 0.2764$)) in comparison to ISSR ($Nm = 1.5610, G_{ST} = 0.2426$). Differences among populations are commonly quantified by the use of one of several statistics, including Wright’s inbreeding coefficient ($F_{ST}$) and Nei’s coefficient of gene variation ($G_{ST}$). Although, they have important conceptual differences, in practice, $F_{ST}$ and $G_{ST}$ are used in similar fashion as indices of genetic difference among populations (Crow, 1986). Based on $F_{ST}$ and $G_{ST}$ estimates of the molecular techniques used in the study, RAPD (0.33796 and 0.2764) was best with highest values than ISSR (0.27063 and 0.2426).

Gene-flow values greater than one is “strong enough” to prevent substantial differentiation due to genetic drift (Slatkin and Barton, 1989). $Nm > 1$ in out crossing species might be due to well developed pollen dispersal mechanisms of the perennial plants. Therefore, the gene flow estimated by all the two molecular techniques was sufficient to counteract the differentiation due to genetic drift in the Zanthoxylum species. But, in the present study, gene differentiation was also found to be high in the Zanthoxylum. The reason might be fragmented populations of a small size and all of them were under human influence, which is likely to lead to drastic reductions of the population size or population isolation and extensive, recurrent gene flow. Ellstrand and Elam (1993) reported that population isolation may lead to stochastic differentiation by genetic drift.

To know the relationship between genome coverage of the RAPD and ISSR marker systems, Mantel test statistics was evaluated. Correlation between the two-marker systems was found to be high ($r = 0.65612$) with a value of determination coefficient 0.43049 ($r^2$). This means that both the markers share a minimum of 43% genomes for evaluation in common which might be due to large variation in genome size due to the difference in the chromosome numbers of the three species (Mehra and Khosla, 1973). Therefore, a comparison of the molecular markers based on the efficiency parameters like multiplex ratio (MR), effective multiplex ratio (EMR) and finally the marker index (MI) of both the techniques were evaluated to know the best suitable method for the evaluation of genetic diversity existing between the Zanthoxylum accessions and the ISSR marker was found to be slightly more efficient (Table 8). MI reveals the amount of information that can be obtained from a particular primer. The higher the MI indicated the more the usefulness and informativeness of the primer.

Management and conservation of the genus, the extents of genetic variation of the species are always influenced by both selective (drift, gene flow) and non-selective (natural selection) in population subdivision. Reduction in population size will lead to an increased influence of selective forces which may lead to the
breakdown of the former population structure which, in turn, can produce negative effects by putting in contact two differentiated gene pools through outbreeding depression or hybridization between locally adapted populations (Holsinger et al., 1999; Lande, 1999; Young et al., 1996). Awareness among the local people to conserve plants like Zanthoxylum which have thorns is the most primary and essential steps to be taken by the forest officials. Propagation and conservation of the species in the forest areas of their place of recent availability like Gibbon wild life sanctuary in Jorhat district, Nambor Wild life Sanctuary, Golaghat and Karbi Anglong District, Podumoni Reserve Forest, and Tinsukia district will decrease the risk of outbreeding depression if any and subsequently prevent the species to be an endangered species in near future.

Conclusion

All the three species of Zanthoxylum that are available in northeast part of India got a high degree of variance but the Z. nitidum comparatively shared higher variation with the other two. We may suggest for using both the low cost and time consuming DNA fingerprinting methods ISSR and RAPD to assess the genetic diversity as no sequence information or DNA synthesis is required for this technique. But, care should be taken to use a large number of primers for more information for the evaluation of genetic diversity in Zanthoxylum Spp. Creating awareness among the local people to conserve the natural population of the genus to reduce to save the existing diversity in the species and to prevent future extinction of the species available in these localities.

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