Genetic diversity and phylogenetic analysis of *Citrus* (L) from north-east India as revealed by meiosis, and molecular analysis of internal transcribed spacer region of rDNA

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**Abstract**

The north-eastern region of India is reported to be the center of origin and rich in diversity of *Citrus* (L) species, where some wild and endangered species namely *Citrus indica*, *Citrus macroptera*, *Citrus latipes*, *Citrus ichagensis* and *Citrus assamensis* exist in their natural and undisturbed habitat. In order to have comprehensive information about the extent of genetic variability and the occurrence of cryptic genomic hybridity between and within various *Citrus* species, a combined approach involving morphological, cytogenetical and molecular approaches were adopted in the present study. Cytogenetic approaches are known to resolve taxonomic riddles in a more efficient manner, by clearly delineating taxa at species and sub species levels. Male meiotic studies revealed a gametic chromosome number of n = 9, without any evidence of numerical variations. Bivalents outnumbered all other types of associations in pollen mother cells (PMCs) analyzed at diplotene, diakinesis and metaphase I. Univalents were frequently encountered in nine species presently studied, though their presence appropriately did not influence the distributional pattern of the chromosomes at anaphases I and II. The molecular approaches for phylogenetic analysis based on sequence data related to ITS 1, ITS 2 and ITS 1 + 5.8 s + ITS 2 of rDNA using maximum parsimony method and Bayesian inference have thrown light on species inter-relationship and evolution of *Citrus* species confirming our cytogenetical interpretations. The three true basic species i.e. *Citrus medica*, *Citrus maxima* and *Citrus*

**Keywords:**
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Among the three true species in sub-genus seed. The trait of adventive nucellar embryony in to 40 adventive embryos by the nucellus (Fusurato, 1957), so that two or more embryos develop in a single position. From our study it is amply clear that C. reticulata also has close relation to C. ichagensis, as these species have clustered together, denoting their close genetic relationship. On the other hand, our studies did not demonstrate a clear differentiation between subgenera Citrus and Papeda at the rDNA level. The combined approach of cytogenetical and molecular analysis did complement our early karyological findings and helped in resolving many a taxonomic riddles.

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Introduction

The genus Citrus L., the sole source of the Citrus fruits of commerce, belongs to the orange subfamily Aurantioidae of the family Rutaceae and is grown in tropical and subtropical areas of the world (Webber, 1967). The genus includes some of the most commercially important fruits viz. mandarin (Citrus reticulata Blanco), sweet orange (Citrus sinensis (L.) Osbeck), grapefruit (Citrus paradisi Macf.), lemon (Citrus limon (L.) Burm. f.) and lime (Citrus aurantiifolia (Christm.) Swingle). India enjoys a remarkable position in the “Citrus belt of the world” due to her rich wealth of Citrus genetic resources, both wild and cultivated (Malik et al., 2013; Nair and Nayar, 1997). The north-eastern region of India is a rich treasure of various Citrus species. Natural and undisturbed populations of Citrus gene pool observed during collection trips from time to time confirms the assumption that this area might be the center of origin of several Citrus species. As many as 17 Citrus species, their 52 cultivars and 7 probable natural hybrids are reported to have originated in the North-eastern region of India (Bhattacharya and Dutta, 1956). Citrus plants growing in deep forests undisturbed by abiotic factors have also been reported from the region, thus bestowing this area with a special status of “treasure house” of Citrus germplasm (Sharma et al., 2004). Citrus is the third most important fruit crop of India with an estimated production of 9441 MT from an area of 1039 ha (Annual reports, 2012). Annual production of Citrus species in North-east is 506.9 tons from 98.3 ha (Annual report, 2010).

Citrus taxonomy and phylogeny are very complicated, controversial and ambiguous (Nicolosi et al., 2000) due to sexual compatibility among species, long history of cultivation, apomixis (adventives nucellar polyembryony), somatic bud mutation etc. Sexual compatibility even between Citrus and related genera like Fortunella, Poncirus etc. (Frost and Soost 1968; Malik et al., 2013) has contributed to the taxonomic confusion. Citrus taxonomy was based mainly on morphological and geographical data and many classification systems have been formulated from time to time. Two of these systems suggested by Swingle and Reece (1967) and Tanaka (1977) have been the most widely accepted ones. The discrepancy between them is shown by the fact that Swingle’s system recognizes just 16 species while Tanaka’s system recognizes 162 species in the genus Citrus. Scora (1975) and Barrett and Rhodes (1976) suggested that there are only three ‘basic’ true species of Citrus within the subgenus Citrus as follows: citron (Citrus medica L.), mandarin (C. reticulata Blanco), and pummelo (Citrus maxima (Burm) Merrill), other species within this subgenus are hybrids derived from these true species, species of subgenus Papeda or closely related genera. Taxonomic characterization leading to unambiguous identification of Citrus species and their genetic resources are essential requisites for Citrus breeding. Citriculture and Citrus industry. In a systematic account on Indian Citrus, Nair and Nayar (1997) followed primarily the scheme of Swingle and Reece (1967) and partly that of Tanaka (1977) including 18 taxa, comprising of eight species under subgenus Citrus, three under subgenus Papeda, and seven other indigenous Citrus varieties with a suspected hybrid origin and uncertain taxonomic affinities.

Most species of the genus Citrus are characterized by polyembryony, which consists of the production of 1 to 40 adventive embryos by the nucellus (Fusurato, 1957), so that two or more embryos develop in a single seed. The trait of adventive nucellar embryony in Citrus has long been a subject of interest to taxonomists. Among the three true species in sub-genus Citrus i.e. C. medica (citron), C. maxima (pummelo) and C. reticulata
first two species are strictly monoembryonic with only sexual offspring whereas *C. reticulata* is polyembryonic.

Meiotic events in *Citrus* and its inter-specific and inter-generic hybrids are quite interesting. However, the meiotic behavior in *Citrus* is mainly regular; irregularities are infrequent (Agarwal, 1989; Iwamasa, 1966; Raghuvanshi, 1962). Analysis of meiotic chromosome pairing in hybrids not only is a classical and authentic approach to understand species relationships but it also helps in genetic stability of polyploids (Yan et al., 1997). Cultivated *Citrus* species have been hybridized with some wild relatives such as *Murraya*, *Severinia*, *Atalantia*, and *Swinglea*, in order to introduce desirable traits, mainly resistance to pests and pathogens (Barrett, 1977; Motomura et al., 1995). The identification of chromosomes of different genomes could be a simple method of identifying *Citrus* hybrids and is thus important for future work (Cameron and Frost, 1968). Therefore, the present study was taken up to resolve the cryptic hybridity of *Citrus* species present in their natural habitat through cytogenetical tools.

Since morphological characters are only of limited use and cytogenetical parameters are time consuming, alternate approaches, including application of appropriate molecular markers, have now been increasingly adopted to address the problems in *Citrus* taxonomy (Kumar et al., 2012). The perplexing condition of the *Citrus* phylogeny has drawn many workers to try and resolve the ambiguities using molecular markers such as isozymes (Herrero et al., 1996), RAPD and PCR–RFLP (Abkenar et al., 2004; Federici et al., 1998; Jena et al., 2009), RAPD and SCAR (Nicolosi et al., 2000), AFLP (Liang et al., 2007; Pang et al., 2007), SSR (Barkley et al., 2006), ISSR (Fang et al., 1998; Shahsavari et al., 2007) and sequence data analysis of ITS region of nrDNA (Kyndt et al., 2010; Pessina et al., 2011; Xu et al., 2006) and non-coding chloroplast DNA (cpDNA) regions (Araujo et al. 2003; Chase et al. 1999; Lu et al. 2011; Morton et al. 2003). The molecular phylogeny of Indian *Citrus* using PCR–RFLP of the trnD-trnT and *rbcL*-ORF 106 regions as well as sequence data analysis of the *trnL*-trnF intergenic spacer region of cpDNA was carried out by Jena et al. (2009) where they supported the recognition of *C. maxima*, *C. medica* and *C. reticulata* as the basal species of cultivated *Citrus*. Therefore, in order to have comprehensive and substantial information about the extent of genetic variability and occurrence of cryptic genomic hybridity among *Citrus* species, a combined approach involving morphological, cytogenetical and molecular phylogenetical approaches has been adopted in the present study. Thus our investigations constitute as a first multi-pronged approach to resolve several issues of *Citrus* taxonomy, which is beleaguered.

**Material and methods**

**Plant materials**

Extensive surveys and exploration trips were conducted in different states of North-East India to collect wild, semi-wild and cultivated species of *Citrus*. Location, state and name of species are provided in Table 1/Fig. 1. Germplasm was collected in the form of fruits, seeds and flower buds. The samples used in the present study are authenticated and are being maintained at the National Bureau of Plant Genetic Resource, New Delhi. The trees were marked and appropriately labeled before flowers and leaves were collected from them, which formed the basic material for detailed male meiotic studies and phylogenetic analysis.

**Meiotic studies**

Flower buds of appropriate size (1–2 cm in diameter) were harvested from mature trees of *Citrus* species and fixed on the spot in freshly prepared 1:3 glacial acetic acid: 95% ethanol mixture for a minimum of 24 h at room temperature and later stored in 70% ethanol at 10 °C. Anthers were squashed in 1% aceticarmine solution with ferric chloride solution as mordant. On average 25–30 PMCs were analyzed at diplotene/diakinesis/metaphase I to estimate the range of chromosome associations and recombination frequencies through chiasma analysis. On average 15–20 cells were analyzed at anaphase I/II to study the distributional pattern of chromosomes and chromatids. For percentage pollen stainability the pollen grains were stained in 1:1 glycerine:aceticarmine mixture and on average ten slides were scored for stainable pollen. Photomicrographs of cytological preparations were taken from temporary slides with Jenoptik CCD camera (Germany) attached to Labomed LX 400 brightfield microscope. The illustrations in
Table 1
Mean number and range of chromosome associations at diplotene/diakinesis/metaphase I in 10 species of *Citrus* accessions.

| Species         | Accession No. | No. of cells analyzed | 2n | Chromosomes associations | Bivalents | Univalents |
|-----------------|---------------|-----------------------|----|--------------------------|-----------|------------|
|                 |               |                       |    | Quadrivalents             | Total     |            |
|                 |               |                       |    |                          | Rings     |            |
|                 |               |                       |    |                          | Rods      |            |
|                 |               |                       |    |                          | No | Mean | Range | No | Mean | Range | No | Mean | Range |
| *C. medica*     | IC 583259     | 25                     | 18 | –                        | 222 | 8.8 ± 0.3 | 8–9 | 147 | 5.88 ± 1.56 | 3–8 | 74 | 2.96 ± 1.54 | 1–6 |
| *C. latipes*    | IC 583263     | 25                     | 18 | –                        | 222 | 8.8 ± 0.33 | 8–9 | 133 | 5.32 ± 1.31 | 3–8 | 89 | 3.56 ± 1.41 | 1–6 |
| *C. macroptera* | IC 558161     | 25                     | 18 | –                        | 223 | 8.92 ± 0.27 | 8–9 | 142 | 5.4 ± 1.24 | 3–8 | 81 | 3.25 ± 1.26 | 1–6 |
| *C. indica*     | IC 558179     | 25                     | 18 | 1                        | 215 | 8.6 ± 0.76 | 7–9 | 141 | 5.64 ± 1.65 | 2–8 | 73 | 3 ± 1.5 | 1–7 |
| *C. maxima*     | IC 583271     | 25                     | 18 | –                        | 223 | 8.92 ± 0.27 | 8–9 | 125 | 5 ± 1.35 | 2–7 | 98 | 3.92 ± 1.41 | 2–6 |
| *C. sinensis*   | IC 558164     | 25                     | 18 | –                        | 217 | 8.68 ± 0.55 | 7–9 | 119 | 4.76 ± 1.71 | 2–8 | 98 | 3.92 ± 1.68 | 1–6 |
| *C. jambhiri*   | IC 278011     | 25                     | 18 | –                        | 212 | 8.83 ± 0.37 | 8–9 | 117 | 4.87 ± 1.16 | 3–7 | 90 | 3.9 ± 1.17 | 1–6 |
| *C. reticulata* | IC 583264     | 25                     | 18 | –                        | 225 | 9 | 9 | 128 | 5.12 ± 1.36 | 3–9 | 97 | 3.88 ± 1.36 | 2–6 |
| *C. limon*      | IC 278013     | 25                     | 18 | –                        | 224 | 8.95 ± 0.2 | 8–9 | 122 | 4.88 ± 1.66 | 2–6 | 102 | 4.08 ± 1.7 | 1–7 |
| *C. limetta*    | IC 583244     | 25                     | 18 | –                        | 214 | 8.56 ± 0.58 | 7–9 | 108 | 4.32 ± 1.21 | 2–7 | 106 | 4.24 ± 1.2 | 1–7 |

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the present investigation were magnified at 1000× to the original dimensions of the image, with no further increase in the magnification during processing stage.

**DNA extraction, amplification reaction and sequencing for nrDNA ITS**

Genomic DNA of *Citrus* species was extracted following Murray and Thompson (1980). The PCR primers ITS 4 and ITS 5 of White et al. (1990) were used to amplify the ITS region (ITS 1, 5.8S, and ITS 2) utilizing same primers for sequencing. The amplification program consisted of one cycle of initial denaturation at 94 °C for 4 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 3 min and 72 °C for 1 min with final extension of 72 °C for 7 min. DNA amplification was performed in a thermal cycler system (Gene Amp® 2700 Applied Biosystems). Amplified PCR products were purified using QIAquick gel extraction kit (QIAGEN, Germany) and sequenced at Xcelris Scientific Pvt. Ltd., India.

**Sequence alignment and indel coding**

The boundaries of the ITS region for all 12 species of *Citrus* were determined by comparing published sequences and on the basis of the angiosperm consensus motif determined by Jobes and Thien (1997). The putative start and end points of 5.8S regions in the aligned sequences were identified. *Atalantia ceylanica* was selected as out group and sequences obtained were subjected to multiple sequence alignment using Clustal X program (Thompson et al., 1997) with default settings. Clustal X generated alignments were further re-aligned manually. Gaps were included into analysis and coded automatically in a binary matrix using SeqState v.1.21 (Müller, 2005) applying the simple indel coding strategy (Simmons and Ochoterena, 2000).

**Phylogenetic analysis**

The sequence characteristics of the ITS region were calculated using MEGA version 4 (Tamura et al., 2007). Maximum parsimony (MP) method was used to analyze the aligned sequence data matrix. The tree
Table 2
Mean number, range of chiasmata and terminalization coefficient in 10 species of Citrus accessions.

| Species  | Accession number | No. of cells analyzed | n | Total chiasma | Terminalised chiasma | Unterminalised chiasma | Terminilization co efficient | Chiasma frequency | Pollen stainability studies (%) |
|----------|------------------|-----------------------|---|---------------|----------------------|------------------------|-----------------------------|------------------|--------------------------------|
| C. medica | IC 583259        | 25                    | 9 | 669           | 26.5 ± 4.18          | 20–33                  | 284 11.45 ± 2.1 9–15        | 385 15.4 ± 1.9   | 12–18 0.42                      | 26.76 44.4        |
| C. latipes | IC 583263       | 25                    | 9 | 628           | 25.12 ± 4           | 19–31                  | 263 10.52 ± 2.6 6–14        | 365 14.6 ± 1.7   | 12–19 0.41                      | 25.12 87          |
| C. macroptera | IC 558161     | 25                    | 9 | 661           | 26.44 ± 3.7         | 19–33                  | 272 10.88 ± 2.29 6–16       | 389 15.56 ± 1.7  | 13–19 0.41                      | 26.44 80          |
| C. indica  | IC 558179        | 25                    | 9 | 673           | 26.92 ± 5.67        | 18–37                  | 281 11.24 ± 2.63 7–14       | 392 15.68 ± 3.22 | 11–21 0.41                      | 26.92 90          |
| C. maxima  | IC 583271        | 25                    | 9 | 610           | 24.4 ± 2.81         | 17–28                  | 240 9.6 ± 1.52 7–13         | 370 14.8 ± 1.6   | 7–13 0.39                       | 24.4 89           |
| C. sinensis | IC 558164       | 25                    | 9 | 572           | 22.88 ± 5           | 17–33                  | 224 8.96 ± 2.68 5–13        | 348 13.92 ± 2.64 | 9–20 0.39                      | 22.88 78.9       |
| C. jambhiri | IC 278011        | 25                    | 9 | 562           | 24.28 ± 3.27        | 18–30                  | 231 9.62 ± 1.68 7–11        | 354 14.75 ± 1.77 | 11–18 0.41                      | 22.48 59.4        |
| C. reticulata | IC 583264      | 25                    | 9 | 634           | 25.36 ± 8.31        | 17–30                  | 269 10.76 ± 2.52 5–14       | 365 14.6 ± 1.5   | 12–16 0.42                      | 25.36 82          |
| C. limon   | IC 278013        | 25                    | 9 | 604           | 24.16 ± 3.92        | 16–33                  | 241 9.64 ± 2.21 5–14        | 365 14.52 ± 1.93 | 11–19 0.40                      | 24.16 63          |
| C. limetta | IC 583244        | 25                    | 9 | 565           | 22.6 ± 3.52         | 17–28                  | 236 9.44 ± 1.95 6–13        | 322 13.32 ± 1.65 | 10–16 0.41                      | 22.6 80           |
was constructed using Phylip (Felsenstein, 2004). Bootstrap analysis was carried out with 999 random seed and 1000 replicates to examine the relative level of support for individual clades on the cladograms of each search. The Bayesian inference (BI) of phylogeny was also conducted using MRBAYES v.3.1.2 (Ronquist and Huelsenbeck, 2003). BI analysis was performed for 1,000,000 generations applying the default settings (MCMC, two runs with four chains each, heating temperature 0.2, saving one tree every 100 generations).

**Result**

**Meiotic studies**

The meiotic divisions in the wild and cultivated species of *Citrus* were studied. The details regarding the ten *Citrus* species analyzed in the present investigation, total number of PMCs analyzed and their association at diplotene, diakinesis and metaphase I are summarized in Table 1. The data on total and mean number of chiasmata and its range along with number of terminalized chiasma, terminalisation co-efficient and its percentage pollen stainability is summarized in Table 2. The distribution pattern of chromosome at anaphase I has been detailed in Table 1. Most of these observations are illustrated in Fig. 2. From the data summarized in Tables 1 and 2, it is amply clear that all the species presently investigated were characteristic in showing nine bivalents at diplotene/diakinesis/metaphase I in all PMCs analyzed. The present study carried out in 10 species of *Citrus* revealed a gametic number of n = 9, without any variation (Fig. 2). The meiotic chromosome behavior in the ten *Citrus* species studied was regular where bivalent associations outnumbered other types of associations in pollen mother cells (PMCs) studied at diplotene, diakinesis and metaphase I. The mean value for ring bivalents ranged between 5.88 (*C. medica*) and 4.32 (*Citrus limetta*) while that of rod bivalents ranged between 4.24 (*C. limetta*) and 2.96 (*C. medica*). We also observed meiotic irregularities including univalents, quadrivalents, laggards, bridges and chromosome stickiness, though at low frequency in the PMC. Univalents were frequently encountered in most of the species namely *Citrus indica*, *C. limetta*, *C. sinensis*, *Citrus jambhiri*, *C. medica*, *C. maxima*, *Citrus latipes* and *Citrus macroptera*, where a maximum of 4 univalents per PMC was found in *C. indica* and *C. limetta*. *C. reticulata* was unique in having no univalents. 1–3 bivalents were observed to be associated with the nucleolus per PMC. An occasional multivalent association in the form of quadrivalents was detected in *C. indica*. Ring bivalents per PMC ranged from 3 to 9 while 1–7 rod bivalents were also recorded per PMC. From the data presented in Table 2 it is apparent that the mean number of chiasmata per cell ranged from 22.6 (*C. limetta*) to 26.5 (*C. medica*). *C. limetta* (13.32) was observed to have minimum terminalized chiasmata whereas *C. indica* recorded the highest value of 15.68. Terminalization co-efficient ranged from 0.39 to 0.42 in all the ten species studied. Five species viz. *C. latipes*, *C. macroptera*, *C. indica*, *C. jambhiri* and *C. limetta* recorded similar value of terminalisation co-efficient of 0.41. The chiasma frequency in PMCs of the ten species studied ranged from 22.48 to 26.92. The highest value was recorded in *C. indica* (26.92) and the lowest in *C. jambhiri* (22.48). Chromosome distribution at anaphases I and II was observed to be regular, but a few laggards were detected in some of the PMC of a few species. Micronuclei or

| Species       | Accession no. | No of cell analyzed | Chromosome distribution | No. of cells | Percentage |
|---------------|---------------|---------------------|-------------------------|--------------|------------|
| *C. medica*   | IC 583259     | 15                  | 9:9                     | 15           | 100        |
| *C. latipes*  | IC 583263     | 10                  | 9:9                     | 10           | 100        |
| *C. macroptera* | IC 558161    | 20                  | 9:9; 9:1:8              | 19           | 95         |
| *C. indica*   | IC 558179     | 20                  | 9:9                     | 20           | 100        |
| *C. maxima*   | IC 583271     | 15                  | 9:9                     | 15           | 100        |
| *C. sinensis* | IC 558164     | 9                   | 9:9                     | 9            | 100        |
| *C. jambhiri* | IC 278011     | 10                  | 9:9                     | 10           | 100        |
| *C. reticulata* | IC 583264    | 15                  | 9:9; 9:1:8              | 14           | 93         |
| *C. limon*    | IC 278013     | 15                  | 9:9                     | 15           | 100        |
| *C. limetta*  | IC 583244     | 15                  | 9:9                     | 15           | 100        |
secondary associations of the chromosomes were also observed in 1–2 species. Pollen stainability in the ten species studied ranged from 44.4 to 90%. *C. medica*, *C. jambhiri* and *C. limon* exhibited low percentage of pollen stainability with 44.4%, 59.4% and 63% respectively.

Fig. 2. Different male meiotic stages in 10 *Citrus* species. 1–3: diplotene, diakinesis & metaphase I in *C. limetta*; 4–5: diakinesis & metaphase I in *C. medica*; 6–8: diplotene, diakinesis & metaphase I in *C. latipes*; 9–10: diplotene & diakinesis in *C. limon*; 11–12: diplotene & metaphase I in *C. sinensis*; 13–14: diplotene & metaphase I in *C. reticulata*; 15–16: diplotene & diakinesis in *C. macroptera*; 17–19: diplotene, diplotene (I) & diplotene (IV) in *C. indica*; 20: metaphase I in *C. jambhiri*; 21: metaphase I in *C. maxima*; 22: anaphase I in *C. reticulata* (laggard); 23: anaphase II in *C. limetta*; 24: anaphase II in *C. sinensis* (laggards). Scale bar, 5 μm (applies to all images).
ITS sequences data

The phylogenetic analysis based on sequence data related to ITS 1, ITS 2 and ITS 1 + 5.8S + ITS 2 loci using maximum parsimony method and Bayesian inference has thrown light on species inter-relationships and evolution of Citrus species. Sequence length in the 12 Citrus accessions ranged from 601 to 694 bp (ITS 1 and ITS 2 partial and 5.8S complete sequence) as compared to 649 bp in A. ceylanica. The ITS 1 and ITS 2 regions of twelve species of Citrus presently investigated showed variable sequence lengths and G + C content (%). The sequence lengths of ITS 1 for all the 12 species ranged from 210 to 310 bp while ITS 2 sequence lengths ranged from 207 to 270 bp (Table 4). All the twelve Citrus species revealed a sequence length of 160 bp for 5.8S region. The ITS sequences were very rich in G + C content ranging from 59.8% (C. medica) to 64.4% (Citrus ichagensis) with an average of 64.2%. The G + C content (%) of ITS 1 was found to be slightly higher as compared to ITS 2 region and average G + C content of 64.3% and 63.2% were recorded for ITS 1 and ITS 2, respectively. The final aligned data matrix of the combination of ITS 1, 5.8S and ITS 2 yielded 732 characters including 385 conserved, 316 variable and 130 parsimony informative sites. For determining sequence statistics among Citrus species, 245 and 268 characters were aligned for ITS 1 and ITS 2 respectively. The addition of A. ceylanica (selected as out-group) resulted in an aligned length of 324 and 296 characters for ITS 1 and ITS 2 respectively. The 5.8S region has been found to be more conserved as evidenced from the number of conserved sites (153 out of 155, 98.71%), followed by ITS 1 (86.12%) and ITS 2 (69.40%). On the contrary, a higher sequence divergence though marginally was recorded for ITS 2 (Table 5).

### Table 4
Summary of nrDNA sequences of 12 species of Citrus and the out-group Atalantia ceylanica.

| Sl. no. | Taxon       | Accessions number | Status | ITS 1 | 5.8S | ITS 2 | Total | G + C (%) |
|---------|-------------|-------------------|--------|-------|------|-------|-------|-----------|
| 1       | C. assamensis | IC 285355         | Wild   | 254   | 160  | 248   | 662   | 63.3      |
| 2       | C. ichagensis | IC 591460         | wild   | 258   | 160  | 240   | 658   | 64.4      |
| 3       | C. indica    | IC 558179         | Wild   | 224   | 160  | 237   | 621   | 63        |
| 4       | C. jambhiri  | IC 278011         | Cultivated | 221  | 160  | 220   | 601   | 63        |
| 5       | C. latipes   | IC 583263         | Wild   | 253   | 160  | 233   | 646   | 62.8      |
| 6       | C. limon     | IC 278013         | Cultivated | 255  | 160  | 261   | 676   | 61.2      |
| 7       | C. limettoides | IC 583244      | Cultivated | 210  | 160  | 270   | 640   | 63        |
| 8       | C. macroptera | IC 558161        | Wild   | 255   | 160  | 220   | 605   | 63        |
| 9       | C. maxima    | IC 583271         | Cultivated | 255  | 160  | 237   | 652   | 62.1      |
| 10      | C. medica    | IC 583259         | Cultivated | 310  | 160  | 224   | 694   | 59.8      |
| 11      | C. reticulata | IC 583264        | Cultivated | 264  | 160  | 237   | 661   | 61.3      |
| 12      | C. sinensis  | IC 558164         | Cultivated | 221  | 160  | 238   | 619   | 60        |
| 13      | Atalantia ceylanica | –          | –    | 256   | 155  | 238   | 649   | 64.4      |

### Table 5
Sequence characteristics of ITS region of rDNA in Citrus species.

| Sl. no. | Parameters             | ITS 1 (bp) | ITS 2 (bp) | 5.8S (bp) | G + C content (%) |
|---------|------------------------|------------|------------|-----------|-------------------|
| 1       | Length range (in-group) | 601–694    | 210–310    | 207–270   | 160               |
| 2       | Length (out-group)     | 649        | 256        | 238       | 155               |
| 3       | Aligned length         | 732        | 324        | 296       | 170               |
|         | including missing data| 385(52.5)  | 181(55.6)  | 183(61.8) | 155(91.1)         |
|         | No. of conserved sites | 316(43.1)  | 113(34)    | 83(28)    | 12(7)             |
|         | No. of variable sites  | 130(25.4)  | 99(30.5)   | 81(27.3)  | 9(1.76)           |
|         | No. of informative sites| 20         | 14         | 9         | 6                 |
| 4       | Indels                 | 59.8–64.4  | 57–69      | 56.6–69.6 | 52.4–55.8         |
| 5       | G + C content mean (%) | 62.24      | 64.3       | 63.2      | 54.2              |
| 6       | Sequence divergence (%) | 26.5       | 34.8       | 28        | 7.05              |
| 7       | Nucleotide frequencies of adenine | 0.208 | 0.185 | 0.185 | 0.25 |
|         | Thymine                | 0.181      | 0.136      | 0.174     | 0.217             |
|         | Cytosine               | 0.310      | 0.346      | 0.333     | 0.263             |
|         | Guanine                | 0.300      | 0.333      | 0.308     | 0.27              |
| 9       | Transition/transversion bias (R) | 0.83 | 6.023 | 1.048 | 1.095 |

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of sequence divergence based on substitution plus indels was 34.8% for ITS 1; 28 for ITS 2 and 7.05% for 5.8S region respectively. ITS 1 recorded highest percentage (30.5%) of parsimony informative sites. The numbers of indels for ITS 1 and ITS 2 were 14 and 9 respectively. In ITS sequence (ITS 1 and ITS 2 partial and 5.8S complete sequence), the nucleotide frequencies were found as 0.208 (A), 0.310 (C), 0.300 (G), and 0.181 (T). Transition/transversion bias (R) was 0.83.

The Maximum Parsimony (MP) and Bayesian Inference (BI) methods were used to assess the phylogenetic relationship of the genus *Citrus* based on the combined nucleotide sequence data of ITS 1, 5.8S and ITS 2 (Figs. 3 and 4). A clear relationship among subgenera is observed in all the trees generated through two phylogenetic methods. ITS sequence (ITS 1 and ITS 2 partial and 5.8S complete sequence), analysis showed moderate rate of nucleotide divergence within and among the *Citrus* taxa and *A. ceylanica*,

Fig. 3. 50% majority-rule consensus phylogenetic trees for illustrating the relationship among Indian representatives of the genus *Citrus* based on ITS of the rDNA sequence data using maximum parsimony method. Bootstrap values given at the nodes. The tree is rooted with the *Atalantia ceylanica*. The meiotic data of chromosome association, chiasma frequency per cell, terminalization co-efficient and pollen stainability followed by number of ring, rod bivalents and univalents is also given.
genetic divergence within Citrus group ranged from 0 to 26.5%. The phylogenetic trees based on both maximum parsimony and Bayesian analyses show a clear separation between the three ‘basic’ species as proposed by Scora (1975) and Barrett and Rhodes (1976). A clear relationship among subgenera in the maximum parsimony analysis is observed and the phylogenetic tree (Fig. 3) has been resolved into three

Fig. 4. Bayesian inference phylogenetic tree of 12 Citrus species based on ITS of the rDNA sequence data. The posterior probability is given on each node. The tree is rooted with the Atalantia ceylanica. The scale bar represents branch length (number of substitutions/site). The meiotic data of chromosome association, chiasma frequency per cell, terminalization co-efficient and pollen stainability followed by number of ring, rod bivalents and univalents is also given.
major clusters. Major cluster I is further resolved into two sub-clusters viz. Sub-cluster Ia which consisted of *C. medica, C. maxima* and *C. limon* and sub-cluster Ib which had *C. limetta, C. jambhiri* and *C. macroptera*. Major cluster II consisted of *C. latipes* and *C. assamensis* while major cluster III is further resolved into two sub-clusters i.e. IIa and IIIb. Sub-cluster IIIa comprised of *C. reticulata, C. ichagensis* and *C. sinensis* while sub-cluster IIb had only *C. indica*. *A. ceylanica* was separately attached at the base of tree as the diverging *Citrus* relative’s lineage.

Bayesian Inference (BI) method was used to assess the phylogenetic relationship of the genus *Citrus* based nrDNA sequences. The phylogenetic tree based on BI method (Fig. 4) has resolved into four major clusters. Major cluster I had only *C. indica* while major cluster II is further resolved into two sub-clusters. Sub-cluster Ila had *C. medica, C. maxima* and *C. limon* while sub-cluster IIb consisted of *C. jambhiri, C. limetta* and *C. macroptera*. Major cluster III comprised of *C. sinensis, C. ichagensis* and *C. reticulata* and lastly major cluster IV consisted of *C. assamensis* and *C. latipes*. *A. ceylanica* was separately attached at the base of tree as the diverging *Citrus* relative’s lineage.

**Discussion**

*Citrus* classification is ambiguous and highly controversial. Various taxonomists have recognized 16 to 162 species in the genus *Citrus* (Swingle, 1943 and Tanaka, 1954). Most of the confusion is due to free hybridization of different species and occurrences of intermediate forms. This study was an attempt to distinguish the intermediate forms through analysis of chromosomal associations and their behavior during meiosis. Interestingly it was found, by and large, normal for all the *Citrus* species investigated where bivalent associations outnumbered other types of associations in pollen mother cells (PMCs) studied at diplotene, diakinesis and metaphase I, the presence of regular bivalent formation in these species indicates that the genome of the species is homologous and does not have large structural differences. Univalents were frequently encountered in most of the species studied. Raghuvanshi (1962) found univalents in 17 out of 25 *Citrus* species analyzed. The presence of univalents in some of the species studied indicates certain degree of structural heterogeneity in the genetic makeup of the bivalents. *C. limetta, C. indica* and *C. sinensis* which recorded highest number of univalents per PMC confirm the heterogeneity within their genomes, which could possibly be of an intermediate nature. Up to 18 univalents were detected in intergeneric hybrids between *Citrus* and *Poncirus*, suggesting a lack of homology of different chromosomes (Iwamasa, 1966), though Raghuvanshi (1962) said this could be due to precocious separation of bivalents. Early separation of synapsed homologues is generally the reason for regular occurrence of univalents in many of the tree species (Kumar et al., 2002; Singh, 1993). This explanation is also given for the presence of univalents in other genera, for examples, in wild *Saccharum* species (Burner, 1991). Univalents may lead to unequal distribution at anaphase and consequently a decrease in fertility (Khazanehdari and Jones, 1997). The distribution of chromosomes at anaphases I and II in all the species studied was normal indicating that the pollen sterility in these species is genic controlled (Agarwal, 1987).

The association of some bivalents (Abkenar et al., 2004; Agarwal, 1984, 1987, 1989) with nucleolus in majority of the PMCs analyzed at diplotene/diakinesis might be indicative of nucleolar nature of representative chromosomes. Secondary association of chromosomes has been considered as an evidence of remote affinity between the chromosomes (Singh, 1993). Further the maximum grouping of these bivalents in groups of three indicates the basic chromosome number for *Citrus* as three, as has been reported earlier by Banerjee (1954) and Agarwal (1984). The sporadic occurrence of quadrivalents may be attributed to plausible partial homology between otherwise non homologous chromosomes arising out of structural rearrangements (Stebbins, 1971). Agarwal (1987) suggested that the presence of tetravalents in four hybrid *Citrus* taxa indicated homology (or homoeology) among different genomes as well as the absence of large chromosomal differences. Chromosome structural/numerical changes apparently did not play any role in *Citrus* speciation and evolution. Most probably variations at gene level might have influenced speciation in *Citrus* as evident from its morphological diversity. Hore and Barua (2004) reported presence of several intermediate types, hinting at natural hybridization. Meiotic behavior of somatic hybrids provides valuable information for their practical utilization in *Citrus* breeding programs (Khan, 2007). Meiotic abnormalities such as chromosome bridges and chromosomes orientated away from the equatorial plate are frequently observed in hybrids resulting in different sizes of pollen grain and generally abnormal tetrad formation and irregular chromosome behavior with univalents or multivalent
pairing which occur in somatic hybrid plants (Chen et al., 2004). However, from our studies none of the species presently investigated from cytogenetical point of view could be regarded as hybrid origin due to lack of stabilized polyplody events in the species. The relationship between the genomes of the parental species has great influence on the determination of the process of chromosome pairing and recombination and thus the extent of meiotic irregularities and viability of the gametes (De Jong et al., 1993). The taxonomic relationships between the genomes of these intergeneric species need to be re-evaluated by observing meiotic behavior and also molecular cytogenetics studies like FISH/GISH can play a main role in ascertaining the true hybrid nature of the intermediate types in more authentic manner.

The phylogenetic analysis based on sequence data related to ITS 1, ITS 2 and ITS 1 + 5.8 s + ITS 2 using maximum parsimony method and Bayesian inference has thrown light on species inter-relationship and evolution of Citrus species. Among all the analyses BI method used for analysis of ITS 1 + 5.8 s + ITS 2 sequenced data has given more convincing information which had the critical support of both morphological and cytogenetical analyses. The three true basic species i.e. C. medica, C. maxima and C. reticulata have resolved into distinct clades. C. indica occupies a unique position in the phylogenetic ladder of the genus Citrus. C. medica, C. limon and C. maxima have resolved into a distinct group, which is on expected lines and receives support from published literature (Jena et al., 2009). C. jambhiri, C. limetta and C. macroptera have resolved into a distinct but separate cluster. It is quite interesting and intriguing to note that C. macroptera considered to be a member of sub-genus Papeda which shows similarity with the members (C. jambhiri and C. limetta) of sub genus Citrus. The grouping of C. reticulata with C. sinensis and C. ichangenesis is duly approved as the latter species are probable derivatives of C. reticulata as reported in literature (Penjor et al., 2010, 2013). C. assamensis which belongs to sub genus Citrus and C. latipes which belongs to sub-genus Papeda are also grouped together. However, both the species exhibit distinct morphological and cytogenetical diversity from each other.

Citrus indica is a true wild species endemic to the Garo Hills in Meghalaya. Tanaka (1928) was the first to describe it as a new species. He placed C. indica in section Acrumen of the Subgenus MetaCitrus. C. indica clustered distinctly from all the other species in the phylogenetic ladder of both MP and BI trees which is also reflected in the meiotic data with the presence of univalents indicating, probably a heterogeneous genome. The presences of quadrivalents in C. indica also echo its unique position. Therefore, elucidating its special taxonomic position as a true species or progenitor species of cultivated Citrus taxa. C. medica (citron), C. reticulata (mandarin) and C. maxima (pummelo) are defined as basic true species by Swingle and Reece (1967) a phylogenetic truth which was later supported by a number of workers (Barrett and Rhodes, 1976; Jena et al., 2009; Kumar et al., 2012; Kyndt et al., 2010; Scora, 1975). This undisputed taxonomical phenomenon also gains support from our investigation, though partially where C. reticulata, C. medica and C. maxima were resolved into separate clusters while C. maxima clustered itself closer to C. medica. The presence of maximum number of ring bivalents indicates the homology and stability of their genome. Univalents were recorded in both C. medica and C. maxima but are low in numbers and there was a total absence of univalents in C. reticulata, all these findings support the position of the three basic species. The chiasma frequency recorded in the C. medica, C. reticulata and C. maxima which indicates cryptic heterozygosity at molecular level which is confirmed by clustering patterns in the ITS analysis (Fig. 3). The species belonging to sub genus Papeda i.e. C. ichangenesis, C. macroptera and C. latipes have clustered separately and show similarity with the members of sub genus Citrus. Kumar et al. (2012) also could not find any clear cut differentiation between subgenera Citrus and Papeda as per Swingle’s system. This supports the earlier findings of Nicolosi et al. (2000) and Pang et al. (2007). Cytogenetically C. macroptera and C. latipes are alike having maximum number of bivalents which are resolved into ring (5.4 and 5.32) and rod bivalents (3.25 and 3.5). The genome size of C. ichangenesis is considerably larger as reflected in chromosome size (unpublished data) as compared to C. latipes and C. macroptera. Thus C. ichangenesis differs from the two papedian taxa significantly and also gets support from the molecular data of ITS analysis.

C. reticulata which is considered as a basal species and possible progenitor of C. sinensis (Kumar et al., 2012; Mabberley, 2004; Moore, 2001) and C. ichangenesis (Penjor et al., 2013) is characteristic of having extensive homogeneity in their genome by showing 100% bivalent association of which more number of ring bivalents and fewer rod bivalents followed by complete absence of univalents. On the other hand, C. sinensis which is supposedly a derivative of C. reticulata does exhibit cryptic structural hybridity in the form of fewer bivalents, with ring and rod in equal proportion followed by the presence of significant
number of univalents which is also reflected in its low pollen stainability. Jena et al. (2009) based on their cpDNA data, also elucidated the involvement of C. reticulata as a maternal parent in the origin of sweet orange (C. sinensis) although few workers suggest that C. maxima may be the maternal parent of sweet orange (Araujo et al., 2003; Nicolosi et al., 2000; Penjor et al., 2013). The molecular data suggest that C. limon and C. maxima are closely related while Nicolosi et al. (2000) hypothesized, from chloroplastic CAPS and nuclear genome analysis, that C. limon was derived from hybridization between C. aurantium (♀) and C. medica (♂). Cytogenetical studies of both the species do not show significant difference either in chromosomes association of chiasma frequency, therefore it is difficult to support the above theory of C. maxima as parental species. However the only parameter which is supportive for this observation is pollen stainability, which is drastically reduced in C. limon as compared to C. maxima.

Thus the phylogenetic inter-relationship of species presently investigated derived the support of molecular analysis of ITS region and male meiotic studies in ten different species of Citrus from north-east India.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mgene.2014.01.008.

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