Chloroplast DNA Evolves Slowly in the Palm Family (Arecaceae)[1]

Mark A. Wilson, Brandon Gaut, and Michael T. Clegg
Department of Botany and Plant Sciences, University of California, Riverside

A survey of cpDNA restriction-site variation for 22 species representing five of the six subfamilies of the palm family was conducted. Phylogenetic reconstructions based on the restriction-site data are in general agreement with conventional analyses based on morphological characters. Base-substitution estimates, derived from the restriction-site data, indicate a potential 5–13-fold decrease in substitution rates within the palms relative to rate estimates for annual plant taxa. Approximately 1,000 bp of the *rbc*L gene (ribulose-1,5-bisphosphate carboxylase, large subunit) were sequenced from species representing two subfamilies of the palms. The complete DNA sequence data are in accord with the restriction-site data and indicate a total rate of nucleotide substitution that is about eightfold lower than that observed for annual plants.

Introduction

The Arecaceae, a family of ~200 genera and ~2,700 species, is one of the best represented angiosperm families in the fossil record. Leaf and stem fossils appear as early as 80–85 Myr ago (Mya), and pollen microfossils are found beginning ~65 Mya (Daghlian 1981; Muller 1981). The palms also are the most morphologically diverse of the monocot families—and, perhaps, of all angiosperms (Moore and Uhl 1982). A wide range of morphological and anatomical features have been used in systematic studies of the Arecaceae. These features have included overall size; growth habit; anatomy and morphology of leaves, stems, and reproductive structures; and chromosome numbers. The evolutionary and phylogenetic implications of these and other characters have been reviewed elsewhere (Moore 1973; Moore and Uhl 1982; Uhl and Dransfield 1987). With such a long evolutionary history and the great morphological diversity of the group, it is not surprising that it has been difficult to develop a phylogenetic classification of the palms. There have been numerous classification schemes proposed, beginning with the works of von Martius (1849–53) and Drude (1889). The most current classification includes 200 genera in six subfamilies—the Coryphoideae, Calamoideae, Nypoideae, Ceroxyloideae, Arecoideae, and Phytelephantoideae (Uhl and Dransfield 1987). The phylogenetic placement of two other distinctive groups, the phoenicoid and caryotoid palms, has been controversial. Uhl and Dransfield include them within the Coryphoideae and Arecoideae, respectively.

Owing to a slow rate of nucleotide substitution, chloroplast DNA (cpDNA) restriction-site variation has been shown to be well suited for studies of genetic relationships at or below the family level. The chloroplast genome of most angiosperms consists of a large and a small region of single-copy DNA separated by a pair of

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Address for correspondence and reprints: Michael T. Clegg, Department of Botany and Plant Sciences, University of California, Riverside, Riverside, California 92521.

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identical but inverted repeat sequences. Chloroplast genome size ranges from ~120 kb to 210 kb, with most being ~150 kb in size. In contrast to the case for nuclear and plant mitochondrial genomes, chloroplast gene sequence rearrangements are quite rare (Palmer 1987). Therefore, restriction-pattern differences between taxa may be interpreted as site changes caused by single base substitutions or as single insertion-deletion events (referred to as "indels"). By relating variation in cpDNA restriction-fragment patterns to specific mutations, either base substitutions or indels, data sets suitable for phylogenetic reconstructions using parsimony analysis may be produced (i.e., see Palmer and Zamir 1982; Sytsma and Gottlieb 1986; Jansen and Palmer 1988).

Restriction-site variation also may be used to estimate total sequence divergence between taxa (i.e., see Palmer and Zamir 1982; Clegg et al. 1984; Sytsma and Gottlieb 1986). Such distance measures may be used to reconstruct phylogenies (i.e., see Sytsma and Gottlieb 1986) or to estimate base-substitution rates by using divergence times inferred from the fossil record. Comparison of these rates may determine whether base-substitution rates are similar (clocklike) or variable across taxa.

We surveyed cpDNA restriction-fragment-length polymorphisms (RFLPs) among 22 palm species representing five of the six subfamilies. In the present paper, we present the evolutionary pattern derived, for the Arecaceae, from these data. Furthermore, we obtained an estimate of the rate of cpDNA nucleotide substitution based on site changes. Finally, we compared rates of evolution as estimated from restriction-site data with estimates from direct DNA sequence comparisons. Our results show a greatly reduced rate of nucleotide substitution in the palm family relative to that in annual plant taxa.

Material and Methods

Tissue Sources and Nucleic Acid Isolation

Fresh leaf tissue, from either newly expanding or young mature leaves, was collected from 26 individuals representing 22 species. Five specimens of *Phoenix dactylifera*—one each of the varieties Amir Hajj, Bentamooda, Hilali, Khadrawy, and Medjool—were assayed as a control to compare within- and between-species variation. Materials were obtained from the University of California, Riverside campus; the San Diego Zoo; the Huntington Botanical Gardens; the USDA Station at Brawley, Calif.; and the Fairchild Tropical Gardens in Miami (see Appendix).

Nucleic Acid Isolation and Restriction-Endonuclease Digestion

Total DNA was extracted using either the method of Wagner et al. (1987) or a modification (Doyle and Doyle 1987) of the method of Saghai-Maroof et al. (1984). The modifications of the Saghai-Maroof et al. method were as follows: One gram of fresh leaf material was ground to a slurry in 8 ml of 2 × extraction buffer [100 mM Tris-(hydroxymethyl)amino-methane-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA (ethylenediaminetetraacetic acid), 2% hexadecyltrimethylammonium bromide, 0.2% 2-mercaptoethanol] at 60°C in a mortar which had also been heated to 60°C. Ground glass was added to aid grinding. The slurry was transferred to a centrifuge tube and incubated at 60°C for 15–30 min. After incubation, the slurry was extracted with 1 vol of chloroform and centrifuged at 6,000 *g* to remove debris. The supernatant was removed to a clean tube, and nucleic acids were collected by precipitation with a two-thirds volume of cold isopropanol. This method yielded 50–100 μg of total DNA per gram fresh weight of leaf tissue. The method of Wagner et al. yielded roughly the same amount of DNA, of slightly higher average molecular weight.

Approximately 1–2 μg of total DNA from each individual was digested with each of five restriction enzymes (*Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, and *Hpa*I). The digested DNAs were size fractionated by electrophoresis on 1% agarose gels in Tris-Acetate pH 8.3 (40 mM Tris Base, 20 mM sodium acetate, 2 mM EDTA) at 25 V until a bromophenol blue marker dye had migrated ~10 cm. The size-fractionated DNAs were transferred to GeneScreen© membrane (New England Nuclear) by alkali transfer. Hybridizations were performed at 42°C, washes were performed at 60°C, and membranes were stripped for rehybridizations according to method I of the GeneScreen procedures. Eight overlapping cloned fragments of cpDNA were individually hybridized to the palm total DNA blots. Seven of the cloned fragments were derived from pearl millet, *Pennisetum americanum* (Thomas et al. 1984), and one was derived from barley, *Hordeum vulgare* (Zurawski et al. 1984). Positions of these clones on the barley cpDNA molecule are shown in figure 1. Probes were labeled by the random priming technique of Feinberg and Vogelstein (1984), except that alpha-^{32}PdATP was used in the reaction instead of alpha-^{32}PdCTP. Kodak XAR film was exposed to the hybridized filters 1–5 d with Dupont Cronex Hi Plus intensifying screens at −70°C. Autoradiographs were developed with a Kodak X-Omat M20 processor.

The nature of RFLPs was determined by physical mapping of variable fragments. These polymorphisms were the result either of loss or gain of restriction sites (presumably base substitutions) or of insertion-deletion of DNA sequences. All mutations were coded as discrete presence-absence characters. Site-change mutations were used to estimate numbers of base substitutions per site (Nei and Li 1979). Base-substitution estimates were used as distance measures for a phylogenetic reconstruction by the Fitch and Margoliash (1967) method. Both analyses were performed by programs contained within the "phylogenetic inference package" (Phylip version 2.6) of J. Felsenstein.

DNA Sequencing

Total DNA from *Serenoa repens* and *Calamus usitatus* was digested to completion with *Eco*RI and ligated into the phage vector Lambda gt10. Clones containing *Eco*RI fragments with the *rbc*L sequence were recovered following plaque hybridization with the plasmid vector pMCSp5 from *Pennisetum americanum,* which includes the *rbc*L gene (Thomas et al. 1984). Positive clones were plaque purified, and DNA was prepared and subcloned into pUC118 and M13. Sequencing reactions were carried out using

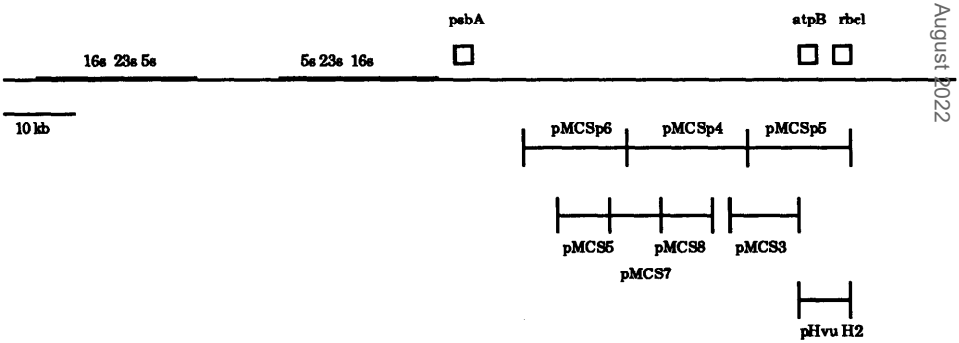

FIG. 1.—Locations on pearl millet chloroplast genome of cpDNA clones used as probes. Inverted repeat regions are indicated by heavy lines.

the dideoxy method of Sanger et al. (1977), with primers synthesized to correspond to conserved internal portions of the gene.

Results and Discussion

Restriction-Site Variation in Palm cpDNA

Restriction-fragment analysis using the five restriction enzymes *Bam*HI, *Cla*I, *Eco*RI, *Hin*dIII, and *Hpa*I, in conjunction with ~50 kb in overlapping cpDNA probes, allowed the identification of 78 restriction sites and seven indels in the 22 species of palms examined. Forty-two of the 78 restriction sites detected were common to all species. The remaining 36 variable sites and the seven indels are listed in table 1. Examples of cpDNA fragments produced by each type of mutation are shown in figure 2.

Ten of the 36 polymorphic sites were shared by two or more genera. The two *Calamus* species shared seven restriction-site changes and two indels not found in any of the other taxa. The five *Phoenix dactylifera* varieties had identical restriction patterns. The 36 variable restriction sites account for 46% of the total number of sites detected. This proportion of variability is greater than that seen in many studies of cpDNA restriction-site variation. For example, Sytsma and Gottlieb (1986) found that 18% of sites were polymorphic in *Clarkia* and *Heterogaura.* Other studies have reported values ranging from ~2%, for comparisons between 14 species of *Triticum* and *Aegilops* (Bowman et al. 1983), to 9.6% between seven species of *Brassica* (Palmer et al. 1983). In contrast, Jansen and Palmer (1988) found that 211 of 213 restriction sites varied among 16 species of the Asteraceae.

Estimates of the number of substitutions per nucleotide, based on the proportion of shared restriction sites between each species pair, were calculated by the method of Nei and Li (1979). Estimates ranged from 0.000 to 0.024 substitutions per base, with an average value of 0.009. Reported base-substitution values in *Brassica* ranged from 0 to 0.024 (Palmer et al. 1983), in *Lisianthus* from 0 to 0.003 (Sytsma and Schaal 1985), in *Clarkia* and *Heterogaura* from 0.002 to 0.016 with a mean of 0.0108 (Sytsma and Gottlieb 1986), and, between *Pennisetum* and *Cenchrus,* from 0 to 0.012 (Clegg et al. 1984). The base-substitution values estimated within the palms are only slightly higher than values reported for the grass species *Pennisetum* and *Cenchrus* and are more or less equivalent to that seen in the *Brassica* and *Clarkia-Heterogaura* studies. This low level of estimated substitution is surprising, both because the proportion of variable restriction sites was at least twice as high as that in other reported studies and because the taxa in this study are more distantly related. The apparent discrepancy between the relatively higher proportion of variable restriction sites and the low number of estimated per-site substitutions may be explained by the fact that 18 of the 36 variable sites in the present study are restricted to only four taxa—*Caryota mitis, Chamaedorea costaricana,* and the two *Calamus* species. The remaining 18 variable restriction sites are distributed more or less equally among the other 18 taxa.

The base-substitution estimates were used as distance measures to produce the Fitch and Margoliash phylogenetic network shown in figure 3. This analysis groups taxa on the basis of their relative distance measures from one another. The length of the line separating taxa is a direct measure of the genetic distance between them. The phylogenetic analysis provides additional insight into the possible evolutionary relationships among the palm subfamilies. The Coryphoideae are presumed to represent an ancestral group in the Arecaceae. An early split then gave rise to the remaining subfamilies. Fossil information suggests that the Coryphoideae, Arecoideae, Nypoideae, and Calamoideae have been distinct evolutionary lines for ≥60 Myr. Although fossil

Table 1
Mutations Detected by Restriction-Endonuclease Analysis

A. Site Changes

	Enzyme	Change	Size	Probe	Species
1.	*Bam*HI	Site gain	4.7kb to 3.7+1.0	pMCSp5	B1 and B2
2.	*Bam*HI	Site gain	6.6kb to 5.7+0.9	pMCSp4	A2, A3, A5, A6, A7, A8, A9, and A10
3.	*Bam*HI	Site gain	6.6kb to 5.0+1.6	pMCSp5	E1
4.	*Bam*HI	Site gain	6.5kb to 5.3+1.2	pMCSp5	A1
5.	*Bam*HI	Site gain	5.7kb to 5.2+0.5	pMCSp5	A8, A9, and A10
6.	*Bam*HI	Site loss	2.3kb+0.5 to 2.8	pMCS3	A8
7.	*Bam*HI	Site loss	2.5kb+0.3 to 2.8	pMCSp6	C1 and E7
8.	*Cla*I	Site gain	5.6kb to 3.3+2.3	pMCSp5	E2
9.	*Cla*I	Site loss	5.6kb+1.0 to 6.6	pMCSp5	E1
10.	*Cla*I	Site loss	2.4kb+1.3 to 3.2	pMCSp4	B1 and B2
11.	*Cla*I	Site loss	7.8kb+3.2 to 11	pMCSp4,5	A2 and A3
12.	*Cla*I	Site gain	2.5kb to 1.9+0.6	pMCSp6	A10
13.	*Cla*I	Site loss	2.7kb+0.6 to 3.3	pMCSp4	E1
14.	*Eco*RI	Site loss	2.7kb+2.2 to 4.9	pHvu H2	D2 and E1
15.	*Eco*RI	Site gain	4.8kb to 4.4+0.4	pMCSp5	C1
16.	*Eco*RI	Site loss	1.0kb+0.5 to 1.5	pMCSp5	B1, B2, and C1
17.	*Eco*RI	Site loss	1.5kb+0.5 to 2.0	pMCSp5	C1
18.	*Eco*RI	Site loss	1.9kb+1.6 to 3.5	pMCS3	B1 and B2
19.	*Eco*RI	Site loss	2.6kb+1.0 to 3.6	pMCSp5	D2
20.	*Eco*RI	Site loss	8.0kb+3.5 to 11.5	pMCSp4	C1
21.	*Eco*RI	Site gain	11.0kb to 8.6+2.4	pMCSp4	B1 and B2
22.	*Eco*RI	Site gain	8.6kb to 4.3+4.4	pMCSp4,6	B1 and B2
23.	*Eco*RI	Site gain	11.0kb to 5.6+5.6	pMCSp4	A8, A9, and A10
24.	*Eco*RI	Site gain	3.5kb to 1.7+1.7	pMCSp4	B1, B2
25.	*Eco*RI	Site loss	3.5kb+4.2 to 8.0	pMCSp4	D2, E2, E3, E4, E5, E6, and E7
26.	*Eco*RI	Site gain	8.0kb to 7.0+1.0	pMCSp4	D2
27.	*Eco*RI	Site gain	11.0kb to 10.0+1.0	pMCS3 and pMCSp4	E6 and E7
28.	*Eco*RI	Site gain	3.5kb to 3.0+0.5	pMCS3 and pMCSp4	E7
29.	*Hpa*I	Site gain	>25kb to >23+2.0	pMCSp5	D2
30.	*Hpa*I	Site loss	11.0kb+4.6 to 15.6	pMCSp5	E1
31.	*Hpa*I	Site gain	7.8kb to 6.2+1.6	pMCSp4,5	D2
32.	*Hin*dIII	Site gain	12.5kb to 5.7+6.8	pMCSp5	E5
33.	*Hin*dIII	Site gain	6.3kb to 4.6+1.7	pMCSp5	D2
34.	*Hin*dIII	Site gain	6.6kb to 5.4+1.2	pMCS3 and pMCSp4	A4 and D2
35.	*Hin*dIII	Site loss	9.0kb+1.8 to 10.8	pMCSp4	B1 and B2
36.	*Hin*dIII	Site gain	9.0kb to 7.2+1.8	pMCSp4	B1 and B2

B. Addition and Deletions

	Change	Size (kb)	Enzyme(s)	Probe	Species
37.	Deletion	0.2	*Cla*I and *Eco*RI	pMCSp6	A8 and A9
38.	Deletion	0.2	*Bam*HI, *Cla*I, and *Eco*RI	pMCSp5	A10
39.	Deletion	0.7	*Cla*I, *Eco*RI, and *Hin*dIII	pMCSp5	B1 and B2
40.	Deletion	0.3	*Cla*I and *Eco*RI	pMCSp6	B1 and B2
41.	Addition	0.2	*Eco*RI	pMCSp5	D1
42.	Addition	0.2	*Bam*HI, *Eco*RI, and *Hpa*I	pMCSp5	E2
43.	Deletion	0.2	*Cla*I	pMCSp4	E7

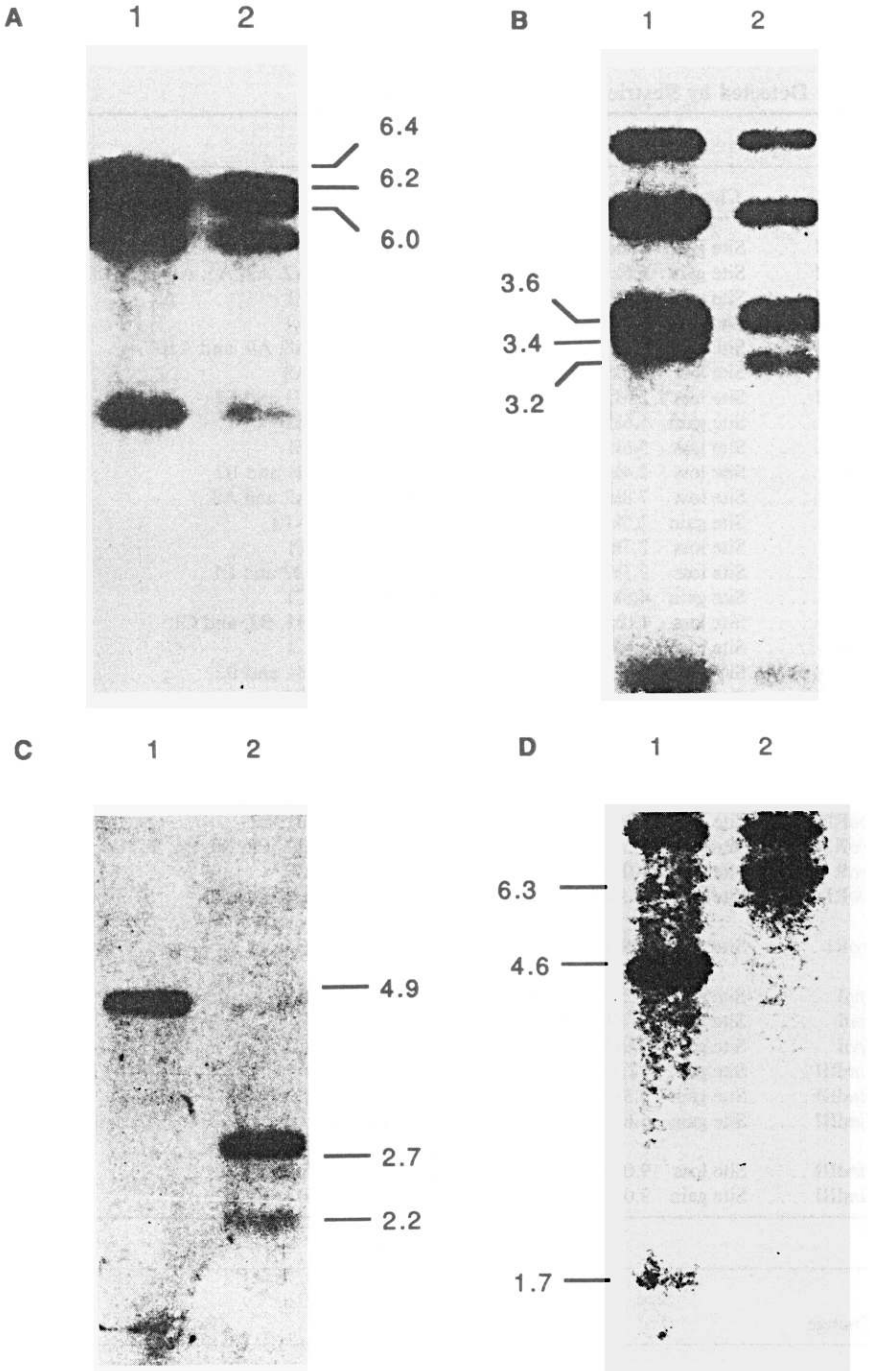

FIG. 2.—Examples of cpDNA restriction-fragment-length polymorphisms detected. Fragment sizes are indicated in kilobase pairs (kbp). A, *Cla*I restriction-enzyme digest hybridized with MC9 probe, showing an ~200-bp deletion. Lane 1, *Phoenix reclinata,* showing the common restriction-fragment band pattern; lane 2, *P. rupicola* with deletion (*P. reclinata,* 6.4 kb, *P. rupicola,* 62 kb; table 2, mutation 38). B, Same deletion event as in panel A but with *Bam*HI. C, *Eco*RI digest hybridized with *atpBE* fragment of pHvu H2. Loss of *Eco*RI site in *Caryota mitis,* forming a single 4.9-kb band in place of the common 2.2- and 2.7-kb bands (table 2, mutation 14). Lane 1, *C. mitis;* lane 2, *Rhapis humilis.* D, Gain of *Hin*dIII restriction site in *Chamaedorea costaricana,* replacing the common 6.3-kb band with bands 4.6 and 1.7 kb in size (table 2, mutation 33). Lane 1, *C. costaricana;* lane 2, *Trachycarpus takil.*

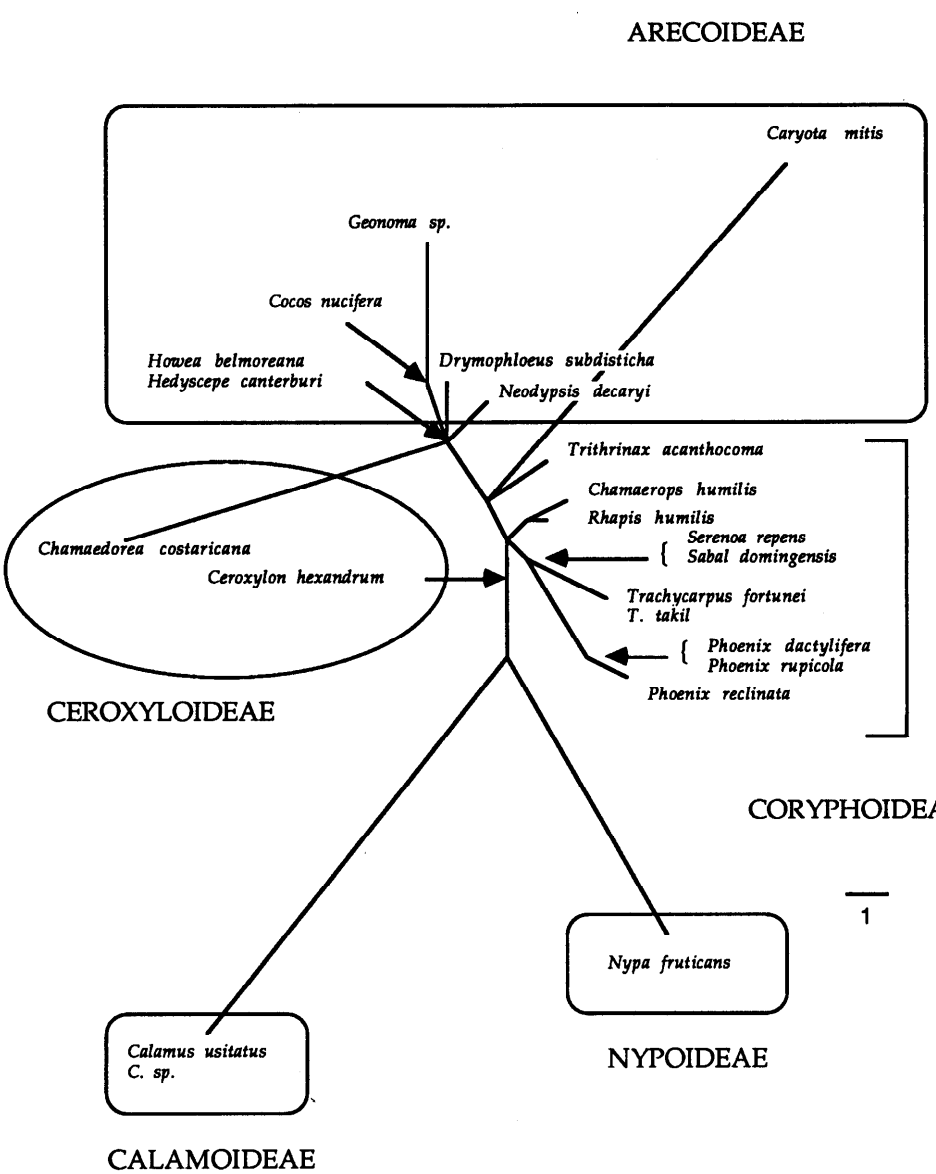

FIG. 3.—Fitch-Margoliash phylogenetic network derived from base-substitution estimates. The length of the line connecting any two taxa is proportional to the estimated number of base substitutions between them. The scale bar equals 1 substitution/1,000 bases. Average % SD over all species in this tree is 17.4.

records do not support such an early separation of the Ceroxyloideae, both the present distribution of living genera and the presence of many primitive features in these palms also suggest ancient derivation of that subfamily (Moore 1973). The same may be true of the caryotoid group. The basic pattern that is presently accepted for the evolutionary history of the palms has been an early radiation into the major groups, with subsequent specialization of a few groups, such as the Phoeiceae within the Coryphoideae. From a broad perspective, the phylogenetic analysis derived from the restriction-site data is generally consistent with these interpretations of the evolution of the palm family.

Estimates of Nucleotide-Substitution Rates

Dividing the per-nucleotide base-substitution estimates obtained from the restriction-site data, by time since divergence as based on fossil records, gives an estimate of nucleotide-substitution rates between taxa. The fossil data available for palms allow comparisons to be made for four of the subfamilies (table 2). Using fossil evidence of a minimum divergence time of 60 Myr between other palms and *Calamus* (Daghlian 1981; Muller 1981), we estimated the base-substitution rate as $\sim 1.3 \times 10^{-10}$ substitutions/site/year. If one assumes that there was an equally early divergence for the Ceroxyloideae, the estimated rate between the ceroxyloid palm *Ceroxylon* and the remaining taxa is 5.2×10^{-11}. This is a 2.5-fold difference in substitution-rate estimates. An overall estimate of 6.7×10^{-10} nucleotide substitutions/site/year was determined between barley and maize cpDNAs by assuming a divergence time of 50 Mya (Zurawski et al. 1984). The estimate was based on direct sequence comparisons of the chloroplast-encoded large subunit gene for ribulose-1,5-bisphosphate carboxylase (1,431 bp), together with ~800 bp of noncoding DNA 5′ to the gene. This rate is more than fivefold greater than that between the calamoid and other palms and is almost 13-fold greater than the estimated substitution rate between the ceroxyloid and other palms.

Restriction-site comparisons may not provide an accurate estimate of substitution rates, because small indels may be confounded with restriction-site changes and tend to be estimated as nucleotide substitutions. Small indels (typically 1–10 bp in length) are a common mutational class in noncoding regions of the chloroplast genome (Zurawski and Clegg 1987). In addition, different regions of the molecule may evolve at different rates. For instance, it is now well established that the inverted-repeat region evolves at a reduced rate relative to other regions (Clegg et al. 1984; Doebley et al. 1987; Palmer 1987). For these reasons, rate comparisons using complete DNA sequence data based on the same gene taken from different taxa are more desirable.

To further characterize the apparent rate differences estimated from the restriction-site data, we sequenced 985 bp of the *rbc*L gene from *Serenoa repens* (Coryphoideae) and *Calamus usitatus* (Calamoideae). The sequence comparisons are shown in figure 4. Only 10 nucleotide differences (five silent mutations and five replacement mutations) were observed between the two palm species. The number of nucleotide substitutions was estimated using the 3ST model of Kimura (1981), which yielded 0.0051 (SE = 0.0072) replacements and the same number of silent substitutions per nucleotide site. If one assumes a divergence time of 60 Mya, the rate estimates (expressed on a per-nucleotide site–per-year basis) are each 4.2×10^{-11}. An estimate for the average

Table 2
Estimates of the Number of Substitutions per Nucleotide Site (following the method of Nei and Li 1979), per Year, Between Palm Subfamilies Represented by Fossil Records (Daghlian 1981)

Subfamily	Fossil Age (Mya)	Substitution rate
Coryphoideae	80	5.8×10^{-11}
Nypoideae	60	1.1×10^{-10}
Arecoideae.....	60	7.8×10^{-11}
Calamoideae	60	1.3×10^{-10}

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Serenoa      0   AATTCCCACT TCTTATTCCA AAACTTTCCA AGGcCCGCCT CATGGCATCC
Calamus      0   ---------- ---------- ---------- ---t------ ----------

Serenoa     50   AAGTTGAAAG AGATAAGTTG AACAAGTATG GTCGTCCTCT ATTGGGATGT
Calamus     50   ---------- ---------- ---------- ---------- ----------

Serenoa    100   ACTATTAAaC CAAAATTGGG ATTATCCGCA AAGAACTACG GTAGAGCGGT
Calamus    100   --------g- ---------- ---------- ---------- ----------

Serenoa    150   TTATGAATGT CTACGCGGTG GACTTGATTT TACCAAGGAT GATGAAAACG
Calamus    150   ---------- ---------- ---------- ---------- ----------

Serenoa    200   TGAACTCACA ACCATTTATG CGTTGGAGAG ACCGTTTCTT ATTTTGTGCC
Calamus    200   ---------- ---------- ---------- ---------- ----------

Serenoa    250   GAAGCAaTTT ATAAAGCGCA GGCCGAAACG GGTGAAATCA AAGGACATTA
Calamus    250   ------c--- ---------- ---------- ---------- ----------

Serenoa    300   CTTGAATGCT ACTGCGGGTA CATGTGAAGA AATGATcAAA AGGGCCgTAT
Calamus    300   ---------- ---------- ---------- ------g--- ------a---

Serenoa    350   TTGCCAGAGA ATTGGGAGTT CCTATCGTAA TGCATGACTA CTTAACTGGG
Calamus    350   ---------- ---------- ---------- ---------- ----------

Serenoa    400   GGATTCACTG CAAATACTAG CTTGGCTCAT TATTGCCGCG ATAACGGCCT
Calamus    400   ---------- ---------- ---------- ---------- ----------

Serenoa    450   ACTTCTTCAC ATCCATCGCG CAATGCATGC AGTTATTGAT AGACAGAAAA
Calamus    450   ---------- ---------- ---------- ---------- ----------

Serenoa    500   ATCATGGTAT GCATTTTCGT GTACTAGCTA AAGCATTACG TATGTCTGGT
Calamus    500   ---------- ---------- ---------- ---------- ----------

Serenoa    550   GGAGATCATA TTCACGcAGG TACAGTAGTG GGTA-ACTGG AAGGGGAACG
Calamus    550   ---------- ------g--- ---------- ---------- ----------

Serenoa    600   TGAGATGACT TTGGGTTTTG TTGATTTATT ACGTGATGAT TTTATTGAAA
Calamus    600   ---------- ---------- ---------- ---------- ----------

Serenoa    650   AAGACCGAAG TCGCGGTATC TTTTTTACTC AAGATTGGGT CTCTATGCCA
Calamus    650   ---------- ---------- ---------- ---------- ----------

Serenoa    700   GGTGTTATaC CCGTGGCTTC AGGGGGTATT CATGTTTGGC ATATGCCTGC
Calamus    700   --------c- ---------- ---------- ---------- ----------

Serenoa    750   CCTGACCGAA ATCTTTGGaG ATGATTCCGT ACTACAGTTT GGCGGAGGAA
Calamus    750   ---------- --------g- ---------- ---------- ----------

Serenoa    800   CTTTAGGACA CCCTTGGGGA AATGCACCCG GTGCAGTAGC TAATCGGGTG
Calamus    800   ---------- ---------- ---------- ---------- ----------

Serenoa    850   GCTTTAGAAG CGTGTGTACA AGCTCGTAAT GAGGGACGTG ATCTTGCTCG
Calamus    850   ---------- ---------- ---------- ---------- ----------

Serenoa    900   TGAAGGTAAT GAAATTATCC GTGAAGCTAG CAAATGGAGC CCTGAACTAG
Calamus    900   ---------- ---------- ---------- ---------- ----------

Serenoa    950   CGTCCGCTTG TGAAGTATGG AAgGaGATCA AATTC
Calamus    950   ---------- ---------- --a-c----- -----
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FIG. 4.—Sequence of 985 bp of the *Serenoa repens rbc*L gene. Differences between *Serenoa* and *Calamus* are indicated by giving the *Calamus* nucleotide below the *Serenoa* sequence.

rate of silent substitution in *rbc*L between annual monocot and dicot species is 1.5×10^{-9} (Zurawski and Clegg 1987).

Direct comparisons between these estimates indicates a 36-fold reduction in the silent rate for the palm taxa. A remarkable feature of the palm data is the equal number of replacement and silent substitutions. As a consequence, there is only a fourfold reduction in the rate of replacement substitutions for the palm taxa relative to the annual dicot and monocot contrast. The total estimate of nucleotide substitution per nucleotide site, taken over all nucleotide positions, is 0.010 (SE = 0.015) for the palm contrast, which compares with a total estimate of 0.067 (SE = 0.007) for barley versus maize (Zurawski et al. 1984). Assuming both that barley and maize diverged 50 Mya and a divergence time of 60 Mya for *Calamus* versus *Seranoa* leads to an estimated sevenfold excess of nucleotide substitutions for the two grass taxa relative

to the two palms. These calculations are consistent with the estimates based on the restriction-site data given above. Ratios like those presented here have large SEs, especially since relatively few mutational events were observed among the palms. Nevertheless, the overall picture is consistent with the restriction-site data in indicating a substantially reduced rate of cpDNA evolution in palms.

Wu and Li (1985) have proposed that the number of cell divisions prior to germline formation may influence rates of mitochondrial DNA (mtDNA) sequence evolution in vertebrates. Humans undergo twice as many cell cycles prior to formation of the germ-line tissue as do rodents, and they appear to display an mtDNA substitution rate half that of mice. The great difference in base-substitution rate estimates between palms and grasses may be an indication of an effect of generation time on cpDNA evolutionary rates. Thus the far greater time to reproduction in long-lived perennial plants, as compared with that in annuals, may play a role in generating these widely divergent estimates of base-substitution rates.

Most studies of cpDNA variation have utilized annuals or short-lived perennial plants. Preliminary investigations of cpDNA restriction-site variation in the Juglandaceae (Smith and Doyle 1986), a family of woody dicotyledons with a relatively long fossil record (>60 Myr), have suggested a considerably slower rate of cpDNA evolution when compared with the current data from mainly annual plants. Further research on long-lived, long-generation-time perennials will provide a much needed base of information from which to examine variation in rates of cpDNA evolution.

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APPENDIX

Species Used for Restriction-Map Comparisons, According to Subfamilial Classification

The letters in parentheses indicate the sources: a = Fairchild Tropical Gardens; b = University of California, Riverside; c = USDA station; d = San Diego Zoo; e = Huntington Gardens.

I. Coryphoideae
- A. Corypheae
  1. *Trithrinax acanthocoma* Drude (a)
  2. *Trachycarpus fortunei* Wendl. (b)
  3. *Trachycarpus takil* Becc. (b)
  4. *Rhapis humilis* Blume (b)
  5. *Serenoa repens* Bartr. (b)
  6. *Sabal domingensis* Becc. (b)
  7. *Chamaerops humilis* L. (b)
- B. Phoeniceae
  8. *Phoenix dactylifera* L. (b)
     var. Amir Hajj (c)
     var. Bentamooda (c)

var. Hilali (c)
var. Khadrawy (c)
var. Medjool (c)
9. *P. reclinata* Jacq. (b)
10. *P. rupicola* T. Anders. (b)

II. Calamoideae
A. Calamae
1. *Calamus usitatus* (d)
2. *Calamus* sp. (a)

III. Nypoideae
1. *Nypa fruticans* Wurmb. (a)

IV. Ceroxyloideae
A. Ceroxylae
1. *Ceroxylon hexandrum* Dugand (e)
B. Hyophorbeae
2. *Chamaedorea costaricana* Orst. (a)

V. Arecoideae
A. Caryoteae
1. *Caryota mitis* Lour. (a)
B. Areceae
2. *Neodypsis decaryi* Jumelle (e)
3. *Hedyscepe canterburi* Wendl. et Dr. (e)
4. *Howea belmoreana* Becc. (e)
5. *Drymophloeus subdisticha* H. E. Moore (a)
C. Cocoeae
6. *Cocos nucifera* L. (a)
D. Geonomeae
7. *Geonoma* sp. (e)

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