Meta Gene 2 (2014) 237–251

Contents lists available at ScienceDirect

Meta Gene

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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ARTICLE INFO

Article history:
Received 6 November 2013
Received in revised form 25 January 2014
Accepted 28 January 2014
Available online 28 March 2014

Keywords:
North-east India
Univalents
Quadrivalents
Bayesian inference
Maximum parsimony
Species diversity
Citrus

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*

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http://dx.doi.org/10.1016/j.mgene.2014.01.008

reticulata with their unique status have been resolved into distinct clades with molecular approaches as well. *C. indica* which occupies a unique position in the phylogenetic ladder of the genus *Citrus* has been resolved as a distinct clade and almost behaving as an out-group. The presences of quadrivalents in *C. indica* also echo and support its unique 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.

Introduction

The genus *Citrus* L., the sole source of the Citrus fruits of commerce, belongs to the orange subfamily Aurantioideae 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* genepool 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*

(mandarin), 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; Shahsavar 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% acetocarmine 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:acetocarmine 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											
				Quadrivalents	Bivalents									Univalents		
					Total			Rings			Rods					
					No	Mean	Range	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	6	0.24 ± 0.66	0–2
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	6	0.244 ± 0.66	0–2
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	4	0.16 ± 0.55	0–2
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	16	0.64 ± 1.38	0–4
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	4	0.16 ± 0.55	0–2
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	16	0.64 ± 1.1	0–4
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	8	0.34 ± 0.74	0–2
C. reticulata	IC 583264	25	18	–	225	9 ± 0	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	2	0.08 ± 0.4	0–2
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	22	0.88 ± 1.16	0–4

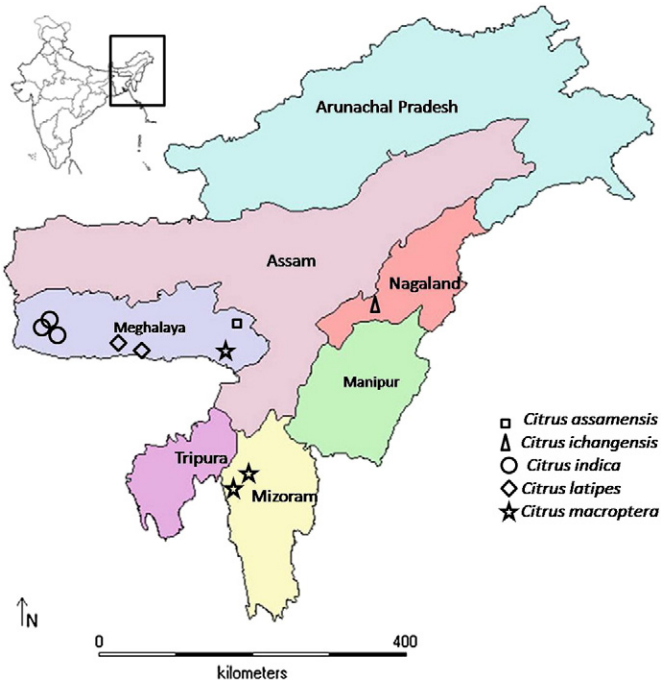

Fig. 1. Collection sites of wild and semi-wild *Citrus* species from North-east India.

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 (%)
				No	Mean	Range	No	Mean	range	No	Mean	Range			
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 ± 3.81	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 1 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 3. 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 limon*, *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

Table 3
Anaphase I distribution in *Citrus* species.

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

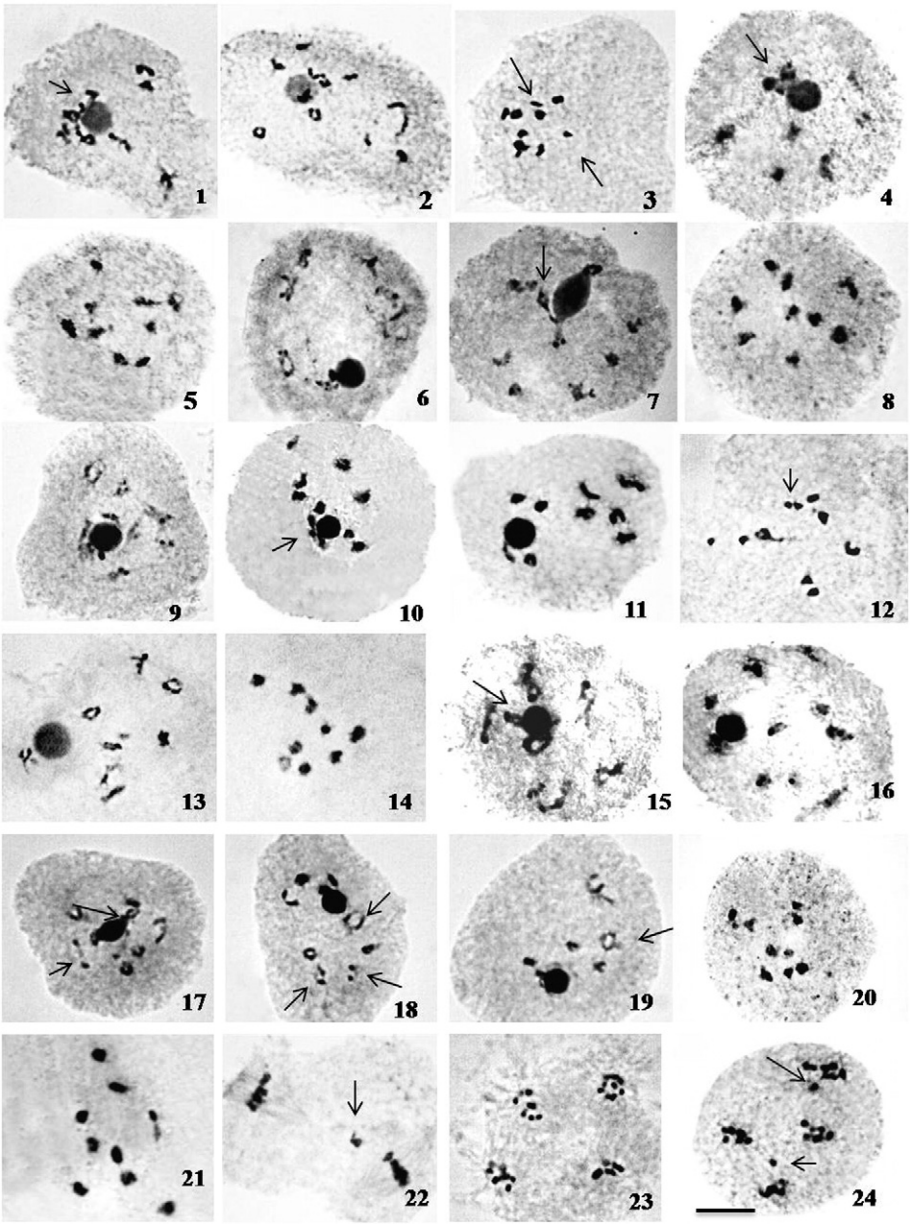

Fig. 2. Different male meiotic stages in 10 *Citrus* species. 1–3: diplotene, diakinesis & metaphase I in *C. limetta*; 4–5: diakinesis & metaphase I in *C. medica;* 6–8: diplotene, diakinesis & metaphase I in *C. latipes*; 9–10: dilpotene & diakinesis in *C. limon*; 11–12: diplotene & metaphase I in *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).

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.

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. limettioides*	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

ITS sequences data

The phylogenetic analysis based on sequence data related to ITS 1, ITS 2 and ITS 1 + 5.8 s + 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). Percentage

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

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

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*,

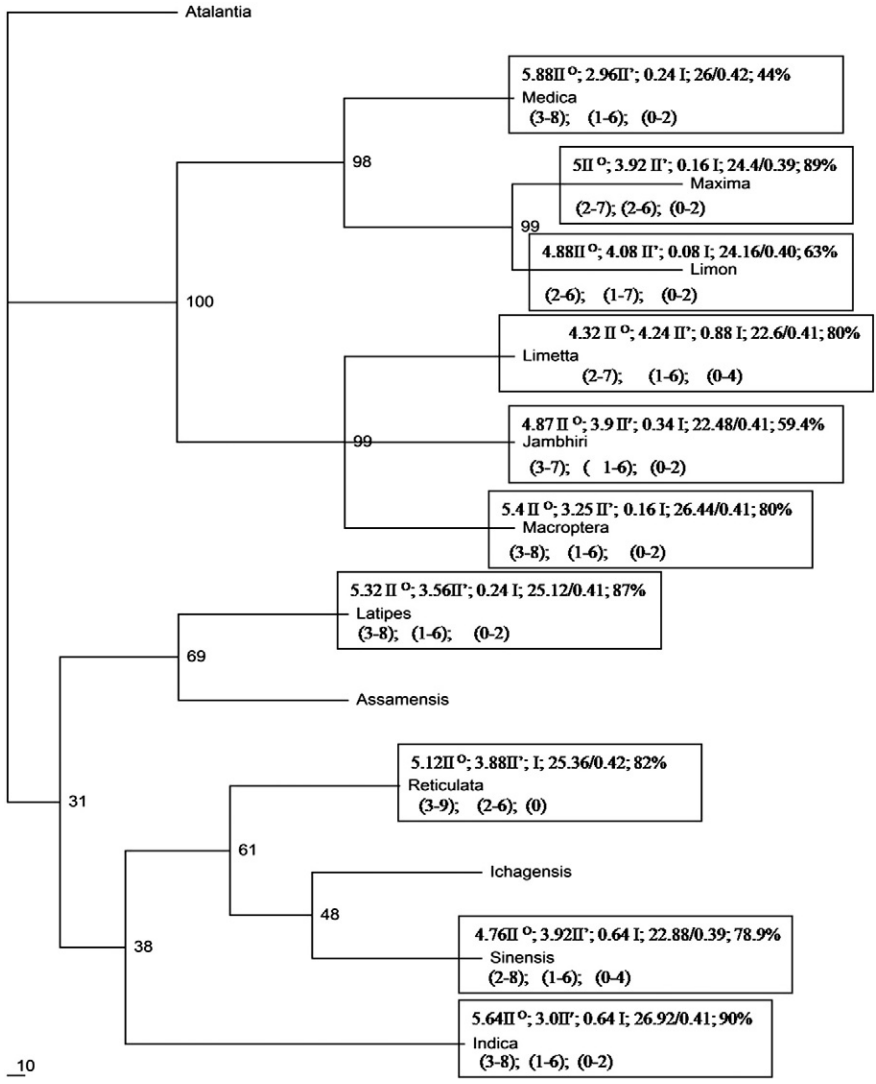

Fig. 3. 50% majority-rule consensus phylogenetic trees for illustrating the relationship among Indian representatives of the genus *Citrus* based on ITS of the rDNA sequence data using maximum parsimony method. Bootstrap values given at the nodes. The tree is rooted with the *Atalantia ceylanica*. The 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.

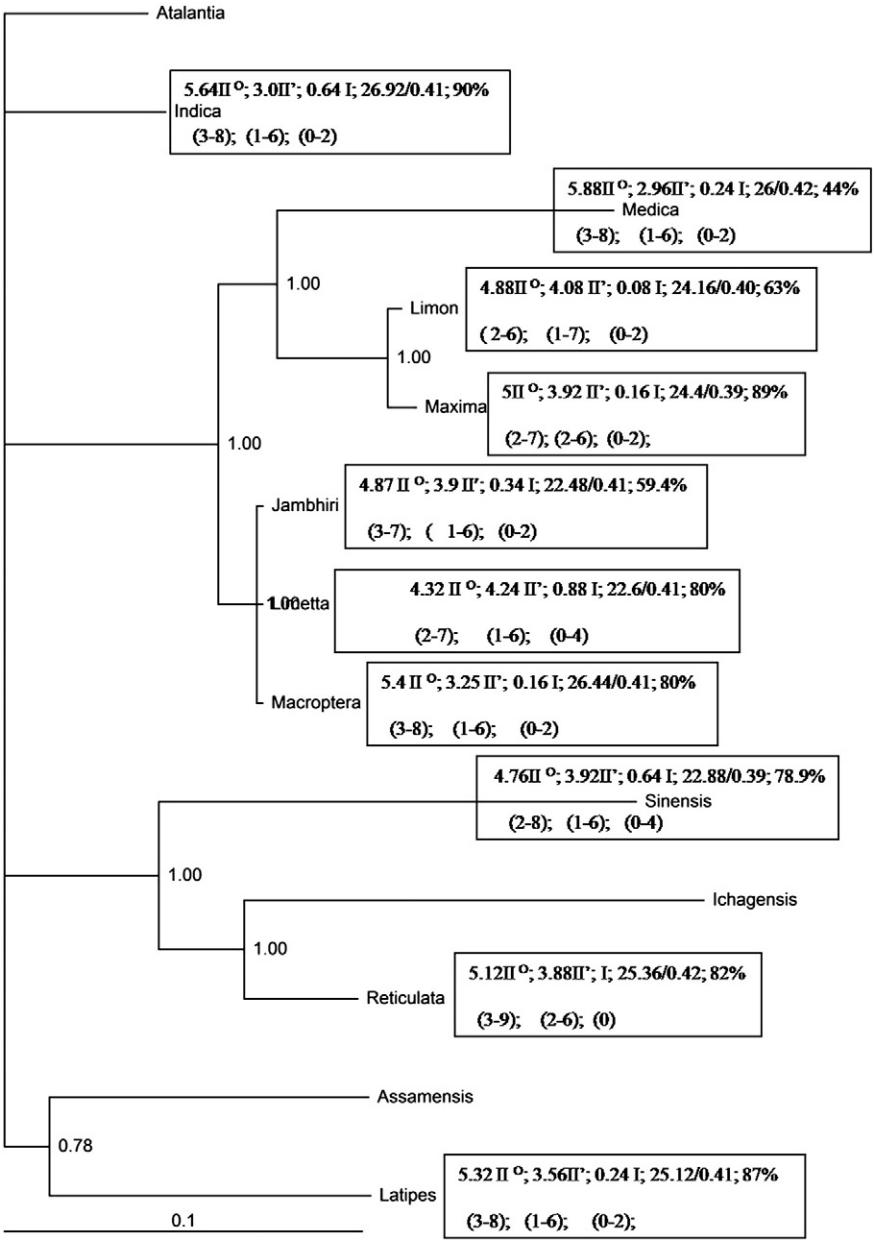

Fig. 4. Bayesian inference phylogenetic tree of 12 *Citrus* species based on ITS of the rDNA sequence data. The posterior probability is given on each node. The tree is rooted with the *Atalantia ceylanica*. The scale bar represents branch length (number of substitutions/site). The meiotic data of chromosome association, chiasma frequency per cell, terminalization co-efficient and pollen stainability followed by number of ring, rod bivalents and univalents is also given.

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

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. IIIa and IIIb. Sub-cluster IIIa comprised of *C. reticulata*, *C. ichagensis* and *C. sinensis* while sub-cluster IIIb 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 IIa 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 polyploidy 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 + 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. marcoptera* 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. ichagenesis* 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. ichangensis*, *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. ichagensis* is considerably larger as reflected in chromosome size (unpublished data) as compared to *C. latipes* and *C. macroptera*. Thus *C. ichangensis* 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. ichangensis* (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.

References

Abkenar, A.A., Isshiki, S., Tashino, Y., 2004. Phylogenetic relationships in the true *Citrus* fruit trees revealed by PCRRFLP analysis of cp DNA. Sci. Hortic. 102, 233–242.

Agarwal, P.K., 1984. Meiosis in an interspecific mandarin hybrid 'Kinnow' (*Citrus nobilis* Lour x *Citrus deliciosa* Tan.). Curr. Sci. 53, 547–548.

Agarwal, P.K., 1987. Cytogenetical investigations in Rutaceae I. Meiotic studies in four *Citrus* species of hybrid origin. Cytologia 52, 753–756.

Agarwal, P.K., 1989. Cytogenetical investigations in Rutaceae V. Cytomorphology of the three intergeneric hybrids of *Citrus* and *Poncirus*. Cytologia 54, 705–708.

Annual Report, 2010. ICAR Research Complex for NEH Region. Umiam-793 103, Meghalaya.

Araujo, E.F., Queiroz, L.P., Machado, M.A., 2003. What is *Citrus*? Taxonomic implications from a study of cp-DNA evolution in the tribe Citreae (Rutaceae subfamily Aurantioideae). Org. Divers. Evol. 3, 55–62.

Banerjee, I., 1954. Morphological and cytogenetical studies in *Citrus grandis* (Osbeck). Phytomorphology 4, 379–389.

Barkley, N.A., Roose, M.L., Krueger, R.R., Federici, C.T., 2006. Assessing genetic diversity and population structure in a *Citrus* germplasm collection utilizing simple sequence repeat markers (SSRs). Theor. Appl. Genet. 112, 1519–1531.

Barrett, H.C., 1977. Intergeneric hybridization of *Citrus* and other genera in *Citrus* cultivar improvement. Proc. Int. Soc. Citriculture 2, 586–589.

Barrett, H.C., Rhodes, A.M., 1976. A numerical taxonomic study of affinity relationship in cultivated *Citrus* and its closed relatives. Syst. Bot. 1, 105–136.

Bhattacharya, S.C., Dutta, S., 1956. Classification of *Citrus* fruits of Assam. *Science Monograph* 20. ICAR, New Delhi 110.

Burner, D.M., 1991. Cytogenetic analyses of sugarcane relatives (Andropogoneae: *Saccharinae*). Euphytica 54, 125–133.

Cameron, J.W., Frost, H.B., 1968. Genetics, breeding and nucellar embryony. In: Reuther, W., Batchelor, L.D., Webber, H.J. (Eds.), The Citrus Industry, vol. II. University of California Press, California, USA, pp. 325–370.

Chase, W., Morton, C.M., Kallunki, J.A., 1999. Phylogenetic relationships of Rutaceae: a cladistic analysis of the subfamilies using evidence from rbcL and atpB sequence variation. Am. J. Bot. 8, 1191–1199.

Chen, C.L., Guo, W.W., Yi, H.L., Deng, X.X., 2004. Cytogenetic analysis of two interspecific *Citrus* allotetraploid somatic hybrids and their diploid fusion parents. Plant Breed. 123, 332–337.

De Jong, J.H., Wolters, A.M.A., Kok, J.M., Verhaar, H., Van Eden, J., 1993. Chromosome pairing and potential for intergeneric recombination in some hypotetraploid somatic hybrids of *Lycopersicon esculentum* (+) *Solanum tuberosum*. Genome 36, 1032–1041.

Fang, D.Q., Krueger, R.R., Roose, M.L., 1998. Phylogenetic relationships among selected *Citrus* germplasm accessions revealed by inter-simple sequence repeat (ISSR) markers. J. Am. Soc. Hortic. Sci. 123, 612–617.

Federici, C.T., Fang, D.Q., Scora, R.W., 1998. Phylogenetic relationship within the genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. Theor. Appl. Genet. 96, 812–822.

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package) Version 3.6. (Distributed by the author) Department of Genome Sciences, University of Washington, Seattle.

Frost, H.B., Soost, R.K, 1968. Seed reproduction: Development of gametes and embryos. In: Reuther, W., Batchelor, L.D., Webber, H.B. (Eds.), The Citrus industry, Vol. 2. Univ. California Press, pp. 290–324.

Fusurato, K., 1957. Studies on polyembryony in *Citrus*. Rep. Kihara Inst. Biol. Res. 8, 40–48.

Herrero, R., Asins, M.J., Carbonell, E.A., Navarro, L., 1996. Genetic diversity in the orange subfamily Aurantoideae. I. Interspecies and intragenus genetic variability. Theor. Appl. Genet. 92, 599–609.

Hore, D.K., Barua, U., 2004. Status of citriculture in north eastern region of India — a review. Agric. Rev. 25 (1), 1–15.

Iwamasa, M., 1966. Studies on the sterility in the genus *Citrus* with special reference to the seedlessness. Bull. Horticult. Res. Sta. Jpn. Ser. 6, 1–77.

Jena, S.N., Kumar, S., Nair, K.N., 2009. Molecular phylogeny in Indian *Citrus* L. (Rutaceae) inferred through PCR–RFLP and trnL-trnF sequence data of chloroplast DNA. Sci. Hortic. 199, 403–416.

Jobes, D.V., Thien, L.B., 1997. A conserved motif in the 5.8S ribosomal RNA (rRNA) gene is a useful diagnostic marker for plant internal transcribed spacer (ITS) sequences. Plant Mol. Biol. Rep. 15, 326–334.
Khan, I.A., 2007. *Citrus* Genetics, Breeding and Biotechnology. CAB International. http://dx.doi.org/10.1079/9780851990194.0000 (160 pp).
Khazanehdari, K.A., Jones, G.H., 1997. The causes and consequences of meiotic irregularity in the leek (*Allium ampeloprasum* spp. *porrum*); implications for fertility. Euphytica 93, 313–319.
Kumar, S., Nair, K.N., Jena, S.N., 2012. Molecular differentiation in Indian Citrus L. (Rutaceae) inferred from nrDNA ITS sequence analysis. Genet. Resour. Crop Evol. http://dx.doi.org/10.1007/s10722-012-9814-x.
Kumar, A., Rao, S.R., Rathore, T.S., 2002. Cytological investigation in some important tree species of Rajasthan IV. Male meiosis studies in the genus Salvadora L. Cytologia. 67, 105–115.
Kyndt, T., Dung, T.N., Goetghebeur, P., Toan, H.T., Gheysen, G., 2010. Analysis of ITS of the rDNA to infer phylogenetic relationship among Vietnamese *Citrus* accessions. Genet. Resour. Crop. Evol. 57, 183–192.
Liang, G., Xiong, G., Guo, Q., He, Q., Li, X., 2007. AFLP analysis and the taxonomy of *Citrus*. Acta Hortic. (ISHS) 760, 137–142.
Lu, Z., Zhou, Z., Xie, R., 2011. Molecular phylogeny of the true *Citrus* fruit trees group (Aurantioideae, Rutaceae) as inferred from chloroplast DNA sequences. Agric. Sci. China 10, 49–57.
Mabberley, D.J., 2004. *Citrus* (Rutaceae): a review of recent advances in etymology, systematics and medical applications. Blumea 49, 481–498.
Malik, S.K., Kumar, S., Singh, I.P., Dhariwal, O.P., Chaudhury, R., 2013. Socio-economic importance, domestication trends and in situ conservation of wild *Citrus* species of Northeast India. Genet. Resour. Crop Evol. http://dx.doi.org/10.1007/s10722-012-9948-x.
Moore, G., 2001. Oranges and lemons: clues to the taxonomy of *Citrus* from molecular markers. Trends Genet. 17, 536–540.
Morton, C.M., Grant, M., Blackmore, S., 2003. Phylogenetic relationships of the Aurantioideae inferred from cpDNA sequenced data. Am. J. Bot. 90, 1463–1469.
Motomura, T., Hidaka, T., Moriguchi, T., Akihama, T., Omura, M., 1995. Intergeneric somatic hybrids between *Citrus* and *Atalantia* or *Severinia* by electrofusion, and recombination of mitochondrial genomes. Breed. Sci. 45, 309–314.
Müller, K., 2005. SeqState: primer design and sequence statistics for phylogenetic DNA datasets. Appl. Bioinforma. 4, 65–69.
Murray, H.G., Thompson, W.F., 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8, 4321–4325.
Nair, K.N., Nayar, M.P., 1997. Rutaceae. In: Hajra, P.K., Nair, V.J., Daniel, P. (Eds.), Flora of India, vol IV. Botanical Survey of India, Calcutta, pp. 229–407.
Nicolosi, E., Deng, Z.N., Gentile, A., Malfa, S.L., Ciotinella, G., Tribulato, E., 2000. *Citrus* phylogeny and genetic origin of important species as investigated by molecular markers. Theor. Appl. Genet. 100, 1155–1166.
Pang, X.M., Hu, C.G., Deng, X.X., 2007. Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. Genet. Resour. Crop. Evol. 54, 429–436.
Penjor, T., Anai, T., Nagano, Y., Matsumoto, R., Yamamoto, M., 2010. Phylogenetic relationships of *Citrus* and its relatives based on *rbcL* gene sequences. Tree Genet. Genome 6, 931–939. http://dx.doi.org/10.1007/s11295-010-0302-1.
Penjor, T., Yamamoto, M., Uehara, M., Ide, M., Matsumoto, N., Matsumoto, R., Nagano, Y., 2013. Phylogenetic relationships of *Citrus* and its relatives based on matK gene sequences. PLoS ONE 8 (4), e62574. http://dx.doi.org/10.1371/journal.pone.0062574.
Pessina, D., Gentili, R., Barcacciab, G., Nicole'b, S., Rossic, G., Barbestia, S., Sgorbatia, S., 2011. DNA content, morphometric and molecular marker analyses of *Citrus limonimedica*, *C. limon* and *C. medica* for the determination of their variability and genetic relationships within the genus *Citrus*. Sci. Hortic. 129, 663–673.
Raghuvanshi, S.S., 1962. Cytologenetical studies in genus *Citrus* IV.Evolution in genus *Citrus*. Cytologia 27, 172–188.
Reports, Annual, 2012. Indian horticulture database. In: Tiwari, R.K., Mistry, N.C., Singh, B., Gandhi, C.P. (Eds.), National Horticultural Board. Ministry of Agriculture, Government of India.
Ronquist, F., Huelsenbeck, J.P., 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19, 1572–1574.
Scora, R.W., 1975. On the history and origin of *Citrus*. Bull. Torrey Bot. Club 102, 369–375.
Shahsavar, A.R., Izadpanah, K., Tafazoli, E., Tabatabaei, B.E.S., 2007. Characterization of *Citrus* germplasm including unknown variants by inter-simple sequence repeat (ISSR) markers. Sci. Hortic. 112, 310–314.
Sharma, B.D., Hore, D.K., Gupta, S.G., 2004. Genetic resources of *Citrus* of north-eastern India and their potential use. Genet. Resour. Crop. Evol. 51, 411–418.
Simmons, M.P., Ochoterena, H., 2000. Gaps as characters in sequence-based phylogenetic analyses. Syst. Biol. 49, 369–381.
Singh, R.J., 1993. Plant Cytogenetics. CRC Press, Inc., Boca Raton, Florida, USA.
Stebbins, G.L., 1971. Chromosomal Evolution in Higher Plants. Columbia University Press, New York.
Swingle, W.T., 1943. The botany of *Citrus* and its wild relatives. In: Webber, H.J., Batchelor, D.L. (Eds.), The Citrus Industry, vol 1. University of California, Berkeley, pp. 128–474.
Swingle, W.T., Reece, P.C., 1967. The botany of *Citrus* and its wild relatives. In: Reuther, W., Webber, H.J., Batchelor, L.D. (Eds.), The *Citrus* Industry, vol 1. University of California, Berkeley, pp. 190–430.
Tamura, K., Dudley, J., Nei, M., Kumar, S., 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. 24, 1596–1599.
Tanaka, T., 1928. On certain new species of *Citrus*. Stud. Citrologia 2, 155–164 (Japanese with English & Latin resume).
Tanaka, T., 1954. Species Problem in *Citrus*. Japanese Society for promotion of Science, Ueno 152.
Tanaka, T., 1977. Fundamental discussion of *Citrus* classification. Stud. Citrologia 14, 1–6.
Thompson, J.D., Gibson, T.J., Plewniak, F., Mongin, F.J., Higgins, D.G., 1997. Clustal X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25, 4876–4882.
Webber, H.J., 1967. History and development of the *Citrus* industry. In: Webber, H.J., Batchelor, D.L. (Eds.), The *Citrus* Industry, vol I. University of California, Berkeley, pp. 1–39.
White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis, M.A., Gelfand, D.H.J., Sninsky, J., White, T.J. (Eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York, pp. 315–322.
Xu, C.J., Bau, L., Zhang, B., Bei, Z.M., Ye, X.Y., Zhang, S.L., Chen, K.S., 2006. Parentage analysis of huyou (*Citrus changshanensis*) based on internal transcribed spacer sequences. Plant Breed. 125, 519–522.
Yan, G., Ferguson, A.R., McNeilage, M.A., Murray, B.G., 1997. Numerically unreduced (2n) gametes and sexual polyploidization in *Actinidia Euphytica*, 96 267–272.