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Phylogenetic relationships within *Hevea brasiliensis* as deduced from a polymorphic mitochondrial DNA region

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Abstract We have cloned a 4.5-kb mtDNA fragment showing a high RFLP polymorphism between various *Hevea* genotypes. Subcloning and sequencing of a 1.4-kb segment of this clone allowed us to design PCR amplification primers to isolate homologous mtDNA segments of about 0.9 kb from 23 representative genotypes of *Hevea*. Complete sequences from 4 genotypes showed between 6.7% and 20.2% of nucleotide diversity, suggesting the presence of a hypervariable, or hot-spot, region. A sequence of 345 nucleotides within this region was determined for the 23 genotypes. The phylogenetic relationships inferred from the sequence comparison are in general agreement with the results obtained from mtDNA RFLP analysis, indicating that this polymorphic mtDNA region is a useful molecular marker for phylogenetic analysis within *Hevea*.

Key words *Hevea brasiliensis* · Mitochondrial DNA · Phylogeny · Hypervariable sequence · Hot spot

Introduction

Cultivated varieties of *Hevea brasiliensis*, the plant used for rubber production, have a very narrow genetic basis, resulting in breeding limitations (Varghese 1992). Several explorations have therefore been carried out in Amazonian forests, with the result that a large number of wild genotypes have become available. In order to facilitate their direct introduction into breeding programs, efforts have been made to estimate the genetic relationships of this collection using agronomic variability and leaf morphology (cited in Chevallier 1988), isozyme polymorphism (Chevallier 1988) and nuclear DNA restriction fragment length polymorphism (RFLP) analysis (Besse 1994). The number of loci identified by isozymes is rather limited, and nuclear DNA RFLP analysis has some drawbacks due to the mixing and recombination of the two parental nuclear genomes that occurs at each generation. As an alternative, the high degree of mitochondrial DNA (mtDNA) RFLP has allowed us to distinguish a large number of *H. brasiliensis* genotypes and to deduce the phylogenetic relationships between them (Luo et al. 1995). However, restriction fragment polymorphism of the mitochondrial genome in most plant species is due, in part, to frequent DNA rearrangement rather than nucleotide substitutions (Palmer and Herbon 1988). This observation made us question whether phylogenetic relationships based on DNA rearrangements would give similar results to those derived from nucleotide substitution. To answer this question, it is necessary to estimate the number of nucleotide substitutions among different genotypes or operational taxonomic units (OTUs). The most reliable approach with the highest resolution is the direct comparison of the nucleotide sequences.

In investigations of phylogenetic relationships in plants, sequence data from numerous species have been obtained for both nuclear and organellar genes. Since plant nuclear genes have a higher rate of nucleotide substitution than organellar genes (Palmer and Herbon 1988; Wolfe et al. 1987), various nuclear gene sequences, such as 5S rRNA (Hori and Osawa 1979; Hori et al. 1985), glyceraldehyde-3-phosphate dehydrogenase (Martin et al. 1989), the phytochrome introns (Barbier et al. 1991) and the calcium-modulated contractile protein, centrin (Bhattacharya et al. 1993), have been used to address specific questions of phylogeny either between or within species. Because of its slow rate of evolution, the chloroplast-encoded gene, ribulose-1, 5-bisphosphate carboxylase/oxygenase (*rbcL*) has been widely employed to study flowering plant evolution at the higher taxonomic level (Soltis et al. 1990; Les et al. 1991; Bousquet et al. 1992; Clegg 1993).

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Unlike mammalian mtDNA, which evolves much faster than nuclear DNA (Brown et al. 1979; Brown et al. 1982), plant mtDNA evolves considerably slower than nuclear and chloroplast DNAs (Wolfe et al. 1987; Palmer 1990). Its application to plant phylogenetic studies is therefore limited, contrasting with the situation in animals, in which mtDNA sequences have been used extensively as powerful molecular marker in phylogenetic studies (Avise 1991; Vigilant et al. 1991). Recently, cytochrome oxidase subunit III (*cox*III) coding sequences (both genomic and cDNA) have been analyzed in representatives of the major groups of land plants (Hiesel et al. 1994), and the derived phylogenetic relationships confirm the monophyletic origin of land plant mitochondria; the data also agree with the general order and descent of land plants deduced by other molecular, physiological and morphological traits. To investigate the phylogenetic relationships in *Hevea* at the sequence level, we have sequenced a polymorphic mtDNA fragment from representative genotypes of *H. brasiliensis*. The phylogenetic relationships obtained from these mtDNA sequences confirm the results deduced by RFLP analysis (Luo et al. 1995) and suggest that mtDNA sequences can be a powerful tool for phylogenetic analysis.

Materials and methods

Plant materials

H. Benthamiana and 22 *H. brasiliensis* genotypes were used, representing major mitochondrial types previously defined by RFLP analysis (Luo et al. 1995). Their geographical origin and mitochondrial type are given in Table 1. The material was provided by the Institut de Recherches sur le Caoutchouc (France and Ivory Coast). Fresh *Hevea* leaves, for the preparation of crude mtDNA, were obtained from our laboratory's greenhouse.

The seeds of the broad bean (*Vicia faba* L.) 'Exelle' used to prepare mitochondrial and (cp) DNA came from the Station d'Amélioration des Plantes (Gembloux, Belgium).

DNA extraction

Total *Hevea* DNA was extracted from 0.15-1 g of dried leaves as previously described (Luo et al. 1995). For genomic library construction, DNA from 1 g of dried leaves was further purified by isopycnic (cesium chloride) centrifugation (Sambrook et al. 1989).

Hevea mtDNA was prepared by a modification of the method of Flamand et al. (1993). By means of a pestle and mortar, fresh leaves (4 g) were ground up in 50 ml of 0.4 *M* sucrose, 50 m*M* Tris-HCl (pH 7.6), 10 m*M* KH_2PO_4, 1 m*M* EGTA, 5 m*M* β-mercaptoethanol, 0.2% (w/v) BSA. The homogenate was filtered through three layers of Miracloth (Calbiochem) and centrifuged first for 10 s when the speed reached 5,000 rpm, then for 10 min at 15,000 rpm in a Sorvall SS34 rotor. The pellet was resuspended in 2 ml of 0.3 *M* mannitol, 10 m*M* K_2HPO_4, 0.2% (w/v) BSA, pH 7.2 (KOH) supplemented with 10 m*M* $MgCl_2$, and 25 µg DNase I. After a 30 min incubation at 4 °C, the mitochondria were pelleted by centrifugation for 10 min at 15,000 rpm in a Sorval SS34 rotor, then lyzed for 30 min at 37 °C in 400 µl of 1.25% (w/v) sarkosyl, 100 m*M* NaCl, 10 m*M* EDTA, 10 m*M* Tris-HCl (pH 8.4), supplemented with 200 µg proteinase K. After phenol/chloroform/isoamyl alcohol (25:24:1) extraction, the nucleic acids were ethanol-precipitated before Southern hybridization analysis.

Broad bean mtDNA and cpDNA were prepared and purified as previously described (Boutry and Briquet 1982; Luo et al. 1995).

Construction and screening of the total DNA library

Total *Hevea* DNA (10 µg) and the cloning plasmid vector pBluescript SK^+ (Stratagene) (5 µg) were digested with *Hin*dIII, treated with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) and ethanol precipitated. The plasmid DNA was further dephosphorylated using alkaline phosphatase according to the supplier's instructions (Boehringer). The DNA fragments were ligated into the vector in an approximate molar ratio of 2:1 overnight at 21 °C in 20 µl of reaction buffer containing 50 m*M* Tris-HCl (pH 7.5), 7 m*M* $MgCl_2$, 10 m*M* dithiothreitol, 5 m*M* ATP and 5 units of T4 DNA ligase (BRL). The ligation products were transformed into competent *E. coli* (JM109) cells following the method of Hanahan (1983), then plated directly onto nitrocellulose filters (Millipore) placed on LB agar plates containing 100 µg/µl ampicillin. After incubation for 16 h at 30 °C, replica filters were prepared by filter-to-filter contact, and the lysis of resulting bacteria colonies and binding of DNA to nitrocellulose filters carried out according to Sambrook et al. (1989). The treated replica filters were pre-washed at 42 °C, with slight agitation, for 2 × 1 h in pre-wash buffer (0.1% (w/v) SDS, 1 *M* NaCl, 1 m*M* EDTA, 50 m*M* Tris-HCl (pH 8.0)), then used for hybridization. Probes consisted of a pool of 14 *Hin*dIII inserts of broad bean mtDNA clones (Scheepers et al. 1990; Luo et al. 1995) that were purified twice from an agarose gel using the Geneclean II kit (Westburg) and labelled with [^{32}P] α-dCTP (Amersham) using a nick translation kit (BRL). Hybridization was performed for 20 h at 42 °C in a buffer containing 6 × SSC (1 × SSC is 0.15 *M* sodium chloride, 0.015 *M* sodium citrate, pH 7.0 (NaOH)), 50% formamide, 1% sodium dodecylsulfate (SDS), 2 × Denhardt's solution (1 × Denhardt's solution is 0.2 mg/ml bovine serum albumin, 0.2 mg/ml Ficoll (MW 400,000), 0.2 mg/ml polyvinylpyrrolidone), 0.02% denatured herring

Table 1 List of the 23 *Hevea* accessions used for mtDNA polymorphism investigation

Mt genotype code[a]	Accession code[b]	Mt genotype code[a]	Accession code[b]	Mt genotype code[a]	Accession code[b]
A1	AC54	AX10	AC.X.20.31	MC17	MT.C.03.05
AB12	AC.B.17.09	R2	RO52	MI3	MT.IT.17.22
AF11	AC.F.07.34	RA1	RO.A.O7.100	MR1	MT.A.21.09
AS1	AC.S.08.78	RC1	RO.C.09.22	MV	MT.VB.25A.11
AS8	AC.S.11.17	RC5	RO.C.08.09	GT1	GT1
AS14	AC.S.08.12	RJ4	RO.J.05.21	W	IRCA18
AS21	AC.S.10.16	MC9	MT.C.05.11	F	F4506
AT12	AC.T.04.19	MC12	MT.C.09.07		

[a] Obtained from the assessment of mtDNA RFLP profile (Luo et al. 1995)

[b] AC, state of Acre, including the following districts: T, Tarauaca; F, Feijo; S, Sena Madureira; B, Brasiléia; X, Xapuri. RO, state of Rondônia, including the following districts: C, Calama; A, Ariquemes; J, Jaru. MT, State of Mato Grosso, including the following districts: C, Juruena; IT, Itauba; VB, Vila Bela. IRCA, GT1, Wickham clones (Luo et al. 1995). F4506, clone *H. benthamiana*.

sperm DNA and 10^6 cpm/ml of DNA probes. The filters were washed twice for 5 min in 2 × SSC and 0.1% SDS at room temperature, once for 15 min at 50 °C in the same medium, and twice for 15 min at 50 °C in 1 × SSC and 0.1% SDS. The membranes were then exposed at −70 °C to Kodak XAR X-ray films, using Du Pont Lightning Plus™ intensifying screens. All positive clones were subjected to a second round of screening by the same procedure.

Southern blot analysis

Samples of DNA (5 µg) were digested with restriction enzymes and the resulting fragments separated by electrophoresis on a 0.8% (w/v) agarose gel. The DNA in the gel was nicked by depurination in 0.25 *M* HCl and denatured in 0.4 *M* NaOH, 0.6 *M* NaCl, then neutralized with 1.5 *M* NaCl and 0.5 *M* Tris-HCl (pH 7.5) prior to transfer to a Hybond N^+ membrane (Amersham) (Sambrook et al. 1989). Probe preparation and hybridization were as described above.

Polymerase chain reaction (PCR)

The two primers designed to amplify a mtDNA fragment which is polymorphic among various *Hevea* genotypes were as follows: 5′-CGAGAATTCGAACACGCTATGATTGACATGTGC-3′ and 5′-CGAGAATTCAATAGAAGCGGAAGATGCGG-3′. The underlined sequences represent the added *Eco*RI restriction site. The reaction mixtures (20 µl total volume), overlaid with a drop of mineral oil, consisted of 50 m*M* KCl, 10 m*M* Tris-HCl (pH 8.8), 1.5 m*M* $MgCl_2$, 0.1% (w/v) Triton X-100, 200 µ*M* each of dATP, dCTP, dGTP and dTTP, 0.5 µ*M* of each primer, 0.2 µg of template DNA and 1 unit of Dynazyme DNA polymerase (TechGen). Amplification was performed in a LEPSCIENTIFIC PREM™ thermal cycler programmed for 30 cycles of 1 min at 94 °C (denaturation), 2 min at 55 °C (hybridization), 3 min at 72 °C (elongation) and a final elongation step at 72 °C for 10 min. The PCR products were treated with proteinase K (Crowe et al. 1991), digested with *Eco*RI, and fractionated on a 1.2% (w/v) agarose gel. The fragment of the expected size was extracted from the gel, purified using the Geneclean II kit (Westburg), then cloned into a bacterial plasmid vector for sequencing.

Sequencing and phylogenetic analyses

Double-stranded DNA was sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977) using T7 DNA polymerase (Pharmacia). Sequences obtained using the universal and reverse primers or synthetic oligonocleotides were edited and aligned using the PC/Gene package from Intelligenetics. Pairwise comparison of sequences was used to calculate similarity values, which were then converted to evolutionary distance estimates (Kimura 1980). Phylogenetic trees based on pairwise distance values were constructed by the Neighbor-joining method (Saitou and Nei 1987; Saitou and Imanishi 1989) because of its much higher performance in tree reconstruction compared to other distance methods. Tree topologies were also determined directly from the sequence data using the most parsimonious method (Felsenstein 1982; Fitch 1977). Statistical tests were performed by the bootstrap method (500 replications) (Felsenstein 1985) to evaluate the pertinence of branching within the tree. All analyses were performed using PHYLIP (Phylogenetic Inference Package) software (Felsenstein, Dept of Genetics, University of Washington, Seattle, Wash. version 3.4, 1991) on a Sun Sparc 10/30 station under SunOs, release 4.1.3.

Results

Polymorphic mtDNA cloning

In a previous study on mtDNA RFLP of *Hevea* genotypes, we found a high degree of polymorphism (Luo et al. 1995). We then hypothesized that a region polymorphic for RFLP would possibly also be polymorphic in nucleotide sequence and decided to identify and clone such mtDNA sequences. Fresh material was only available in small quantities, and the techniques in use at that time in the laboratory to prepare purified mtDNA from plant species did not give intact *Hevea* mtDNA. We therefore screened a plasmid library prepared from total DNA of a *Hevea* genotype (IRCA 18). The probes used consisted of a pool of the 14 broad bean mtDNA fragments previously used for *Hevea* mtDNA RFLP analysis (Luo et al. 1995). After two rounds of screening, six independent clones were analyzed in more detail. About 250 bp were sequenced at both insert ends. This partial sequence detected the presence of mitochondrial genes (26S rRNA, *nad*4, *nad*5, *nad*1) in four clones (data not shown). To identify the clone revealing the most polymorphic region, we hybridized each clone with *Hin*dIII-cut total DNA prepared from 17 *Hevea* genotypes previously shown to belong to distinct mtDNA types (Luo et al. 1995). Clones no. 12, containing a 4.5-kb insert, revealed the highest polymorphism, as seven different RFLP profiles were detected in the 17 genotypes analyzed (Fig. 1). The clones was therefore chosen for further study.

To confirm its mtDNA identity, the insert was hybridized with total and crude mitochondrial *Hevea* DNAs and with purified broad bean nuclear, chloroplast or mitochondiral DNAs (Fig. 2). The fragment did not hybridize with broad bean nuclear or chloroplast DNAs (lanes 1 and 2), but produced two strong signals when hybridized with mtDNA (lane 3). When *Hevea* total or crude mtDNAs were hybridized with this fragment, the latter, despite the smaller amount on the gel and its partial degradation, gave much stronger signals than did the former, demonstrating that this insert is indeed a mtDNA fragment.

The next step consisted of cloning, by PCR amplification, the polymorphic region from several *Hevea* genotypes. As the clone no. 12 insert was too large for PCR amplification, we subcloned a 1.4-kb *Hin*dIII-*Bam*HI fragment that still revealed restriction polymorphism among *Hevea* clones (data not shown). This sub-clone (no. 12B) was sequenced at both ends (see below) allowing the design of two primers aimed at the PCR amplification of a 934-bp fragment.

Cloning and sequencing of PCR-amplified fragments

Amplification of the specific mtDNA fragment by PCR was carried out on total DNA of 23 *Hevea* genotypes. The amplified fragments showed the expected size (about 0.9 kb) and were cloned for sequencing. The reliability of PCR amplification was checked by sequencing three cloned fragments, each amplified from two independent PCR reactions from the same genotype. No errors were detected along the 450 bp of sequences analyzed, thus excluding the possibilities of the

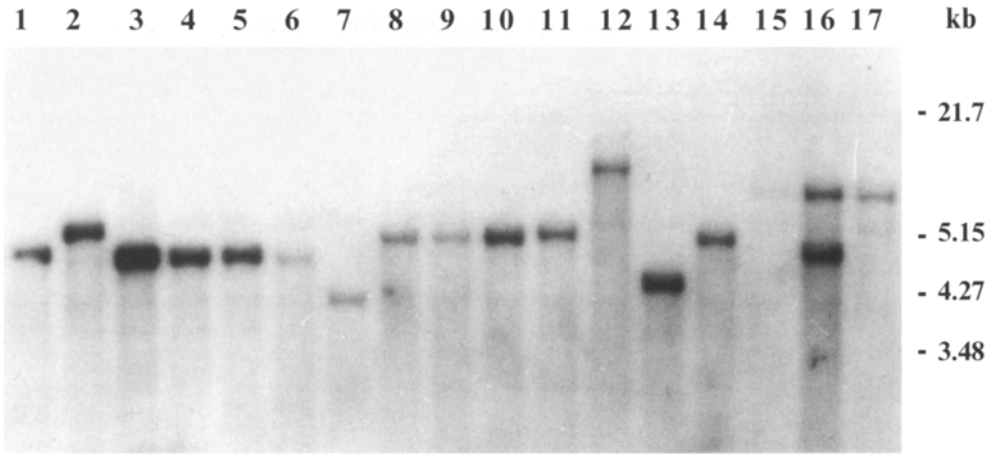
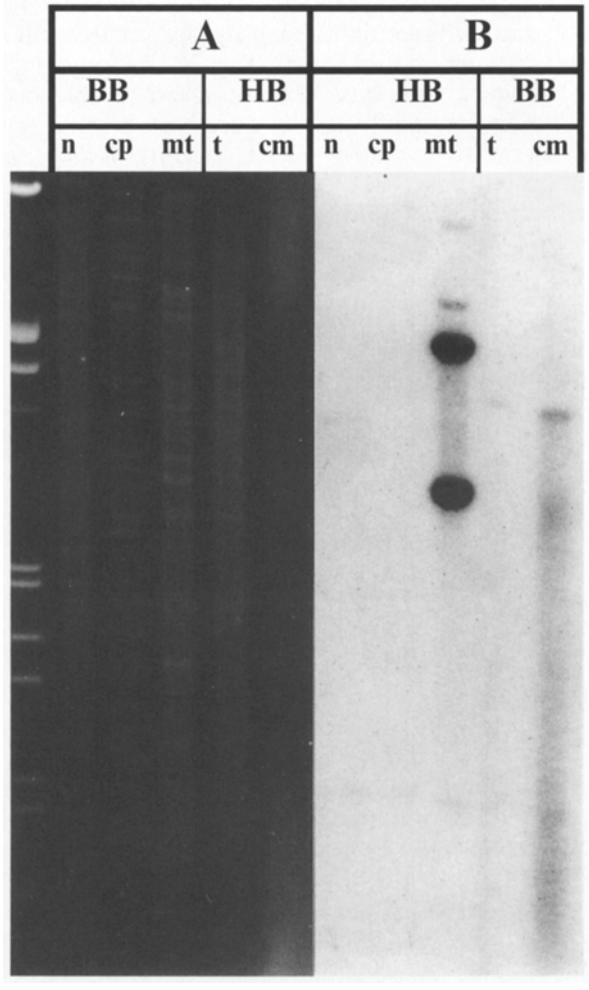


Fig. 1 MtDNA RFLP of *Hevea* detected by clone no. 12 of mtDNA. Total DNA (5 μg) of 17 *Hevea* genotypes (*1* AT12, *2* AS1, *3* AS8, *4* AS21, *5* AB12, *6* A1, *7* W, *8* MI3, *9* MC12, *10* MV, *11* MC17, *12* MR1, *13* RC5, *14* RA1, *15* R2, *16* GT1, *17* F) was digested with *Hin*dIII, electrophoresed, transferred to a nylon membrane and probed with clone no. 12 of *Hevea* mtDNA. The molecular size (kb) is shown on the right

Fig. 2 Mitochondrial origin of clone no. 12 identified by Southern blot analysis. DNAs were digested with *Hin*dIII, electrophoresed (0.8% agarose), transferred to a nylon membrane and probed with clone no. 12 of *Hevea*. The Ethidium bromide staining (**A**) and autoradiography (**B**) are shown. The molecular size (kb) is shown on the *left* top to bottom = 21.70, 5.15, 5.00, 4.27, 3.48, 1.98, 1.90, 1.59, 1.37, 0.94, 0.83, 0.56. Broad bean (*BB*): *n* purified nuclear DNA (5 μg), *cp* purified chloroplast DNA (0.5 μg), *mt* purified mitochondrial DNA (0.5 μg); *H. brasiliensis* (*HB*): *t* total DNA (5 μg), *cm* crude mitochondrial DNA (0.1 μg)



production of a high frequency of mutation. In fact, even if mutations were to be introduced during amplification (and wrongly considered as polymorphism), they would be present at a very low level in comparison with the number of substitutions due to actual polymorphism. The error would therefore be negligible.

The complete sequence of the amplified fragments from 4 genotypes was obtained (Fig. 3). Sequence alignment revealed slightly different lenghts due to several deletions in some genotypes: 886 (MC12), 891 (AS14), 892 (AF11) and 912 bp (F) and showed 79.8–93.3% nucleotide identity. Moreover, sequence comparison of this polymorphic fragment did not reveal any homology with datebase sequences. Some short direct repeats (bold in Fig. 3) were identified around deleted sequences, and these could possibly be involved in excision/addition due to uneven crossing-over between two direct repeats (Ogihara et al. 1992), resulting in length modification of this fragment between different *Hevea* genotypes.

For the other genotypes, we restricted the sequencing to the first 345 nucleotides (Fig. 3). Although the more downstream part of the fragment appears slightly more polymorphic, the presence of several insertions/deletions in this region makes the phylogenetic analysis more difficult.

Phylogenetic analysis

Phylogenetic trees predicting the genetic relationships of all of the genotypes analyzed were constructed by the Neighbor-joining (Fig. 4A) and most parsimonious (Fig. 4B) methods. We analyzed the data using 500 bootstrap replicates, and the tree obtained provides confidence intervals for the nodes. Since our purpose was to compare phylogeneitc relationships predicted from either nucleotide substitution or RFLP, we took the mtDNA RFLP data for the 23 *Hevea* genotypes analyzed here from a previous analysis (Luo et al. 1995). A phylogenetic tree was then constructed by the Neighbor-joining method (Fig. 4C).

```
      1                                                  50
AS14  TTGACATGTG CCAGGGTATG TCATGACAGT CCTGTACATC CCAATCTCCA
AF11  .......... .......... .......... .......... ..........
MC12  .......... .......... .......... .......... ..........
F     .......... .......... .......... .......... ..........
AX10  .......... .......... .......... .......... ..........
AS8   .......... .......... .......... .......... ..........
RC1   .......... ....A..... .......... G......... ..........
A1    .......... ....A..... .......... G......... ..........
AB12  .......... ....A..... .......... .........G GG........
AS21  .......... ....A..... .......... .......... ..........
MC17  .......... .......... .......... .......... ..........
W     .......... .......... .......... .......... ..........
RJ4   -......... .......... .......... .......... ..........
R2    -......... .......... .......... .......... ..........
MC9   .......... .......... .......... .......... ..........
MV    .......... .......... .......... .......... ..........
MI3   .......... .......... .......... .......... ..........
RA1   .......... .......... .......... .......... ..........
MR1   .......... ...CCC.... .......... .......... ..........
GT1   .......... .......... .......... .......... .T........
AT12  .......... T......... .......... .......... ..........
RC5   .......... .......... .......... .......... ..........
AS1   .......... .T..A..... .......... G......... ...G......

      51                                                 100
AS14  CCGTTGATCT CACTTGTAAG GATGAATCC- GGAACCCTTG GATCAAAAGA
AF11  .........C .......... ..CA..C... .........A .....GG...
MC12  .........C .......... ..CA..C... .........A ......G...
F     .........C .......... ..CA..C... .........A ......G...
AX10  .......... .......... .......... .......... ..........
AS8   .......... ......C... .......T.. .......... ..........
RC1   .......... .G........ .......... .......... ..........
A1    .......... .G........ .......... .......... ..........
AB12  .......... .......... .......... .......... ..........
AS21  .......... .......... .......... .......... ..........
MC17  .........C .......... ..CA..C... .........A ......G...
W     .........C .......... ..CA..C..A .........A .....GG...
RJ4   .........C .......... ..CA..C... .........A ......G...
R2    .........C .......... ..CA..C... .........A ......G...
MC9   .........C .......... ..CA..C... .........A ......G...
MV    .........C .......... ..CA..C... .........A ......G...
MI3   .........C .......... ..CA..C... .........A ......G...
RA1   .........C .......... ..CA..C... ...G.....A ......G...
MR1   .........C .......... ..CA..C... .........A ......G...
GT1   .........C .......... ..CA..C... .........A ......G...
AT12  .........C .......... ..CA..C..A -..G.....A ......G...
RC5   .........C .......... ..CA..C... .........A ......G...
AS1   .......... T......... .....GC... ...G.....A .......GA.

      101                                                150
AS14  GAAGATGATA ATACCAGCGT TTTTCACTCT CAGCCCTCAG ATCGTTCCGG
AF11  .....C..C. .......... C......... T.......G. ..........
MC12  .....C..C. .......... C......... T.......G. ..........
F     .....C..C. .......... C....G.... T.A.....G. .........A
AX10  .......... .......... .......... .......... ..........
AS8   .....C..C. .......... .......... T......... ..........
RC1   .....C..C. .......... .......... T......... ..........
A1    .....C..C. .......... .......... T......... ..........
AB12  .....C..C. .......... .......... .......... ..........
AS21  .....C..C. .......... .......... .......... ..........
MC17  .....C..C. .......... C......... T.......G. ..........
W     .....C..C. .......... C......... T.......G. ..........
RJ4   .....C..C. ......A... C....G.... T.A.....G. ..........
R2    .....C..C. ......A... C....G.... T.A.....G. ..........
MC9   .....C..C. .......... C......... T.......G. ..........
MV    .....C..C. .......... C......... T.......G. ..........
MI3   .....C..C. .......... C......... T.......G. ..........
RA1   .....C..C. .......... C......... T.......G. ..........
MR1   .....C..C. .......... C....G.... T.A.....G. ..........
GT1   .....C..C. .......... C....G.... T.A.....G. ..........
AT12  .....C..C. .......... C....G.... T.......G. ..........
RC5   .....C..C. .......... C......... T.......G. ..........
AS1   .....C..C. .G......AC C......G.C .........G. ....CC.TT.

      151                                                200
AS14  ACAAGAGGAT TCAAGACCGT CGGATTAAAA GAGGAAAAGG ACAAATACTT
AF11  .....GAA.. .TGG...... .A...GG... ..A....... .......T..
MC12  .....GAA.. .TGG...... .A...GG... ..A....... .......T..
F     ......AA.. .TGG...... .A...GG... ..A....... .......T..
AX10  .......... .......... .......... .......... ..........
AS8   .......... .......... .A........ .......... .......T.C
RC1   .......... .......... .A........ .......... ......GT.C
A1    .......... .......... .A........ .......... ......GT.C
AB12  .......... .......... .......... .......... .......T..
AS21  .......... .......... .......... .......... .......T..
MC17  .....GAA.. .TGG...... .A...GG... ..A....... .......T..
W     .....GAA.. .TGG...... .A...GG... ..A....... .......T..
RJ4   .T...GAA.. .TGG...... .A...GG... ..A....... .......T..
R2    .....GAA.. .TGG...... .A...GG... ..A....... .......T..
MC9   .....GAA.. .TGG...... .A...GG... ..A....... .......T..
MV    .....GAA.. .TG....... .A...GG... ..A....... ..........
MI3   .....GAA.. .TGG...... .A...GG... ..A....... .......T..
RA1   G....GAA.. .TGG...... .A...GG... ..A....... .......T..
MR1   .....GAA.. .TTG...... .A...GG... ..A....... .......T..
GT1   .....GAA.. .TTG...... .A...GG... ..A....... .......T..
AT12  .T...GAA.. .TGG...... .A...GG... ..A....... .......T..
RC5   .....GAA.. .TGG...... .A...GG... .......... .......T..
AS1   ......AA.. .TTG.G.... .A.....G.. ...AG..... .....A.T.C

      201                                                250
AS14  TGAGAAGCGA TCTCAGCCAT TCGTTTTGAT TCTCTTTAAT T-CCTGAACA
AF11  ...AG..T.. .....A.... ..A...C... .........- .........C
MC12  ...AG..T.. .....A.... ..A....... .......... .........C
F     ...AG..T.. .....A.... ..A....... .......... .........C
AX10  .......... .......... .......... .......... ..........
AS8   C......... .......... .......... .......... ..........
RC1   ....G..... .......... ..A.C..... ...T...... ..........
A1    ....G..... .......... ..A.C..... ...T...... ..........
AB12  .......... ..A....... .......... .......... ..........
AS21  .......... ..A....... .......... .......... ..........
MC17  ...AG..T.. .....A.... ..A...C... .......... .........C
W     ...AG..T.. .....A.... ..A...C... .......... .C.......C
RJ4   ...AG..T.. .....A.... ..A....... .......... .........C
R2    ...AG..T.. .....A.... ..A....... .......... .........C
MC9   ...AG..T.. .....A.... ..A....... .......... .........C
MV    ...AG..T.. .....A.... ..A....... .......... .........C
MI3   ...AG..T.. .....A.... ..A....... .......... .........C
RA1   ...AG..T.. .....A.... ..A....... .......... .........C
MR1   ...AG..T.. .....A.... ..A....... .......... .........C
GT1   ...AG..T.. .....A.... ..A....... .......... .........C
AT12  ...AG..T.. .....A.... ..A....... .......... .C.......C
RC5   ...AG..T.. .....A.... ..A...C... .......... .........C
AS1   ...A-..G.. .........GG ..C..A.... .......G.. .C..-A.G.C

      251                                                300
AS14  TCCGATCATG TACTTCAAAA TCCTGACCCT CCATCTCTCT CAAAATGAAT
AF11  .......... .C....GTG. ..TC...... .......C.. .-....A...
MC12  .......... .C....G.G. ..TC...... .......C.. .-....A...
F     .......... .C....G... ..TC...... .......C.. .-....A...
AX10  .......... .......... .......... .......... ..........
AS8   .......... .......... ..T....... ......TC.. .....A...
RC1   .......... .C........ ..T....... .......C.. .-....A...
A1    .......... .C........ ..T....... .......C.. .-....A...
AB12  .......... .......... ..T....... .......C.. ..........
AS21  .......... .......... ..T....... .......C.. ..........
MC17  .......... .C....GTG. ..TC...... .......C.. .-....A...
W     .......... .C....GTG. ..TC...... .......C.. .-....A...
RJ4   .......... .C....G.G. ..TC...... .......C.. .-....A...
R2    .......... .C....C... ..TC...... .......C.. .-....A...
MC9   .......... .C....G.G. ..TC...... .......C.. .-....A...
MV    .......... .C....G.G. ..TC...... .......C.. .-....A...
MI3   .......... .C....G.G. ..TC...... .......C.. .-....A...
RA1   .......... .-....G.G. ..TC...... .......C.. .-....A...
MR1   .......... .C....G... ..TC...... .......C.. .-....A...
GT1   .......... .C....G... ..TC...... .......C.. .-....A...
AT12  .......... .C....G... ..TC...... .......C.. .-....A...
RC5   .......... .C....G.G. ..TC...... .......C.. ......A...
AS1   ..A..A.... .C...T.... ....A..... .......C.. .G....A...
```

Fig. 3

The phylogenetic trees based on sequence data were deduced from the first 345 nucleotides. In order to evaluate whether the use of a partial sequence would affect phylogenetic reconstruction, two independent sequences (nucleotides 1–150 and nucleotides 151–345 of Fig. 3) were also used to construct independent phylogenetic trees using the Neighbor-joining method.

The topologies obtained from these two sub-sets of sequence data were similar to the tree obtained with the 345-nucleotide long sequence (result not shown).

The topologies of the trees derived from both the Neighbor-joining (Fig. 4A) and parsimony (Fig. 4B) methods based on sequence data are congruent with one other and in general agreement with that obtained from

```
      301                                                        350
AS14  CCTGACCCTT CATGGGGAGC ACCCGATTCC TATAAATACC TGCATGAAAA
AF11  .......... ..C.AA.... .T........ .......... ..........
MC12  .......... ..C.AA.... .......... .......... G....-....
F     .......... ..C.AA.... .......... .......... .....-....
AX10  .......... .......... .......... .......... .....
AS8   .......... .......... .......... .......... .....
RC1   .......... ..C....... .......... .......... .....
A1    .......... ..C....... .......... .......... .....
AB12  .......... .......... .......... .......... .....
AS21  .......... .......... .......... .......... .....
MC17  .......... ..C.AA.... .T........ .......... .....
W     ....-..... ..C.AA.... .T........ .......... .....
RJ4   .......... .TC.AA..-. .......... .......... .....
R2    .......... ..C.AA..-G CA........ .......... .....
MC9   .......... ..C.AA.... .......... .......... G....
MV    .......... .TC.AA.... .......... .......... G....
MI3   .......... ..C.AA.... .......... .......... G....
RA1   .......... ..C.AA.... .......... .......... G....
MR1   .......... ..C.AA.... .......... .......... ....C
GT1   .......... ..C.AA.... .......... .......... .....
AT12  .TGA--.... ..C.AA...- --A.CGA.T. .......... .....
RC5   .......... .......... .......... .......... .....
AS1   .......... ..C.AA.... .T........ .......... .....

      351                                                        400
AS14  ACTGTTCAGG GGG-GGACAA AAAAATATAG GCAAGAGGCT GCTATTATCG
AF11  .........C .......... .......--. A.G....... .T...    A
MC12  ........A. ..AC..A.GG ........--. A.G....... .T......A
F     ........A. ..AC..G.A- ........--. A.......... .T..     A.

      401                                                        450
AS14  -TGGAGGAAC TCTGAAACTT CATTC-AGTT TGTTACTCTG CTGTTTTGTA
AF11  CAA..... .......G.. ....TT.... .......... ..A...A.A.
MC12  CAA..... .......G.. ....T..... .......... ..A...A.A.
F     CA-..... .......T.. ....T..... ..C....... ..AC..A.A.

      451                                                        500
AS14  GT---GATTA AAAATACTTT TGAAACTAGT TTTCAGGAGT GTTTT-CTTG
AF11  ..ATC...C. ....G..... T......... ....T.A... ..........
MC12  ..GTT..... ....G..... G......... ....T.A... ..........
F     ..GTCA..C. ...-G..... T......... ....C.A... .....T....

      501                                                        550
AS14  AAAAAGATTT TGAATATTGG AACTCTACAT TTCCAGTTTG TTCTTTATCT
AF11  G...-....C ......C... .......... .......... ...A......
MC12  ..G.-..... ......C... .......T.. .......... ...A......
F     ....-....C ......C... .......... .......... ...A......

      551                                                        600
AS14  GGTCATACTC TCATCTTCT- -CTGCTTTCC TTTC------ CTCTGTTCAA
AF11  .......... ...CT....G T..TTTA..A .C..TGTTTT .A...CC...
MC12  A......... ...CT....G T..TTTA..A .C..TGTTTT .A...CC...
F     .....C.... ...CT....G C..TTTA..A .C..TGTTTT .AT...C...

      601                                                        650
AS14  GCTCAACTTC CTTTCACCTG GTTCTTACC- -------CGG TT-ACCTTTT
AF11  .A..A..... A.A....TCA T.....T--. .......--- ----------
MC12  .A..G..... A.A....TCA T.....T--. .......--- --.-------
F     ....A..... A.TC.....G G.....TT.A TCTTGAT.T. A.CG.A....

      651                                                        700
AS14  AGCTAAAGCA TCTCTCGGTT TCACGACAGA CATCAACTCT CAGGCTGATC
AF11  ......G--T A.C...CAG. ...T..AGA. ..C..C.C-. ......A...
MC12  ......G--T A.C...CAG. ......AGA. ..C..C.C-. ......A...
F     ......G--C A.C...CAG. ......GGA. ..C..C.C.. .....CA...

      701                                                        750
AS14  AATCCGC--- --CATCACTG TTTACCACTT CACAGTTATT TCAATATATT
AF11  .G..TA.TTT CT....C... ..CG....CC .GT.AC.... .T.G..G...
MC12  .G..TA.TGT CT....C... ...G....CC .GT.AC.... .T.G..G.CC
F     .C....TTGC ..TG.....A ...GA...CC .AT....... .T.G..G...

      751                                                        800
AS14  TGGCTCCTCA CAGGTAAGAG TTCATACTAC TGTTT----- -------ACG
AF11  ........T. .......... C......... .....TATTA AGTGCTT..A
MC12  CA........ .......... ........G. .....----- -------..A
F     ........T. .......... C........T .....TATTA AGTGCTT..A
```

Fig. 3 (continued)

```
      801                                                        850
AS14  TTTGTTTCCT GCACTTCCTT TGTTCTTCAT ACATCTGCAA CTTTCTTCAT
AF11  ...AC..T.. ...T..T... G.......TC .......TG. ......A.--
MC12  ...A...T.. .......... .......... .......... .........A
F     ...AC..T.. ...T..T... .......TC .......TG. ......A.--

      851                                                        900
AS14  GGGTATTTTG TACAAGAGAA CCCAAGAGAA AATATTGTTA TAAGAGTTCA
AF11  -A---..... C....AC..T ...C.-.-.G ...G..AC.G ..........
MC12  .AT..... .......... .......-.. ...G.....G ..........
F     .A---./... C....AC..T ...C.-.-.G ......AC.. ......C...

      901                                                934
AS14  TGTTACTGTTTTATTAAGCGTCTACGTTTATTTC
AF11  .A.C..........C...T....G.A...TC..T
MC12  ..C.............T.............
F     .A.C..........CG.......G.A....C...
```

Fig. 3 Alignment of the nucleotide sequences of the PCR-amplified polymorphic mtDNA fragment (no. 12B) for 23 *Hevea* genotypes. The sequence of the whole fragment is reported for four genotypes: AS14, AF11, MC12 and F. For the other genotypes, only the first 345 nucleotides were determined. Numbering corresponds to the AS14 sequence. *Dots* represent nucleotides identical to the AS14 sequence, *hyphens* represent gaps introduced for maximal alignment, *bold-faced letters* denote the putative direct repeats around deleted region

RFLP data with Neighbor-joining (Fig. 4C), or most parsimonious and maximum likelihood methods (not shown). Both the mtDNA RFLP and nucleotide sequence data are concordant in suggesting a branching (cluster no. 1 in Fig. 4) of all genotypes from three contiguous geographic districts of state Acre (Luo et al. 1995) (Brasiléia (AB12), Xapuri (AX10) and Sena Maduriera (AS1, AS8, AS14, AS21)), and another Acre genotype, A1 as well as RC1, belonging to the district of Calama in the state of Rondônia. Cluster no. 2 is rather heterogeneous, containing a genotype of *H. benthamiana* (F), a genotype (GT1) characterized by a male-sterile phenotype, and other genotypes that are supposed to have an interspecific origin (Luo et al. 1995). Cluster no. 3 contains clones mainly from the states of Rondônia and Mato Grosso, a cultivated clone (w) and an Acre genotype from the district of Feijo (AF11). Here, a minor topological difference was found between the parsimony tree and the Neighbor-joining tree. All genotypes in this cluster are unambigously grouped together in the parsimony tree (Fig. 4B), whereas in the Neighbor-joining tree, some appear to be closer to cluster 2 (Fig. 4A). However, this discrepency may not be real as the percentage of confidence for the nodes at that position is low (25%) (Fig. 4A). Since the Neighbor-joining method is based on distance matrix derived from the sequence data, the low confidence may result from the possible loss of information during the transformation from sequence data to genetic distances. In addition, 1 genotype, MC17, fell in cluster no. 3 (Fig. 4A, B)), whereas by RFLP analysis, it was grouped in cluster no. 1 (Fig. 4C), although close to cluster no. 3.

Discussion

A polymorphic mtDNA fragment has been isolated from *Hevea brasiliensis*. Considerable sequence variation among various genotypes provided insights into phylogenetic relationships. The resulting dendrograms mirror those obtained from mtDNA RFLP data. Indeed, most of the genotypes were found to be clustered

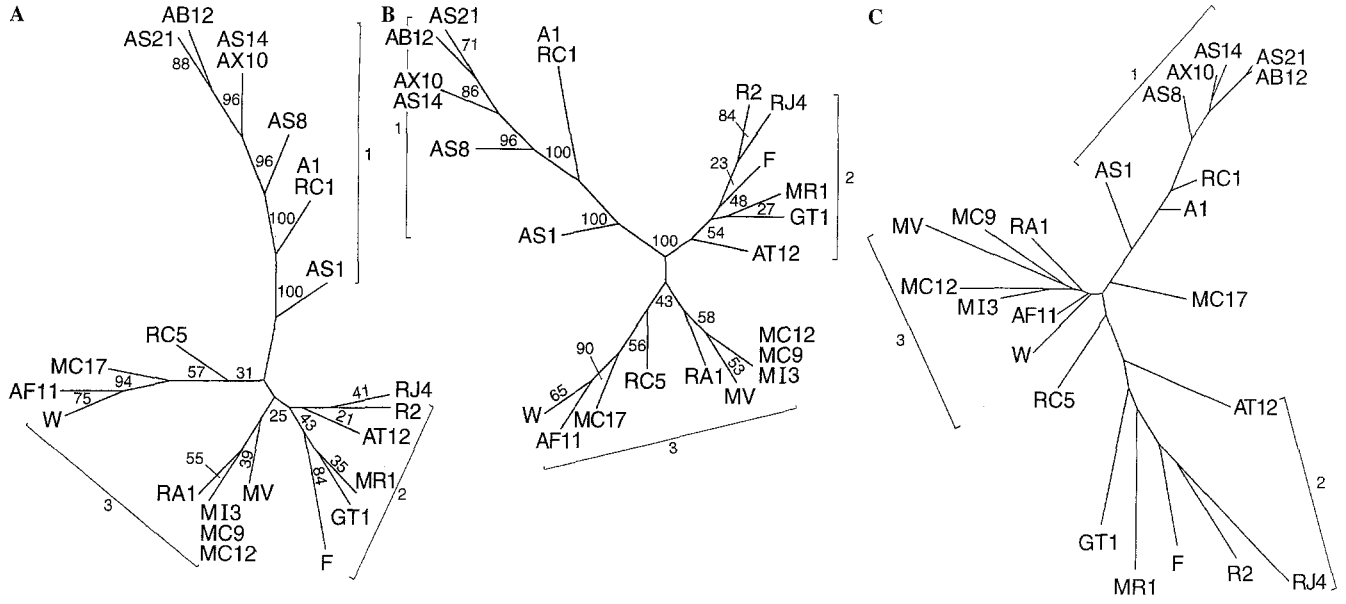


Fig. 4A–C Phylogenetic trees derived from the mtDNA sequence and from RFLP data. **A** Network constructed by the Neighbor-joining method based on sequence data; **B** network constructed by the most parsimonious method based on sequence data. *H. benthamiana* (*F*) was used as outgroup. Similar results was obtained when other genotypes were used as outgroup. For **A** and **B**, bootstrap percentage values from 500 resamplings of the sequence data are shown at the interval nodes. **C** Network constructed by the Neighbor-joining method based on mtDNA RFLP data taken from Luo et al. (1995)

according to their geographic origin. This conclusion was previously reached in the nuclear DNA (Besse et al. 1994) and mtDNA (Luo et al. 1995) RFLP surveys. This indicates that plant mtDNA RFLP, despite resulting, in most plant species, more from DNA rearrangements than from nucleotide substitutions (Palmer and Herbon 1988), is still a valuable tool for classification and phylogeny.

The striking sequence divergence of the fragment analyzed within *Hevea brasiliensis* is remarkable, since it has long been believed that the nucleotide sequence of mtDNA, despite its frequent rearrangement, evolves much more slowly than its nuclear and chloroplast counterparts (Palmer and Herbon 1988; Wolfe et al. 1987).

From our previous mtDNA RFLP data covering about 38 kb of the *Hevea* mitochondrial genome (Luo et al. 1995), we can estimate an average frequency of nucleotide substitution of 0.96×10^{-2} (Table 2) according to the method of Nei (1978). In contrast, the average frequency of nucleotide substitution estimated from the sequence data of the polymorphic mtDNA fragment in terms of Kimura's two-parameter method (Kimura 1980) is 8.52×10^{-2} (ranging from 0.88×10^{-2} to 21.50×10^{-2}) among the 23 *Hevea* genotypes analyzed (Table 2). The substitution rate of this polymorphic region is almost 9 times greater than that estimated from the RFLP data for a mitochondrial genome region of about 38 kb. The polymorphic region we have sequenced can therefore be considered to be hypervariable sequence.

Can we relate these figures to data available in the literature? No report of an extensive comparison of plant mtDNA sequences within a species has yet been published. However, cases of intraspecific mtDNA sequence variation have been reported. For instance, nucleotide changes have been indentified in the ORF219/221 gene, which exists as a single copy in four maize cytoplasms analyzed (Stamper et al. 1987). The nucleotide substitution of the ORF219/221 sequence varies between 0.27×10^{-2} and 2.17×10^{-2} (Table 2). This ORF219/221 comparison corresponds to an intraspecific analysis. Now, to broaden our comparison, we have analyzed the sequence data for ten known mitochondrial genes among 15 plant species. The nucleotide substitution rate calculated from sequence comparison varies between 1.03 and 5.13×10^{-2} within monocots, 1.48 and 11.80×10^{-2} within dicots and 5.00 and 12.25×10^{-2} between monocots and dicots (Table 2). These rates are still lower than those found within *Hevea brasiliensis* from the mitochondrial polymorphic DNA sequence data. In these comparisons, however, the functional constraints of the coding sequences probably slow down the substitute rate. Indeed, analysis of the sequence data of the intergenic region of plant mitochondrial 18S and 5S rRNA genes gives rise to higher nucleotide substitution rates, most exceeding 20.00×10^{-2} (Tables 2). Thus, the substitution rate observed for the polymorphic region within *H. brasiliensis* is close to the substitution rate identified for the ribosomal intergenic spacer between monocot and dicot species. The lack of sequence conservation in the *H. brasiliensis* mtDNA fragment that we have identified may simply indicate a lack of associated function, forming a so-called "hot-spot" region, which can tolerate more changes than other regions. This has been

Table 2 Comparison of nucleotide substitutions per site in plant mitochondrial genomes

Data type	Taxa compared	L[a]	K[a] × 100
	Within species		
RFLP data[b]	*Hevea*	–	0.19 to 1.68
Sequence	*Hevea* (clone no.12)	345	0.88 to 21.5
	Maize (ORF219/221)[e]	660	0.27 to 2.17
	Between species		
Coding sequences[c]	Within monocots	8,654	1.03 to 5.13[f]
	Within dicots	7,873	1.48 to 11.80[g]
	Monocots vs. dicots	9,005	5.00 to 12.25[h]
Spacer region[d]	Maize vs. wheat	114	5.99
	Soybean vs. lupine	190	3.60
	Hevea vs. oenothera	633	21.50
	Maize vs. soybean	160	23.09
	Wheat vs. soybean	160	21.50
	Maize vs. lupine	190	27.09
	Wheat vs. lupine	190	23.88

[a] L: number of nucleotides analyzed, K: nucleotide substitutions per site

[b] Based on the mtDNA RFLP data (Luo et al. 1995)

[c] Coding sequences of ten mitochondrial genes (*atp6*, *atp9*, *atpA*, *cob*, *coxI*, *coxII*, *coxIII*, *rps13*, *rps19*, *rrn18S*) in various plant species (sorghum, maize, rice, oenothera, *Vicia faba*, rapeseed, sugarbeet, wheat, *Raphanus sativus*, *Nicotina tabacum*, pea, potato, petunia, soybean and carrot) obtained from GenEMBL

[d] Intergenic region of 18S and 5S rRNA genes (see text for details)

[e] Sequence of ORF219/221 in four cytoplasms (Stamper et al. 1987)

[f] 1.03×10^{-2} is an averaged substitution rate of the *coxI* coding sequence (1,550 bp) in maize, rice, sorghum and wheat; 5.13×10^{-2} is an averaged substitution rate of the *atp6* coding sequence (756 bp) in sorghum, maize and rice

[g] 1.48×10^{-2} is the substitution rate of the *coxIII* coding sequence (967 bp) between soybean and *Vicia faba*; 11.80×10^{-2} is an averaged substitution rate of the *atp9* coding sequence (204 bp) in oenothera, *Raphanus sativus*, *Nicotiana tabacum* and petunia

[h] 5.00×10^{-2} is an averaged substitution rate of the *coxIII* gene (967 bp) between monocot species (rice, wheat and maize) and dicot species (soybean and *Vicia faba*); 12.25×10^{-2} is an averaged substitution rate of the *atp6* gene (756 bp) between monocot species (sorghum, maize and rice) and dicot species (oenothera, *Vicia faba* and *Raphanus sativus*)

reported in the chloroplast genome, where mutations occur frequently at specific sites. For instance, a 16.0-kb region around the *rbcL* and *petA* (cytochrome *f*) genes has been found to be a variation hot spot (Morton and Clegg 1993; Ogihara et al. 1991). Sequence comparison of this hypervariabale region among wheat groups, and between wheat groups and other grasses, showed high nucleotide divergence (Ogihara et al. 1991). All of these data reinforce the idea that the polymorphic fragment we have identified in the *Hevea* mitochondrial genome is located in hypervariable hot-spot region.

In conclusion, this work has allowed the identification of a highly polymorphic region of the *Hevea* mitochondrial genome. The available sequence data for the polymorphic mitochondrial DNA region have provided phylogenetic information within *Hevea* and have enabled us to elucidate genetic relationships between different genotypes within one species. The results from this approach are very similar to those derived from mtDNA RFLP analysis, suggesting that both methods are useful tools for predicting genetic relationships.

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