




Article

# Molecular and Morphological Phylogenetic Analyses of New World Cycad Beetles: What They Reveal about Cycad Evolution in the New World

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**Abstract:** Two major lineages of beetles inhabit cycad cones in the New World: weevils (Curculionioidea) in the subtribe Allocorynina, including the genera *Notorhopalotria* Tang and O'Brien, *Parallocorynus* Voss, *Protocorynus* O'Brien and Tang and *Rhopalotria* Chevrolat, and beetles in the family Erotylidae, including the genus *Pharaxonotha* Reitter. Analysis of the 16S ribosomal RNA (rRNA) mitochondrial gene as well as cladistic analysis of morphological characters of the weevils indicate four major radiations, with a probable origin on the cycad genus *Dioon* Lindl. and comparatively recent host shifts onto *Zamia* L. Analysis of the 16S rRNA gene for erotyloid beetles indicates that an undescribed genus restricted to New World *Ceratozamia* Brongn. is the most early-diverging clade, and this lineage is sister to a large radiation of the genus *Pharaxonotha* onto *Zamia*, with apparent host shifts onto *Dioon* and *Ceratozamia*. Analysis of beetles are in accord with current models of continental drift in the Caribbean basin, support some proposed species groupings of cycads, but not others, and suggest that pollinator type may impact population genetic structure in their host cycads.

**Keywords:** Belidae; Oxycoryninae; Erotylidae; Pharaxonothinae; cycad pollination

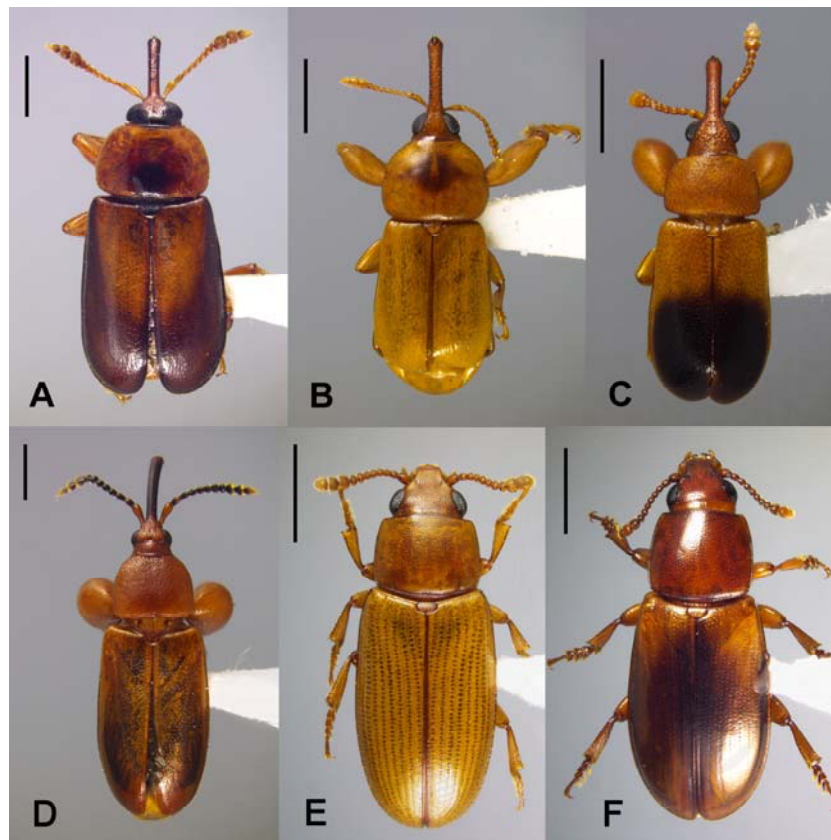
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## 1. Introduction

Over the last three decades, evidence has accumulated that insect pollination is widespread in New World cycads. This evidence includes wind and insect exclusion experiments on ovulate cones of three species in the cycad genus *Zamia*, detailed observations of the life cycle and behavior of the beetles that inhabit them [1–5], as well as observations on other New World cycad genera *Ceratozamia*, *Dioon*, and *Microcycas* (Miq.) A. DC. [6–8]. Similar experiments and observations on other continents indicate the same for other genera of cycads [9–19]. In a recent seminal work on guidelines for cycad classification, insect symbionts of cycads were identified as having a potentially important impact on cycad classification: “Insects appear to be the primary vectors for pollination [ . . . ] evidence is accumulating to suggest coevolutionary processes between cycads and their pollinators. Once these processes are uncovered, resulting data will probably have a significant impact on how cycad taxa are classified” [20]. During the 6th International Conference on Cycad Biology, a coordinated global effort was organized to collect and study the insect pollinators of cycads [21]. One of the explicit goals was to use this information to understand cycad evolution. In this paper, we report on results of this insect survey effort in the New World, present phylogenetic analyses of the insects found, and discuss implications for cycad taxonomy.

The majority of cycads in the New World host more than one species of beetle in their cones and some host as many as three species. These beetles fall into two distinct and not closely related groups: (1) Weevils of the subtribe Allocorynina (Coleoptera: Curculionoidea: Belidae: Oxycoryninae: Oxycorinini; higher-level classification follows Bouchard et al. [22]) associated with *Dioon* and *Zamia*; these include the genera *Notorhopalotria*, *Parallocorynus*, *Protocorynus*, and *Rhopalotria* [8,23] (see Figure 1A–D) and (2) Erotylidae (Coleoptera: Cucujoidea) in the subfamily Pharaxonothinae associated with *Ceratozamia*, *Dioon*, *Microcycas*, and *Zamia* (Cycadales: Zamiaceae; classification follows Calonje et al. [24]); these include the genus *Pharaxonotha* and an undescribed genus [7,25–27] (see Figure 1E,F).

O’Brien and Tang [8] recently described or reviewed all known species of Allocorynina, but they did not present a detailed phylogenetic analysis of the species. All known species inhabit and develop in cones of New World cycads. Six species of New World Pharaxonothinae have been described, but many remain undescribed [7,27]. New World forms are closely related to the recently described genus *Cycadophila* found on the Asian cycad genus *Cycas* [26,28]. The lack of phylogenetic frameworks for the New World groups of cycad beetles hinders the proper allocation of biological information and host/beetle associations and limits what can be interpreted from them. For instance, Maldonado-Ruiz and Flores-Vazquez [29] catalogued beetles found with a species of *Dioon* in Mexico, however, due to lack of keys for identification or prior phylogenetic work, they were not able to assess how many species of Allocorynina and Pharaxonothinae they were dealing with. In this paper, phylogenetic analyses were conducted on both morphological characters and DNA data of Allocorynina and DNA data of Pharaxonothinae beetles collected from cycad cones. The resulting trees from these analyses are used to generate hypotheses about cycad biogeography and evolutionary patterns at genus, species, and population levels [30,31].



**Figure 1.** Representatives of the six major lineages of Coleoptera inhabiting New World cycads, dorsal views: (A) *Protocorynus bontai* O'Brien and Tang, male; (B) *Notorhopalotria montgomeryensis* O'Brien and Tang, male; (C) *Rhopalotria (R.) dimidiata* Chevrolat, male; (D) *Parallocorynus (Neocorynus) schiblii* Tang and O'Brien, male; (E,F) *Pharaxonotha* sp. and Erotylidae, undescribed genus inhabiting a male cone of *Ceratozamia vovidesii* Pérez-Farr. and Iglesias; scale bars = 1 mm.

## 2. Materials and Methods

Beetles were collected from cycad cones in habitat by the authors and other cooperators and include previously described cycad-associated beetle species, as well as many undescribed beetle taxa [8,27]. Total number of cycad taxa sampled include 3 of the 31 recognized species of *Ceratozamia*, 13 of the 15 species of *Dioon*, and 29 of the 77 currently recognized species of *Zamia* [24]. In total, 89 cycad populations or localities yielded beetles with useable DNA for this study. The monotypic Cuban cycad genus *Microcycas* was not sampled, however, a species of *Pharaxonotha* has been described from this host [7]. For Allocorynina, institutions for deposition of specimens are listed in O'Brien and Tang [8]. Pharaxonothinae used are deposited in the Florida State Collection of Arthropods, Division of Plant Industry, Gainesville, FL, USA.

### 2.1. Morphological Analysis

For the Allocorynina weevils, external morphology and morphology of genitalia were studied and photographed using Nikon<sup>®</sup> SMZ1500 stereoscopic and Eclipse 80i compound microscopes mounted with Nikon<sup>®</sup> DS-Fi1 digital cameras. For morphological characters, taxonomic description of species and specimens used, see Appendix A and O'Brien and Tang [8]. For the outgroup, *Oxycraspedus cornutus* Kuschel (Belidae: Oxycoriniinae: Oxycorini: Oxycraspedina), currently placed in the same subfamily and tribe as the Allocorynina [32], was chosen. A matrix of 89 characters based mainly on morphology, but also host-associations and behavior such as diet and pupation sites, was built (see Figure A1 in Appendix B). A phylogenetic tree was generated using maximum parsimony (MP) implemented by TNT [33] using

default settings, and a strict consensus tree was generated from the ten most parsimonious trees found. Bootstrap support values were generated based on 1000 replicates. Multi-state characters were treated as non-additive; and all characters were weighed equally in the analysis. Morphological analyses of cycad-associated erotylid taxa are under way [27], but are not presented here.

## 2.2. DNA Analysis

The quality of DNA preservation of the beetles available for this study varied and was often poor, therefore, only short sections of DNA could be consistently sequenced in the samples available. We selected a fragment of the mitochondrial 16S ribosomal RNA (rRNA) gene with a combined sequence length ranging from 311–316, varying with additions and deletions of sections. The aligned data set contained 318 sites, with 222 constant, 93 variable, and 68 parsimony informative sites. As seen in other arthropods, the 16S rRNA gene is highly AT-rich with average nucleotide frequencies of thymine (T) 42.9%, cytosine (C) 7.9%, adenine (A) 35.0%, and guanine (G) 14.3%. The 16S rRNA gene has been used widely in insect molecular systematics and its utility in discerning species groups and deeper divisions in beetles and other holometabolous insects is well-founded [34–38]. It has been proposed for use as a standard for insect phylogenetics [39]. Total DNA was extracted from individual beetles, either adults or larvae, using Epicenter Master Complete DNA and RNA Purification Kits (Epicenter Technologies, Madison, WI, USA) following the manufacturer protocols and dissolved in 30  $\mu$ L H<sub>2</sub>O. The mitochondrial 16S rRNA was amplified using the following primers: 73Forward-AGATAGAAACCARCCTGGCT, 98Forward-CGGTYTRAACTCAGATCATGTA, and 430Reverse-AAGACGAGAAGACCCTATAG [26,28]. Reactions were carried out in 25  $\mu$ L volumes containing 1  $\mu$ L DNA, 5  $\mu$ L 5X buffer, 4  $\mu$ L of 25  $\mu$ M MgCl<sub>2</sub>, 1  $\mu$ L of 10 mM dNTPs, 1  $\mu$ L of 10  $\mu$ M of each primer, and 0.2  $\mu$ L 5 U/ $\mu$ L of Taq polymerase (Promega, Madison, WI, USA). PCR was performed using an Eppendorf ep mastercycler (Eppendorf, Westbury, NY, USA) using the following DNA denaturation, annealing and replication protocol: 94 °C for 1 min, then 40 cycles of 94 °C for 15 s, 50 °C for 15 s, and 72 °C for 40 s. Amplified products were cleaned up with the ExoSAP-IT kit (USB, Cleveland, OH, USA) and sequenced bidirectionally on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

DNA sequences were deposited into GenBank with accession numbers MF990634-MF990709 for weevils and KR005722, KR005724, KR005725, KY365240, KY365243, and MG256677-MG256758 for Erotylidae. Specimens of *Oxycraspedus cornutus*, used as the outgroup for the morphological analyses, did not yield usable DNA, therefore, for the phylogenetic analysis for weevils using DNA, we chose as outgroups *Hypera postica* (Curculionidae), *Ischnoptera pironi* (Brentidae), *Rhinotia haemoptera* (Belidae), *Anthribus albinus*, and *Anthribus nebulosus* (Anthribidae), with 16S sequences obtained from GenBank, accession numbers: U16967.1, KY084146.1, AJ495455.1, AJ495448.1, and AJ495449.1, respectively.

For weevils, nucleotide sequences were aligned using MAFFT version 7 [40]. The aligned sequences were then phylogenetically analyzed via: (1) maximum parsimony (MP) implemented by TNT [33] using default settings: a strict consensus tree was generated from the ten most parsimonious trees found; (2) maximum likelihood (ML) implemented in RAxML version 8 [41]: the best tree from 20 independent searches was selected and bipartition bootstrap values were written from 500 bootstrap replicates; (3) bayesian inference (BI) implemented in Mr Bayes version 3.2 [42], which was run with two simultaneous searches each using four chains for 1 million generations: trees were sampled every 100 generations, and the first 25% were discarded as burnin.

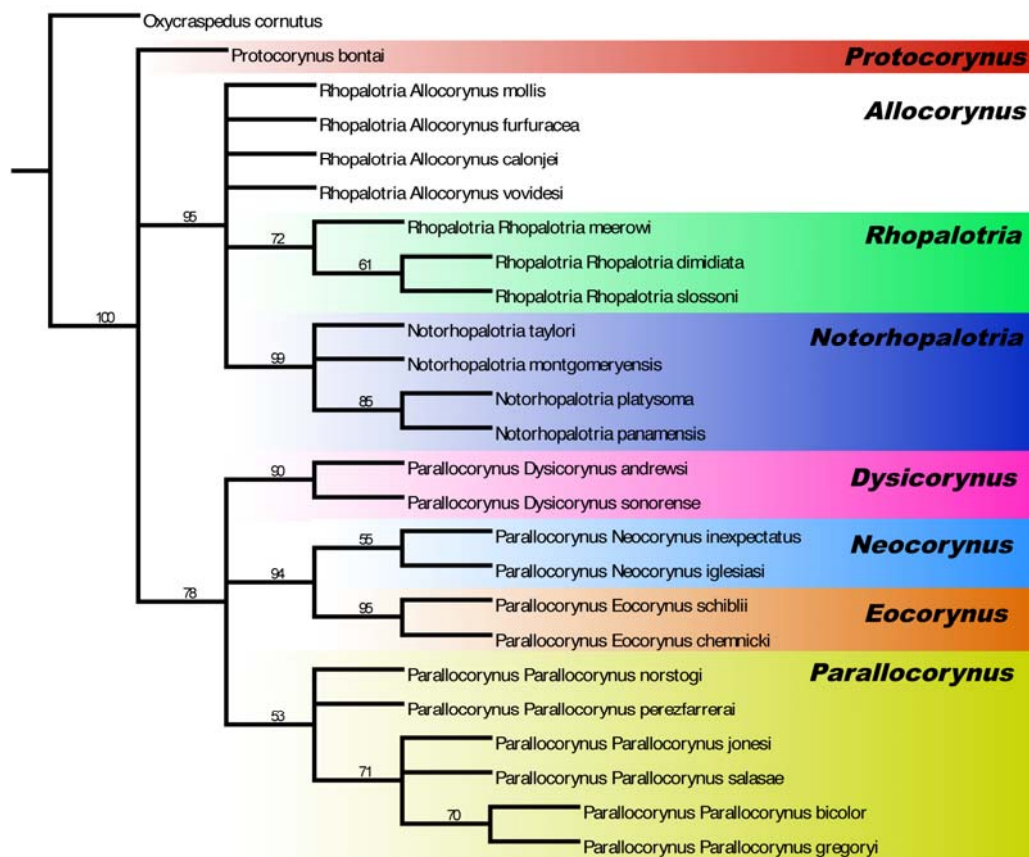
For erotylid beetles, two Asian species of Pharaonothinae that inhabit the genus *Cycas* were used as outgroups: *Cycadophila* (*C.*) *debaonica* and *C. (Strobilophila) tansachai* [26,28], GenBank accession numbers KR005715, KY365223, respectively. Multiple-sequence alignments were conducted with CLUSTAL W [43]. Phylogenetic trees were generated using maximum parsimony (MP), neighbor joining (NJ), and maximum likelihood (ML) methods as implemented in PAUP 4.0b10 [44] and MEGA5 [45]. Bootstrap support values were generated based on 1000 replicates. The best fit model

of sequence evolution employed in the ML analysis was the Tamura 3-parameter+G model with log likelihood  $-1448.98$  and Gamma distribution  $0.2653$ .

### 3. Results and Discussion

#### 3.1. Allocorynina Trees

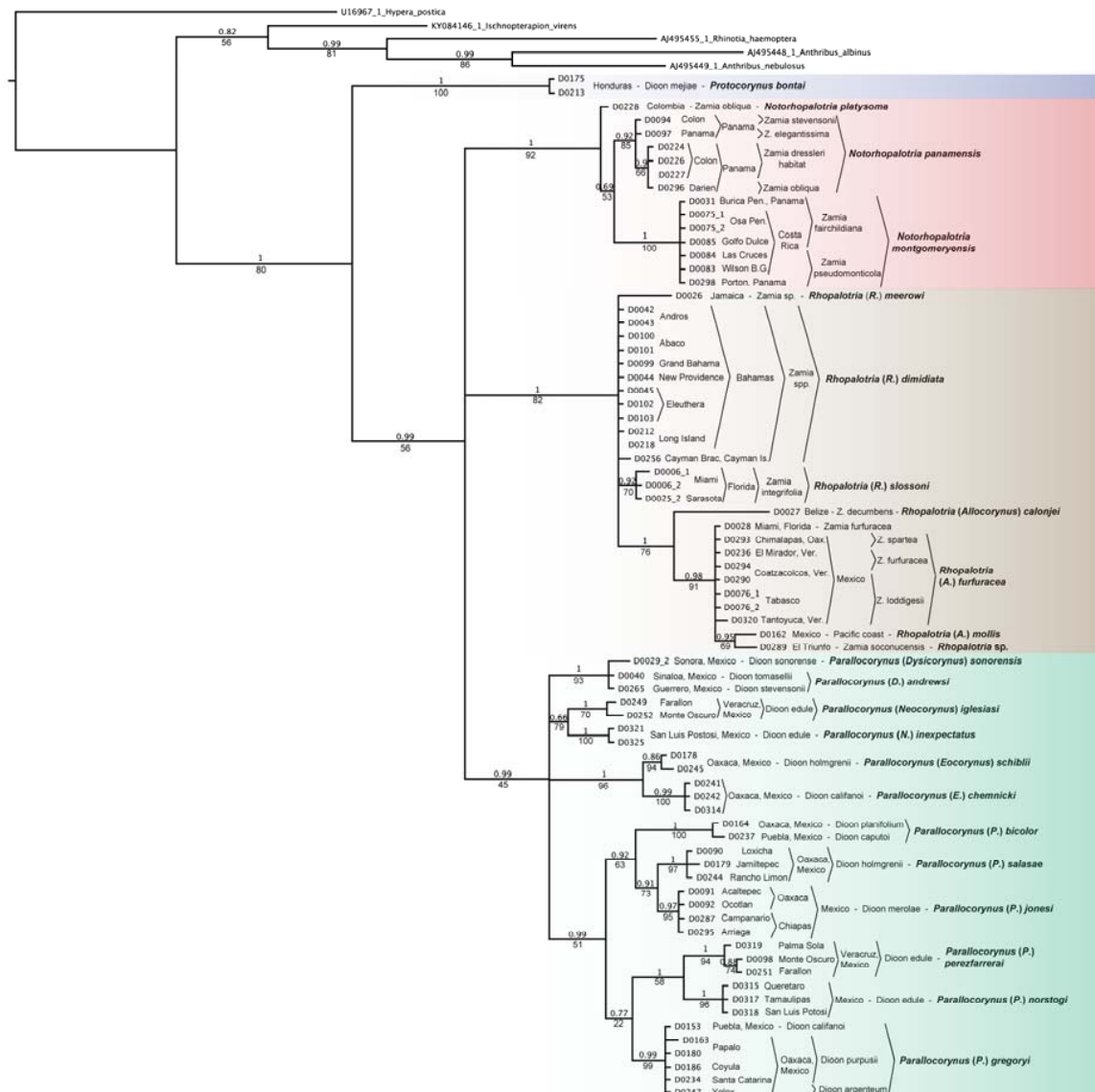
The phylogenetic tree based on MP analysis of the morphological, behavioral, and host-association data set is displayed in Figure 2. This tree generally supports the genera and subgenera recognized by O'Brien and Tang [8]. Genera *Protocorynus*, *Neocorynus*, and *Parallocorynus* and subgenera *Rhopalotria*, *Dysicorynus*, *Neocorynus*, *Eocorynus*, and *Parallocorynus* are monophyletic. Only the monophyly of genus *Rhopalotria* and its subgenus *Allocorynus* is not supported. The three molecular trees for the Allocorynina generated from MP, ML, and BI analyses of the 16S rRNA data set were almost identical, and any slightly conflicting clades are linked to very low support values. Therefore, in Figure 3, only the molecular tree from the BI analysis is presented, with ML support values annotated underneath the branches. Also, in this tree, each analyzed sample displays corresponding host cycad species and the geographic region where they were collected. All genera (and most subgenera) are well supported, but the relationships between them are not. The main difference between the two trees is that in the molecular tree subgenus *Rhopalotria* is paraphyletic with respect to *Allocorynus*, whereas in the morphology tree, the situation is reversed and subgenus *Allocorynus* is paraphyletic, but neither of them refute the monophyly of the other analysis. Another major difference in the molecular tree is the strong support for *Protocorynus* to be sister to the remaining three genera. These differences between the two trees may, in part, be attributed to different choices in outgroups.



**Figure 2.** Phylogenetic tree for the Allocorynina based on maximum parsimony analysis of the matrix of morphological, behavioral, and host characters in Appendix B; *Oxycraspedus cornutus* is used as the outgroup; numbers are bootstrap values.

The morphological analysis and the resultant tree are valuable in answering how each of these weevil lineages differ and what may be driving evolution in this group. Each recognized genus and subgenus is distinguished by differences in their genitalia and also in the spination of their profemora. Behavioral observations [3,4] indicate that the profemora are used during courtship battles between males. The spination on their profemora appear to function in grasping an opposing male during these mating struggles. Sexual selection, larval feeding sites, pupation sites, as well as host genus (*Dioon* vs. *Zamia*) appear to account for many of the morphological differences between the genera and subgenera. For details on synapomorphic characters for major clades see Appendix C.

This phylogenetic analysis of the Allocorynina is the most extensive to date and greatly expands upon previous efforts [31,32], while generally reaffirming the sub-/generic concepts erected by O'Brien and Tang [8].



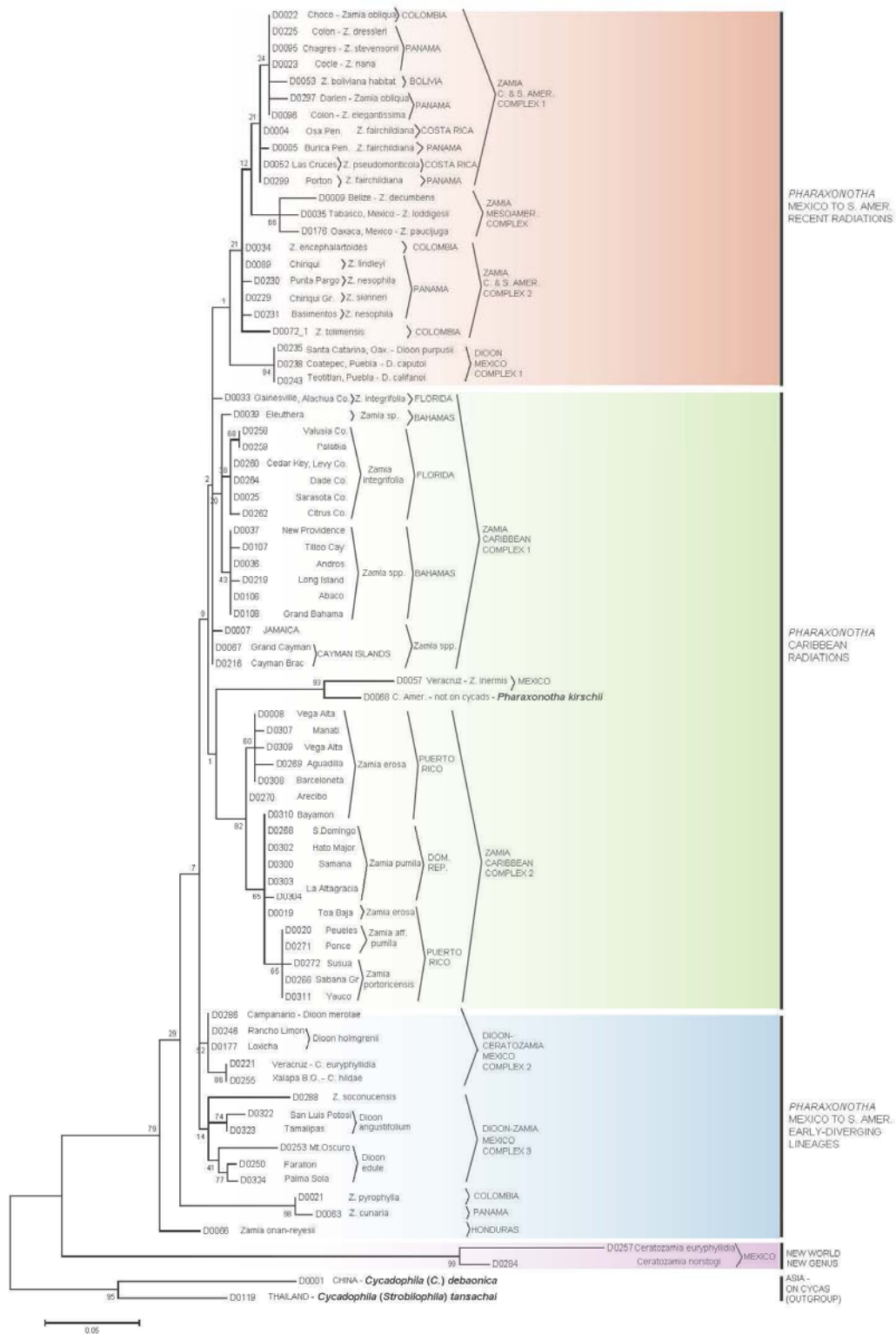
**Figure 3.** Phylogenetic tree for the Allocorynina synthesized from maximum likelihood (ML) and bayesian inference (BI) analyses of 16S ribosomal RNA (rRNA) gene sequences; numbers above the branches are posterior probabilities from Mr Bayes and the numbers below the branches are bootstrap support from RAXML. Localities and host cycad species are indicated for each sample.

### 3.2. Erotylidae Tree

The trees produced from MP, NJ, and ML analyses of 16S rRNA sequences for Erotylidae were similar, therefore, only the ML tree is displayed in Figure 4. The MP and NJ trees are provided in Appendix D as Figures A2 and A3. The ML tree, as well as the MP and NJ trees, can be divided into four sections: (1) The most early-diverging clade is an undescribed genus residing in cones of *Ceratozamia* (purple color in Figure 4); the remainder of the New World taxa are sister to this clade and are tentatively assigned to the genus *Pharaxonotha*. (2) This *Pharaxonotha* clade can be broadly assigned to three sections; the first of these are labeled “Early-diverging lineages Mexico to South America” (blue color in Figure 4). Within this division, the most early-diverging branches reside in cones of *Zamia onan-reyesii* C. Nelson and Sandoval, an aerial-stemmed species from Honduras and subterranean-stemmed *Z. cunaria* Dressler and D. W. Stev. and *Z. pyrophylla* Calonje, D. W. Stev. and A. Lindstr., in the eastern Panama-northern Colombia region. Also among these early diverging lineages are species of *Pharaxonotha* that reside in other *Ceratozamia*, *Dioon*, and *Zamia* hosts. (3) A third broad division consist of *Pharaxonotha* inhabiting cones of the *Zamia pumila* L. species complex in the Caribbean, specifically on islands of the Greater Antilles, the Bahamas archipelago, and the Florida peninsula (green color in Figure 4). This division consists of two clades; one in the easternmost section of the Greater Antilles on Puerto Rico and Hispaniola and the other clade in the western Greater Antilles, Florida, and the Bahamas. (4) Lastly, there is a fourth major division, consisting of a more recent radiation of *Pharaxonotha* that also extends from Mexico to South America and inhabits *Zamia* and *Dioon* (red color in Figure 4). Although bootstrap support for many of these clades are weak, they fall consistently into these four broad categories in the ML, MP, and NJ trees, with only one exception: the *P. kirschii* Reitter lineage, discussed below.

Although no morphological analyses are presented in this paper for the Erotylidae, Xu et al. [28], Skelley et al. [26], and Tang et al. [27] have identified a number of external morphological and genital characters which support many of the clades revealed here (Figure 4) through phylogenetic analysis of the 16S rRNA gene. All species discussed herein are assigned to a single subfamily, the Pharaxonothinae, of the Erotylidae. Of special interest is the taxon *Pharaxonotha kirschii*, the type species for the genus and the only known Pharaxonothinae in the New World that is a generalist feeder. Although it has been found with *Zamia*, it is the only known member of the New World Pharaxonothinae that is not an obligate inhabitant of a cycad during its life cycle and is widely distributed in Central America [26]. Our DNA analysis indicates that the *Pharaxonotha* associated with *Z. inermis* (specimen D0057 in Figure 4), while a distinct species, is related to *P. kirschii*. The specimen used for DNA analysis was a larva and an adult female associated with that larva matches the morphology of *P. kirschii*. This specimen and *Pharaxonotha kirschii* may be part of a species complex, with different branches of this complex inhabiting either cycads or non-cycad hosts. In the ML tree, this *P. kirschii* clade is associated with the Caribbean group, however, in the MP and NJ trees (Figures A2 and A3), these two taxa are associated with the early diverging lineages from Mexico to South America.

While all *Zamia* that have been closely sampled have yielded *Pharaxonotha* beetles from their cones, this is not so for all *Dioon* and *Ceratozamia* populations examined. The cycad genus *Dioon* was especially well-sampled in this study and in one species, *Dioon mejiae* Standl. and L.O. Williams, no erotylids were detected in three separate samples consisting of a total of many hundreds of beetles (only Allocorynina weevils found). Furthermore, the erotylid beetles sampled here from seven other *Dioon* species cluster in three disparate branches in the erotylid trees, suggesting separate colonization events of *Dioon* by this group of beetles.



**Figure 4.** Phylogenetic tree for the Erotylidae: Pharaxonothinae on the cycads of the New World based on maximum likelihood analysis of 16S rRNA gene sequences; scale bar indicates base pair substitutions per nucleotide position; numbers on branches are bootstrap values.



### 3.3. Implications for the Evolution of Cycad Hosts

#### 3.3.1. Using Beetle Trees to Generate Hypotheses of Cycad Evolution

While the 16S rRNA gene is considered to provide fairly accurate phylogenies for beetles and other insects, the search for plastid and nuclear genes of similar utility in the construction of phylogenies for New World cycads has had equivocal results [46–49]. Genetic analysis of beetles that have coevolved with cycads may reveal evolutionary patterns in cycads that are much more difficult to discern from direct examination of cycad genomes. The following discussions will be confined mainly to the generation of hypotheses about cycad evolution in the New World based on the interpretation of the beetle trees produced in this study.

Before proceeding, we offer some caveats and limitations as to what can or cannot be deduced about cycad evolution from coevolving insect pollinators. Cycads typically form colonies or groves widely dispersed from other populations of conspecific or closely related species [50], and in such situations, flight distances of seed dispersers or pollinators between populations may be typically measured in kilometers or tens of kilometers. Cycad seeds are relatively large and heavy compared to available animal dispersers, and field studies indicate that the great majority of cycad seeds fall within two meters of their mother [51–53], suggesting that gene flow between distant populations via seed dispersal is rare. These population structures and seed dispersal constraints may configure insect pollinators as the primary gatekeepers of gene flow in most cycad populations. Thus, when two cycad populations host different species of beetles believed to be involved in pollination, we can use this to infer that the cycads in question may be genetically isolated, as little or no gene flow is likely to occur via exchange of pollinators. When the scenario is reversed and cycad populations share the same pollinator, we cannot automatically assume gene flow is occurring between the cycad populations via pollinators. Studies by Donaldson et al. [9] and Terry [13] show that pollen loads on insects that leave cycad cones drop rapidly with distance and time from the source cones. Crowson [54,55] illustrated deep antennal pockets in the antennae of *Allocoynina* weevils and hypothesized that these might be structures that function to hold pollen. Closer examination of these pockets with SEM [8] indicate that their entrances are occluded with hairs with a probable sensory function and that these pockets are unlikely to facilitate pollen transport. While a cycad pollinator may be able to fly between populations of different host cycad species and interbreed with other populations of its own species, during this process, it may not be transferring any cycad pollen between distant host cycad populations. A phylogenetic study of thrips pollinators of the Australia cycad *Macrozamia* suggests that both cycad and pollinator populations fragment together as a result of climate change and aridification [56] and that co-diversification of the host and pollinator in allopatry appears to be the process affecting diversity. Furthermore, that study concluded that distinct cycad species separated by short distances may continue to share the same pollinator species. Similarly, in New World cycads, O'Brien and Tang [8] recognized that some cycad beetles inhabit more than one host cycad species: (1) *Parallocorynus* (*P.*) *gregoryi* O'Brien and Tang inhabits the cones of three closely related *Dioon* species, *D. argenteum* T.J.Greg., Chemnick, Salas-Mor. and Vovides, *D. califanoi* De Luca and Sabato and *D. purpusii* Rose; (2) *Rhopalotria* (*R.*) *dimidiata* inhabits cones of many species of the *Zamia pumila* complex living in the Bahamas, Cuba, and Cayman Islands, and (3) *Notorhopalotria panamensis* O'Brien and Tang inhabits at least three species of *Zamia* in central Panama with adjoining geographic distributions, *Z. dressleri* D. W. Stev., *Z. elegantissima* Schutzman, Vovides and R. S. Adams and *Z. stevensonii* A. S. Taylor and Holzman.

Examination of these DNA-based trees indicates that host-shifts of pollinating beetles have occurred during the evolution of cycads. We cannot assume strict co-speciation of beetle lineages with their corresponding host cycad lineage. Host-shifts of cone beetles between cycad genera and between cycad species within a genus can occur. Although this limits our ability to make inferences about coevolution of cycads and their beetles, the host-shifts in themselves are interesting and informative about evolutionary processes in cycads [57].

### 3.3.2. Cycad Hypotheses Based on the Allocorynina Trees

From the DNA-based tree for Allocorynina (Figure 3) in combination with morphological data (Appendix A, [8]) we generate these hypotheses about cycads:

- (1) The presence of one or two species of Allocorynina in all species of *Dioon* sampled compared with its absence from many *Zamia* species and its complete absence in the other New World cycad genera *Ceratozamia* and *Microcycas* suggests that *Dioon* is the earliest host lineage colonized by Allocorynina weevils, with one or possibly two host-shifts onto *Zamia*. In this hypothesis, Allocorynina are the original pollinators in the genus *Dioon*, while erotyloid beetles are later colonists in *Dioon*.
- (2) Based on the morphological and genetic analyses of its pollinator *Dioon mejiae*, located on the Chortis block, a tectonic terrane roughly corresponding to the country of Honduras [58], is hypothesized to be one of the earliest bifurcating lineages within *Dioon*.
- (3) Species of narrow-leaflet *Dioon* in Mexico form four lineages with distinct biogeographic distributions: (A) western Mexico lineage along the Pacific drainage of the Sierra Madre Occidental from Sonora to Guerrero consisting of *D. sonorensis* (De Luca, Sabato and Vázq.Torres) Chemnick, T.J.Greg. and Salas-Mor., *D. stevensonii* Nic.-Mor. and Vovides and *D. tomasellii* De Luca, Sabato and Vázq.Torres; (B) eastern Mexico lineage along the Sierra Madre Oriental from Nuevo Leon to Veracruz consisting of *D. angustifolium* Miq. and *D. edule* Lindl.; (C) south central Mexico lineage consisting of *D. argentium*, *D. califanoi*, and *D. purpusii*; (D) southern Mexico lineage along the Pacific drainage of Oaxaca to Chiapas, consisting of *D. caputoi* De Luca, Sabato and Vázq.Torres, *D. holmgrenii* De Luca, Sabato and Vázq.Torres, *D. merolae* De Luca, Sabato and Vázq.Torres and *D. planifolium* Salas-Mor., Chemnick and T. J. Greg.
- (4) For the eastern Mexico lineage (group 3B above) *Dioon edule* (including *D. angustifolium*) north of the Trans-Mexican Volcanic Belt in the states of Nuevo Leon, Queretaro, San Luis Potosi, and Tamaulipas is likely a distinct species from *D. edule* south of this mountain range in Veracruz; the two lineages of Allocorynina and the lineage of erotyloids inhabiting this cycad species group support this division.
- (5) The absence of Allocorynina in the periphery of the geographic range of *Zamia* (e.g., eastern part of the Greater Antilles and much of Panama and South America) suggests that colonization of *Zamia* by Allocorynina is relatively recent and perhaps an ongoing ecological and evolutionary process. The alternate hypothesis is that there may have been a more widespread distribution on *Zamia*, but these weevils have suffered selective extinction in parts of their range.
- (6) The shift of Allocorynina from *Dioon* onto *Zamia* may have occurred during major tectonic events in the formation of Central America when landmasses were moving through the region, emerging from the sea, and/or colliding with Mesoamerica [59,60]; during this time, lineages of cycads (including *Zamia* and/or possibly other extinct cycad lineages) and the Allocorynina associated with them migrated in three directions: (A) south into Central America, (B) east into the Caribbean islands, and (C) within Mesoamerica.
- (7) *Zamia obliqua* A. Braun in the Choco of Colombia is likely a different species from *Z. obliqua* in the Darien of Panama based on genetic differences in the Allocorynina inhabiting their respective cones.

### 3.3.3. Cycad Hypotheses Based on the Erotylidae Tree

From the DNA-based tree of the Erotylidae (Figure 4) we generate these hypotheses:

- (I) The most early-diverging lineage of cycad-associated erotyloid pharaxonothine beetles in the New World is confined to the genus *Ceratozamia*. This branching pattern is consistent with fossil evidence indicating that the *Ceratozamia* lineage may have first evolved in Europe in the mid Cenozoic and then migrated to North America prior to the complete separation of these

continents [61]. In addition, the apparent close relation between cycad-associated erotyliids of the New World with those found on *Cycas* in Asia, suggest that these beetles may have an ancient Laurasian association with cycads that predates the breakup of Laurasia.

- (II) Two early-diverging erotyliid beetle lineages associated with *Zamia* are located in: (a) Honduras on *Z. onan-reyesii* and (b) The northern South America-Darien region on *Zamia cunaria* and *Z. pyrophylla*. We may hypothesize that these host lineages of *Zamia* are among the earliest to diverge for the genus and are likely relicts from an earlier radiation of *Zamia* throughout these regions.
- (III) The presence of erotyliid beetles in the cones of all species of *Zamia* that have been carefully sampled suggests that these were the original pollinators of *Zamia*. In this hypothesis, the Allocorynina weevils are later colonists of *Zamia*.
- (IV) In addition to old relictual clades in hypothesis II, the existence of three separate derived clades of *Pharaxonotha* beetles on *Zamia* suggests that at least three recent and separate radiations of *Zamia* have occurred in the following regions: (a) A radiation into the eastern islands of the Greater Antilles, which includes Hispaniola and Puerto Rico, probably beginning when these landmasses were more closely associated with Central America [60]; (b) A more recent radiation into the western islands of the Greater Antilles, including Cuba, Cayman Islands and Jamaica and neighboring landmasses of the Bahamas and Florida; and (c) Sister to these two Caribbean lineages, a recent radiation onto *Zamia* in Mesoamerica, Central America, and northern South America.
- (V) The separation of *Pharaxonotha* beetles, that inhabit *Dioon* cones, into three distinct and not closely related clades and the absence of erotyliids on one species, *D. mejiae* in Honduras, at the periphery of the range of *Dioon*, suggests that erotyliids colonized *Dioon* from the *Zamia* lineage in three separate host shift events and that these host shifts have occurred relatively recently compared to the radiation of Allocorynina in *Dioon*.
- (VI) At least one recent host-shift of *Pharaxonotha*, originating from *Zamia*, have occurred onto *Ceratozamia*. A larger and wider sampling of *Ceratozamia* beetles may reveal more than one host shift. These coexist within *Ceratozamia* cones with the more ancient erotyliids beetles discussed in hypothesis I, so that now two disparate lineages of *Pharaxonothinae* coinhabit *Ceratozamia* cones. This host-shift radiation is allied with those in *Dioon*, suggesting important watershed periods in cycad evolution when exchange of pollinators occurred among cycad genera in the New World. Deeper study of these periods may be crucial in understanding the relatively recent resurgence of cycad evolution that have been proposed [62–64].
- (VII) The pattern of population genetic variation of *Pharaxonotha* beetles that presumably pollinate *Zamia* in Puerto Rico and Hispaniola mirrors to a great extent the population genetic variation exhibited by the *Zamia* on those islands [65]. These mirroring patterns suggest that the mobility and/or abundance of a cycad's pollinator may influence gene flow in its host cycad and consequently the speciation pattern of its host. For example, observations [66] suggests that unlike other cycad beetles, this lineage of *Pharaxonotha* is highly sensitive to human disturbance of its vegetative habitat and easily becomes rare or locally extinct as a result. This susceptibility to disturbance and the low ability to recolonize its host from nearby populations suggests low mobility and low ability to mediate gene flow in its host *Zamia*. The resulting effect is to produce local reproductive isolation of cycad populations that appear on casual observation to have continuous distributions.
- (VIII) Based on population genetic variation of beetles discussed in hypothesis VII, the *Zamia* populations near Bayamon and Toa Baja, Puerto Rico may be conspecific with *Zamia pumila* in Hispaniola; furthermore, *Z. pumila* populations in Hispaniola may be recent colonists from an ocean dispersal event originating from Bayamon and Toa Baja.

### 3.3.4. Independent Tests of Beetle Generated Hypotheses

Many of these beetle-generated hypotheses about cycad evolutionary patterns in the New World may be tested with data sets of genetic and/or morphological characters from their host *Ceratozamia*, *Dioon*, or *Zamia*. Our tree for erotyloid beetles (Figure 4) contains samples from 61 populations of *Zamia* along with collection localities. A phylogenetic tree for *Zamia* based on sequences of one gene combined with morphological data published by Caputo et al. [67] is available for comparison. Their tree contains 22 species of *Zamia*, of which only ten correspond with populations in our tree, nevertheless, some broad tests can be made of our erotyloid hypotheses II and IV. Their tree shows some congruence with the erotyloid trees presented here. For instance, in their tree as in ours, the Caribbean *Z. pumila* group is sister to many Mesoamerican and South American *Zamia*. Their tree differs significantly from our erotyloid tree, however, in that a large branch of the Central American *Zamia* forms a clade that is sister to the previously described clades. Our erotyloid tree suggests that their Central American clade should form part of their Mesoamerican and South American *Zamia* clade. In addition, our erotyloid tree suggests that *Z. soconuscensis* Schutzman, Vovides and Dehgan belongs in a clade that is an early diverging relative to the Caribbean clade, however, *Z. soconuscensis* appears as a member of their Mesoamerican and South American clade that is sister to the Caribbean clade. If both beetle and *Zamia* host phylogenies are accurate, we would conclude that host and symbiont beetles are not co-speciating in a perfectly parallel fashion and that host shifts have occurred at important junctures in both *Zamia* and beetle radiations. The converse may be true, that the beetle phylogenies do reflect the evolutionary patterns of their host *Zamia* accurately, and that a more extensive genetic data set for *Zamia* may be required for a more accurate comparison.

A maximum likelihood tree produced by Nagalingum et al. [68] using three genes is also available for testing of hypothesis IV. Their tree contains 35 species of *Zamia*, of which 17 correspond with host populations in our tree and is broadly congruent with ours. In their tree, the Caribbean clade is sister to other Mesoamerican and Central and South America clades. None of our identified early diverging host *Zamia* lineages, however, were sampled in their study, so hypothesis I cannot be tested with their dataset.

Hypotheses may be tested by comparing trees from different beetle groups that are cohabiting in the same hosts. For example, we can test hypotheses 3A–D generated using Allocorynina weevils that there are four species groups of narrow-leafed *Dioon* that inhabit four distinct geographic regions. Our erotyloid tree, based on less extensive samples from *Dioon* than that for Allocorynina, exhibits three distinct clades for *Dioon* erotyloids that largely correspond to the three Allocorynina clades in hypotheses 3B–D. The only exception is the erotyloid beetle on *D. caputoi*, which suggest a single host shift has occurred between our proposed regions 3C and 3D. Also, no usable DNA was extracted for erotyloids from region 3A, so no comparison can be made for hypothesized region 3A. A recently published phylogeny for *Dioon* based on DNA by Gutiérrez-Ortega et al. [49] also provides a test of some of the Allocorynina generated hypotheses. Their tree [49] supports Allocorynina hypothesis 1 that *D. mejiae* is one of the early diverging lineages of the genus and the geographic regions proposed in hypotheses 3A–B. Their tree, however, does not support the distinction between hypothesized *Dioon* regions 3C and 3D, and in their tree, the *Dioon* species of 3C and 3D form an integrated clade. Their tree also does not support our hypothesis 4, that *D. edule* and *D. angustifolium* north of the Trans-Mexican Volcanic Belt form a distinct clade from *D. edule* south of the belt. Another published phylogeny for *Dioon* based on combined molecular and morphological data by Moynihan et al. [48] supports our hypotheses 3A–D, except their tree shows *D. caputoi* as a distinct branch separate from the other four.

A test of erotyloid hypothesis VII, that mobility of a pollinator may influence the population genetic structure of its host, can be partly tested with the beetles inhabiting *Zamia* in the six island groups of the Bahamas. Although the Allocorynina beetles sampled from these islands all display the same 16S rRNA haplotype, suggesting panmixia or alternatively recent introduction to the islands from a single source, the *Pharaxonotha* beetles on three of these islands, Eleuthera, Long Island, and Tiloo Cay (near Abaco), exhibit haplotypes distinct from the rest. The hosts of these three *Pharaxonotha* haplotypes,

*Z. angustifolia* Jacq., *Z. lucayana* Britton, and *Z. sp.* “Tiloo Cay”, possess distinctly narrower, broader, or more coriaceous leaflet phenotypes than *Zamia* on other Bahamian islands. Genetic analysis of Bahamian *Zamia* populations [69] supports the possibility of extended genetic isolation of the Long Island *Z. lucayana* population, but not of the Eleuthera and Tiloo Cay *Zamia* populations. In this case, genetic patterns in *Pharaxonotha* mirror the phenotypic traits displayed by their host *Zamia* better than the genetic analysis of the *Zamia* populations themselves. Possibly, restricted gene flow mediated by the *Pharaxonotha* may have been masked by gene flow mediated by *Allocorynina* pollinators at a later stage, since both pollinators are present in these *Zamia* populations.

Hypothesis VII also predicts that low mobility of cycad pollinators may result in high genetic variation among cycad populations that are in relatively close proximity. Lazcano-Lara (66) demonstrated that successful pollination of *Z. portoricensis* Urb. in Puerto Rico decreases dramatically when a female plant is beyond 1.9 m of a male plant, suggesting that its sole beetle pollinator, *Pharaxonotha portophylla* Franz and Skelley, provides relatively ineffective long range pollination, and this appears to contribute to the high degree of genetic differentiation exhibited between neighboring *Zamia* populations on this island [65,66].

#### 4. Conclusions

It is widely understood that insect pollination is a critical facet of cycad biology and conservation [30,31,70,71]. In addition to their importance in cycad reproductive biology, the morphological and genetic analysis of pollinators can provide evidence for supporting or refuting hypotheses about cycad taxonomy and biogeography. As more extensive phylogenetic studies of New World cycads become available, the hypotheses presented here can be tested in more depth. Our hypotheses and analyses can also be refined in future work with a broader sampling of mitochondrial as well as nuclear genes from cycad beetles and a wider geographic sample of beetles that corresponds more closely with the host cycads on which phylogenetic studies have been conducted. Also, analyses of beetles can be improved with divergence time estimates, which require fossils. Recently, putative fossil relatives in both *Allocorynini* and *Pharaxonothinae* have been described from amber deposits [72,73], however, with only crude age estimates ranging from Eocene to Miocene. Hypotheses about cycad evolution generated through study of their associated insects can be novel and unexpected, with examples above, and provide new insights into the evolutionary history of New World cycads. This first attempt at reciprocal illumination of New World cycads through study of their pollinators indicates that this is a fruitful avenue of endeavor.

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#### Appendix A

Morphological, behavioral, and host characters of *Allocorynina* used in the matrix in Appendix B.

- (1) Labial palp: (0) 3 segments; (1) 2 segments. The presence of 2-segmented labial palps is a synapomorphy for the *Notorhopalotria-Rhopalotria* clade.
- (2) Mean male rostral length/pronotal length (RL/PL): (0) <1.0; (1) >1.0, and <1.30; (2) >1.30. High male RL/PL are characteristic of *Paralloccorynus* subgenus *Eocorynus* and the subgenus *Paralloccorynus bicolor-jonesi-salasae-gregoryi* clade, while low male RL/PL (rostral length < pronotal length) is found in the genus *Notorhopalotria* and two species of *Rhopalotria* subgenus *Allocorynus*.

- (3) Mean female RL/PL: (0) <1.25; (1) >1.25, and <1.50; (2) >1.50. High female RL/PL are characteristic of *Parallocorynus* subgenera *Eocorynus* and *Neocorynus*, but has arisen independently in females of other taxa including *Protocorynus bontai*, *Notorhopalotria montgomeryensis*, *Rhopalotria vovidesi*, *Parallocorynus* (*Parallocorynus*) *bicolor*, and *P. (P.) gregoryi*.
- (4) Interocular width/head width at eye: (0) = or >0.5; (1) = or <0.4; (2) >0.4, and <0.5. Short interocular widths are indicative of large eyes and are found in the genera *Protocorynus* and *Notorhopalotria* and in *Rhopalotria* in the *mollis-furfuracea* species group; long interocular distances are indicative of smaller eyes and are characteristic of *Parallocorynus* in the subgenus *Eocorynus* and the subgenus *Parallocorynus bicolor-jonesi-salasae-gregoryi* clade.
- (5) Male: mean post-ocular head width/head width at eye (POW/HW): (0) = or <0.95; (1) >0.95 and = or <1.0; (2) >1.0. Wide male post-ocular head width is characteristic of *Notorhopalotria*, *Parallocorynus* subgenus *Eocorynus*, the subgenus *Parallocorynus P. bicolor-jonesi-salasae-gregoryi* clade, and *Rhopalotria calonjei*.
- (6) Female: mean POW/HW: (0) = or < 0.95; (1) >0.95 and = or < 1.0; (2) >1.0. Medium to short female postocular head widths are characteristic of the Allocorynina compared with the narrow width in the outgroup genus *Oxycraspedus*.
- (7) POW/HW: sexually dimorphic (no overlap): (0) No (dimorphism absent); (1) Yes (dimorphism present). Strong sexual dimorphism in post-ocular head width is characteristic in *Parallocorynus* subgenus *Eocorynus*, but has arisen six separate times in other genera and subgenera.
- (8) Transverse postocular groove: (0) Absent; (1) Present. Character 1 is a synapomorphy for the genus *Parallocorynus*.
- (9) Antennal insertion shape: (0) Foveiform to slightly oval; (1) Sulciform.
- (10) Antennal club, connection of antennomeres: (0) Distinct, 9–10 and 10–11 loosely connected; (1) Distinct, 9–10 loosely connected, 10–11 tightly joined.
- (11) Number of pockets on each side club antennomeres: (0) 1; (1) 2; (2) 3. One pocket is characteristic for *Parallocorynus* and *Protocorynus*; two pockets is characteristic of *Rhopalotria* subgenus *Rhopalotria*.
- (12) Antennal club pocket shape: (0) Half circle (autapomorphy for *Protocorynus*); (1) Oval to round; (2) Elongate oval with irregular outline. Character state 2 is a synapomorphy for *Rhopalotria* subgenus *Rhopalotria*.
- (13) Funicular antennomere 1 in females: (0) Approximately symmetrical; (1) Strongly asymmetrical. Synapomorphy for the *Parallocorynus bicolor-jonesi-salasae-gregoryi* clade.
- (14) Mean scape length in males: (0) >1.1X and <1.8X eye length; (1) <1.1X eye length; (2) >1.8X eye length. Scape length shorter than 1.1X eye length in males is characteristic for *Notorhopalotria* and *Rhopalotria*. Scape length relative to eye length is also a synapomorphy for the *Parallocorynus bicolor-jonesi-salasae-gregoryi* clade; this is the only clade where the scape length routinely exceeds 2X eye length.
- (15) Mean scape length in males: (0) <1.27X length of funicular antennomeres 1 and 2; (1) >1.27X length of funicular antennomeres 1 and 2. Scape length shorter than 1.27X length of funicular antennomeres 1 and 2 in males is characteristic for *Rhopalotria*.
- (16) Gular suture: (0) Entirely separated; (1) Fused.
- (17) Sulcus at posterior margin of eye: (0) Absent; (1) Present and extending around dorsal margin of eye.
- (18) Collar on anterior pronotal margin: (0) Present; (1) Absent. Character shared between *Protocorynus* and *Parallocorynus*.
- (19) Pronotal apex: (0) Without constriction; (1) With constriction.
- (20) Lateral margin of pronotum: (0) Not carinate; (1) With carinae.
- (21) Lateral pronotal margin: (0) Not crenulate; (1) Crenulate.
- (22) Shape of prothorax: (0) Anterior lateral angles not produced; (1) Anterior lateral angles produced forward.

- (23) Male: mean pronotal width/pronotal length (PW/PL): (0)  $< \text{or} = 1.35$ ; (1)  $>1.35$  and  $= \text{or} < 1.5$ ; (2)  $>1.5$ . Within the Allocorynina a relatively narrow pronotum in males is characteristic for *Parallocorynus* except for an inferred reversal in the subgenus *Dysicorynus*.
- (24) Female: mean PW/PL: (0)  $<1.25$ ; (1)  $>1.25$  and  $<1.45$ ; (2)  $>1.45$ . Within the Allocorynina, a relatively wide pronotum is characteristic in *Protocorynus* and *Rhopalotria* (except *R. vovidesi*).
- (25) PW/PL: (0) Overlap between sexes; (1) No overlap between sexes. Strong sexual dimorphism in this character is characteristic of *Parallocorynus* subgenus *Eocorynus*, but has arisen independently twice in *Notorhopalotria* and *Rhopalotria*.
- (26) Anterior pronotal setal fringe: (0) Present; (1) Obsolete between eyes (not protruding beyond margin). Shared character between *Notorhopalotria* and *Rhopalotria* (except for *R. vovidesi*).
- (27) Fovea on pronotum: (0) Absent; (1) Present.
- (28) Notopleural suture reaching anterior margin of pronotum: (0) Yes; (1) No. Characteristic for *Rhopalotria* and with a reversal for *Parallocorynus chemnicki*.
- (29) Mean distance from procoxa to anterior margin of prosternum/distance from procoxa to posterior margin of prosternum: (0)  $>2.2$  and  $<3.8$ ; (1)  $<2.2$ ; (2)  $>3.8$ . High ratios indicate that the procoxae are inserted on the posterior side of the prosternum and is a synapomorphy for *Parallocorynus* subgenera *Eocorynus* and *Neocorynus*.
- (30) Procoxae separated by: (0) Broad sclerite; (1) Sclerotized septum; (2) Not separated. The lack of septum is a synapomorphy for *Parallocorynus*.
- (31) Forecoxae: (0) Partially open laterally; (1) Completely closed laterally.
- (32) Male profemur: (0) Not enlarged; (1) Enlarged. Enlarged profemora in males appears to have arisen independently in *Notorhopalotria*, *Rhopalotria*, and the *Eocorynus-Neocorynus* clades.
- (33) Male profemur granular field: (0) Absent (1) Present. Presence is a synapomorphy for the *Eocorynus-Neocorynus* clade.
- (34) Male profemoral ventrodiscal spine number: (0) Absent; (1) One; (2) Two; (3) More than two. Profemoral spines in males appear to have arisen three times independently in the *Notorhopalotria*, *Rhopalotria*, and the *Eocorynus* clades.
- (35) Male profemoral spine position at ventrodiscal pit: (0) Absent; (1) At proximal apex; (2) Lateral.
- (36) Male profemoral spine location from margin of ventrodiscal pit: (0) Absent; (1) At margin; (2) Away from margin. Spine location away from pit is a synapomorphy for *Notorhopalotria*.
- (37) Male profemur with a longitudinal ventroproximal ridge: (0) Absent; (1) Present. Synapomorphy for *Notorhopalotria*.
- (38) Male: profemora with ventrodiscal angulation: (0) Absent; (1) Present. Synapomorphy for subgenus *Neocorynus*.
- (39) Meso- and metafemora: (0) Not conspicuously compressed; (1) Strongly compressed.
- (40) Meso- and metafemora: (0) Without dorsal crenulation; (1) With dorsal crenulation.
- (41) Tibial spurs: (0) Present and articulated; (1) Present but fused.
- (42) Basal tarsal segment: (0) Subequal to second segment; (1) Much shorter than second and almost concealed.
- (43) Pronotum, frons, and dorsal surfaces of profemora with fine reticulation: (0) No; (1) Yes. Synapomorphy for *Notorhopalotria-Rhopalotria* clade (except for a reversal in *R. vovidesi*).
- (44) Pronotum compressed: height  $< 0.4X$  width: (0) No; (1) Yes.
- (45) Pronotum consistently bicolored: (0) No; (1) Yes. Characteristic of *Protocorynus*, with one independent reversal in *N. montgomeryensis*.
- (46) Punctures on elytra: (0) Irregularly distributed; (1) Ordered longitudinally but not in perfect striae.
- (47) Elytra: (0) With wing locking mechanism, closing to apices, concealing pygidium; (1) Without wing locking mechanism, rounded at apex, pygidium visible.

- (48) Elytra bicolored: (0) No; (1) Yes. This character has arisen independently three times within Allocorynina.
- (49) Color of frons black (vs. brown): (0) No; (1) Yes. This character has arisen twice in Allocorynina in *Protocorynus* and *Parallocorynus chemnicki*.
- (50) Metasternum color black (vs. brown): (0) No; (1) Yes. Within the Allocorynina, this character has arisen once in the *Parallocorynus* subgenus *Parallocorynus bicolor-jonesi-salasae-gregoryi* clade.
- (51) Meso- and metafemur always black: (0) No; (1) Yes.
- (52) Tibia and femur colors often differ: (0) No; (1) Yes. This character is found in *Parallocorynus* in the subgenus *Eocorynus*, and in the subgenus *Parallocorynus bicolor-jonesi-salasae-gregoryi* clade.
- (53) Rostrum color: (0) Brown; (1) Black. A black rostrum has arisen independently twice in *Parallocorynus*.
- (54) Mesoventrite: (0) Flat with intercoxal process strongly projected  $\sim 45^\circ$  angle; (1) Slightly proclinate with intercoxal process on same level.
- (55) Metaventrite: (0) Convex; (1) Disk flattened.
- (56) Metaventrite, latero-posteriorly: (0) Gently rounded; (1) Sharply declined.
- (57) Wing vein rm: (0) Not sclerotized (obsolete); (1) Sclerotized.
- (58) Wing vein Mr spur: (0) Present; (1) Absent.
- (59) Wing vein 1A<sub>2</sub> length: (0) >1A<sub>1</sub>; (1) <1A<sub>1</sub>; (2) Missing. Missing vein is a synapomorphy for the *Notorhopalotria* and *Rhopalotria* clade.
- (60) Wing vein 1A<sub>1</sub>: (0) Present; (1) Missing. Missing 1A<sub>1</sub> vein is a synapomorphy for the *Notorhopalotria* and *Rhopalotria* clade.
- (61) Wing vein 3A: (0) Extends beyond confluence with 2A; (1) Obsolete beyond confluence with 2A. Character state 1 is a synapomorphy for the *Notorhopalotria*.
- (62) Aedeagus apex subtruncate: (0) No; (1) Yes. Synapomorphy for *Rhopalotria*.
- (63) Aedeagus apex length: (0) Approximately equal to own width; (1) Twice own width. Character state 1 is a synapomorphy for *Parallocorynus* subgenus *Dysicorynus*.
- (64) Gonopore with sclerotized knob: (0) No; (1) Yes. Synapomorphy for the *Parallocorynus norstogi-perezfarrerae* clade.
- (65) Gonopore position: (0) Dorsal; (1) Ventrolateral.
- (66) Aedeagus internal sac with ventral strut: (0) No; (1) Yes. Synapomorphy for the *Parallocorynus* subgenus *Parallocorynus*.
- (67) Aedeagus internal sac with transfer apparatus: (0) No; (1) Yes. Synapomorphy for *Rhopalotria*.
- (68) Aedeagus internal sac w/dart: (0) No; (1) Yes. Synapomorphy for *Parallocorynus*.
- (69) Aedeagus internal sac with dorsal pleats: (0) Absent; (1) Present. Synapomorphy for *Parallocorynus* subgenus *Parallocorynus*.
- (70) Aedeagus with prominent sclerotized transverse bridge: (0) No; (1) Yes. Synapomorphy for *Parallocorynus*.
- (71) Aedeagus shape: (0) Trough-shaped; (1) Flattened.
- (72) Tegmen dorsal bridge length from base to its junction with the apical plate extends <1/2 length of apical plate (vs. greater than): (0) No; (1) Yes.
- (73) Tegmen apical setae: (0) Absent or length < width of apical plate; (1) Length > width of apical plate. Character state 1 is a synapomorphy for *Notorhopalotria*.
- (74) Tegmen apical visor: (0) Absent; (1) Present. Synapomorphy for *Rhopalotria*, with the character arising independently in *Protocorynus* where the visor extends across lateral and part of ventral margin.
- (75) Tegmen apical visor curled laterally: (0) No; (1) Yes.
- (76) Tegmen apical plate curls transversely: (0) No; (1) Yes. Synapomorphy for *Notorhopalotria*.
- (77) Tegmen apodeme height: (0) <width of apical plate; (1) >width of apical plate.



- (78) Female: sternum VIII distal half of arms: (0) Strongly converge; (1) Mostly parallel. Character state 1 is a synapomorphy for the *Notorhopalotria* and *Rhopalotria* clade.
- (79) Female: sternum VIII arm length: (0) About equal to length of apodeme; (1) >1.5 length of apodeme. Character state (0) is found only in the *R. furfuracea*-*R. mollis* clade and has arisen independently in *Protocorynus*.
- (80) Female: sternum VIII arms: (0) Curved evenly; (1) With sharp angulate bend. Angulate bends is characteristic of *Paralloccorynus* with a reversal in the subgenera *Eocorynus* and *Neocorynus*.
- (81) Female sternum VIII: junction of arms: (0) Diverging at angle <90°; (1) Forming transverse bar.
- (82) Female: spermathecal tube length: (0) <sternum VIII length; (1) >sternum VIII length. Long tube is characteristic of *Notorhopalotria* and *Paralloccorynus* subgenus *Dysicorynus*.
- (83) Spermatheca: (0) Present and falciform; (1) Absent.
- (84) Spermathecal gland: (0) Tapering to spermathecal duct; (1) Forming common tube with duct.
- (85) Larval feeding site: (0) Female cone; (1) Male sporophyll; (2) Male cone axis.
- (86) Pupation site: (0) Female cone; (1) Male cone; (2) Outside of cone. Pupation site outside of cone is a synapomorphy for the *Eocorynus*-*Neocorynus* clade.
- (87) Host plant family: (0) Araucariaceae; (1) Zamiaceae. Synapomorphy for *Alloccorynina*.
- (88) Host genus: (0) *Araucaria*; (1) *Dioon*; (2) *Zamia*.
- (89) Adult gut contents: (0) Mainly cone tissues other than pollen; (1) Predominately pollen. Character state 1 is a synapomorphy for *Paralloccorynus*.



## Appendix C

Synapomorphic characters separating genera and subgenera of Allocorynina.

The monophyly of *Notorhopalotria* is supported by the following synapomorphies:

- (1) Male profemur with a ventroproximal ridge.
- (2) Male profemoral spine(s) located distantly from margin of profemoral apical pit.
- (3) Wing veins 1A<sub>1</sub>, 1A<sub>2</sub>, and 3A obsolete and not reaching margin of wing.
- (4) Tegmen apical setae longer than width of tegmen.
- (5) Tegmen apical plate curls transversely.

The monophyly of *Parallocorynus* is supported by the following synapomorphies:

- (1) Transverse postocular groove.
- (2) Antennal insertion pointing ventrad.
- (3) One oval sensory pocket on each side of club antennomeres 1 and 2.
- (4) Procoxae not separated by septum.
- (5) Wing vein 1A<sub>1</sub> present and shorter than 1A<sub>2</sub>.
- (6) Aedeagal internal sac with a dart.
- (7) Adults feeding primarily on pollen.

The monophyly of monotypic *Protocorynus* is supported by the following autapomorphies:

- (1) Single semicircular-shaped pit on each side of club antennomeres 1 and 2.
- (2) Pronotal maculation that extends to base of pronotum.
- (3) Tegmen with an apical visor that extends from the dorsal region to part of ventral margin.
- (4) Aedeagus dorsoventrally flattened.
- (5) Spermathecal tube covered with filaments (versus smooth in other Allocorynina [8]).

The monophyly of *Rhopalotria* is supported by these synapomorphies:

- (1) Wing vein 1A<sub>1</sub> missing, but 1A<sub>2</sub> and 3A retained.
- (2) Aedeagal apex subtruncate.
- (3) Aedeagal internal sac with transfer apparatus.
- (4) Tegmen with a dorso-lateral apical visor.

Within the genus *Rhopalotria* two subgenera are distinguished by the following combinations of characters:

Subgenus *Rhopalotria*:

- (1) Male profemora with a single spine at base of profemoral apical pit.
- (2) Two elongate oval sensory pits on each side of club antennomeres 1 and 2.
- (3) Average scape length < length of funicular antennomeres 1 and 2.

Subgenus *Allocorynus*:

- (1) Male profemora with pair of spines at either side of base of the profemoral apical pit.
- (2) Three round sensory pits on either side of club antennomeres 1 and 2.

Within the genus *Parallocorynus*, four subgenera are supported by the following combination of characters:

Subgenus *Dysicorynus*:

- (1) Profemora without granules or spines.
- (2) Female RL/PL >1.27 and <1.44.

- (3) Length of aedeagal apex twice own width.
- (4) Larvae feed and pupate inside of male cone sporophylls.

Subgenus *Eocorynus*:

- (1) Male profemora with granular field and spine.
- (2) Female RL/PL >1.77 and <1.95.
- (3) Larvae feed along cone axis.
- (4) Pupation outside of cone.

Subgenus *Neocorynus*:

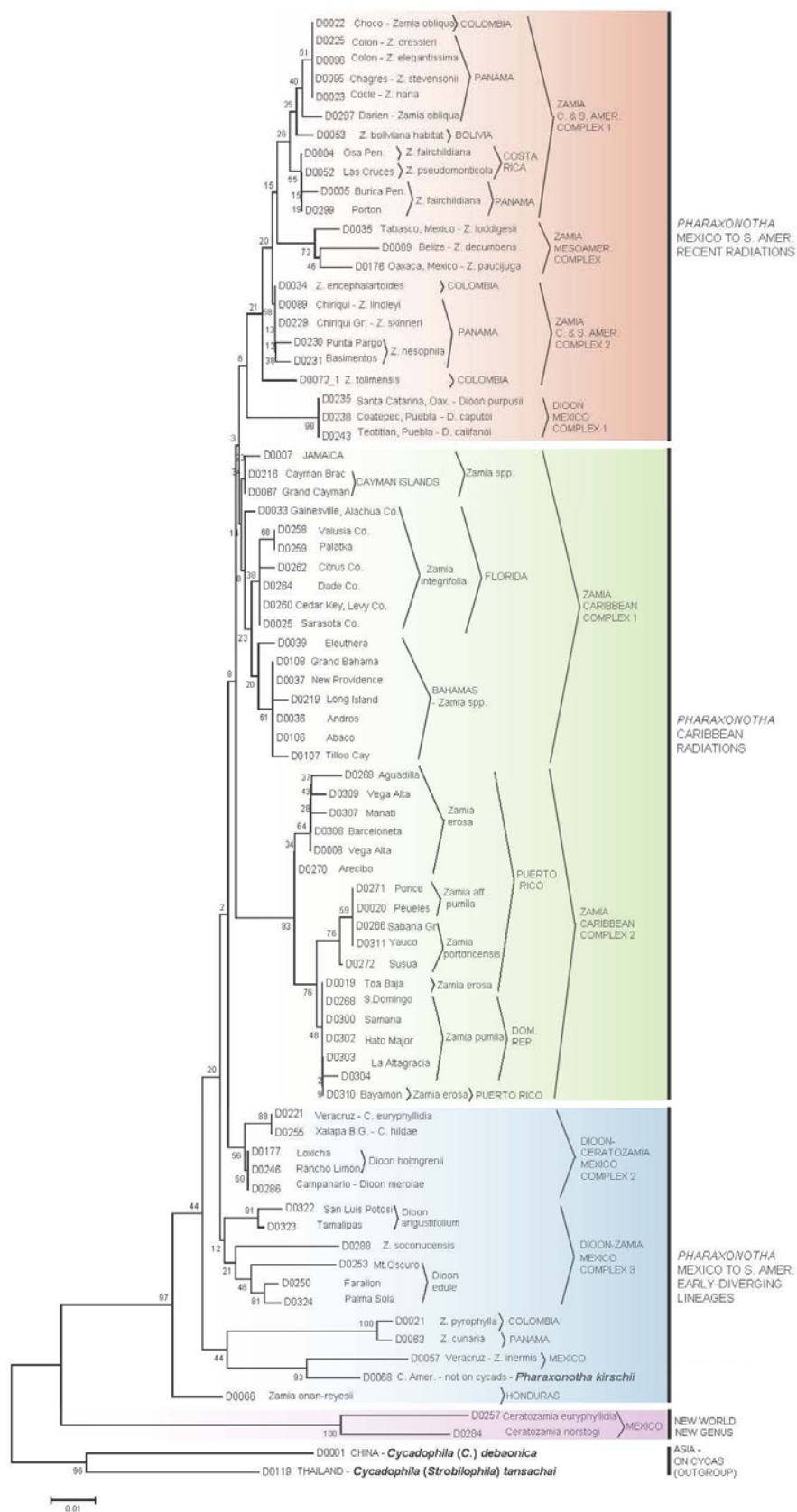
- (1) Male profemora with granular field and no spine.
- (2) Female RL/PL >1.55 and <1.76.
- (3) Larvae feed along cone axis.
- (4) Pupation outside of cone.

Subgenus *Parallocorynus*:

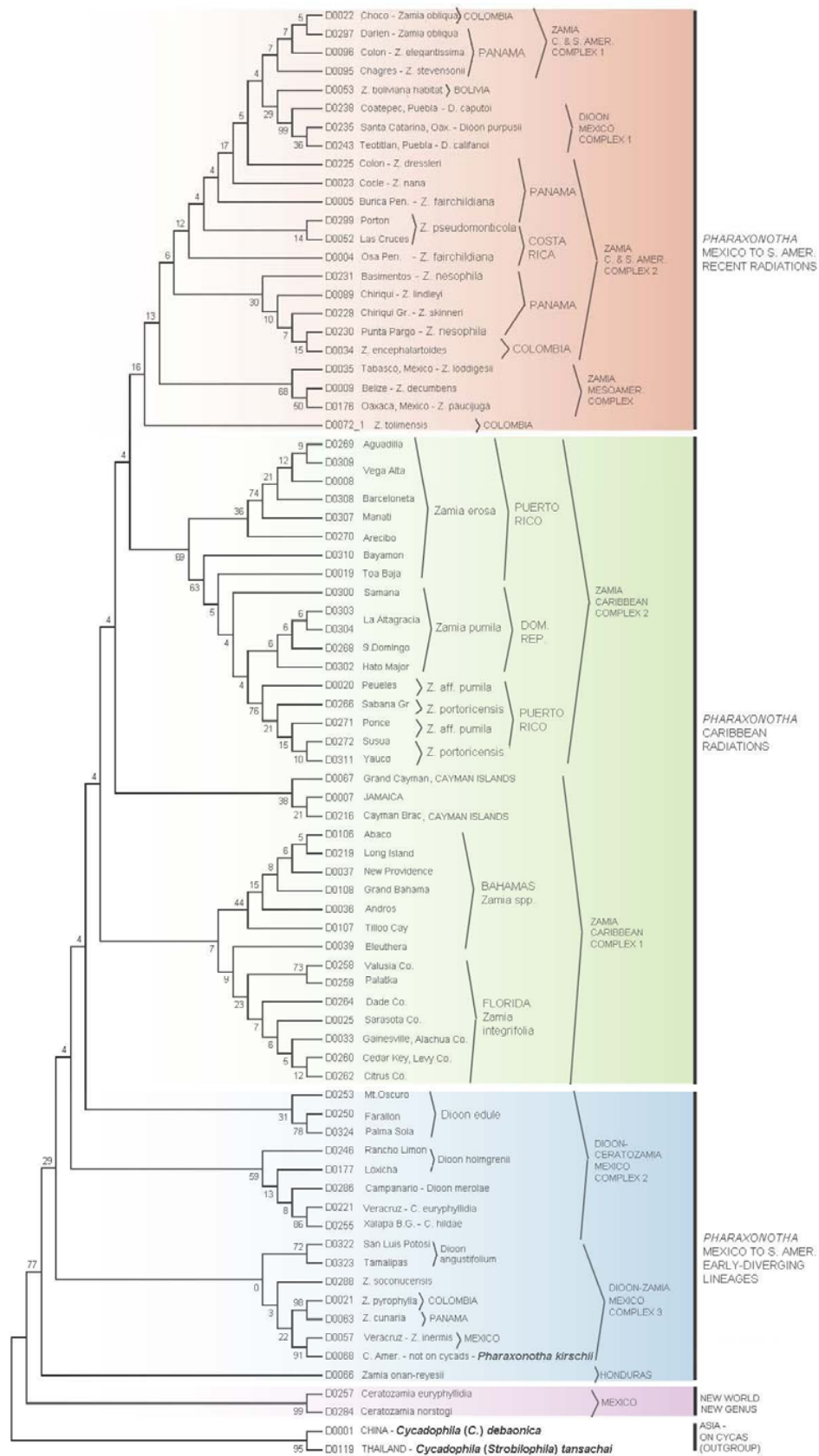
- (1) Profemora without granules or spines.
- (2) Female RL/PL >1.27 and <1.65.
- (3) Aedeagus with internal sac with ventral strut and dorsal pleats.
- (4) Larvae feed and pupate inside of male cone sporophylls.

## Appendix D

Additional phylogenetic trees for Erotylidae.



**Figure A2.** Phylogenetic tree for the Erotylidae: Pharaxonothinae on the cycads of the New World based on neighbor joining (NJ) analysis of 16S rRNA gene sequences; scale bar indicates base pair substitutions per nucleotide position; numbers on branches are bootstrap values.



**Figure A3.** Phylogenetic tree for the Erotylidae: Pharaxonothinae on the cycads of the New World based on maximum parsimony (MP) analysis of 16S rRNA gene sequences; numbers on branches are bootstrap values.

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