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Isolation and Restriction Analysis of Chloroplast DNA from Hevea

CHEONG KAY FONG*, KOH CHONG LEK** AND CHEW NYU PING*

A procedure for the isolation and purification of chloroplast DNA (ctDNA) from Hevea *is described. Differential centrifugation of* Hevea *leaf homogenates separates most of the plastids from nuclei. By means of sucrose density gradient centrifugation, chloroplasts of high integrity have been obtained. These chloroplasts were apparently nuclei-free when observed by phase-contrast microscopy. DNA was prepared and purified from the isolated chloroplasts by organelle lysis and density gradient isopycnic centrifugation. The purified ctDNAs obtained were amenable to digestions with restriction endonucleases. The low background of UV-fluorescent material in the electrophoretograms of restricted* Hevea *ctDNA was consistent with minimal nuclear DNA contamination in the ctDNA preparations. The average genome size of* Hevea *ctDNA as estimated by summation of* Hind*III*-, Pvu*II*-, Sal*I*-, *or* Xho*I*-*generated fragments was 152.3 kbp.*

In plants, chloroplasts are important organelles playing an exclusive role in the photosynthetic fixation of carbon dioxide. These specialised organelles themselves contain genomes comprising covalently closed circular and double-stranded DNAs. The chloroplast genomes of a wide variety of plants have been extensively studied and characterised[1-3] by recombinant DNA technology. For *Hevea brasiliensis* Muell. Arg., however, little is known about the molecular architecture and organisation of its chloroplast genome and the expression and regulation of chloroplast genes.

The prerequisite and initial step for such studies to be undertaken is the availability of pure *Hevea* chloroplast DNA (ctDNA) isolated from leaves. For this purpose, several published methods[4-7] for ctDNA isolation and purification had been tested but they were found to present practical problems for *Hevea*. Often, such preparation methods suffered from several disadvantages, one of which was the low and variable ctDNA yields. In addition, the presence of nuclear DNA (nDNA) in the isolated ctDNAs prevented the yield of consistent restriction enzyme digestion results, thus making restriction fragment pattern analysis and other studies difficult.

We have modified and combined the procedures described by Palmer[8] and Herrmann *et al.*[9] to establish a method that has been used for the routine preparation of ctDNAs from *Hevea* clones and species at the Rubber Research Institute of Malaysia (RRIM). Here, we report the protocol used to isolate and purify *Hevea* ctDNA amenable to restriction endonucleases digestions. The genome size of *Hevea* ctDNA was estimated to be 152.3 kbp.

MATERIALS AND METHODS

Unless stated, all chemicals used were purchased from Sigma Chemical Co., USA. Restriction endonucleases (Type II) were purchased from New England Biolabs (USA), Pharmacia (Sweden), and Promega (USA).

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Hevea leaves were obtained from clonal source bushes in the RRIM Experiment Station, Sungai Buloh, Selangor Darul Ehsan. Leaves of *H. brasiliensis* clone PB 86 and *H. camargoana* were obtained from *Field 15* and *Field 45*, respectively. The leaves of *H. brasiliensis* clones RRIM 600, RRIM 727, Tjir 1, GT 1, and PB 86 were harvested from *Field 49*.

Leaves were blended with a Virtis (USA) 45K homogeniser fitted with the Macro-Shear blade assembly.

Refractive index ($RI_{25°C}$) measurements of cesium chloride (CsCl) solutions were performed with an ABBE refractometer (Atago, Japan). Optical density ($OD_{260\ nm}$ and $OD_{280\ nm}$) measurements to quantify and determine the purity of isolated *Hevea* ctDNA were carried out in quartz microcuvettes (100 µl volume) on a Beckman (USA) DU65 spectrophotometer.

Isolated ctDNAs were digested with restriction endonucleases as described by the vendors. Electrophoretic separation of ctDNA fragments and staining with ethidium bromide (EtBr) were performed as described by Sambrook *et al.*[10] Molecular sizes of restricted ctDNA fragments were calculated from standard molecular weight curve (plotted with the 'Harvard Graphics Version 2.1' computer software. Software Publishing Corp., USA) derived from the electrophoretic mobilities of *Hin*dIII-digested phage lambda DNA fragments.

Chloroplast Isolation

Leaves were harvested in the morning, usually just after light. Only healthy (free from fungal infection), fresh-green, young and expanded leaves were selected.

The procedure for *Hevea* chloroplast isolation was modified from Palmer[8]. Leaves (200 g) were washed under running tap water and then twice in distilled water. They were dried in between clean paper towels, de-ribbed, and sliced into strips (approximately 2 mm width) in ice-cold Buffer A (4 ml/g fresh weight of leaves) containing 0.3 *M* D-sorbitol, 0.05 *M* Tris-HCl, 0.003 *M* EDTA, 0.1% β–mercaptoethanol, 0.3% polyvinylpyrrolidone (PVP), and 0.05% bovine serum albumin (BSA), pH 8.0. The leaf slices were blended with five pulses (3 s each) on high setting (80–100). Subsequent steps were conducted at 0°C – 4°C. The homogenate was filtered through three layers of cheesecloth and then through one layer of nylon mesh (20 µ, Spectramesh, USA). The filtrate was centrifuged at 1000 × g for 5 min on a high speed centrifuge with a swing-out bucket rotor. The supernatant was collected and re-centrifuged at 1000 × g for 15 min. The resulting crude chloroplast pellet was then suspended in 15 ml of Buffer B (0.3 *M* D-sorbitol, 0.05 *M* Tris-HCl, 0.025 *M* EDTA, 0.3% PVP, and 0.05% BSA, pH 8.0). Sucrose density gradients with stirred interface and freshly prepared in Buffer B were according to Palmer[8]. Chloroplast suspension was gently applied onto the gradients (6 ml of suspension per gradient) and then centrifuged in a swing-out rotor (TST 28, Kontron, Switzerland) at 25 000 r.p.m., 4°C for 35 min. The lower of the two green chloroplast bands was collected by means of a sterile Pasteur pipette and pooled. Five volumes of Buffer B was added, mixed and re-centrifuged at 1500 × g for 15 min. The pellet was washed twice by suspending it in the same volume of Buffer B and recentrifuging as before. The final pellet was suspended in 20 ml of Buffer C (0.3 *M* D-sorbitol, 0.05 *M* Tris-HCl, and 0.025 *M* EDTA, pH 8.0).

Purification of ctDNA by CsCl-EtBr Density Gradient Equilibrium Centrifugation

Hevea ctDNAs from isolated chloroplasts were purified by a procedure modified from Herrmann *et al.*[9]

Protease (Type XXV from *Streptomyces griseus*, DNase-free, 10 mg/ml prepared in Buffer C, self-digested at 37°C for 2 h) was added to the chloroplast suspension at one-tenth its volume. After incubating at ambient temperature for 30 min, one-tenth volume of lysis buffer (20% N-lauroylsarcosine, sodium salt, in TE buffer, pH 8.0) was added and the solution gently mixed by inversions. It was allowed to stand at 4°C for 2 h with inversions made every 15 min.

Saturated CsCl solution (in TE buffer, pH 8.0) was added to the lysate until its measured $RI_{25°C}$ was between 1.355 to 1.360. After standing at 4°C for 2 h, the mixture was centrifuged in a fixed-angle rotor (SS-34, Sorvall, Dupont, USA) at 10 000 r.p.m., 4°C, for 15 min to remove starch grains and organellar debris. The supernatant was collected and more saturated CsCl solution added until its $RI_{25°C}$ was 1.366. Each CsCl gradient comprised 15 ml of CsCl-EtBr solution (38 g CsCl in 40 ml of TE buffer, pH 8.0, and EtBr, 10 mg/ml, added at 1 ml per 100 ml of CsCl solution) and 5 ml of gently-layered lysate supernatant. The gradients were centrifuged in a swing-out rotor (TST 28, Kontron, Switzerland) at 25 000 r.p.m., 15°C, for 10 h.

After centrifugation, the ctDNA bands were gently withdrawn with a syringe fitted with a 19-G needle, under long wavelength UV illumination. The collected ctDNAs were pooled and its $RI_{25°C}$ adjusted to 1.388 by the addition of solid CsCl. A second CsCl gradient was formed in a fixed-angle rotor (TFT 80.13, Kontron, Switzerland). After topping up with CsCl-EtBr solution, the polyallomer tubes were capped and centrifuged at 55 000 r.p.m., 15°C, for 28 h. The UV-fluorescent ctDNA bands were withdrawn and pooled as before. Removal of EtBr was by repeated partitioning against an equal volume of isopropanol-CsCl-H_2O mixture (top layer from a mixture of equal volumes of saturated CsCl and isopropanol). The ctDNA was dialysed in prepared tubing (Bethesda Research Laboratories, USA) against 0.2 × SSC at 4°C with at least three changes of 5 litres each over 48 h. Isolated ctDNAs were stored at 4°C with a drop (20 µl) of chloroform added.

RESULTS AND DISCUSSION

Hevea ctDNA could be successfully isolated when fresh, young, and healthy (free from fungal infection) leaves were used. New, expanded and light-green leaves that sprouted soon after cutback of source bushes were found to be most suitable. From our experience, the more matured and dark-green leaves often presented difficulties during homogenisation owing to their tough fibrous nature. In addition, older leaves were observed to give low ctDNA yields and $OD_{260/280\ nm}$ ratios *(Table 1)*.

TABLE 1. UV ABSORPTION READINGS OF CHLOROPLAST DNA ISOLATED FROM MATURED AND YOUNG LEAVES OF *H. BRASILIENSIS*, CLONE RRIM 600

	Matured leaves	Young leaves
$OD_{260\ nm}$	0.116	0.340
$OD_{280\ nm}$	0.096	0.178
$OD_{260/280\ nm}$	1.2	1.91
ctDNA (µg/ml)	5.8	17.0

In order to minimise the presence of leaf starch in ctDNA isolation, we used leaves that were harvested in the early morning (not long after light). However, for *Hevea*, we found that the amount of ctDNAs isolated from de-starched leaves was not significantly different from that from normal leaves.

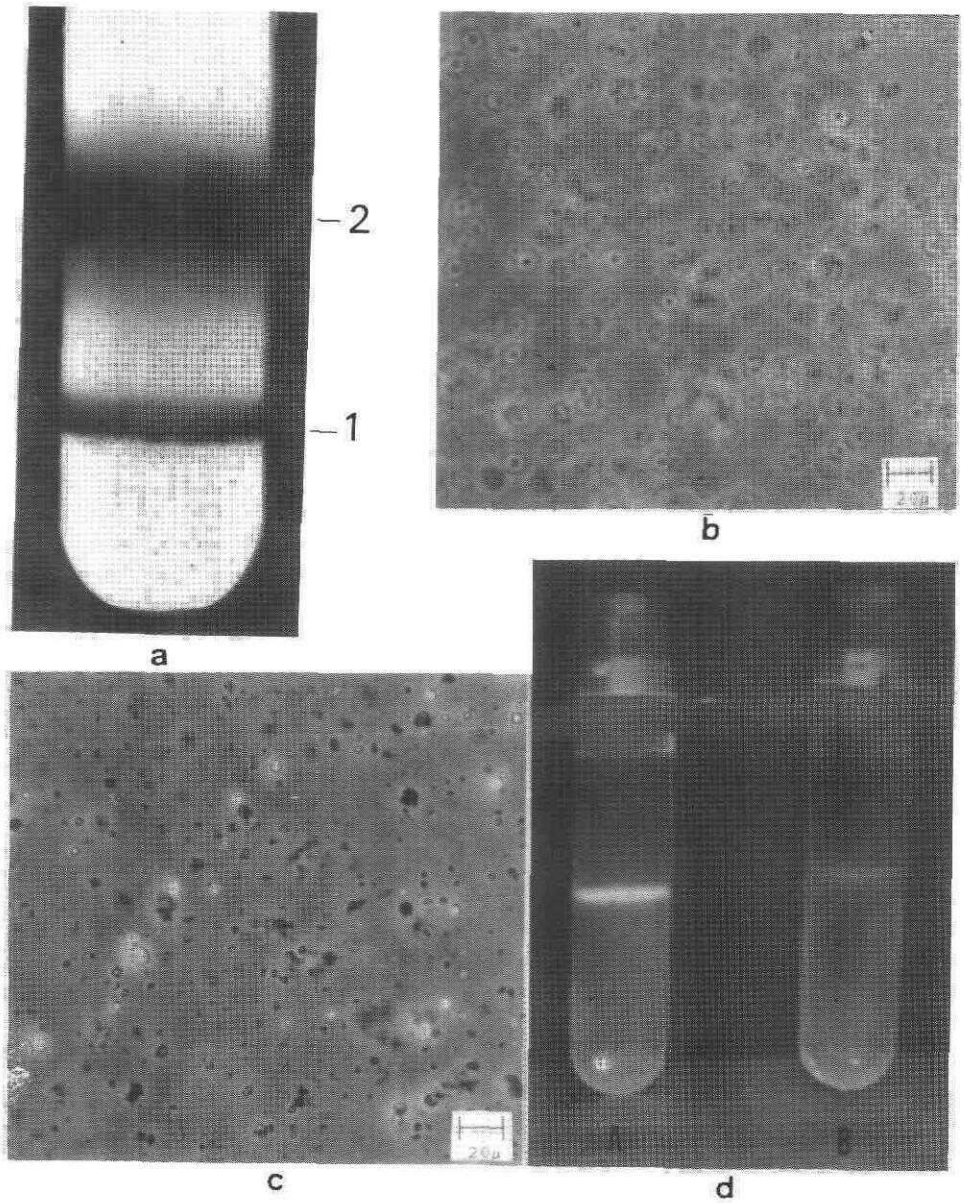

Figure 1. (a) Distribution of chloroplasts into bands 1 and 2 after the centrifugation of Hevea *leaf homogenates in sucrose density gradients. (b) Phase-contrast microscopy of chloroplasts from band 1. Note the predominant presence of intact organelles 'surrounded by halos'. (c) Phase-contrast microscopy of chloroplasts from band 2. Note the predominant presence of cellular debris. (d) UV-fluorescent* Hevea *ctDNA bands obtained after CsCl-EtBr density gradient equilibrium centrifugation: ctDNA isolated from bands 1 (A) and 2 (B) chloroplasts. Note the presence of the UV-fluorescent RNA pellet.*

Figure 1a shows the distribution of *Hevea* chloroplast bands after centrifugation in a sucrose density gradient. Two green chloroplast bands were clearly visible. Although the lower band (band 1) appeared lighter green and less prominent than the upper band (band 2), phase-contrast microscopic examination of the former revealed the predominant presence of intact chloroplasts that were characterised by 'surrounding halos' *(Figure 1b)*. Band 2, on the other hand, contained mainly ruptured chloroplasts, chlorophyllous material not associated with intact chloroplasts, and cellular debris *(Figure 1c)*. Chloroplasts intactness may be measured by the ferricyanide test as described by Lilley *et al.*[11] or as modified by Edwards *et al.*[12]

Under phase-contrast microscopy, intact *Hevea* chloroplasts appeared similar to spinach class I chloroplasts as described by Spencer and Wildman[13]. Class I chloroplasts were characterised by a reflective appearance without clear resolution of the grana. They were also reported as being surrounded by halos under phase-contrast microscopy[14]. Similar observations on intact spinach chloroplasts were also described by Lilley *et al.*[11]; in this case, referred as class A chloroplasts. Class II chloroplasts, on the other hand, were found to possess distinct grana. However, in contrast to class I chloroplasts, they did not retain their 'jackets'[15]. Further verification studies, perhaps similar to those described by Karlstam and Albertsson[14], and Motoyoshi[16], need to be undertaken to substantiate that isolated *Hevea* chloroplasts were indeed nuclei-free.

During the isolation of intact *Hevea* chloroplasts, it was important to avoid overloading the gradient with the applied leaf homogenate. Besides causing organelle aggregation, the overloading of gradients often presented difficulty in chloroplast lysis. This resulted in low ctDNA yield and nDNA contamination. In addition, a non-diffused interface between the overlay (30% sucrose) and underlay (52% sucrose) of the gradient always inevitably resulted in poor separation of chloroplast bands 1 and 2. As suggested by Palmer[8], effective separation of chloroplast bands in the gradient required the interface to be made diffused by gentle stirring or allowing the gradient to stand for 24 h at 4°C.

In the purification of *Hevea* ctDNAs by CsCl gradient centrifugation, the $RI_{25°C}$ of the CsCl-chloroplast lysate must be adjusted to 1.366 and 1.388 for the first and second gradient centrifugations, respectively. These were crucial for effective separation of covalently closed circular ctDNAs (intact) from linear DNAs (sheared ctDNA and nDNA). After the first CsCl gradient centrifugation, ctDNA was distributed as a single band at the lysate-cushion interface when illuminated by long wavelength UV light. An $RI_{25°C}$ of 1.388 adjusted for the pooled ctDNAs (derived from the first centrifugation) gave effective ctDNA purification and concentration as a sharp and intensely UV-fluorescent DNA band in the gradient *(Figure 1d)* after the second centrifugation in a fixed-angle rotor (TFT 80.13, Kontron). This critical requirement is probably an attribute of higher plant ctDNAs being less similar in densities[17] as opposed to the densities of ctDNAs from other lower plants such as the algae.

RNase and α-amylase were used for the removal of RNA and polysaccharides, respectively, from ctDNA preparations of safflower (*Carthamus tinctorius* L.)[18]. For Hevea ctDNA preparation, however, RNase treatment was unnecessary because any RNA present was pelleted during the second CsCl gradient centrifugation *(Figure 1d)*. In addition, no interference by polysaccharides to the electrophoresis of the restricted *Hevea* ctDNA fragments had been encountered.

Treatment of isolated chloroplasts with DNase had been shown to remove contaminating nDNA completely[5]. As a safeguard against the contamination of isolated *Hevea* ctDNA by nDNA, we pre-incubated the washed and unlysed chloroplasts with DNasel (10 mg/ml) as described by Wells and Birnstiel[19]. This was done even though band 1 chloroplasts were strongly believed to be relatively free of nDNA contamination. However, no significant difference in ctDNA purity was observed between DNase-treated and untreated chloroplasts. In fact, DNase-treated *Hevea* chloroplasts yielded much less ctDNA than untreated chloroplasts. Similar results had been reported for tobacco *(Nicotiana tabacum)* chloroplasts although the organelles were morphologically intact[13]. Because of such ctDNA loss, DNase treatment was excluded from the procedure for the isolation of *Hevea* ctDNA.

The possible presence of linear DNA molecules in isolated *Hevea* ctDNA preparations was also not discounted. However, rather than contamination by nDNA molecules, these were most likely attributable to random breaks suffered by the large and fragile ctDNAs during their isolation and purification. Any bulk nDNA contamination of the *Hevea* ctDNA preparations would have already been removed during CsCl gradient centrifugation. Further characterisation of isolated *Hevea* ctDNAs may be pursued with established techniques such as ctDNA photoelectric scans/electron microscopy studies to address questions pertaining to DNA circularity, contour length, monomer size, and molecular species[15] and renaturation characteristics for the calculation of ctDNA complexity[7] as well as criterion of purity[10].

The amount of ctDNA extracted from band 1 chloroplasts was generally about three-fold more than that recovered from band 2 *(Table 2)*. On average, *Hevea* ctDNA yields

TABLE 2. COMPARISON OF *HEVEA* CHLOROPLAST DNA YIELDS BETWEEN BAND 1 AND BAND 2 CHLOROPLASTS

Clone	ctDNA yield (µg/100 g leaves)	
	Band 1	Band 2
GT 1	20	6
RRIM 600	20	7
Tjir 1	18	6
PB 86	20	6

TABLE 3. CHLOROPLAST DNA YIELDS FROM *HEVEA*

Clone	Chloroplast DNA yield (µg/100 g leaves)
GT 1	20
Tjir 1	17, 18
PB 86	18
RRIM 600	20, 23
RRIM 727	22
H. camargoana	22

obtained from this procedure were between 18 and 20 µg per 100 g of leaves homogenised *(Table 3)*. For most of the ctDNAs isolated, the measured $OD_{260/280\ nm}$ ratios were between 1.8 and 2.1.

In order to characterise the isolated *Hevea* ctDNAs, we digested the nucleic acid with some restriction endonucleases. The restricted DNA fragments were separated by EtBr-agarose gel electrophoresis and photographed over short wave-length UV light. DNAs isolated from band 1 chloroplasts produced well-resolved and discrete fragments *(Figure 2)*. In most instances, background smearing of the restriction profiles by nDNA

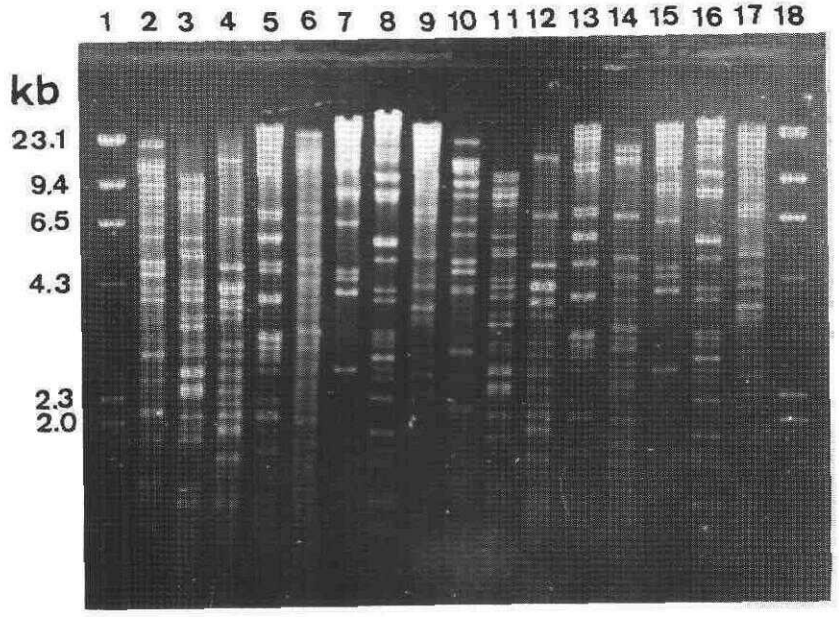

Figure 2. EtBr-stained agarose gel (0.7%, w/v) of restriction endonuclease-digested ctDNAs from H. brasiliensis, *clones GT 1 and RRIM 600. Lanes 1 and 18: phage lambda DNA-*Hin*dIII marker; lanes 2 to 9 are GT 1 ctDNA digested with* Hin*dIII,* Hin*cII,* Eco*RI,* Bam*HI,* Dra*I,* Pvu*II,* Cla*I, and* Bgl*II, respectively; lanes 10 to 17 are RRIM 600 ctDNA digested with* Hin*dIII,* Hin*cII,* Eco*RI,* Bam*HI,* Dra*I,* Pvu*II,* Cla*I, and* Bgl*II, respectively.*

was minimal. This was consistent with the negligible presence of contaminating nDNA. On the other hand, ctDNA isolated from band 2 chloroplasts persistently generated restriction profiles with heavy background smearing owing to contamination by sheared ctDNA and nDNA.

Generally, a basic restriction pattern was observed when *Hevea* ctDNAs of different clones were completely digested with a restriction endonuclease and electrophoresed in agarose gels. However, closer examination of the restriction profiles generated by certain restriction endonucleases revealed that some clones and species exhibited subtle variations from the basic pattern *(Figure 3).* Usually, the variation involved the absence or presence or small size differences of one or two DNA bands on the restriction profile. For instance, the *Hin*dIII-generated restriction profiles of ctDNAs from clones GT 1 and RRIM 600 are different due to variation exhibited by one DNA band. Similar variation in *Hin*dIII-generated restriction profiles was also observed from the ctDNAs of *H. camargoana* and *H. brasiliensis*, clone RRIM 600.

The size of the *Hevea* (RRIM 600) chloroplast genome was estimated by digesting to completion the ctDNA with each of *Hin*dIII, *Pvu*II, *Sal*I, or *Xho*I. Summation of sizes of the

TABLE 4. APPROXIMATE MOLECULAR SIZE AND COPY NUMBER OF *HIN*DIII-, *PVU*II-, *SAL*I-, OR *XHO*I-GENERATED CHLOROPLAST DNA FRAGMENTS OF *H. BRASILIENSIS*, CLONE RRIM 600

Mol. size (kbp)	*Hin*dIII	*Pvu*II	*Sal*I	*Xho*I
57.6	-	-	+	-
44.4	-	-	-	+
39.4	-	-	+	-
29.5	-	+	-	-
28.8	-	-	-	++
23.4	-	+	-	-
22.7	-	-	+	-
18.9	-	-	+	-
15.1	-	+	-	-
14.1	+	-	-	-
13.8	-	+	-	-
12.0	-	++	-	-
9.4	+++	-	-	-
9.1	+	+	-	-
8.7	+	-	-	-
8.3	-	+	-	-
7.9	++	-	-	-
7.7	-	-	-	++
7.6	-	-	-	+
6.8	+	-	-	-
6.3	+	+	-	-
5.8	-	-	++	-
5.6	+	-	-	-
4.7	++	-	-	-
4.6	-	+	-	-
4.5	++	-	-	-
4.4	-	+	-	-
4.2	+	-	-	-
4.0	+	-	-	-
3.8	-	++	-	-
3.7	-	-	-	++
3.6	-	-	+	-
3.5	-	-	-	++
3.4	+	-	-	++
3.1	-	-	-	+
3.0	+	-	-	-
2.8	++	-	-	-
2.6	-	-	-	+
2.5	-	++	-	+
2.3	+	-	-	-
2.0	++	-	-	-
1.8	+	-	-	-
1.7	+	-	-	-
1.3	+	-	-	-
1.2	+	-	-	-
1.1	+	-	-	-
1.0	++	-	-	-
0.8	+	-	-	-
0.6	+	-	-	-
Genome size (kbp)	150	151.1	153.8	154.4

fragments generated in these digestions gave a mean molecular size of not less than 152.3 kbp for the chloroplast genome of *H. brasiliensis*, clone RRIM 600 *(Table 4)*. This size was in agreement with the sizes of many other higher plant chloroplast genomes[2].

The determination of the molecular size of the *Hevea* chloroplast genome by restriction analysis is just an estimation. Its accuracy is limited primarily by the choice of the restriction endonuclease used and the difficulty encountered in determining the sizes of large DNA

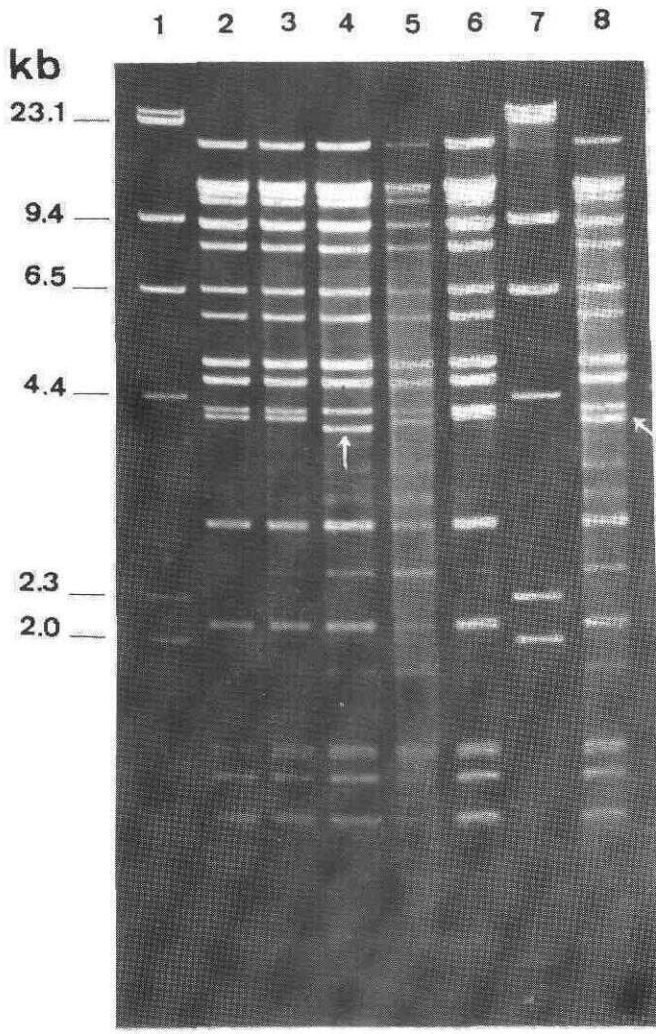

Figure 3. EtBr-strained agarose gel (0.7%, w/v) of Hind*III-digested ctDNAs from* H. brailiensis *clones and* H. camargoana. *Lane 1 and 7:* Hind*III-phage lambda DNA marker; lanes 2,3,4,5 and 6: clones RRIM 612, RRIM 600, GT 1, PB 86 and Tjir 1, respectively. Lane 8:* H. camargoana. *DNA band variations are arrowed.*

fragments. Molecular sizes of ctDNAs determined by summation of the DNA fragments generated after cleavage with a '6-base cutter', such as *Bam*HI, *Eco*RI, *Hin*cII, *Hin*dIII, or *Pvu*II *(Figure 2)* are generally underestimations[15]. For *Hevea* ctDNA, these restriction endonucleases generated numerous small fragments, which may not be detected during electrophoresis, thus making molecular size estimations inaccurate. On the other hand, the summation of sizes of the relatively small number of fragments generated by cleavage with *Sal*I or *Xho*I may very likely represent the molecular size of the entire *Hevea* chloroplast genome. For these reasons, the genome size estimated by the summation of *Hin*dIII- or *Pvu*II-cleaved fragment sizes was shorter than the estimations based on *Sal*I- or *Xho*I-cleaved fragment sizes.

CONCLUSION

A procedure for the isolation and purification of *Hevea* chloroplasts and ctDNA is described. The method was derived by a combination and modification of published procedures and is routinely used in the molecular biology laboratory of the RRIM Experiment Station. Based on phase-contrast microscopy observation and preliminary electrophoreto- gram examinations of its isolated chloroplasts and restricted ctDNAs, respectively, we conclude that the isolated *Hevea* ctDNA was of sufficient purity for restriction analysis. The mean molecular size of *Hevea* chloroplast genome was 152.3 kbp in size.

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On page 210, *Figure 4* should be replaced by the following:

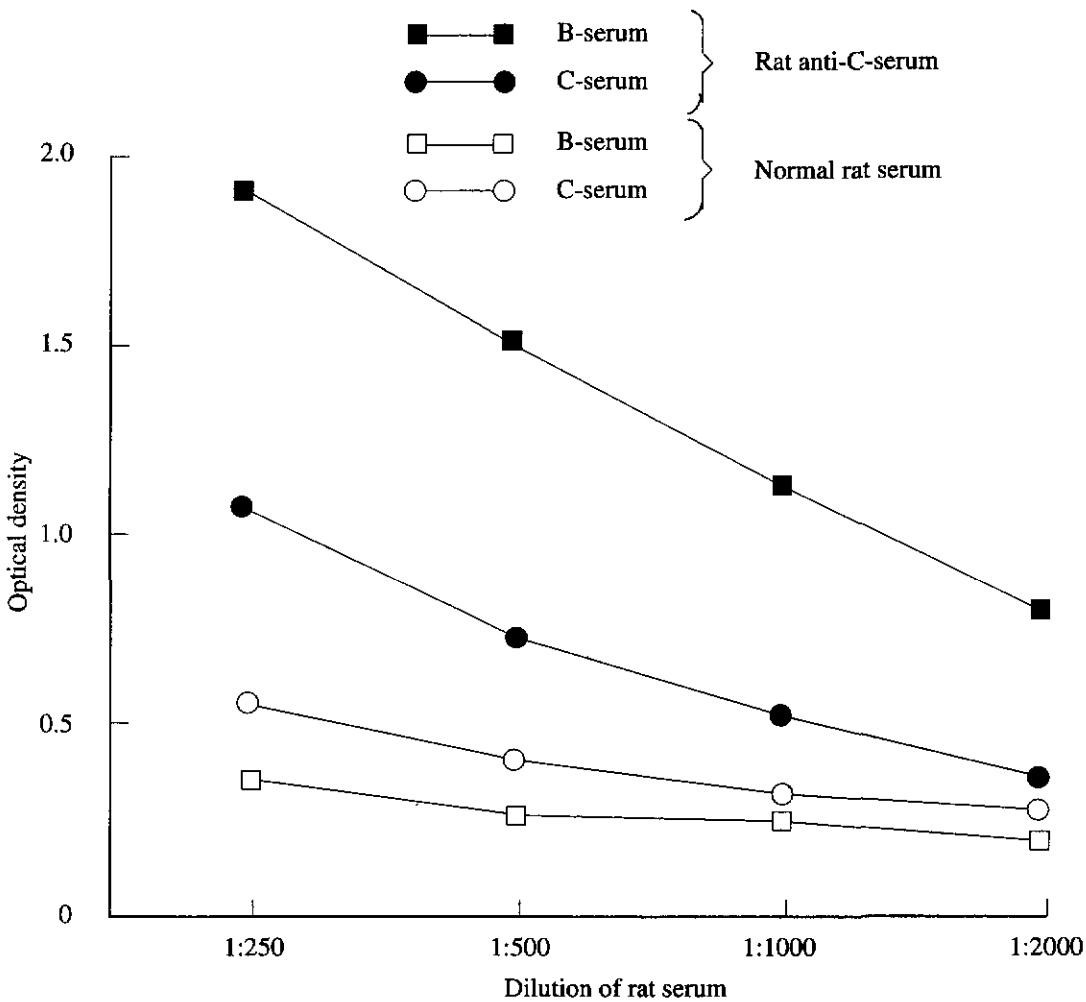

Figure 4. Indirect ELISA showing the reactivity of B-serum and C-serum when measured using a rat antiserum prepared against C-serum as immunogen. The ELISA wells were coated with 2.4 µg B-serum or C-serum and reacted with two fold serial dilutions of rat antiserum against C-serum or normal rat serum.