Systematic Botany (2002), 27(4): pp. 654–661

Low Intralineage Divergence in *Ceratozamia* (Zamiaceae) Detected with Nuclear Ribosomal DNA ITS and Chloroplast DNA *trnL-F* Non-coding Region

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Communicating Editor: Thomas G. Lammers

ABSTRACT. The genus *Ceratozamia* comprises about 16 species with restricted distribution north and south of the Eje Neovolcanico in Mexico. Phylogenetic relationships were explored among species of *Ceratozamia*, using 24 exemplars and seven putative outgroup taxa. We examined variation at the molecular level in two non-coding regions from the chloroplast and nuclear genomes. Although the rate of change of the ITS and *trnL-F* non-coding regions is considered appropriate to recover variation at this taxonomic level, only 33 nucleotide positions were informative out of 2081. Despite low levels of variation, analyses showed that *Ceratozamia* is monophyletic and identified *Zamia* as sister group. The molecular phylogeny of *Ceratozamia* revealed three main clades, which allow for biogeographic interpretations. The most basal clade suggests a probable ancestral geographic area for *Ceratozamia* in Southeast Mexico. Another implication is that speciation within the genus appears to be associated with the post-Pleistocene spread of floristic communities from proposed Pleistocene tropical refugia located in S. E. Mexico towards north of the Eje Neovolcanico.

The genus *Ceratozamia* or ''horned *Zamia*,'' as the name suggests, are cycads largely restricted to Mexico. *Ceratozamia* comprises about 16 species with a distribution range from Honduras, in Central America to the state of Tamaulipas, at its extreme northern limit, in Mexico. The genus is found mainly in dense moist tropical woodlands (Norstog and Nicholls 1997) such as cloud-forests and evergreen tropical rain-forests. It also occurs in mid-elevation pine-oak forests. Some *Ceratozamia* species are arborescent with stems rarely more than about one meter tall, often leaning or curved and rarely branching. Others are semi-hypogeous and often branching (Norstog and Nicholls 1997).

Ceratozamia presents morphological variation both within and between species. Miquel (1870) noted differences in leaflet shape and size. He also observed varying sizes of cones with age of any given species of *Ceratozamia* but stability in micro- and megasporophylls. Dyer (1882–86) commented: ''This variableness (sic) with age makes the separation of nearly allied forms an all but hopeless task.'' To date, species descriptions within the genus are based largely on gross morphological characters, strobilus indument coloring, and geographical distribution. Chromosome number and karyotype within the genus is extremely stable (Vovides 1983, 1985; Moretti 1990), which contrasts with chromosome variation found in other genera such *Zamia* (Moretii and Sabato 1984; Vovides and Olivares 1996). Furthermore, Vovides (1983, 1985) noted differences in secondary constriction or satellite number and position within *Ceratozamia*.

Molecular phylogenetic analyses on New World cycads are scarce, and those so far published have examined RFLP variation and sequence data among genera (Caputo et al. 1991, 1993; Moretti et al. 1993; De Luca et al. 1995; Bogler and Francisco-Ortega in press). According to Crane (1988) and Stevenson (1985) *Ceratozamia*, *Zamia*, and the Cuban endemic *Microcycas* are phylogenetically related. In order to provide an insight into phylogenetic relationships within the Mexican genus *Ceratozamia*, we undertook a study of variation in sequences of non-coding regions from the chloroplast and nuclear genomes. We selected two regions, ITS and *trnL-F*, that have been used in phylogenetic studies at intrageneric level for a variety of plants (Gielly and Taberlet 1994, 1996; Baldwin et al. 1995; Schilling et al. 1998; Potter et al. 2000). The sequence data generated in this study was used to evaluate: 1) monophyly of *Ceratozamia*, 2) phylogenetic relationships to other genera of cycads, and 3) species relationships.

MATERIALS AND METHODS

Exemplars of Ceratozamia and Outgroup Selection. Twenty-four exemplars of *Ceratozamia* were sequenced in this study. They represented all described species (one with a duplicate), one presumed new species, and four unclassified specimens. We were unable to identify these specimens due the lack of female and male cones in the plants. We also sequenced one species each of *Dioon*, *Cycas*, *Microcycas*, *Lepidozamia*, *Zamia*, *Stangeria*, and *Encephalartos* as putative outgroup taxa, representing seven out of 11 described genera within Cycadales (Table 1). In previous cladistic studies with RFLPs of cpDNA, a clade containing *Microcycas*, *Chigua*, and *Zamia* resulted as sister group of *Ceratozamia* (Caputo et al. 1991, 1993). We represented this clade with sequence data from one exemplar of *Zamia* and *Microcycas* but not from *Chigua*.

DNA Extraction. Genomic DNA of all taxa was extracted from field-documented live plants held at the Clavijero Botanic Garden (Instituto de Ecologia, Xalapa). An optimized protocol developed in our lab for DNA extraction was used as follows: 0.1 gr of fresh or dried leaf tissue was surface sterilized with 70% ethanol, rinsed with distilled water, cut in small pieces, and put into 1.5 ml tube.

TABLE 1. List of 31 exemplars used for cladistic analysis of ribosomal DNA internal transcribed spacer (ITS 1 and 2), and *trnL-F* cpDNA non-coding region, locality, voucher number and GenBank accession number. * = incomplete sequence data. Details for each sample are provided in the following order: exemplar species, locality, voucher, GenBank Acc. # ITS, *trnL-F*.

Ceratozamia kuesteriana, Tamaulipas, 83-465.17, AF407285, AF407316; ***C. hildae,*** San Luis Potosi, 77-197.2, AF407284, AF407315; ***C. zaragozae,*** San Luis Potosi, 79-127.05, AF407302, —; ***C. euryphyllidia,*** Veracruz, 86-318, AF407282, AF407313; ***C. morettii,*** San Luis Potosi, 76-014, AF407293, AF407324; ***C. mexicana*** var. ***robusta,*** Veracruz, 76-038, AF407290, AF407321; ***C. mexicana*** var. ***mexicana,*** Veracruz, 76-019, AF407288, AF407319; ***C. microstobila,*** San Luis Potosi, 78-407, AF407291, AF407322; ***C. sabatoi,*** Hidalgo, 91-041, AF407295, AF407326; ***Ceratozamia*** (#1), Veracruz, 82-439.01, AF407287, AF407318; ***Ceratozamia*** (#2), Queretaro, 99-045.01, AF407297, AF407328; ***Ceratozamia*** (#3), Queretaro, 99-049.01, AF407308, —; ***Ceratozamia*** (#4), Hidalgo, 99-055.01, AF407298, AF407329; ***Ceratozamia*** sp nov. (inedit), Tabasco, 85-011.01, AF407280, AF407311; ***C. miqueliana,*** Veracruz, 81-853.01, AF407305, —; ***C. alvarezii,*** Chiapas, 96-012.01, AF407279, AF407310; ***C. matudai,*** Chiapas, 86-098.01, AF407286, AF407317; ***C. norstogii*** (#1), Chiapas, 93-010.01, AF407294, AF407325; ***C. norstogii*** (#2), Chiapas, 96-063.07, AF407299, AF407330; ***C. mirandai,*** Chiapas, 98-091.03, AF407306, —; ***C. zoquorum,*** Chiapas, 97-016.01, AF407309, —; ***C. whitelockiana,*** Oaxaca, 00-028, AF407301, —; ***C. mixeorum,*** Oaxaca, 00-027, AF407307, —; ***C. brevifrons,*** Veracruz, 98-043.05, AF407304, —;

Cycas rumphii, Asia, 90-032, AF407283, AF407314;
Microcycas calocoma, Cuba, 90-019.01, AF407281 *, AF407312 *;
Dioon edule, Veracruz, 82-328.01, AF407289, AF407320;
Stangeria eriopus, South Africa, 90-031-01, AF707300 *, AF407331;
Lepidozamia peroffskyana, Australia, 90-054, AF407296, AF407327 *;
Encephalartos altensteinii, South Africa, 83-340, AF407303, —;
Zamia herrerae, Chiapas, 85-012.02, AF407292 *, AF407323;

The tissue was suspended in 0.6 ml of 2% CTAB extraction buffer (1.4 M NaCl, 100 mM Tris [pH 8.0], 20 mM Na_2EDTA, 2% [w/v] CTAB) and placed in a water bath at 65°C for 30–60 min, mixing occasionally. The 0.6 ml of extraction buffer was transferred to a clean 1.5 ml tube, saving the tissue for repeated extractions. This solution was extracted twice for 5 min, with an equal volume of chloroform-isoamyl alcohol 24:1 (v/v) and centrifuged at 13200 rpm for 15 min. The upper aqueous layer was mixed with 0.1 volumes of 3 M sodium acetate; cold absolute ethanol (1.8 volumes) was then added to precipitate DNA for at least one hr. The pellet was collected, rinsed with 70% ethanol, vacuum dried and re-suspended in TE buffer (10 mM Tris-Cl, pH 8.0 1.0 mM EDTA). The tissue was kept in the refrigerator at 4.0°C for future DNA re-extractions. We recovered good quality DNA from the same tissue up to three consecutive extractions.

DNA Amplification and Sequencing. Prior to amplification, genomic DNA was purified on a 0.6% low-melting-point agarose gel, then removed from the gel and dissolved up to 0.001 μg per μl in distilled water. Approximately 0.01 μg of purified DNA was amplified by the polymerase chain reaction (PCR). Reactions for PCR amplification were performed in a 50 μl mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 200 μM of each of the four deoxynucleoside triphosphates, 5 pmol of each primer, 10 μl of template and 2.5 units of *Taq* polymerase. Amplifications were performed with a Perkin-Elmer Thermal Cycler 480 (Norwalk, CT). The cycle parameters were: an initial denaturation at 96°C for 5 min, followed by 25 cycles consisting of denaturation at 96°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, and a final extension for 7 min at 72°C.

The primers named ''c'', ''e'' and ''f'' were used to amplify and/or sequence the *trnL-F* region of cpDNA (Taberlet et al. 1991). The oligonucleotide primers ITS1, 2, 3, and 4 (White et al. 1990) were used for amplification and sequencing of the ITS region. Owing to the length of the ITS 1 region being greater than 600 nucleotides we designed two new primers, ''cera-373'' (5′-CTATCAGTACACCCCTCTGCCT-3′) and ''cera-394-R'' (5′-GAGGCAGAGGGGTGTACTGATA-3′), for the species of *Ceratozamia*.

PCR products were purified with Geneclean (BIO 101). Amplified DNA was sequenced using a thermo sequenase dye terminator cycle sequencing pre-mix kit (Amersham Life Science) as described by the manufacturer. Sequencing products were separated in a 4.75% polyacrylamide gel using an ABI-373A automated sequencer (Perkin-Elmer, Foster City, CA).

Difficulties were encountered obtaining complete sequence data for *Ceratozamia moretti* and some outgroup taxa despite the inclusion of DMSO (5%) in the reaction mixtures. Consequently, the identities of several nucleotides (approximately 3.0%) in the ITS 1 and *trnL-F* regions were not determined.

Sequence Analyses. The aligned data set included sequences from the ITS region of the rDNA for 24 exemplars of *Ceratozamia* and seven outgroup taxa. *Dioon*, *Cycas*, *Lepidozamia*, and *Encephalartos* had complete sequence data, while *Stangeria*, *Zamia*, and *Microcycas* had only partial sequence data for the ITS 1 region. Sequences from the *trnL-F* non-coding region of the cpDNA are complete for 16 out of 24 exemplars of *Ceratozamia*, and four outgroups (*Dioon*, *Cycas*, *Stangeria*, and *Zamia*). *Lepidozamia* and *Microcycas* had only partial sequence data. We determined the boundaries of the ITS 1 and ITS 2 by comparing our sequences with those previously published for some angiosperms (Baldwin 1993; Soltis et al. 1996; Eriksson and Donoghue 1997).

Sequences were aligned using the Clustal V program (Higgins et al. 1992) within the Megalign software package (Lasergene, DNASTAR Inc.), and later adjusted by visual examination. Only informative characters were included in phylogenetic analyses. Gaps were treated as missing data.

Sequence distances among taxa were measured with the HKY85 model of sequence evolution proposed by Hasegawa et al. (1985). This model allows transitions and transversions to occur at different rates, and allows base frequencies to vary as well. Distances were calculated for each data set (ITS and *trnL-F*) and in combination. All characters and all taxa, even those with partial sequence data, were included in these analyses.

Phylogenetic Analyses. All analyses were performed using the maximum parsimony criterion in PAUP*, ver. 4.0b4 (Swofford 2000). We did a ''branch and bound'' search with simple addition of taxa. Only exemplars with complete sequence data were included in these analyses. Branch support was assessed by bootstrap analysis (Felsenstein 1985) based on 500 heuristic replicates. Decay indexes were calculated with AutoDecay ver. 4.0 (Eriksson 1998).

We used the program MacClade ver. 3.04 (Maddison and Maddison 1992) to optimize the data from localities for each of the 24 exemplars of *Ceratozamia*. Three states were used to code all taxa. Exemplars with localities south of the Eje Neovolcanico had state 0. Specimens with localities at the Eje Neovolcanico had state 1, and those with localities north of the Eje Neovolcanico had state 2.

RESULTS

Sequence Analyses. Sequences of the ITS for 24 exemplars of *Ceratozamia* were very similar. Distance values varied from 0.00 to 0.02530 (Fig. 1). In contrast, the sequences of seven outgroup taxa were completely different among them and among the *Ceratozamia* spp. Including seven outgroup taxa in the analyses, distance values increased up to 1.1879 between *Zamia herrerae* and *Microcycas calocoma*.

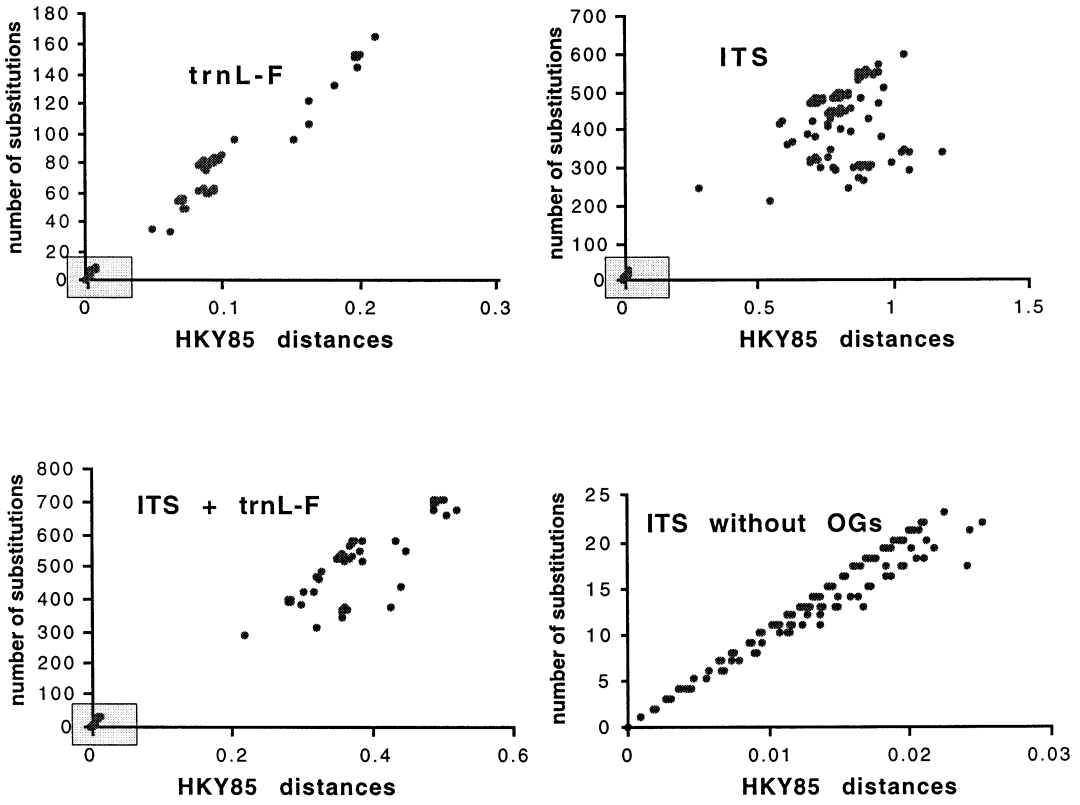


FIG. 1. Corrected distances among all taxa under the HKY85 model of sequence evolution. Distances were calculated for each data set and combined. Inside boxes are the distances only between pairs of species within *Ceratozamia*. All characters and all taxa, even those with partial sequence data, were included in these analyses.

Sequences of the *trnL-F* for 16 exemplars of *Ceratozamia* were almost alike. Distance values ranged from 0.00 to 0.00827 (Fig. 1). The sequences for six outgroup taxa were also very similar to the sequences of *Ceratozamia*. Distance values did not change considerably when we included 6 outgroup taxa in the analyses. The values ranged from 0.00 to 0.21257 (Fig. 1).

The length of the ITS sequences for all 24 exemplars of *Ceratozamia* is 1079 bp from the 5′ end of the ITS 1 to the 3′ end of the ITS 2. The ITS 1, 5.8S, and ITS 2 are 609 bp, 166 bp, and 256 bp respectively. These lengths are similar to those found in other cycads (Liston et al. 1996). The alignment of the ITS region generated 1083 characters. From these, only 31 sites were phylogenetically informative. Three gaps were needed to align all species of *Ceratozamia*.

Analyses with the ITS region were performed using only *Zamia* as outgroup. The inclusion of only one outgroup taxon was due to the ambiguous alignment of the ITS sequence data between ingroup and outgroup taxa. The selection of this exemplar was based on the results obtained in our analyses with the *trnL-F* noncoding region, where *Zamia* was sister group of *Ceratozamia*. The inclusion of *Zamia* generated 88 additional characters. From those, only two were informative.

The length of the *trnL-F* for 16 *Ceratozamia* species was 987 nucleotides. However, *C. norstogii* (Table 1, #2) had 937 and *C. mexicana* var. *robusta* had 997. Four gaps were needed in this region. One large gap of 50 bases was inserted to align *C. norstogii* (#2) because the sequence length was 937 bp. A gap of 10 bases was required for *C. mexicana* var. *robusta* with a sequence of 997 bp, and two gaps of one base were necessary for aligning *Ceratozamia* sp. nov. (inedit), *C. alvarezii*, *C. matudai*, and two exemplars of *C. norstogii*. The alignment for a subsample of 16 *Ceratozamia* sequences generated 998 characters, from which only two were informative. The addition of four outgroups with complete sequence data generated 1017 sites with 57 additional informative characters.

Phylogenetic Analyses. A comparison of general features of trees found with ''branch and bound'' searches is summarized in Table 2. Parsimony analysis of the ITS sequence data set yielded 112 trees of 51 steps (CI = 0.8235; RI = 0.9151; RC = 0.7536). The strict consensus of these trees is shown in Fig. 2 (with bootstrap and decay values) and one randomly chosen minimal length tree is presented to show branch lengths.

Phylogenetic analyses of the ITS characters support-

TABLE 2. Comparisons of results with two sequence data sets. Analysis 1 includes 24 exemplars of *Ceratozamia.* Analyses 2 and 3 are for 16 exemplars. Other columns are: number of trees found in branch and bound analyses, length, consistency index (CI), retention index (RI), re-scaled consistency index (RC).

Analyses	Number of characters	Informative	%	# trees	Length	CI	RI	RC
1) ITS + one outgroup	1171	33	2.81	112	51	0.8235	0.9151	0.7536
2) *trn*L-F + four outgroups	1017	59	5.80	6	88	0.7841	0.8273	0.6487
3) ITS + *trnL-F* + four outgroups	2184	461	21.10	1276	947	0.7867	0.6990	0.5499

ed three main clades within *Ceratozamia* (A, B, C in Fig. 2). Clade ''A'' comprises *C. matudai* and *C. mixeorum* (bootstrap = 92%, decay = 1). Clade ''B'' comprises *Ceratozamia* sp. nov. (inedit), *C. euryphyllidia*, *C. miqueliana*, *C. zoquorum*, *C. whitelockiana*, *C. alvarezii*, two exemplars of *C. norstogii*, and *C. mirandai* (bootstrap = 64%, decay = 1). Clade ''C'' comprises thirteen exemplars of *Ceratozamia* (bootstrap = 67%, decay = 1). Clade ''A'' is sister to clades ''B'' and ''C'' together but with less than a 50% bootstrap value. In addition, ITS characters support internal clades within clade ''B'' and ''C''. Clade ''B'' shows *Ceratozamia* sp. nov. (inedit), *C. euryphyllidia*, and *C. miqueliana* as being sister to a clade formed by *C. zoquorum*, *C. whitelockiana*, *C. mirandai*, *C. norstogii*, and *C. alvarezii*. This clade corresponds to the *C. norstogii* complex. In clade ''C'', one unclassified exemplar of *Ceratozamia* (Table 1, #1) is sister to a clade grouping twelve exemplars (Fig. 2). In this clade only the relationships between *C. mexicana* var *mexicana* and *C. morettii*, also *C. mexicana* var. *robusta* and *C. brevifrons* are resolved with a bootstrap value above 50%.

Analysis of a subsample of *Ceratozamia*, and four outgroup taxa for the *trnL-F* non-coding region yielded six trees of 88 steps (CI = 0.7841; RI = 0.8273; RC = 0.6487, Fig. 3). The branch separating outgroup genera is long, whereas within *Ceratozamia* branch lengths are very short. Although there was almost no variation in the *trnL-F* non-coding region, *Zamia* is resolved as sister group of *Ceratozamia* a relationship strongly supported (bootstrap = 100%, decay = 25).

Analysis of the combined data set for sixteen exemplars of *Ceratozamia* and four outgroup taxa resulted in 1276 trees of 947 steps (CI = 0.7867; RI = 0.6990; RC = 0.5499). The consensus is consistent with the tree generated with the ITS data set alone but less resolved (Fig. 4).

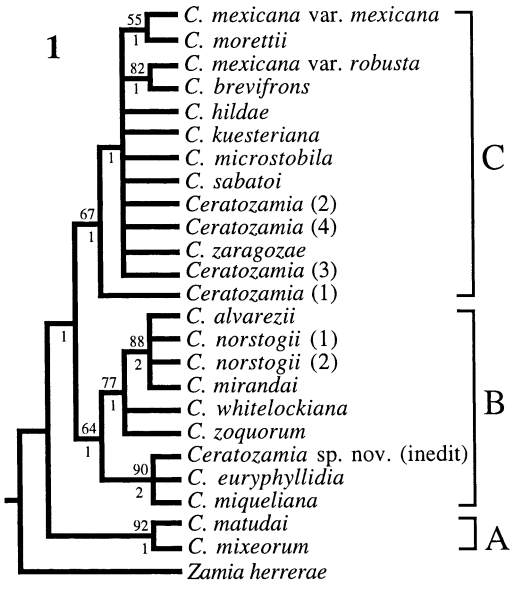
When we optimized the data for the localities for each exemplar of *Ceratozamia* in the cladogram the direction of the transformation series is equivocal for three clades. Therefore, transformation either may go from south through center of the Eje Neovolcanico and then north of it or it may go from south all the way through north and then to the center of it (Fig. 5).

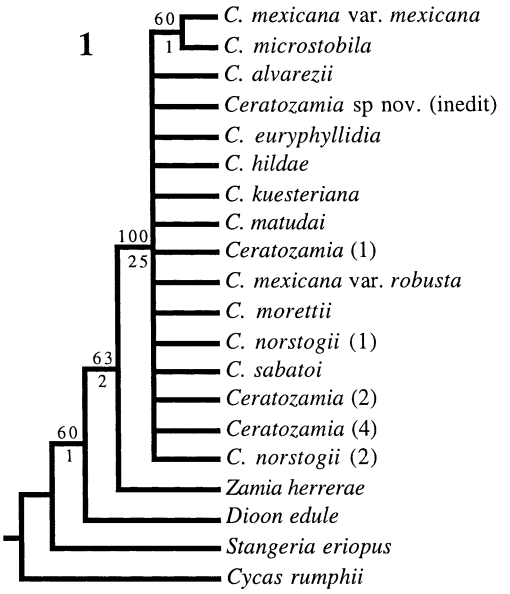
DISCUSSION

The nuclear and chloroplast DNA sequence data exhibited low levels of variability within species of *Ceratozamia*. Variability in the *trnL-F* region is lower than in the nuclear genome ITS. The variation, although limited, allowed some resolution of interspecific relationships, which gives the first insight into relationships within Mexican cycads of the genus *Ceratozamia*. Clade ''A'' grouped *C. mixeorum* and *C. matudai*, which are basal to the rest of the *Ceratozamia* species. Clade ''B'' included *Ceratozamia* sp. nov. (inedit), *C. euryphyllidia*, *C. miqueliana*, *C. zoquorum*, *C. whitelockiana*, *C. alvarezii*, two exemplars of *C. norstogii*, and *C. mirandai*. Clade ''C'' included the four unclassified exemplars of *Ceratozamia* (1, 2, 3, 4), *C. zaragozae*, *C. sabatoi*, *C. microstobila*, *C. kuesteriana*, *C. hildae*, *C. mexicana* var *mexicana*, and *C. morettii*, also *C. mexicana* var. *robusta* and *C. brevifrons*. The molecular data provided support for the monophyly of *Ceratozamia* and for its sister group relationship to *Zamia*.

Sequence variability found among species of *Ceratozamia* appears to be in agreement with certain anatomical characters; for example, *C. mexicana* var. *mexicana*, *C. mexicana* var *robusta*, and *C. morettii* appear to differ in length and width of palisade parenchyma cells and cuticle thickness. *Ceratozamia matudai* is separated from both *C. kuesteriana* and *C. microstrobila* by cuticle thickness, length of palisade parenchyma cells, and number of sclereid cells associated with and not associated with vascular bundles. However, there appears to be homogeneity for other anatomical characters such as length of leaflet adaxial epidermal cells, width of pallisade chlorenchyma cells of leaflet, and thickness of abaxial leaflet cuticle (S. Avendaño unpubl. data).

Non-coding regions have been presumed to be more useful at low taxonomic ranks because they are less functionally constrained and are therefore free to vary, thereby potentially providing more phylogenetically informative characters per unit of sequencing effort (Clegg et al. 1994; Small et al. 1998). However, the ITS and the *trnL-F* non-coding regions in *Ceratozamia* exhibited less phylogenetically informative characters than do most other plant species assayed with comparable methods (Baldwin et al. 1995). The number of





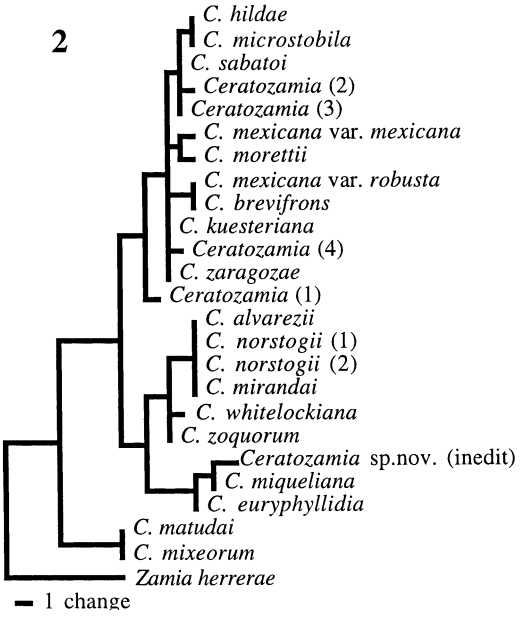


FIG. 2. Phylogenetic trees showing relationships within *Ceratozamia* derived from the ITS sequence data. 1) Strict consensus of 112 equally most parsimonious trees (length 51, CI = 0.8235, RI = 0.9151, RC = 0.7536). Numbers above branches are bootstrap estimates for 500 replicate analyses. Numbers below are decay values. 2) One random chosen tree with the branch lengths proportional to the number of changes.

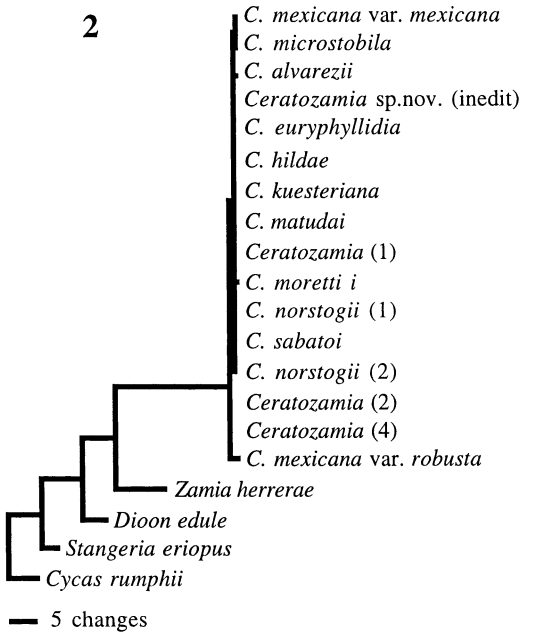


FIG. 3. Phylogenetic trees showing relationships within *Ceratozamia* derived from the *trnL-F* sequence data for 16 taxa. 1) Strict consensus of 6 equally most parsimonious trees (length 88, CI = 0.7841, RI = 0.8273, RC = 0.6487). Numbers above branches are bootstrap estimates for 500 replicate analyses. Numbers below are decay values. 2) One random chosen tree with the branch lengths proportional to the number of changes.

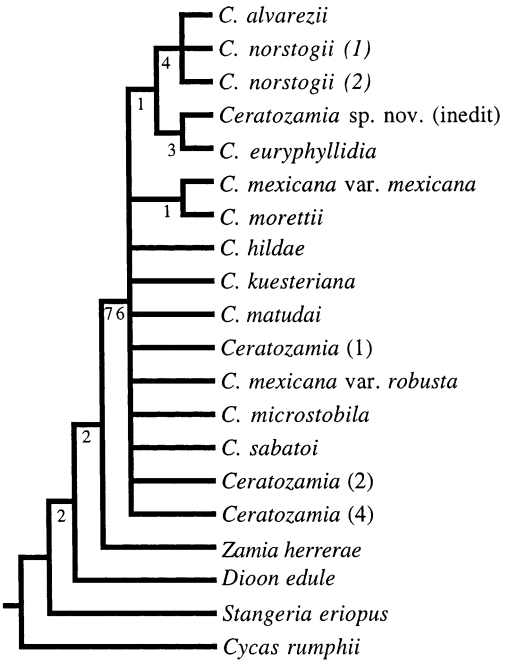


FIG. 4. Strict consensus of 1276 most parsimonious trees based on a combined data set of *trnL-F* and ITS sequence data for sixteen exemplars of *Ceratozamia* and four outgroups (length 947, CI = 0.7893, RI = 0.7039, RC = 0.5556). Numbers below branches are decay values.

informative characters found within *Ceratozamia* in the ITS region (31) and in the *trnL-F* region (2) is low compared with other studies reporting few informative characters in these regions. For example, 73 samples of *Helianthus* had fifty four informative characters in the ITS region, and 43 accessions of *Fragaria* had forty one in the ITS and three in the *trnL-F* region (Schilling et al. 1998; Potter et al. 2000).

We were unable to resolve fully the relationships within clade "B" (Fig. 2). This clade contains two previously defined species complexes: the *C. miqueliana* complex (*C. euryphyllidia* and *C. miqueliana*) and the *C. norstogii* complex (*C. mirandai*, *C. norstogii*, and *C. alvarezii*). This was probably due the limited number of informative characters obtained with these sequence data sets.

Molecular phylogeny of members of *Ceratozamia* provides insights into the biogeography of this group. The three main clades within *Ceratozamia* are consistent with distributional ranges of the species included in each clade (Fig. 5). The two basal clades ("A" and "B") contain species distributed in southern and southeastern Mexico, at and south of the Neovolcanic mountain range of Pliocene-Quaternary (Pleistocene) age. Clade "C" contains a group of species present in localities north and northeast of the Neovolcanic range. This is in agreement with findings of Marshall and Liebherr (2000) who identified two biogeographic as-

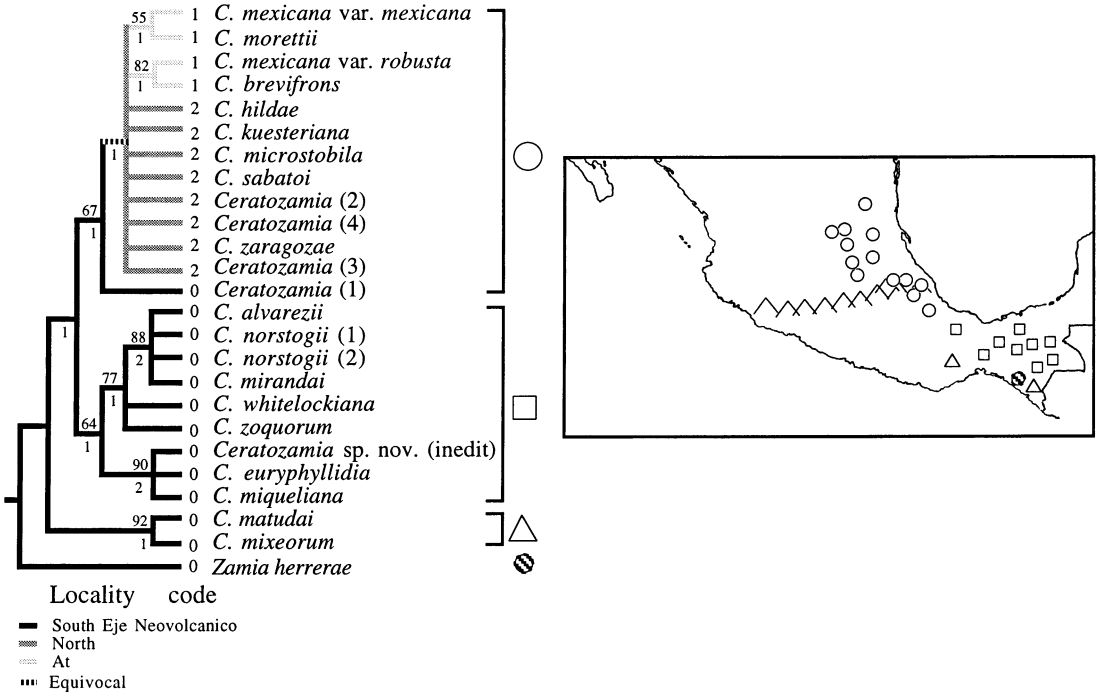


FIG. 5. Map showing distribution of main clades within *Ceratozamia* in relation to the biogeographic boundary of the Eje Neovolcanico. Branch colors indicate the locality of each exemplar. Numbers after each branch show the code used to optimize the localities with the program "MacClade."

semblages, one north of the Neovolcanic range and another south of this.

The history of vegetation from southern Mexico and Belize has been studied by Lundell (1945) and Miranda (1957, 1959). Both agree that the region contains relict floral elements of great age. Also, the existence of very old (40,000 years) floristic and faunistic refuges has been postulated in southern Mexico (Brown 1976; Toledo 1982, 1988), but these were apparently absent in the areas north of the Neovolcanic mountain range.

From the cladistic analyses presented in this study we suggest the hypothesis that *Ceratozamia* has a southern/south eastern Mexico origin. Two species, *C. matudai* and *C. mixeorum* (clade ''A''), are from the Sierra Madre del Sur, Chiapas, and the Sierra Norte de Oaxaca, respectively. *Ceratozamia euryphyllidia*, *C. miqueliana*, *C. whitelockiana*, and *Ceratozamia* sp. nov. (inedit), are from southern Veracruz, northern Chiapas and Oaxaca, and southern Tabasco, respectively, forming what might be called the *C. miqueliana* complex. This complex appears to lie within the arc refuge area of Wendt (1987). *Ceratozamia alvarezii*, *C. norstogii*, and *C. mirandai* are from western Chiapas and *C. norstogii* also appears in the adjacent northern mountains of Oaxaca (Fig. 5).

Clade ''C'' includes twelve taxa distributed at the Neovolcanic range and north of it. Eight taxa are from the northeast of the Neovolcanic belt in the states of Tamaulipas, San Luis Potosi, Queretaro and Hidalgo. Four taxa (*C. mexicana* var. *mexicana*, *C. morettii*, *C. mexicana* var. *robusta*, and *C. brevifrons*) are from central Veracruz on the Neovolcanic belt. Lastly, one unclassified exemplar of *Ceratozamia* (Table 1, #1) is from the Cordoba refuge just south of the Neovolcanic belt, which appears basal to this unresolved clade (Fig. 5).

When the data presented are considered from a biogeographical point of view and in the light of past historical events, a logical pattern appears to be present. Current diversity in *Ceratozamia* appears to be the result of speciation processes and expansion that probably began during Tertiary times with a northwards migratory pattern following a general warming of climate. Species occurring at Neovolcanic range and north of it may be the most recent speciation processes. However, when we optimized the locality data of each of the 24 exemplars of *Ceratozamia* in the cladogram using the program ''MacClade'' the direction of the transformation series is equivocal. Consequently the transformation either may go from south through center of the Eje Neovolcanico and then north of it or it may go from south through north and then to the center of the Eje Neovolcanico.

The small amount of mutations giving rise to the three clades shown in Fig. 2 could be partially explained by the considerable long life cycle in *Ceratozamia* and the reduced number of generations. Generation times (germination, maturity, seed set) under optimal cultivation conditions span at least fifteen years and this period can be safely doubled for conditions in the wild. Therefore only about 300 generations of a putative *Ceratozamia* would have occurred since the end of the Pleistocene ten thousands years ago.

ACKNOWLEDGEMENTS. This research was supported by a grant from CONACyT (No. 29379 N) and account No. 902–14 from the Instituto de Ecología, A.C. We thank Efraín De Luna for the critical review of this manuscript and Cristina Bárcenas for technical assistance.

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