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RESEARCH ARTICLE

Chloroplast and Mitochondrial DNA Assay in Solving Issues Related to the Taxonomy of Beveragial Tea Clones

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Abstract Despite utilization of multidisciplinary approaches including nuclear DNA assay, the taxonomy of world beveragial tea clones is still contentious and elusive. They are considered to be constituted by 1–34 *Camellia* species. In the present study physical mapping of the chloroplast genome in combination with six restriction endonucleases, and restriction fragment length polymorphism of the six (*nad*5, *nad*3, *atp*A, *rrn*26, *cox*I and *cox*III) mitochondrial genes in combination with six restriction endonucleases was analysed in 50 divergent beveragial tea clones. In chloroplast genomes, only six out of the 230 restriction sites identified by 186 enzyme-probe combinations exhibited variation among the tea clones. The six mutations were site mutations. The 36 enzyme-probe combinations in mitochondrial genome yielded 122 fragments that hybridized to the probe. Nine combinations generated monomorphic profiles across all the 50 clones while remaining 27 combinations produced 94 (77.0 %) polymorphic bands in 20 clones. To understand genetic relationships among the clones, Jaccard's similarity coefficient and UPGMA clustering algorithm were applied to the cpDNA and mtDNA data. Strong correlation was observed between the two data. Both data grouped the clones into three clusters with very little or no heterogeneity within the clones forming two clusters. The present study makes it abundantly clear that the beveragial tea is not constituted by 34 species. In fact, the origin of the present day beveragial tea clones, cultivated in ~30 countries, lies in the origin of India hybrid tea as a result of extensive hybridization between closely related Assam (*Camellia assamica* ssp *assamica*) type and China (*C. sinensis*) type germplasm when the seeds of the latter were introduced in north east India.

Keywords Beveragial tea clones · Chloroplast genome · Mitochondrial genome · Diversity · Taxonomic implications

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Introduction

Tea (*Camellia* L.; Camelliaceae) is world's most popular non alcoholic healthy soft beverage [1]. The brewing quality, flavor, liquor colour, and its many therapeutical values make it an ideal drink worldwide. The tender shoots of tea plant comprising of first 2–3 leaves and the bud are used for making commercial tea. The tea industry contributes significantly to the economy of several African and Asian countries. Approximately 80 % of the tea cultivation is concentrated in south and south-east Asian countries, namely India, China, Srilanka, Bangladesh, Japan, Myanmar and Thailand [2]. In 2007, world tea production grew by 3 % to reach 3.6 million metric tons, with China being the largest producer (11,86,500 tons) followed by India (9,49,220 tons) [3]. India and China contribute >50 % of the total tea production. Most of the tea produced in China and Japan is processed as unfermented or green tea. Tea grown in other countries and 40 % of that of China is fermented or black tea.

The taxonomy of beveragial tea is highly divergent and inconsistent [2, 4–10]. It is considered to be constituted by one to 34 species. Sealy [4] recognized only one species, *C. sinensis* with two well marked variants, *C. sinensis* var. *sinensis*, to which 'China type' were assigned, and *C. sinensis* var. *assamica*, the 'Assam type'. Wight [7], on the other hand, recognized the variants as three distinct species and subspecies, *C. sinensis*, the 'China type', and *C. assamica*, the 'Assam type' and *C. assamica* ssp. *lasiocalyx,* the 'Cambod type'. Since then, taxonomists have proposed several modifications to the above classification. Notably, Chang [11] reported that the beveragial tea is constituted by 17 species and three varieties of *C. sinensis*. Tan [6] added another 17 species and one variety of *C. sinensis* to the above list, and so on and so forth. The cultivated tea taxa are self-sterile and allogamous diploids ($2n = 2x = 30$). The spontaneous occurrence of triploid, tetraploid and hexaploid cytotypes have also been reported [2]. The outbreeding nature coupled with frequent spontaneous hybridization that takes place between different types of tea has further complicated the taxonomic treatment of tea. It is not possible to classify the tea clones into small finite number of taxa which are clearly circumscribed on morphological, karyotypic, biochemical, and (or) crossability features [12–16]. Despite the abundance of published information on characterization of cultivated tea by various DNA markers originating from nuclear genome [17–38], the correct identification and nomenclature of cultivated tea remains as elusive as ever. Such kind of classification is very important not only to taxonomists but also to plant breeders looking for sources of genetic variation. According to Stuart "The history of our botanical knowledge of the plant that yields the tea of commerce presents such as picture of ignorance, confusion and arbitrariness that it has deterred many a botanist from thorough revision of the literature" [2]. Due to unresolved taxonomic riddles aforementioned, the beveragial tea for brevity purposes are most commonly referred to as 'China type', [*Camellia sinensis* (L.) O. Kuntze], 'Assam type' [*C. assamica* ssp. *assamica* (Masters) Wight], 'Cambod type' [*C. assamica* ssp. *lasiocalyx* (Planch Ms)] and their 12 intermediary morphotypes, such as Assam Cambod, China Cambod, Cambod China, etc. [2, 30, 39, 40].

An increasing number of studies have compared organelle genomes with a dual purpose of not only characterizing variation and relatedness among cytoplasms but also to determine the scope of variability in chloroplast and (or) mitochondrial genomes in assessing the phylogenetic relationships between and within plant taxa. Such studies have been altogether ignored for the cultivated tea clones or *Camellia* as such.

The chloroplast genome variation is useful tool for assessing phylogenetic relationships at inter-species level [41–44]. Highly conservative mode of evolution with small (120–217 bp) genome size makes chloroplast DNA (cpDNA) most suitable for phylogenetic studies. The distinctive features with regard to the evolution of plant mitochondrial genome make it also a powerful tool for aforementioned analysis [45–49]. In the present study, comprehensive chloroplast and mitochondrial DNA assay was undertaken to obtain new information which has a bearing on resolving the taxonomic riddles of the beveragial tea clones, and to identify/determine the types of cytoplasm involved in the evolution of the clones.

Material and Methods

Plant Material

The main features of the representative 50 tea clones investigated are given in Table 1. The clones, vegetatively propagated by nodal cuttings, were obtained from United Planters Association of South India (UPASI) Tea Research Foundation, Valparai, Coimbatore, India. Majority of these clones are the progenies of plants and (or) seed stocks brought from Assam, China and other geographically unattributed sources that were initially planted for experimental purposes in hilly areas of Nilgiris and other parts of south India. Consequent upon natural selections made from the tea growing areas in south India, and (or) intensive hybridization programme, UPASI released 27 clones (UPASI 1-UPASI 27) for mass cultivation in south India. TRI clones were released by Tea Research Institute, Sri Lanka. The high yielding clones TRF 1 and TRF 2 are biclonal seed stocks produced by crossing two clones UPASI 21 and TRI 2025. Similarly, clones BSS I and BSB I are also biclonal sections.

Total DNA Isolation, Restriction Endonuclease Digestion and Southern Blotting

Total genomic DNA of individual clones was separately extracted from young leaves following the modified CTAB method described by Sue et al. [50]. Total DNA of each clone was digested separately with restriction endonucleases according to manufacturer's instructions, and fractioned by 0.85 % Agarose gel electrophoresis (AGE) in 1× TAE buffer [51]. Lambda phase DNA digested with Hind III was loaded on each gel as a size standard. The digested DNAs were allowed to migrate on gel for about 7 cm. After AGE, the DNA was blotted onto a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, UK) by the alkaline transfer method [52].

Table 1 List of the tea clones used in the present study

Clone	Source of the material	Remarks	Codes used in figures
UPASI 1[a]	Brooklands Estate, The Nilgiris	Good rooter, drought resistant, thrive at mid elevation	UP
UPASI 2	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, drought resistant, thrive at all elevations	UP
UPASI 3	Brooklands Estate, The Nilgiris	Good rooter, drought resistant, high yield, excellent spread, thrive at all elevations	UP
UPASI 4	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, thrive at all elevations	UP
UPASI 5	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, thrive at all elevations	UP
UPASI 6	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, drought resistant, flower during drought	UP
UPASI 7	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, drought resistant, thrive at all elevations	UP
UPASI 8	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, thrive at all elevations	UP
UPASI 9	Brooklands Estate, The Nilgiris	Excellent rooter, excellent spread, drought resistant, thrive at all elevations	UP
UPASI 10	Brooklands Estate, The Nilgiris	Excellent rooter, drought resistant, thrive at all elevations, flower during drought	UP
UPASI 11	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, thrive at mid elevation	UP
UPASI 12	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, thrive at mid elevation	UP
UPASI 13	Brooklands Estate, The Nilgiris	Good roote, excellent spread, good tea quality	UP
UPASI 14	Singara Estate, The Nilgiris	Good rooter, excellent spread, blister blight resistant, good tea quality	UP
UPASI 15	Springfield Esate, The Nilgiris	Good rooter, drought resistant, frost resistant	UP
UPASI 16	Brooklands Estate, The Nilgiris	Good rooter, drought resistant, frost resistant	UP
UPASI 17	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, good tea quality, thrive at all elevations	UP
UPASI 18	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, drought resistant	UP
UPASI 19	Brooklands Estate, The Nilgiris	Slow rooter, excellent spread, drought resistant, frost resistant, Good tea quality	UP
UPASI 20	Brooklands Estate, The Nilgiris	Good rooter, drought resistant, good tea quality	UP
UPASI 21	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, good tea quality	UP
UPASI 22	Brooklands Estate, The Nilgiris	Excellent rooter, excellent spread, average yield, good tea quality	UP
UPASI 23	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, drought resistant	UP
UPASI 24	Brooklands Estate, The Nilgiris	Good rooter, excellent spread, drought resistant	UP
UPASI 25	Kulikkadu, UPASI TRF, Anamallias	Good rooter, excellent spread, blister blight resistant, average yield	UP
UPASI 26	Devarshola Estae, Nilgiri-Wynaad	Good rooter, drought resistant, high yield	UP
UPASI 27	Anaimudi Estate, Anamallais	Good rooter, excellent spread, drought resistant, high yield	UP
TRI 2024	Tea Research Institute, Sri Lanka		TR 24
TRI 2025	Tea Research Institute, Sri Lanka		TR 25
TRI 2026	Tea Research Institute, Sri Lanka		TR 26
TRI 2043	Tea Research Institute, Sri Lanka		TR 43
AKK 1	Akkamalai, Anamallais		AK 1
ATK	Attikuna, Nilgiri-Wynaad	Good rooter, excellent spread, good tea quality, drought resistant	ATK
CR 6017	Craigmore, The Nilgiris	Drought resistant, good tea quality	CR 17
C 17	BBTC, Singamapatty	Average yield	C 17
BSB 1	UPASI-10 × TRI 2025	Slow rooter, high yield, good tea quality	BSB
BSS 1	UPASI-10 × TRI 2025	Excellent spread, drought resistant	BSS 1
K 18			K18
TTL 1	Tata Tea Limited, Munnar	Good tea quality	TTL 1
TTL 2	Tata Tea Limited, Munnar	Good tea quality	TTL 2
CH1	UPASI TRF, Anamallais	Average yield	CH 1
TRF 1	Arrapetta, Wynaad	Highest yield, drought resistant, good tea quality	TRF 1
TRF 2		Average yield, excellent spread, good tea quality	TRF 2
NLT 17/10		Average yield, good tea quality	N/17

Table 1 continued

Clone	Source of the material	Remarks	Codes used in figures
SMP	High Wayves, Tea Estates India	Blister blight susceptible, excellent spread	SMP
TES 34			TES
B/5/163			B/5/163
Wild 1	UPSAI TRF, Anamallias		WILD 1
Wild 2	UPSAI TRF, Anamallias		WILD 2
SA 6	High Wayves, Tea Estates India		SA 6

TRF Tea Research Foundation

[a] *UPASI* United Planters Association of South India

Probe Preparation and Labeling

The details of chloroplast and mitochondrial DNA probes used in the present study are given in Tables 2 and 3. The probes were prepared from gel purified restriction fragments to obtain inserts free of the cloning vectors. The probes were denatured and chemically labeled overnight at 37 °C with fluorescein -11-dUTP (FI-dUTP) (Amersham Pharmacia Biotech) in the presence of exonuclease free klenow, random nanomer primers and fluorescein nucleotide mix (Amersham Pharmacia Biotech). The labeled probes were precipitated with cold (−80 °C) ethanol for 3 h and dissolved in appropriate volume of TE buffer and kept at 4 °C for further use.

Table 2 Tobacco chloroplast probes used for mapping cp DNA in tea clones

S. no	Probe	Vector	Cloning site	Size of insert fragment (kb)
1	Bam 2	pBR322	*Bam*HI	18.1
2	Bam 5b	pBR322	*Bam*HI	7.1
3	Bam 11b	pBR322	*Bam*HI	3.27
4	Bam 26b	pBR322	*Bam*HI	0.48
5	Bam 22b	pBR322	*Bam*HI	1.23
6	Bam 6b	pBR322	*Bam*HI	5.16
7	Bam 14b	pBR322	*Bam*HI	2.95
8	Bam 24b	pBR322	*Bam*HI	1.11
9	Bam 23b	pBR322	*Bam*HI	1.19
10	Bam 7	pBR322	*Bam*HI	5.21
11	Bam 1	pBR322	*Bam*HI	19.6
12–16	S 6	pBR322	*Sal*I	15.4
17	X 9	pBR322	*XhoI*	5.6
18	Bam 9b	pBR322	*Bam*HI	4.56
19	Bam 9a	pBR322	*Bam*HI	4.46
20	Bam 19	pBR322	*Bam*HI	2.1
21	Bam 10b	pBR322	*Bam*HI	3.63
22	Bam 25	pBR322	*Bam*HI	1.06
23	Bam 12a	pBR322	*Bam*HI	3.25
24	Bam 13	pBR322	*Bam*HI	3.0
25	Bam 4	pBR322	*Bam*HI	8.8
26	Bam 10a	pBR322	*Bam*HI	3.62
27	Bam 26c	pBR322	*Bam*HI	0.42
28	Bam 15	pBR322	*Bam*HI	2.87
29	Bam 8	pBR322	*Bam*HI	5.1
30	Bam 24a	pBR322	*Bam*HI	1.1
31	Bam 23a	pBR322	*Bam*HI	1.2

Prehybridization, Hybridization and Immunological Detection

Prehybridization of the membrane containing restriction fragments of total cellular DNAs was performed at 68 °C overnight in 5 % (w/v) Dextran sulphate, 0.1 % (w/v) SDS, 1/20 dilution of liquid block, 5× SSC and 100 µg/ml sheared denatured heterologous DNA. Hybridization was done in the same condition with hybridization solution containing denatured labeled probe. Blots were washed for 15 min each in pre-heated (60 °C) solution of 0.1 % SDS in 1× SSC (Wash 1), and 0.1 % SDS in 0.5× SSC (Wash II). The blots were finally rinsed in a solution of 100 mM Tris-HCl (pH 9.5), 300 mM NaCl (Buffer A) for 1 h at room temperature. Blots rinsed in Buffer A were treated with appropriate volumes of solution containing 1/5000 Anti-fluorescein—AP conjugate, 0.5 % (w/v) BSA fraction V in Buffer A for 1 h at room temperature. After this, the membrane was given three changes of washing in 0.3 % (v/v) Tween 20 in buffer A for 10 min at room temperature. The membrane free of excess of wash buffer was treated with appropriate volume of detection reagent (Amersham Pharmacia Biotech), and subsequently exposed to X-Omat^{TM} film (Kodak).

Sequential Overlapped Physical Mapping of Chloroplast Genome

Physical restriction maps for various restriction enzymes were constructed for 42 clones presently investigated. Each

Table 3 Mitochondrial DNA probes used in the present study

Probe	Informational content	Cloning site	Size of insert fragment (kb)	Origin of sequence
Cytochrome oxidase genes				
*cox*I	Cytochrome C oxidase subunit I	*Bam*HI + *Eco*RI	0.7	*Oenothera*
*cox*III	Cytochrome C oxidase subunit III	*Eco*RI + *Pst*I	1.1	*Oenothera*
Ribosomal RNA genes				
*rrn*26	26S ribosomal RNA gene	*Bam*HI + *Sal*I	5.1	Wheat
NADH genes				
*nad*3	NADH dehydrogenase subunit 3	*Bgl*II	1.4	Wheat
*nad*5	NADH dehydrogenase subunit 5	*Hin*dIII	5.6	Wheat
ATP Synthase gene				
*atp*A	Subunit A of the ATPase complex	*Eco*RI + *Hin*dIII	1.5 kb	Pea

nylon membrane bearing the restriction fragments for all the clones, for the given endonuclease were sequentially probed with each of the 31 cloned cpDNA fragments (Fig. 1; Table 2). The resulting RFLP patterns were grouped together for each of the specific enzyme-probe combination. The general order of single digestion fragments was indicated by their homology with specific cpDNA probes. Fragments known hybridizing to the adjacent probes (identified on the basis of known tobacco chloroplast gene map), and restriction site changes among cpDNA fragment of different taxa within probe regions were used to arrange fragments in linear order. The chloroplast genome was mapped in this manner for various restriction enzymes. Clone by clone comparison of these maps for each enzyme facilitated scoring of restriction sites with precision.

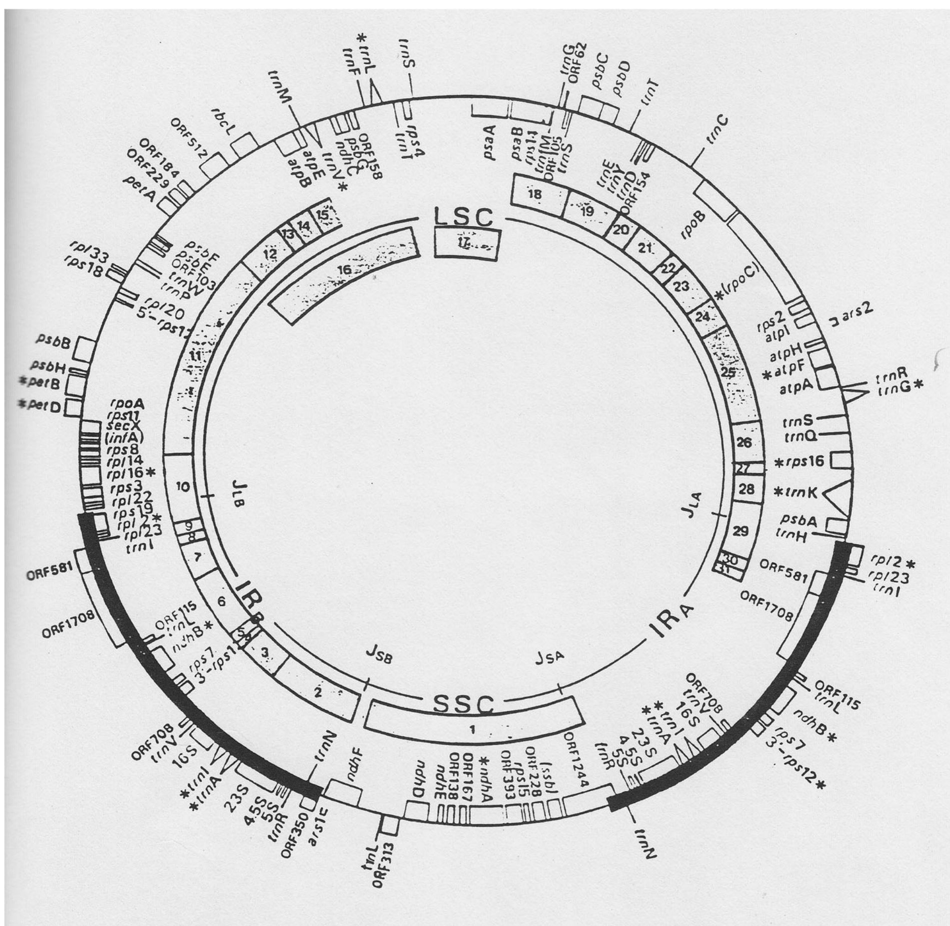

Fig. 1 Circular map of chloroplast DNA of *Nicotiana tabacum*. Position of 31 probes used in the present study are given inside the *circle*. Probe numbers given correspond to those given in Table 2

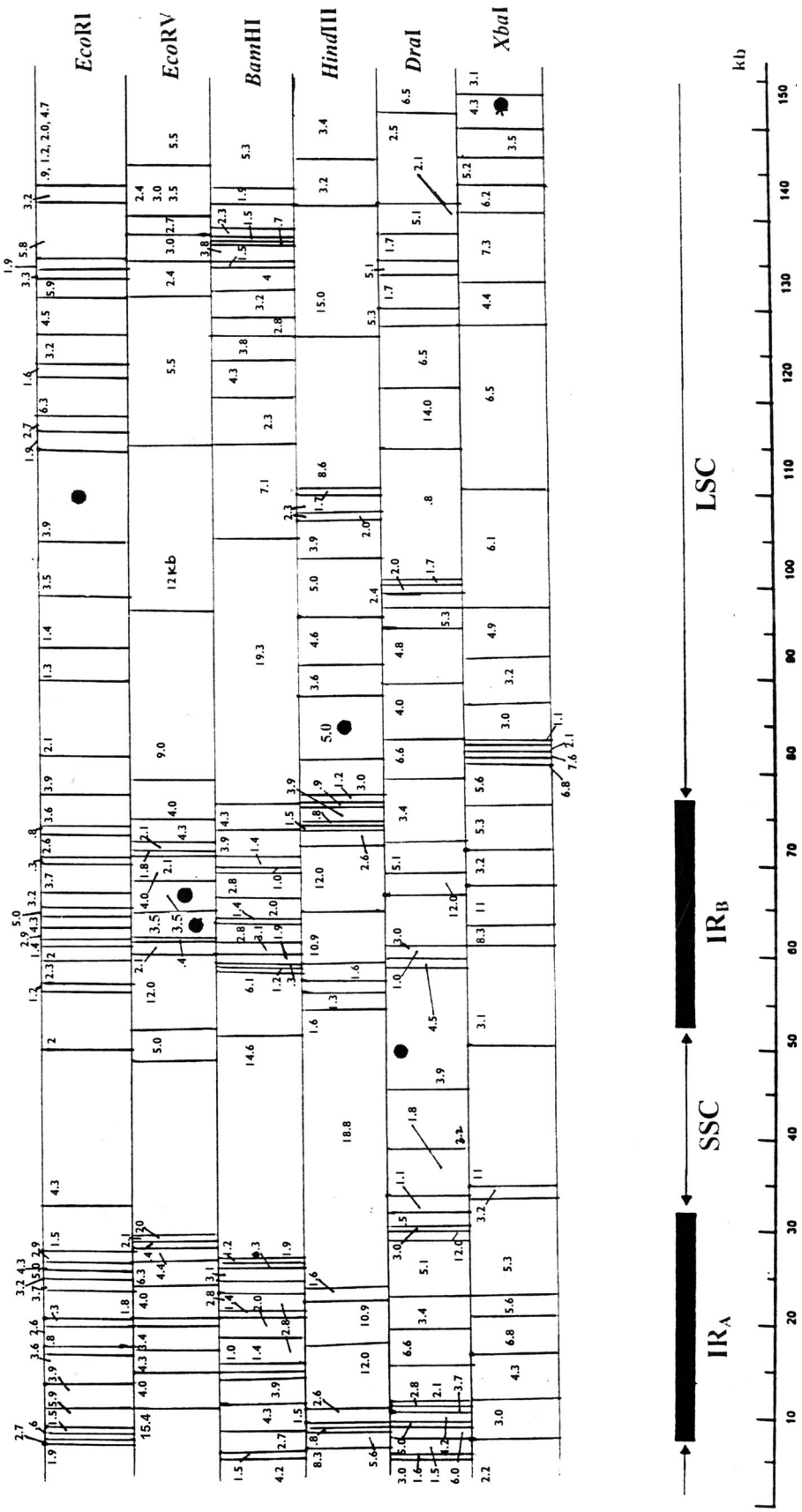

Fig. 2 Linearized physical map of UPASI 2 tea clone chloroplast DNA constructed by probing total genomic DNA restriction pattern of six restriction endonucleases with heterologous probes of tobacco cpDNA. IR_A and IR_B are the inverted repeat regions. The *numbers* written in maps are size of fragment in base pairs and they correspond to those presented in Table 4. *Bullet* in maps represents site mutations

Table 4 Size in kbp of chloroplast DNA restriction fragment of the tea clones. The number of fragment with identical sizes is given in parenthesis

Fragment no.	*Bam*HI	*Eco*RI	*Eco*RV	*Dra*I	*Hind*III	*Xba*I
1	19.3	6.3	20.0	14.0	18.8	11.0 (2)
2	14.6	5.9 (2)	15.4	12.0 (2)	15.0 (2)	8.3
3	7.1	5.8	12.0 (2)	6.6 (2)	12.0 (2)	7.6
4	6.1	5.0 (2)	9.0	6.5	10.9 (2)	7.3
5	5.3	4.8	6.3 (3)	6.0	8.6	6.8 (2)
6	4.3 (3)	4.7	5.5	5.3 (2)	5.0	6.5
7	4.2	4.5	5.0	5.1 (4)	4.6	6.2
8	4.0	4.3 (2)	4.4 (2)	5.0	4.2	6.1
9	3.9 (2)	3.9 (3)	4.3 (2)	4.8	3.9 (3)	5.6 (2)
10	3.8 (2)	3.7 (2)	4.0 (4)	4.5	3.6	5.3 (2)
11	3.2	3.6 (2)	3.5	4.2	3.4	5.2
12	3.1 (2)	3.5	3.0 (2)	4.0	3.2	4.9
13	2.8	3.3	2.7	3.9	3.0	4.4
14	2.78 (4)	3.2 (4)	2.4 (2)	3.7	2.6 (2)	4.3 (2)
15	2.7	2.9 (2)	2.1 (4)	3.4 (2)	2.3	3.5
16	2.3 (2)	2.7 (2)	1.8 (2)	3.2	2.0	3.2 (3)
17	2.0 (2)	2.6 (2)	0.4 (2)	3.0 (3)	1.7	3.1 (2)
18	1.9 (3)	2.3		2.8	1.6	3.0 (2)
19	1.5 (2)	2.1		2.5	1.5	2.2
20	1.4 (4)	2.0 (3)		2.4	1.3	2.1
21	1.2 (2)	1.9 (3)		2.1 (2)	1.2	1.1
22	1.0 (3)	1.6		2.0	0.9	
23	0.7	1.5 (2)		1.8	0.8	
24	0.5	1.4 (2)		1.7 (3)		
25	0.3 (2)	1.3		1.6		
26		1.2 (2)		1.5		
27		0.9		1.1		
28		0.8 (2)		1.0		
29		0.6		0.8		
30		0.3 (2)		0.5		

Table 5 Chloroplast DNA mutation analysis in tea clones

Restriction enzyme	Probe	Site loss	Site gain	Tea clones
Xba I	B25-Ba4	4.4 (3.9 + 0.5)	–	UP26, CHI, CR17, C17, TRF1, TRF2
*Eco*RI	S6	3.9 (3.2 + 0.7)	–	CHI
Eco RV	B18–Ba11b	–	3.0 + 0.5 (3.5)	CHI
Eco RV	B18–Ba6b		3.0 + 0.5 (3.5)	CHI
*Hind*III	Ba1	5.0 (4.3 + 0.7)	–	UP26, C17, CR17
Dra I	Ba2	3.9 (3.5 + 0.4)		UP14, SA6

Data Analysis

The molecular size of each fragment was estimated using Bio-1D software (Image Analysis Software, Vilber Lourmat, France). Presence and absence of bands were scored as binary unit character (1 = presence, 0 = absence). Genetic dissimilarities based on Jaccard's coefficient were calculated using DARwin 5.0.158 software (http://Darwin.cirad.fr/); [53]. The resulting dissimilarity matrices were then clustered using UPGMA [54] and neighbour

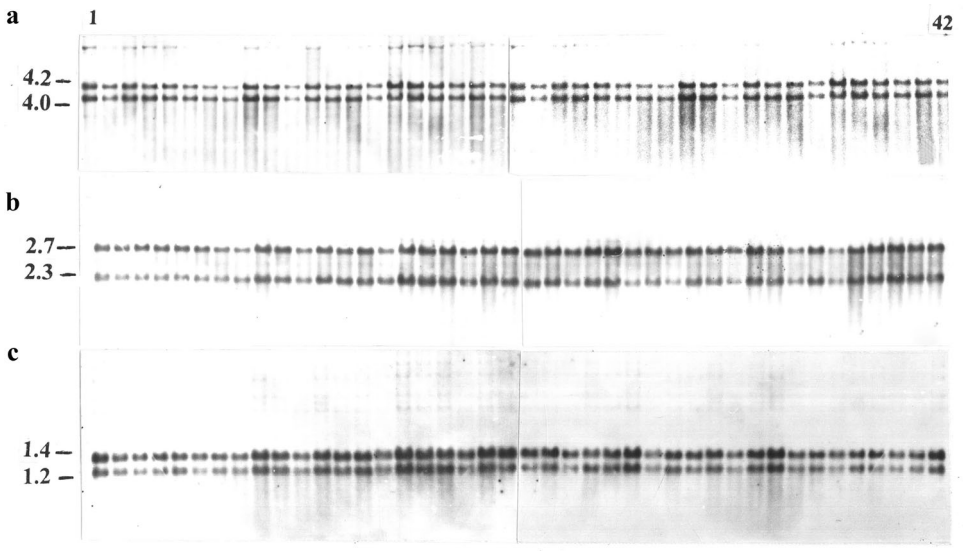

Fig. 3 *Bam*HI restriction fragments of total genomic DNA hybridized to cpDNA probes, Ba7 (**a**), Ba6b (**b**), Ba24b (**c**)

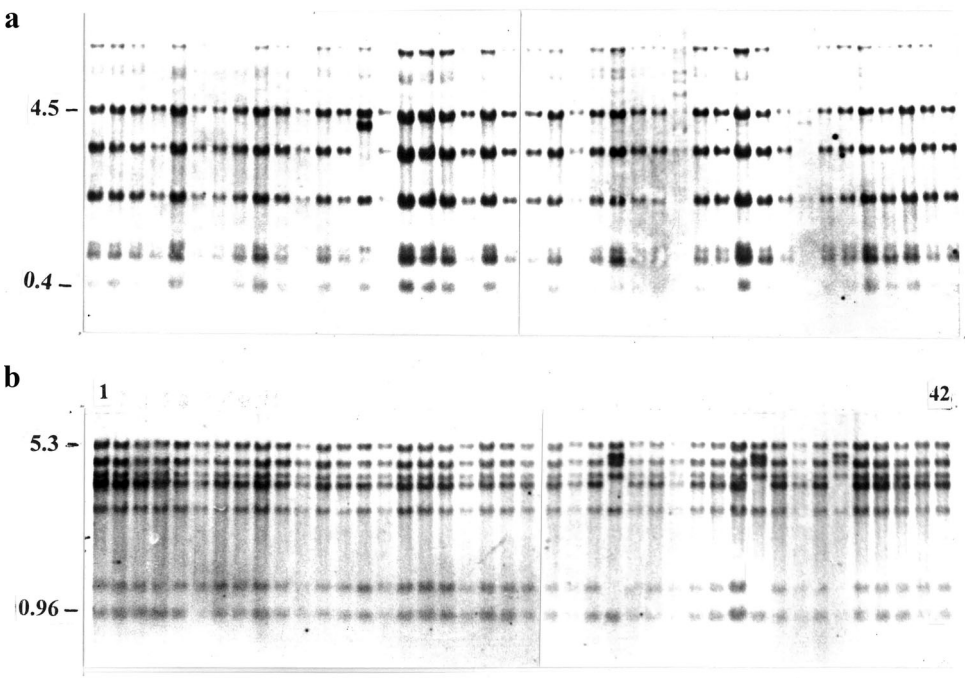

Fig. 4 *Dra*I (**a**) and *Hind* III (**b**) restriction fragments of total genomic DNA hybridized to cpDNA probes, Ba2 (**a**) and Ba1 (**b**)

joining [55] clustering methods. The robustness of dendrograms was evaluated by bootstrapping [56] with 1000 replications.

Results and Discussion

Chloroplast Genome

A diagrammatic representation of restriction sites in clone UPASI 2 mapped by six enzymes is given in Fig. 2.

Out of the six restriction enzymes used, EcoRI produced the maximum (52) number of hybridized fragments while HindIII produced the minimum (29) number of bands. BamHI, EcoRV, DraI and XbaI produced, 45, 32, 42 and 30 fragments each, respectively. Molecular weights of the bands generated by six restriction endonucleases ranged from 20.0 to 0.3 kb (Table 4). The size of the chloroplast genome was estimated on the basis of molecular weight of individual restriction fragments and their copy number (Table 4). Genome size determined averaged to 156.7 kb. Chloroplast genome size in tea is comparable to that of tobacco.

Mapping data showed that cpDNA of tea, like most of the angiosperm taxa, has four sectors and two inverted repeats, the large and small single copy regions. The

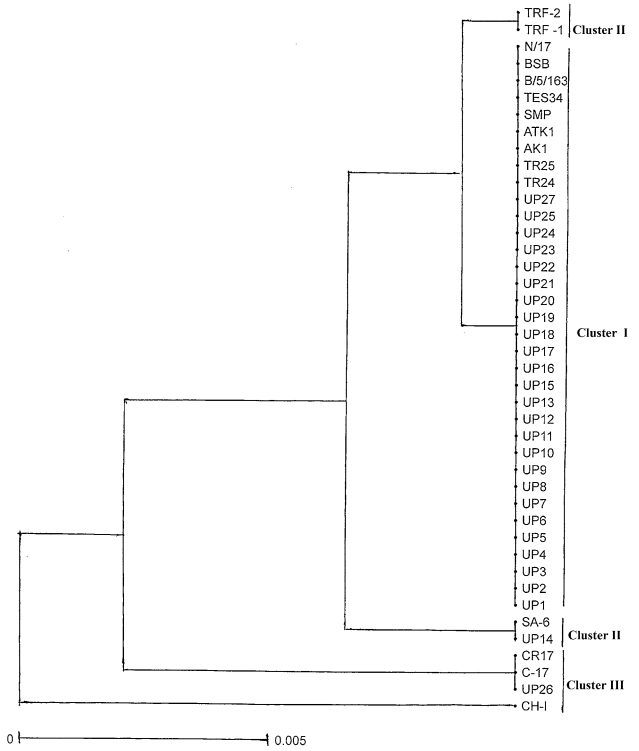

Fig. 5 Dendrogram, generated using UPGMA tree, of the tea clones based on chloroplast restriction site data

approximate sizes of the inverted repeat (IR), the large single copy region (LSC) and small single copy region (SSC) was determined to be 30, 80 and 20 kb, respectively. Interestingly, only six out of the 230 restriction sites identified by as many 186 combinations (31 probe and 6 enzymes) exhibited variation among the 42 tea clones. BamHI restriction fragment patterns that hybridized to the 3 probes were monomorphic in all the clones analysed (Fig. 3a, b, c). DraI showed one site mutation in clones SA 6 and UPASI 14 (Fig. 4a) when probed with Ba2. In these two clones due to site loss, two bands of 3.5 and 0.4 kb were replaced by one band of 3.9 kb (Table 5; Fig. 4a). Probe Ba1 in combination with HindIII detected one site mutation in UPASI 26, C-17 and CR 6017 clones (Fig. 4b) Single site loss in them resulted in replacement of two fragments of 4.3 and 0.7 kb by a band of 5.0 kb (Table 5). EcoRI digestion on probing with probe S6 detected one site mutation in clone CHI, where one band of 3.9 kb was obtained instead of two bands of 3.2 and 0.7 kb due to a site loss at this locus. EcoRV digestion detected two site mutations with Ba6b and Ba11b probes in clone CHI, where a single site gain resulted in replacement of 3.5 kb fragment by two fragments of 3.0 and 0.5 kb (Table 5). One site loss gave a fragment of 4.4 kb instead of two fragments of 3.9 and 0.5 kb in clones UPASI 26, CHI, CR 6017, C-17 (Selection A and Selection B in B 25-Ba4 and Xba I combination).

The two clustering methods, UPGMA and neighbour-joining, resolved 42 clones into three major clusters (Figs. 5, 6). Cluster I comprised of thirty four (UPASI 1–13, UPASI 15–25, UPASI 27, B/5/163, TRI 2024, TRI 2025, ATK, AKK, NLT/17/10, SMP, TES 34, BSB) clones. The remaining eight clones were delineated into cluster II and cluster III containing 4 (CH-1, CR-6017, C-17 and UPASI 26) and 4 (UPASI 14, SA 6, TRF-1 and TRF-2) clones each, respectively. All 34 clones in cluster I were grouped together at 0 % dissimilarity distance whereas in cluster II distance at which four clones were clustered ranged from 0.000025 to 0.39. Clone CH-1 was the most distantly related to this cluster. Clones in Cluster III showed dissimilarity distance of 0.0135. Cluster II and III were further divided into two subclusters each. In cluster II subcluster I contained three clones CR, C-17 and UPASI 26 clustered at 0 % dissimilarity distance. Subcluster II within cluster II had only one clone CH-1 which is grouped with subcluster I at 100 % bootstrap confidence. Cluster III also showed two subclusters. Clones SA6 and UPASI 14 formed one subcluster at 0 % dissimilarity distance grouped with the other subcluster (clones TRF-1 and TRF-2) at 65 % bootstrap value. Cluster II and cluster III were clustered together at 98 % confidence value. Grouping of cluster I with cluster II and cluster III was supported at 63 % bootstrap value.

Mitochondrial Genome

Thirty six enzymes (*EcoRI*, *DraI*, *XbaI*, *BamHI*, *HindIII* and *EcoRV*) probe (*nad*5, *nad*3, *atp*A, *rrn*26, *cox*1 and *cox*III) combinations produced a total of 122 bands in the 50 tea clones. The fragment size varied from 0.89 to 24.8 kb. The number of bands produced by a single-enzyme probe combination ranged from 1 to 7. Ninety four bands from a total of 122 were polymorphic giving 77.0 % polymorphism (Table 6). Out of the total 36 combinations used, nine (EcoRV + coxIII, DraI + rrn 26, XbaI + rrn 26, BamHI + cox III, EcoRI + nad5, EcoRI + rrn26, HindIII + nad3, Hind III + rrn26, HindIII + cox III) did not show any polymorphism across all the 50 clones analysed. Infact, the remaining 27 combinations also produced entirely monomorphic bands in 30 clones (UPASI 1–8, UPASI 10, UPASI 11, UPASI 12, UPASI 13, UPASI 13, UPASI 14, UPASI 15, UPASI 19, UPASI 22, UPASI 24, UPASI 25, UPASI 27, NLT 17/10, BSB I, SMP, SA 6, B/5/163, TES 34, Wild 1, Wild 2, K 18, BSS I). In other words, the polymorphism was observed to occur in the 20 clones (UPASI 26, CHI, CR 6017, C-17, TRF 1 TRF 2 TRI 2024, TRI 2025, TRI 2026, TRI 2043, TTL1, TTL2, AKK, ATK, UPASI 9, UPASI 16, UPASI 17, UPASI 18, UPASI 19, UPASI 20 AND UPASI 23). A representative survey of the fragment patterns produced by enzyme-probe

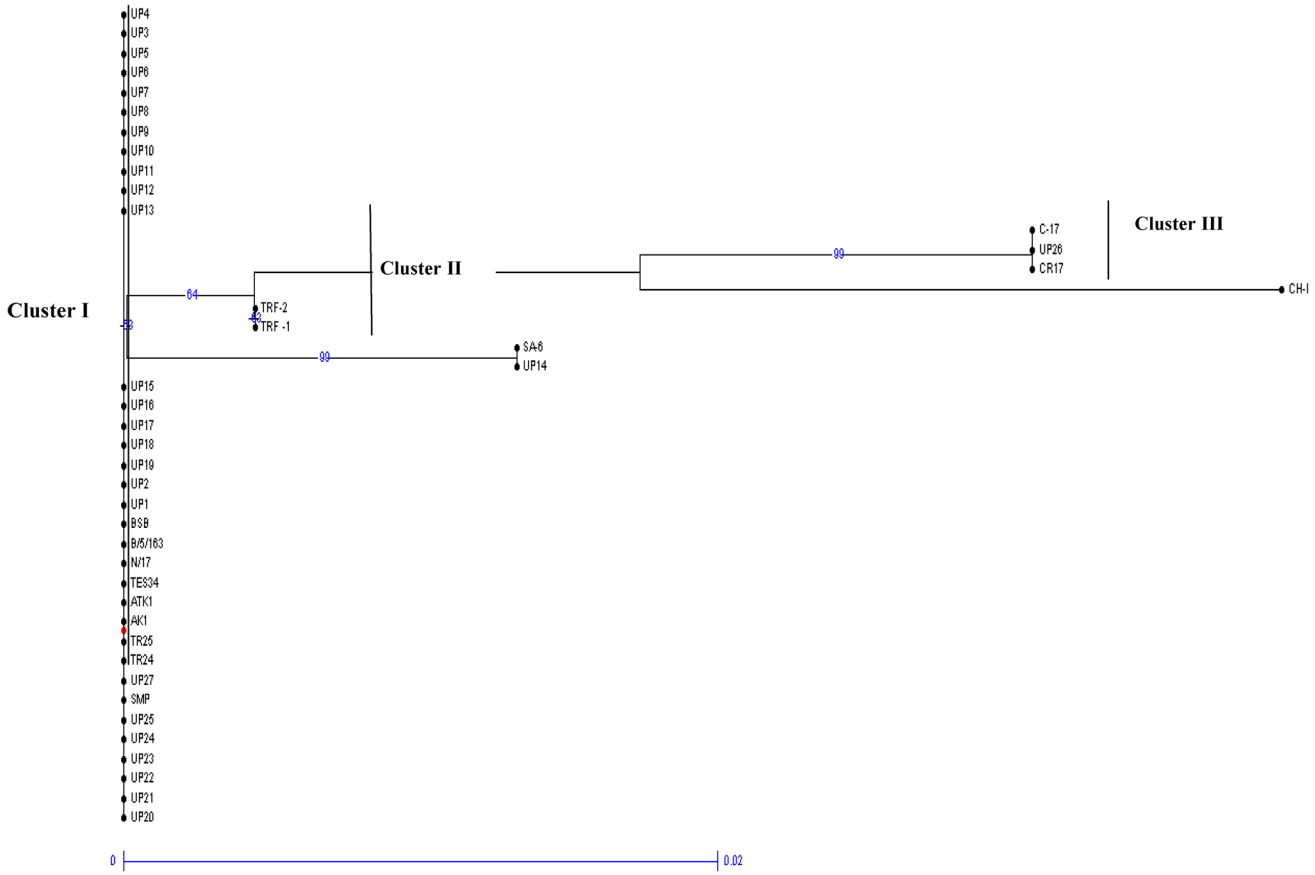

Fig. 6 Dendrogram, generated using NJ tree, of the tea clones based on chloroplast restriction site data

combinations is shown in Fig. 7 to illustrate several salient features of tea mitochondrial DNAs.

The two clustering methods (UPGMA and Neighbour-joining) resolved 50 clones in three major clusters (Figs. 8, 9). Dissimilarity coefficient values ranged from 0.000034 to 0.52206 among clones. Cluster I comprised of forty two (UPASI 1–25, UPASI 27, B/5/163, SA6, NLT/17/10, SMP, TES 34, BSB) clones and remaining nine clones were delineated into cluster II and Cluster III containing 4 (CH-1, CR-6017, C-17 and UPASI 26) and 6 (TRI 2024, TRI 2025, AKK 1, ATK, TRF-1, and TRF-2) clones each, respectively. All 32 clones in cluster I were grouped together at 0 % dissimilarity distance whereas in cluster II distance at which four clones were clustered ranged from 0.018 to 0.53. Clone CH-1 was the most distantly related to this cluster. Clones in Cluster III showed dissimilarity distance ranging from 0.01289 to 0.059. Cluster III was further divided into three subclusters. Clones TRI 2025, AKK and clones TRF-1, TRF-2 and TRI 2025 were grouped together into subcluster I and II, which were further clustered with AKK representing third subcluster within cluster III. In cluster II grouping of C-17 and UPASI 26 to clone CR 6017 was supported by 92 % bootstrap confidence and these clones were then grouped with CH-1 at 86 % bootstrap value. Subcluster I and II showed 78 and 66 % bootstrap values and their clustering with clone ATK showed a high bootstrap confidence of 93 %. Grouping of Cluster II with Cluster III showed 100 % bootstrap support whereas their relation with cluster I had 62 % bootstrap confidence.

Evolutionary changes in cpDNA can be categorized into two distinct classes, nucleotide substitutions (point mutation) and structural rearrangements (insertions, deletions, inversions and translocations). Both classes can be utilized for phylogenetic relationships. Point mutations, resulting from nucleotide substitutions are the most common sources of DNA variation. Nucleotide substitutions can be detected by restriction site analysis and direct sequence comparison. The use of restriction site variation has been widely used for inferring phylogenetic relationships in angiosperms [41, 57].

In the present study, comparative restriction site mapping of cpDNA was first time successfully investigated in 42 divergent beveragial tea clones by sequential 31 cpDNA

Table 6 Restriction fragment length polymorphism in mitochondrial genome of the tea clones

Enzyme	Probe	Total no. of hybridized bands	Monomorphic bands	Polymorphic bands	Size (kb)
*Eco*RV	*nad*5	3		3	21.4, 17.4, 16.5
	*nad*3	8		8	23.1, 18.4, 9.8, 8.4, 8.2, 7.7, 6.4, 5.4
	*cox*I	3		3	10.0, 9.8, 5.6
	*rrn*26	2	1	1	5.7, 4.8
	*atp*A	4		4	9.0, 8.7, 5.0, 2.5
	*cox*III	1	1		3.1
*Dra*I	*nad*5	2		2	13.8, 11.8
	*nad*3	6		6	13.6, 13.0, 12.4, 9.8, 7.5, 7.0
	*cox*I	4		4	8.9, 8.2, 6.0, 5.8
	rrn26	2	2		16.0, 11.2
	*atp*A	7		7	18.6, 17.9, 14.8, 13.9, 13.0, 2.3, 1.2
	*cox*III	3		3	18.7, 17.7, 14.5
*Xba*I	*nad*5	2		2	12.6, 10.1
	*nad*3	5	1	4	9.4, 8.9, 8.0, 7.7, 4.0
	*cox*I	4		4	17.2, 16.9, 11.7, 11.0
	*rrn*26	2	2		24.8, 19.5
	*atp*A	4	4	4	16.5, 15.8, 4.7, 2.5
	*cox*III	3		3	8.2, 7.9, 7.3
*Bam*HI	*nad*5	4	1	3	14.3, 13.5, 6.2, 2.5
	*nad*3	5	1	4	4.9, 3.9, 3.7, 2.6, 2.4
	*cox*I	4	4		7.9, 6.9, 3.7, 2.0
	*rrn*26	5	1	4	9.5, 9.4, 8.6, 5.3, 5.2
	*atp*A	3	2	1	16.2, 5.4, 3.3
	*cox*III	1	1		4.1
EcoRI	*nad*5	1	1		4.1
	*nad*3	5	1	4	7.3, 6.4, 2.3, 1.4, 4.1
	*cox*I	5		5	8.1, 8.0, 7.6, 6.9, 6.4
	*rrn*26	3	3		6.2, 4.6, 2.3
	*atp*A	2	1	1	4.7, 2.4
	*cox*III	3		3	4.2, 3.7, 3.2
HindIII	*nad*5	2		2	5.6, 5.4
	*nad*3	1	1		*2.2*
	*cox*I	5	3	2	5.4, 5.3, 5.0, 5.1, 4.9
	*rrn*26	4	4		2.0, 1.3, 1.0, 0.89
	*atp*A	3	3		5.4, 5.2, 4.5
	*cox*III	1	1		3.2
	36 combination*s*	122	28	94	0.89–24.8

probes (constituting 90 % of the cp genome) in combination with six restriction endonucleases, enabling sequential mapping of restriction enzyme cleavage sites (Fig. 2). It also permitted a more critical analysis of site mutations, discriminated from length mutations.

The chloroplast genome in the tea clones was characterized by very little variation. Hardly six mutations among recorded 230 restriction sites were observed. The six site mutations observed (Table 5) were interpreted as loss or gain of restriction sites. The interpretation was straight forward as the additive patterns of fragment sizes expected from restriction site loss/gain mutations was observed in each case. These mutations were not specific to certain region of the genome. Five were located in LSC region and one in SSC region. No other type of mutation(s) like deletion, insertions or inversion was observed in tea chloroplast genomes.

The variable genome size in conjugation with rapid evolution has made plant mt genome potentially powerful

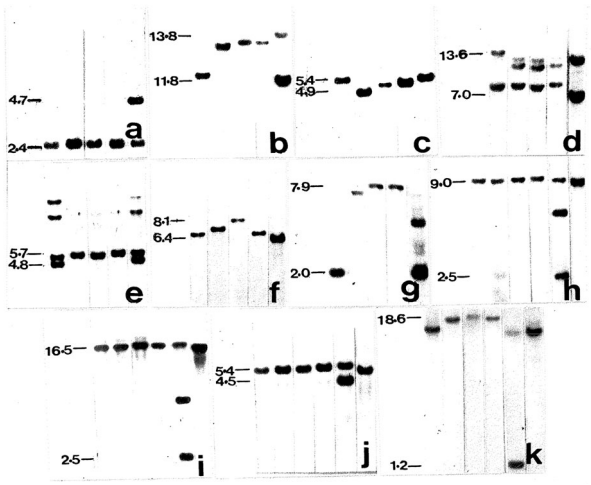

Fig. 7 **a**–**k** Representative restriction fragment length polymorphism patterns in tea clones digested and probed with *Eco*RI + atpA (**a**) *Dra*I + nad5 (**b**) *Hind*III + coxI (**c**) *Dra*I + nad3 (**d**) *Eco*RV + r-rn26 (**e**); *Eco*RI + coxI (**f**); *Bam*HI + coxI (**g**) (*Lanes* 1, 2,3,4, 5 represents cluster I; subcluster IIc, IIa, IIb and cluster III), Figs. 8 and 9; *Eco*RV + atpA (**h**); *Xba*I + atpA (**i**); *Hind*III + atpA (**j**); *Dra*I + atpA (**k**) (*Lanes* 1 2, 3, 4, 5, 6 represent cluster I, cluster II and subcluster IIIa and IIIb Figs. 8, 9)

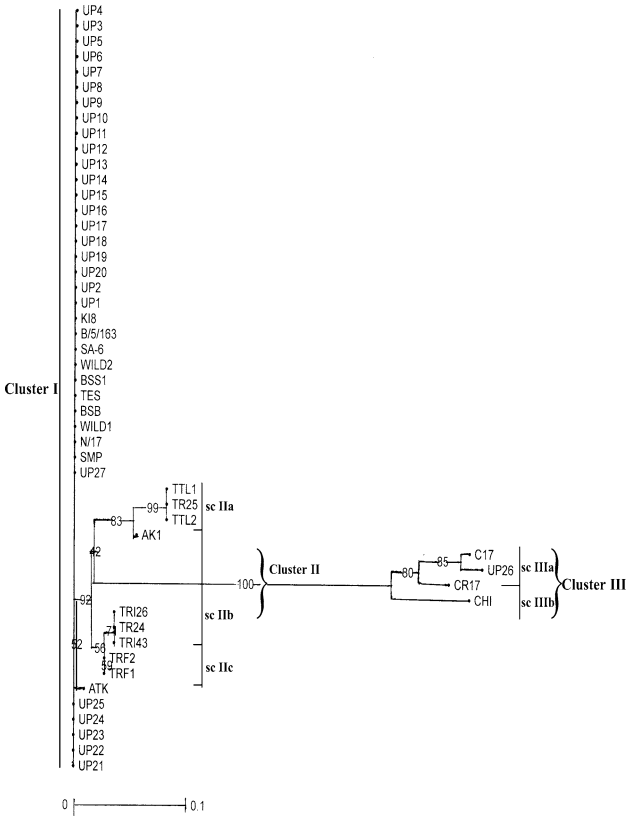

Fig. 8 Dendrogram, generated using NJ tree, of the tea clones based on mitochondrial DNA restriction fragment length polymorphism

tool for analysis of within and/or among population genetic structure and (or) phylogenetic relationships. The mtDNA might evolve unusually very rapidly, and in such cases, the phenetic classification based mtDNA RFLP pattern cannot index the divergence in critical manner. Such variation may be most suitable for population differentiation. In many cases, however, the amount and pattern of mtDNA diversity is such that it permits to quantitatively and qualitatively differentiate the species. [47], for example, it is pointed out that mt DNA RFLP patterns in *Triticum* and *Aegilops* were useful in clarifying phylogenetic relationships between different accessions of a species or even between species that had otherwise very similar chloroplast genome. Similarly, in *Vicia*, all the accessions within species had identical mtDNA phenotypes but RFLPs patterns between species were quiet distinct [58], and so on. The results presented in the present paper on mt genome are significant in that it can be said that the sort of variation found in the 50 clones establishes beyond doubt that there is considerable homeology or homology between the analysed mitochondrial genomes. The astonishing complexity in morphological diversity [59], and great range of variability to be found in nuclear genomes as assessed by multitude of DNA markers [17–22, 24–27, 29, 30, 38] is not reflected in mitochondrial genomes of the tea clones. In the present study, forty two clones in cluster I, showed completely homogenous mitochondrial genome. It indicates that the female parent of the drought resistant and vigorously growing segmental allotriploid [16] UPASI 3 is the same as that of other diploid clones clustered in cluster I. The mitochondrial RFLP patterns for UPASI 26, UPASI 27 and UPASI 3 obtained in the present study corresponded with the patterns reported by Devarumath et al. [60]. The clone TRF 1, a very recent selection, is considered to be a "wonder clone" as it is very high yielding quality tea clone most suitable for south India. The pattern of mt genome diversity and hence maternal lineage of this clone is similar to the other two clones (TRF2, TR2024) clustered in cluster III.

The clustering patterns obtained with chloroplast and mitochondrial DNA data showed distinctive congruence with each other. All the four dendrograms (Figs. 5, 6, 8, 9) grouped the beveragial tea clones into three main clusters. The representative clones, with few exceptions, in the three clusters were same for the mitochondrial and chloroplast genome. Eight more clones studied for mt genome are clustered in cluster I. The congruence between marker systems originating from two different genomes may be dependent on the type of sequence variation recognized by each marker system [61]. The morphologically most

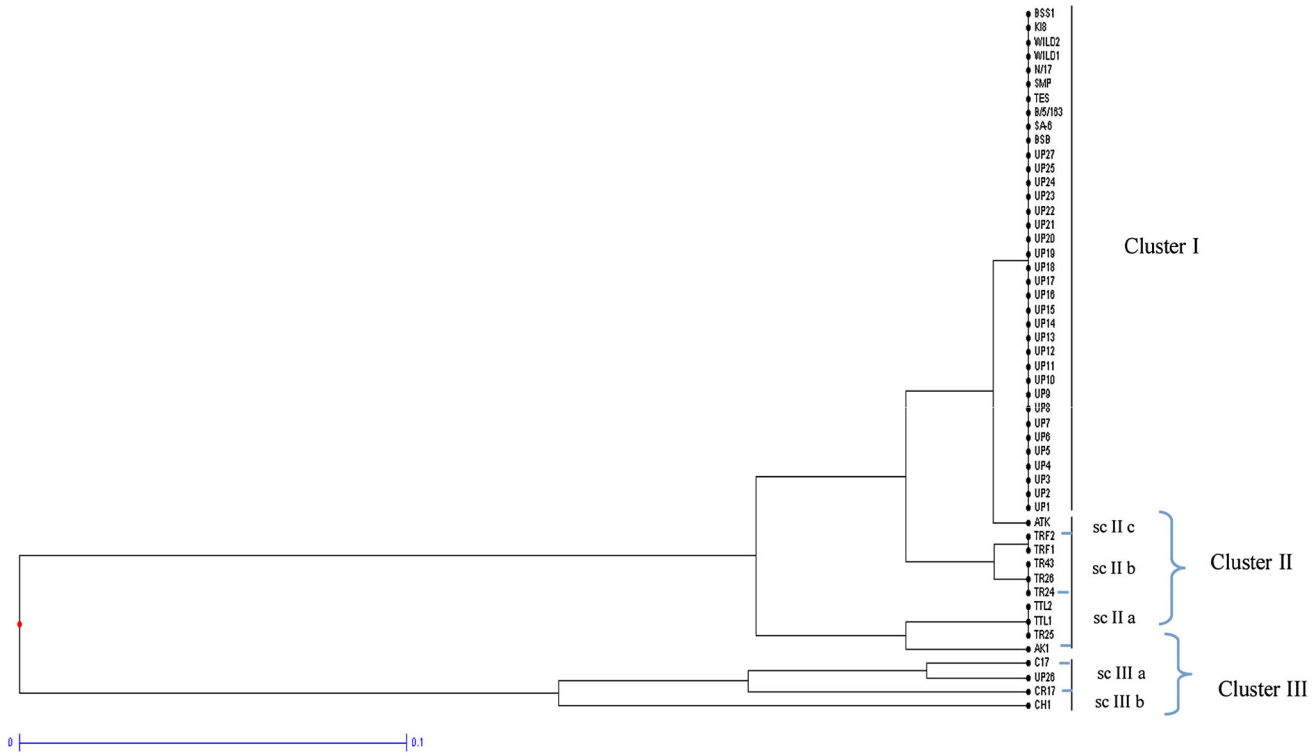

Fig. 9 Dendrogram, generated using UPGMA tree, of the tea clones based on mitochondrial DNA restriction fragment length polymorphism

distinct clone CH-1 was also most distantly related to other clones in both mt DNA and cp DNA data based dendrograms (Figs. 5, 6, 8, 9).

It is thus clear that the involvement of as many as 17 [16] or 34 *Camellia* species [6] in the origin of world beveragial tea is not supported by the present data.

The intrinsically low level of mutation events observed in tea clones in the present study approximate to the values obtained for within species variation and on this basis it seems reasonable to relegate the China type (*Camellia sinensis*); the Assam type (*C. assamica* ssp. *assamica*) and the Cambod type *C. assamica* ssp. *lasiocalyx* to a single species. This supports the widely accepted view that the origin of present day commercial tea clones cultivated in ~30 countries lies in the origin of India hybrid tea derived from extensive hybridization between Assam type tea (*C. assamica* ssp. *assamica*) and China type tea (*C. sinensis*) in north east India [1, 2, 30]. The hybridization between China type and Assam type tea germplasm resulted in many recognizable "specificness" intergrades that can be arranged in a cline based on morphological (morphotypes) characters extending from China type through intermediates like China type, China hybrid, China Assam, China Cambod, Assam hybrid, Assam China hybrid, Assam Cambod to Assam type and so on [30]. The present information could be of great help for plant breeders looking for beveragial tea improvement.

Conclusion

On the basis of DNA assay of both mitochondrial and chloroplast genomes, it is clear that the beveragial tea is not constituted by 34 species as reported. The present study demonstrates narrow genetic diversity in the beveragial tea clones. It supports the view that the origin of tea clones lies in extensive hybridization between closely related Assam (*Camellia assamica* ssp *assamica*) type and China (*C. sinensis*) type germplasm.

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