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Beetles (Coleoptera) in cones of cycads (Cycadales)
of the northern hemisphere: diversity and evolution

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Beetles (Coleoptera) in cones of cycads (Cycadales) of the northern hemisphere: diversity and evolution

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Abstract. Three groups of beetles inhabit cones of cycads (Cycadales) in the northern hemisphere and are believed to be involved in their pollination. The primitive weevil subtribe Allocorynina (Coleoptera: Belidae) is restricted to the New World cycad genera *Dioon* Lindl. and *Zamia* L. One group of weevils (Curculionidae), found only in *Cycas* L., appears to be a relatively recent colonizer of northern hemisphere cycads. Members of the beetle subfamily Pharaxonothinae (Erotylidae) occur in all Asian and New World cycad genera. Phylogenetic trees of these beetles, based on DNA analysis and supported with morphological studies, are compared to patterns of continental drift and cycad phylogenies. Laurasian origins are suggested for these beetle groups with high latitude dispersal for at least one of these groups during periods of global warm climates.

Key words. Cycad pollination, Cucujoidea, Curculionoidea, symbiosis

Introduction

Field surveys in recent decades have identified three major lineages of beetles in the northern hemisphere that have life cycles intricately tied to the male cones of cycads (Tang 1987a; Vovides 1991; Norstog et al. 1992; Oberprieler 1995, 2004; Tang et al. 1999; Franz and Skelley 2008). Dissection of adults and/or larvae living in the cones of these cycads indicates that they eat cycad pollen or other cone tissues and may sequester cycad toxins (Norstog and Fawcett 1989; O'Brien and Tang 2015; Xu et al. 2015; Skelley et al. 2017; Tang et al. 2018b). Taxonomic reviews of some of these beetle lineages have recently been completed. One is a primitive weevil subtribe, Allocorynina (Belidae), found only in the New World, consisting of four genera: *Notorhopalotria* Tang and O'Brien, *Parallocorynus* Voss, *Protocorynus* O'Brien and Tang, and *Rhopalotria* Chevrolat (O'Brien and Tang 2015). The second major lineage is a group of weevils (Curculionidae) found in southern Asia, consisting of the genera *Nanoplaxes* Heller, *Tychiosoma* Wollaston and other as yet undescribed genera (Oberprieler 1995; Tang et al. 1999), hereafter referred to as the *Nanoplaxes* group. The third major lineage consists of cucujoid beetles belonging to the subfamily Pharaxonothinae (Cucujoidea: Erotylidae) and have been found on all cycad genera in the northern hemisphere and include the genus *Cycadophila* Xu, Tang and Skelley, which is found with *Cycas* L. in Asia (Xu et al. 2015; Skelley et al. 2017), *Pharaxonotha* Reitter found on *Ceratozamia* Brongn., *Dioon* Lindl., *Microcycas* (Miq.) A. DC., and *Zamia* L. in the New World (Chaves and Genaro 2005; Tang et al. 2018b) and *Ceratophila* Tang, Skelley and Pérez-Farrera found only on *Ceratozamia* in Mesoamerica (Tang et al. 2018a). These beetle lineages, like their cycad hosts are largely confined to the northern hemisphere. Where their distributions are sympatric, members of these beetle lineages will co-inhabit male cones of cycads, with up to two of these three major lineages represented in the same cone. Between 1–7 species may occur in one species of cycad cone, but typically 2–3 species will share the cone habitat and observations indicate spatial and temporal niche partitioning among co-inhabiting beetles (O'Brien and Tang 2015; Xu et al. 2015; Tang et al. 2018a).

Wind tunnel tests on female cones of northern hemisphere species of *Cycas*, *Dioon*, and *Zamia* (Niklas and Norstog 1984), wind and insect exclusion experiments on female cones of *Zamia* and *Cycas* (Norstog et al. 1986; Tang 1987a; Yang et al. 2010; Valencia-Montoya et al. 2017), as well as observations of the life cycle and behavior of beetles on other northern hemisphere cycad genera *Ceratozamia*, *Dioon*, and *Microcycas* (Vovides 1991; Chaves and Genaro 2005; O'Brien and Tang 2015; Tang et al. 2018a) indicate these beetle groups are the principle pollination agents of northern hemisphere cycads. Similar experiments and observations on southern hemisphere cycads in South Africa and Australia indicate that the cycad genera there are also insect pollinated, but by different lineages of beetles or by thrips (Forster et al. 1994; Donaldson et al. 1995; Donaldson 1997; Mound and Terry 2001; Terry 2001; Wilson 2002; Hall et al. 2004; Suinyuy et al. 2009, 2012).

Recently phylogenies of Allocorynina cycad beetles have been reconstructed based on cladistic analyses of morphological characters or DNA sequences (Tang et al. 2018b). Phylogenies of Asian (Xu et al. 2015; Skelley et al. 2017) and New World pharaxonothine cycad beetles (Tang et al. 2018a) have also been reconstructed using DNA. Here we summarize previous work and present reanalysis of accumulated DNA data, with either larger combined datasets, alternative outgroups and/or different phylogenetic tests. Additionally, we examine here, for the first time, a DNA-based phylogeny for the *Nanoplaxes*

weevil group that has radiated on Asian *Cycas*. These beetle phylogenies will be compared with those generated for some of their Asian and New World cycad hosts and hypotheses are proposed on the likely origins of these beetle lineages, patterns of adaptive radiation and possible patterns of host shifts.

Materials and Methods

Materials

In total, 133 populations of cycads were sampled in Asia and the New World. In each population beetle adults and larvae were either collected from the surface of male cones or were extracted from inside of microsporophylls. In some cases, adults were taken from between cataphylls or megasporophylls from strobili at the crown of the stem. The sampling in Asia extended from China south to Vietnam and Thailand, and from the Philippines westward to India (see Xu et al. 2015; Skelley et al. 2017 for collection locality details). In the New World sampling extended from Florida to islands in the Bahamas and Greater Antilles and from Mexico south to Colombia and Bolivia (see O'Brien and Tang 2015; Tang et al. 2018a, 2018b; Skelley and Segalla 2019 for collection locality details). Four of the five northern hemisphere cycad genera were sampled; the number of host species include three of the 31 species of *Ceratozamia*, 20 of the 116 species of *Cycas*, 13 of the 16 species of *Dioon* and 29 of the 78 species of *Zamia* currently recognized (Calonje et al. 2018). DNA was also extracted from museum specimens of *Pharaxonotha* sp. (Bolivia) and *P. kirschi* Reitter (Honduras) from the Florida State Collection of Arthropods, Division of Plant Industry, Gainesville, Florida, USA (FSCA). Immature specimens used for analysis were destroyed during DNA extraction, however, for most adult specimens only the abdomen was removed and the remainder of the bodies are deposited as vouchers at FSCA. The monotypic Cuban cycad genus *Microcycas* was not sampled, however, a species of *Pharaxonotha* has been described from this host (Chaves and Genaro 2005).

Morphology

Beetles were preserved in 75–95% ethanol. Specimen dissection techniques followed that of Hanley and Ashe (2003). Genitalia were cleared of surrounding muscle and fat tissue by immersion in a 10–20% solution of KOH. Photographs and measurements were taken digitally with a Nikon DS-Fi2 camera attached to either a NIKON SMZ-1500 stereoscope or NIKON Eclipse 80i compound scope.

DNA analysis

For DNA analysis we used a section of the 16S rRNA mitochondrial 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 (Wink et al. 1997; Whitfield and Cameron 1998; Hosoya and Araya 2005; Sobti et al. 2007; Aruggoda et al. 2010). It has been proposed for use as a standard for insect phylogenies (Caterino et al. 2000).

Total DNA was extracted from individual beetles, either adults or larvae, using Epicenter Master Complete DNA and RNA Purification Kits (Epicenter Technologies, Madison, WI) following the manufacturer protocols and dissolved in 30 μ l H₂O. The mitochondrial 16S rRNA was amplified using the following primers: 73Forward–AGATAGAAACCCARCCTGGCT, 98Forward–CGGTYTAACTCAGATCATGTA, and 430Reverse–AAGACGAGAAGACCCTATAG (Xu et al. 2015; Skelley et al. 2017). Reactions were carried out in 25 μ l volumes containing 1 μ l DNA, 5 μ l 5 \times buffer, 4 μ l of 25 μ M MgCl₂, 1 μ l of 10mM dNTPs, 1 μ l of 10 μ M each primer, 0.2 μ l 5 U/ μ L of Taq polymerase (Promega, Madison, WI). PCR was performed using an Eppendorf ep mastercycler (Eppendorf, Westbury, NY) 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, Ohio) and sequenced bidirectionally on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA).

DNA sequences have been deposited into GenBank with accession numbers MF990634–MF990709 for Allocorynina weevils, MT482347–MT482403 for the Asian *Nanoplaxes* weevils and KR005715–KR005730, KY365217–KY365252 and MG256677–MG256758 for Erotylidae. For analysis of the Allocorynina weevils we used outgroups: *Acanthopygus griseus* Montrouzier, *Anthribus albinus* Linnaeus (Anthribidae), *Rhinotia haemoptera* (Kirby) (Belidae), *Phrissotrichum tubiferum* (Gyllenhal) (Brentidae), *Sitophilus oryzae* Linnaeus (Curculionidae: Dryophthorinae), (GenBank, accession numbers KX683366, AJ495448.1, AJ495455.1, KY084237, KX641892). Due to the uncertain placement of the Asian *Nanoplaxes* weevil group, outgroups were chosen from various Curculionoidea: *Ischnopterapion virens* (Herbst) (Apioninae), *Sitophilus oryzae*, *Sphenophorus* sp. (Dryophthorinae), *Hypera postica* Gyllenhal (Hyperinae), *Cylydrorhinus caudiculatus* Fairmaire (Entiminae), *Gleodema* sp. (Cossoninae), and *Eucryptorrhynchus chinensis* (Olivier) (Cryptorrhynchinae) (GenBank accession numbers KY084146.1, KX373615, BYU-CO246, U16967.1, EF214094, LN888365, KP410324). For the Pharaxonothinae analysis, outgroups were chosen from members of other subfamilies of the Erotylidae: Languriinae sp. and *Cryptophilus* sp. (GenBank accession numbers DQ202523, DQ202560).

Multiple-sequence alignments were conducted with CLUSTAL W. Phylogenetic trees were reconstructed using the maximum likelihood (ML) method as implemented in PAUP 4.0b10 (Swofford 2002) and MEGA5 (Tamura et al. 2011). Bootstrap support values were generated based on 1000 replicates. Based on the lowest Bayesian Information Criterion (BIC) scores the following best models were chosen for tree construction: for the Allocorynina weevils, HKY+G+I: Hasegawa-Kishino-Yano with Gamma distribution and invariable sites; for the *Nanoplaxes* weevil group, HKY+G; for the Pharaxonothinae, GTR+G.

Results and Discussion

Allocorynina weevils

The maximum likelihood (ML) tree for Allocorynina (Fig. 1) exhibits a topology similar to that of the DNA tree synthesized from Bayesian inference and ML analyses produced by Tang et al. (2018b). The data set used to generate the tree here is the same, except four duplicate samples from populations already included in the data set were excluded and a different more phylogenetically diverse set of outgroup Curculionoidea was employed. This tree supports the four genera and the species proposed by O'Brien and Tang (2015) that were confirmed by phylogenetic analysis of morphological characters (Tang et al. 2018b). As in the earlier DNA tree there is strong support for the monotypic genus *Protocorynus*, from Honduras on *Dioon mejiae* Standl. and L. O. Williams, being sister to the other genera, *Notorhopalotria*, *Parallocorynus*, and *Rhopalotria*. Only one lineage in the Allocorynina, genus *Rhopalotria*, is found on more than one host genus, living on *Dioon spinulosum* Dyer ex Eichler in Mexico as well as on *Zamia* species in Mexico, Belize, across the islands of the Greater Antilles, the Bahamas, and Florida. *Rhopalotria* have likely dispersed in the Caribbean on host *Zamia* plants that rafted between islands (Eckenwalder 1980). The Mexican genus, *Parallocorynus*, is subdivided into four subgenera that have radiated only on *Dioon*. The Central American *Notorhopalotria* is found only on *Zamia* from Costa Rica to the Chocó of Colombia.

Nanoplaxes weevil group

A maximum likelihood tree for the Asian cycad weevils (*Nanoplaxes* group) generated from molecular data is published here for the first time (Fig. 2). In previous assessments of this group, these weevils were tentatively assigned to the genus *Tychiodes* Wollaston (Oberprieler 1995; Tang et al. 1999). The genus *Tychiodes* was originally described from “Awasima Island off the northwest coast of Nippon” (Wollaston 1873), a locality today that most closely corresponds to the island of Awashima off the northwest coast of Shikoku, one of the four main islands of Japan. Awashima is 270 km northeast of

the northernmost population of *Cycas revoluta* Thunb. (Osborne and Tomiyama 1995), the only cycad native to Japan. A detailed survey of the Coleoptera inhabiting *C. revoluta* in Yomaguni Island (Okinawa, Japan) revealed no *Tychiodes* beetles (Kono and Tobe 2001), therefore we can confidently assume that the type species for *Tychiodes* does not use *Cycas* as a host and likely does not belong in this clade, although it may be related. Here we use the genus *Nanoplaxes*, described from specimens collected in Pegu, India (now Myanmar) and Luzon, Philippines (Heller 1913), to represent this clade. Examination of the type specimens of *Nanoplaxes*, located in Dresden, by the senior author indicates a close match with specimens collected recently in Thailand and Vietnam on *Cycas* cones. Tang et al. (1999) divided this group tentatively into three clades: A) Group A corresponds with the genus *Nanoplaxes*, a group of relatively large, dorso-ventrally compressed weevils (Fig. 2); B) Group B consists of a group of smaller weevils, with more cylindrical shape (Fig. 2); in this analysis group B does not form a monophyletic lineage, but rather four distinct clades, some which are sister either to *Nanoplaxes* or Group C, but not both simultaneously; C) Group C consists of morphologically distinctive, narrowed-bodied weevils with long snouts; this analysis supports Group C as being monophyletic (Fig. 2); D) Specimens matching *Tychiosoma* Wollaston appear in our tree as the sister lineage to these other groups in this analysis (Fig. 2). Overall the *Nanoplaxes* weevil group is well supported as a natural, monophyletic clade in this analysis (bootstrap support = 94). Oberprieler (1995) reluctantly retained this group of weevils within the subfamily Cossoninae while indicating a possible placement in Curculioninae: Molytini. Tang et al. (1999) placed it in Molytinae: Trypetidini. In this analysis the *Nanoplaxes* complex does not sort with species of Cossoninae, Dryophthorinae, Entiminae, Hyperinae, or Apioninae used as outgroups, but may be closer to Cryptorhynchinae. A recent phylogeny of Curculionidae by McKenna et al. (2009), based on a multi-gene sample, places the Trypetidini near Ceutorhynchini. In their tree, however, Cossoninae, Curculioninae, and Molytinae all appear polyphyletic, suggesting that many of the morphological characters that tie members of these subfamilies together are the result of convergent morphological evolution and that much of the subfamilial and tribal taxonomy of the family Curculionidae requires reassessment.

Pharaxonothinae

The maximum likelihood tree for Pharaxonothinae associated with northern hemisphere cycads is shown in Figure 3. This tree exhibits the same general clades produced in previous, smaller trees for Asian (Xu et al. 2015; Skelley et al. 2017) and New World species (Tang et al. 2018b), but with some differences in the branching of the clades relative to one another. Three genera, *Ceratophila*, *Cycadophila*, and *Pharaxonotha*, form the three main branches. The Asian genus, *Cycadophila*, is subdivided into subgenera *Cycadophila* Xu, Tang and Skelley and *Strobilophila* Skelley, Xu and Tang and subgenus *Cycadophila* is further subdivided into four species groups: *lata*, *fupingensis*, *nigra*, and *papua* (see Skelley et al. 2017). As in a previous DNA analysis of the New World forms (Tang et al. 2018b) the genus *Pharaxonotha* can be divided into three groups: A) Early-diverging lineages from an older radiation that are distributed from Mexico to Colombia; B) A Caribbean radiation; C) A second, more recent, radiation from Mexico to Colombia and Bolivia. Bootstrap support for the position of some of these clades in the tree is weak and further analysis with a greater sampling of genes will help to improve their phylogenetic placement.

Latitudinal radiation of cycads and their beetles

There are good biogeographic reasons to consider the cycad beetle fauna of the northern hemisphere separately from that of the southern hemisphere. But for a few exceptions (discussed below) genera of northern hemisphere cycads are different from those of the southern hemisphere (Goode 2001; Hill and Osborne 2001; Whitelock 2002). For much of the late Mesozoic and early Cenozoic the Tethys Seaway separated Africa and Australia from Eurasia (Seton et al. 2012) and the Proto-Caribbean Seaway separated North and South America (Pindell and Kennan 2009). These seaways served as significant barriers to migration between north and south. Phylogenetic reconstructions of modern cycad lineages based on analyses of their DNA and calibrated with fossils (Nagalingum et al. 2011; Salas-Leiva et al. 2013) indicate Mesozoic or Paleozoic divergence times between primarily northern hemisphere lineages and

those restricted to the southern hemisphere. In addition, studies by Nagalingum et al. (2011), Salas-Leiva et al. (2013), and Condamine et al. (2015) reveal a global synchronous radiation and spread of extant cycad genera and species in the later part of the Cenozoic. When current species richness of cycads is measured globally from north to south pole, their species numbers, unlike other gymnosperms, has a strong bimodal distribution, being highest near the Tropics of Cancer and Capricorn, around latitude 19°N and 25°S (Fragnière et al. 2015) and low near the equator. Taken together these patterns indicate cycad beetles in the north and south have for the most part evolved on separate hosts and diversified separately. A prior survey of cycad beetle diversity by Oberprieler (1995) also indicates different cycad beetle groups occupying the northern and southern hemisphere.

In the northern hemisphere, in the New World, there are four genera of cycads, *Ceratozamia*, *Dioon*, *Microcycas*, and *Zamia*. The first three have distributions centered mainly south of the Tropic of Cancer, in Mesoamerica (Mexico to Honduras) for *Ceratozamia* and *Dioon* and in Cuba for *Microcycas*. The fourth genus, *Zamia*, spans from Florida, Mexico and the Greater Antilles across the equator into central South America. When we compare the distribution of Allocorynina weevils with that of their host cycads we find an agreement in the distribution of diversity. Three of the four genera in the Allocorynina, *Parallocorynus*, *Protocorynus*, and *Rhopalotria*, are also confined to the region of Mesoamerica, Greater Antilles and Florida. Only one genus, *Notorhopalotria*, has a more equatorial range, south of 10°N, from Costa Rica to Colombia. The phylogenetic analysis of these beetles (Fig. 1) indicates that the most early-diverging lineage within the Allocorynina is *Protocorynus*, which occurs only in the Chortis block (roughly corresponding to the country of Honduras). The cycad species of Honduras are unique to that country, indicating a relatively long period of isolation (Whitelock 2002). The Chortis block is a continental fragment which has moved gradually southeastward from a position along the west coast of central Mexico to its present location on the southeastern tip of Mesoamerica (Coates 1997; Pindell and Kennan 2009).

The other cycad beetle group in the New World is the Pharaxonothinae and is represented by two genera, *Ceratophila* and *Pharaxonotha*. As noted earlier, *Ceratophila* is confined to *Ceratozamia* and has not been detected in any other cycad host, despite extensive surveys in the northern hemisphere. *Ceratophila* is currently known only from southern Mexico, however, its distribution likely extends farther south with its host genus to Honduras. *Pharaxonotha* has the largest geographic as well as host range of any cycad beetle, living in the cones of most species of all four New World cycad genera. They have been found on all species of *Zamia* that have been carefully sampled and the monotypic *Microcycas* (Chaves and Genaro 2005; Tang et al. 2018b), but not all species of *Ceratozamia* or *Dioon*, suggesting that *Zamia* or its ancestor may be the original host for this genus. The Caribbean and recent radiations of *Pharaxonotha* (Fig. 3) correspond well with the most recent phylogenetic tree generated for *Zamia* using 10 genes (Calonje et al. 2019), both with a Caribbean radiation that is sister to a recent clade with three branches: one in Mesoamerica and two in Central and South America. The crown age of *Zamia* (age of the last common ancestor of extant *Zamia* species) is estimated to be ca. 10 million years (Calonje et al. 2019), however, a fossil from Panama, verified as belonging to *Zamia* based on epidermal morphology, has an age of ca. 30 million years (Erdei et al. 2018). Taken together the phylogenetic analysis of *Zamia* and this fossil *Zamia* indicates that a period of *Zamia* evolution between 10 and 30 million years ago cannot be reconstructed using DNA. Our *Pharaxonotha* tree possesses early radiations associated with species of *Ceratozamia* and *Dioon*, as well as some early-diverging lineages of *Zamia*, such as *Z. soconuscensis* Schutzman, Vovides and Dehgan, suggesting that part of the early evolution of *Pharaxonotha* may have taken place in these host groups during this gap of evolutionary history of *Zamia* between 10 and 30 million years ago. Also in the early-diverging lineages of *Pharaxonotha* is *P. kirschii*, a generalist feeder that is found on non-cycad as well as cycad hosts, suggesting that the *Pharaxonotha* lineage may have descended from an ancestor that did not feed exclusively on cycads. Indeed, an analysis by Leschen and Buckley (2007) indicates that fungivory may have been the ancestral state in the Erotylidae. *Zamia* and *Pharaxonotha* trees do not align perfectly as there are some relict, early-diverging lineages amongst *Pharaxonotha* with hosts that are not considered early diverging lineages in the *Zamia* tree of Calonje et al. (2019), such as *Z. cunaria* Dressler and *D. W. Stev.* These relict *Pharaxonotha* lineages may have host-shifted from older cycad lineages that have since gone extinct. Estimates of the crown age of *Dioon* vary between 14–56 million years (Condamine et al. 2015; Gutiérrez-Ortega et al. 2018) with potentially greater time for *Pharaxonotha* to be associated with this genus and this may explain why there are several early-diverging lineages of *Pharaxonotha* that inhabit *Dioon*. There are both early-diverging as well as

recent radiations of *Pharaxonotha* onto *Dioon* on disparate parts of the tree indicating that *Pharaxonotha* continues to actively host-shift between cycad genera (see Fig. 3, Table 1). *Ceratozamia* has a fossil record extending back some 25 million years (Kvaček 2002, 2004, 2014; Walther and Kvaček 2008; see below) and early diverging lineages of *Pharaxonotha* are also associated with this lineage. Finally, while the range of *Zamia* extends south of the equator in South America, the DNA-based phylogenetic tree of *Zamia* produced by Calonje et al. (2019) indicates these are recently evolved species that are likely to have dispersed to these regions recently. The one species of *Pharaxonotha* sampled in this study from south of the equator, from Bolivia (see Fig. 3), also appears to be a recently evolved species and like their host *Zamia* do not appear to be a lineage with ancient habitation in the southern hemisphere.

In Asia, there is a single cycad genus, *Cycas*. *Cycas* is divided into six sections, four of these, *Asiorientales* J. Schuster, *Indosinenses* J. Schuster, *Panzhihuaenses* (D. Yue Wang) K. D. Hill, and *Stangerioides* Smitinand, are restricted to mainland and near shore southeast Asia and have distributions centered

Table 1. Northern hemisphere cycad beetles and their corresponding cycad hosts.

Cycad group		Cycas sect. <i>Stangerioides</i>	Cycas sect. <i>Indosinenses</i>	Cycas sect. <i>Wadeae</i>	Cycas sect. <i>Cycas</i>	<i>Ceratozamia</i>	<i>Dioon</i>	<i>Microcycas</i>	<i>Zamia</i>
Beetle family/ subtribe/genus↓	Beetle subgroup↓								
EROTYLIDAE									
<i>Cycadophila</i>	<i>lata</i>	X							
	<i>fupingensis</i>	X							
	<i>nigra</i>	X	X						
	<i>papua</i>			X	X				
	<i>Strobilophila</i>		X						
<i>Ceratophila</i>	<i>Ceratophila</i>					X			
	<i>Voidesa</i> *					X			
<i>Pharaxonotha</i>	Early-diverging					X	X	X	X
	Caribbean								X
	Recent radiation						X		X
CURCULIONIDAE									
<i>Nanoplaxes</i> complex	A		X						
	B1				X				
	B2		X						
	B3		X						
	B4			X	X				
	C		X						
	<i>Tychiosoma</i>				X				
BELIDAE									
Allocorynina	<i>Notorhopalotria</i>								X
	<i>Parallocorynus</i>						X		
	<i>Protocorynus</i>						X		
	<i>Rhopalotria</i>						X		X

*See Tang et al. (2018a) for the original description and details of this subgenus.

along the Tropic of Cancer or that are completely north of it (Hill 2008; Lindstrom et al. 2008; Liu et al. 2018). A fifth section, *Wadeae* K. D. Hill and A. Lindstrom, is restricted to an island system that originated on the continental shelf of Asia near the Tropic of Cancer and then rifted to its present position around latitude 10°N, probably carrying its endemic *Cycas* lineage with it (Hall 1998; Tang 2004b). The sixth and youngest section of the genus, *Cycas*, is believed to have been the most recent to evolve (Hill 2008; Liu et al. 2018) and spread into the southern hemisphere and Australia only recently via ocean dispersal of seeds (Dehgan and Yuen 1983; Hill 1996). It has a tropical distribution, completely south of the Tropic of Cancer. For the seven species of *Cycas* in sections *Asiorientales*, *Panzhihuaenses*, and *Wadeae*, only *Wadeae* is host to members of our three beetle groups (*Nanoplaxes* group and *Pharaxonothinae*). Only two species in these sections, however, have been surveyed for cone insects; the rest have either not been sampled or have been poorly sampled. We will therefore focus our analysis on sections *Stangerioides*, *Indosinenses*, and *Cycas*, where 95% of the species diversity occurs in this cycad genus (116 species total in the genus). For the Asian beetle genus *Cycadophila*, including its subgenus *Strobilophila* and four of the five species groups of its nominate subgenus *Cycadophila*, distributions coincide with the Tropic of Cancer. Only the papua species group is restricted to the tropics (Xu et al. 2015; Skelley et al. 2017, see Fig. 3, Table 1). Furthermore, the fupingensis and lata species groups of subgenus *Cycadophila* are confined to *Cycas* section *Stangerioides* and subgenus *Strobilophila* is restricted to *Cycas* section *Indosinenses* (see Table 1). Both these *Cycas* sections are host to the nigra species group of beetles. The papua species group appears to be confined to *Cycas* section *Cycas*, however, there may be sampling bias due to low number of collections for the papua species group. In general, the subgenus/species group diversity of these beetles coincides with those *Cycas* sections with the highest species diversity.

Weevils of the *Nanoplaxes* group occur sympatrically with *Cycadophila*, but only within three of the five sections of *Cycas*, namely sections *Wadeae*, *Indosinenses*, and *Cycas*. They have not been detected in cones of section *Stangerioides*, which are inhabited solely by *Cycadophila* species groups, namely fupingensis, lata, and nigra (see Table 1). The tree for the *Nanoplaxes* weevil group (Fig. 2) suggests an origin in the Philippines on section *Cycas*, the latest section of the genus *Cycas* to evolve, with a spread westward. It has spread into section *Indosinenses*, but not into the older section *Stangerioides*. Unlike in the *Pharaxonothinae*, this pattern suggests a more recent adaptive radiation within the host group, which started on islands on more recently evolved *Cycas* species and then spread to mainland Asia onto older lineages. The *Nanoplaxes* group has also radiated to fill multiple niches within cones, with as many as five species co-inhabiting cones in populations of *Cycas pectinata* Buch.-Ham. in Thailand, along with as many as two species of *Cycadophila*. Greater sampling in more southern regions, such as Indonesia and Australia, may reveal more species living on *Cycas* farther to the south (Oberprieler 1995).

Dispersal of cycads and beetles between continents

Fossils indicate that Eurasia and North America shared cycad genera during the early and middle Cenozoic. Fossils of the extinct cycad genus *Dioonopsis* Horiuchi et Kimura, have been found in the Paleocene of Japan and the Eocene of western North America (Erdei et al. 2012) and fossils of the extinct cycad genus *Eostangeria* Barthel are known from the Eocene of central Europe and the Paleocene of Wyoming and Eocene in Oregon in North America (Kvaček and Manchester 1999). The leaf epidermal morphology of these two fossil genera distinguish them from any living genera. Although the gross morphology of the leaves of *Eostangeria* appears similar to that of the living genus *Stangeria* T. Moore in South Africa, the epidermal structure suggests closer affinity to the New World genus *Zamia* (Kvaček and Manchester 1999) and gross similarity may be attributable to convergence. Fossils of the extant genus *Ceratozamia*, currently native only to Mesoamerica, are confirmed from the Oligocene and Miocene of central Europe (Kvaček 2002, 2004, 2014; Walther and Kvaček 2008) based on leaf epidermal structure. Together these distributions indicate periods in the early Cenozoic when warmer global temperatures allowed dispersal of cycad genera across high latitude land bridges of Laurasia (Erdei et al. 2012). These fossil cycad distribution patterns are in alignment with the high degree of similarity at the genus level between the flora of North America and those of Europe and Asia (Wen 1999; Qian 2001; Zhu et al. 2012) that have also been attributed to migrations across the Bering and North Atlantic land bridges. Insects that are dependent on cycad hosts to complete their life cycle can be expected to accompany their

hosts as they spread geographically, and many insect and other animal groups of Eurasia and North America display distribution patterns similar to those of plants (Sanmartín et al. 2001). Of the northern hemisphere cycad beetles, only the Pharaxonothinae have a distribution that spans both the Old and New World. Below, we compare current distributions and phylogenies of Pharaxonothinae beetles with ancient and modern cycad floras of Asia and the New World.

The phylogenetic tree presented here for Pharaxonothinae (Fig. 3) suggests that its three cycad-associated genera, *Ceratophila*, *Cycadophila*, and *Pharaxonotha*, had a common origin in a time when cycads and their associated cone beetles could disperse more freely within a more interconnected Laurasian landmass. While sharing similarities these genera have diverged in several aspects of their morphology. In *Ceratophila* and *Cycadophila* the male genitalia (aedeagus and tegmen) are cylindrical to slightly laterally compressed, a shape typical for the Erotylidae (Leschen 2003), however, their orientations are not typical. The male genitalia of *Cycadophila* are twisted like a corkscrew and are oriented laterally when at rest in the abdomen (Xu et al. 2015; Skelley et al. 2017; Fig. 4A). In *Ceratophila* they are not twisted, but are oriented upside down (Tang et al. 2018a; Fig. 4B). In *Pharaxonotha* the male genitalia are also oriented upside down, but unlike other Erotylidae are dorso-ventrally compressed (Tang et al. 2018a; Fig. 4C). These shapes and orientations are interpreted here as adaptations for mating in the tight confines typically found in male cycad cones. Adults of these beetles generally appear within male cones as their sporophylls begin to separate, an environment with insufficient vertically space to allow for male beetles to mount females, the typical fashion by which beetles mate (Thornhill and Alcock 1983; Shuker and Simmons 2014). Due to the shapes of their male genitalia these cycad beetles likely mate by some variant of side to side or end to end and indicate that each of the three lineages have solved this space constraint independently, each by evolving male genitalia of unique shape or orientation. In Figure 3, *Ceratophila*, the beetle lineage confined to New World *Ceratozamia*, has a closer association with the Asia beetle lineage *Cycadophila* found only on the Asia host *Cycas*, rather than the other New World beetle lineage *Pharaxonotha*. This makes sense when we consider the fossils of *Ceratozamia* in Europe, which suggest that both host and beetle lineages may have originated in Eurasia.

Finally, we discuss possible dispersal of southern hemisphere cycad beetle lineages into the northern hemisphere. Extant Boganiidae beetles are known only from the southern hemisphere, in Australia, New Caledonia and South Africa with several species associated with cycad cones (Crowson 1990; Lawrence and Ślipiński 2010; Escalona et al. 2015). Recent phylogenetic analyses of beetles based on the analysis of 95 and 89 genes respectively (Zhang et al. 2018; McKenna et al. 2019) place the Boganiidae and Erotylidae in an early-diverging cucujoid clade that is sister to all other cucujoid beetles. Boganiidae appear to be pollen feeders and recently a fossil species was discovered in amber from northern Myanmar associated with pollen of the *Cycadopites* form genus, which closely matches pollen of several modern cycad genera (Cai et al. 2018). This Myanmar amber deposit has been dated to the mid-Cretaceous, about 99 million years ago, and paleogeographic reconstructions as well as the presence of marine mollusks in this deposit indicate that it was located on the West Burma Block, which appears to have been an island at the time or was in the process of colliding with the Asian continent (Clarke et al. 2019; Yu et al. 2019). Araucarioid wood and leaf fragments, as well as other floral and faunal evidence, indicate the West Burma Block originated from Eastern Gondwana (North-western Australia) and drifted to its present position, carrying a distinct beetle fauna with it (Clarke et al. 2019). An additional fossil boganiid is known from the mid-Jurassic of China (Liu et al. 2017). Although it was not found associated with any cycad part, phylogenetic analysis places it most closely to extant boganiid genera that are associated with the cones of modern-day cycads. Fossil boganiids that reveal ancient cycad beetle distributions extending across both the northern and southern hemisphere are mirrored in the Erotylidae. Besides the pharaxonothine beetles found on northern hemisphere cycads (described above), erotylid beetles are also found on southern hemisphere cycads, including those in Australia [placed in the subfamily Xenoscelinae by Leschen (2003)] and another undescribed group found in South Africa [not belonging to the Pharaxonothinae (Skelley, unpub. data)]. The origins of the Boganiidae and Erotylidae have been estimated in the early to middle Jurassic (Zhang et al. 2018; McKenna et al. 2019), early in the geological breakup of Pangea, when there were fewer oceanic barriers to the dispersal of both cycads and their pollinators. At this earlier stage in their evolution a more globally uniform cycad flora and cycad beetle fauna may have existed, before subsequent isolation, radiations and extinctions shaped the modern cycad flora and cycad beetle faunas.

Hypotheses on the origin of cycad beetle faunas

Multiple hypotheses have been proposed for the origin and evolution of cycad beetles. Considering the fossil record of cycads, which extends back to the Permian, Norstog and Nichols (1997) hypothesized Mesozoic origins for beetles involved in cycad pollination. Based on early phylogenetic analyses of beetle lineages, which suggested origins of most beetle groups in the Cretaceous (Farrell, 1998), Oberprieler (2004) hypothesized that beetles only colonized cycad cones more recently, in the Cenozoic. Newer phylogenies of beetles, calibrated with recently discovered beetle fossils (McKenna et al. 2015, 2019; Toussaint et al. 2016; Zhang et al. 2018) suggests much earlier origins for the beetle superfamilies Curculionoidea and Cucujoidea, in the Triassic or Jurassic, and therefore the possibility of much earlier associations with cycads. Pellmyr et al. (1991) suggested that insects colonized cones first as herbivores before coevolving as pollinators. Fossilized beetle droppings have been found in a fossil Triassic cycad cone indicating herbivorous and possible pollination activity by beetles in cycads of this age (Klavins et al. 2003). Tang (2003, 2004a) hypothesized that several insect and beetle lineages have colonized cycad cones over their long evolutionary history, each colonization event occurring in different time periods (cycad hotel hypothesis). According to this hypothesis male cones, which are filled with starch and lipids (Tang et al. 1987), present a resource that attract many insect visitors and many lineages become permanent residents. Cycad cones are under selective pressure to adapt traits associated with pollination, such the strength and pattern of their heat and odor production (Tang 1987b, 1993) to manipulate their most effective pollen vectors while discouraging visitation by those insects which provide minimal pollination service. Over time new insect lineages appear on cycads, while others may go extinct, so that at any one period in the course of evolutionary time a different array of beetles will reside in a cycad's cone. In effect, any given cycad species will have a number of guests in its cone "hotel", with heat and odor signals adjusted to the most effective pollinators (Terry et al. 2007). In many ways this last hypothesis is a hybrid of previous ones and accounts for many factors that may impinge on the evolution of cycad cone insect faunas during the evolutionary history of the host cycad.

Oberprieler (2004) hypothesized that the original hosts of New World cycad weevils may have been parasitic angiosperms that possess inflorescences with shapes similar to cycad cones. More recently Salzman et al. (2018) found that insects that feed on cycads, despite belonging to disparate phylogenetic lineages, shared a common core gut microbiome, with some of these gut bacteria belonging to rare extremophilic taxa. They suggested that the ability to assimilate a specialized gut flora enabled insects that feed on cycads to cope with the specialized toxins within this plant lineage, such as in the case of sequestration of toxins in the hindgut by *Rhopalotria* weevils (Norstog and Fawcett 1989; Vovides et al. 1993). In the New World there are several cases where cycad cone beetles have shifted from one cycad genus to another, and except for the case of *Pharaxonotha kirschii*, little evidence of any shift between angiosperms to cycads or vice versa. In Asia, there appears to have been but a single major radiation of insects, of the *Nanoplaxes* weevil complex, onto cycads from a probable ancestral angiosperm host. It is likely that chemical barriers are more important than any visual similarities in allowing host shifts and that chemical barriers and the necessary adaptations to specialized gut microflora to cope with these toxins have largely confined radiation of cycad beetles to cycads and minimized the number of host shifts from angiosperms.

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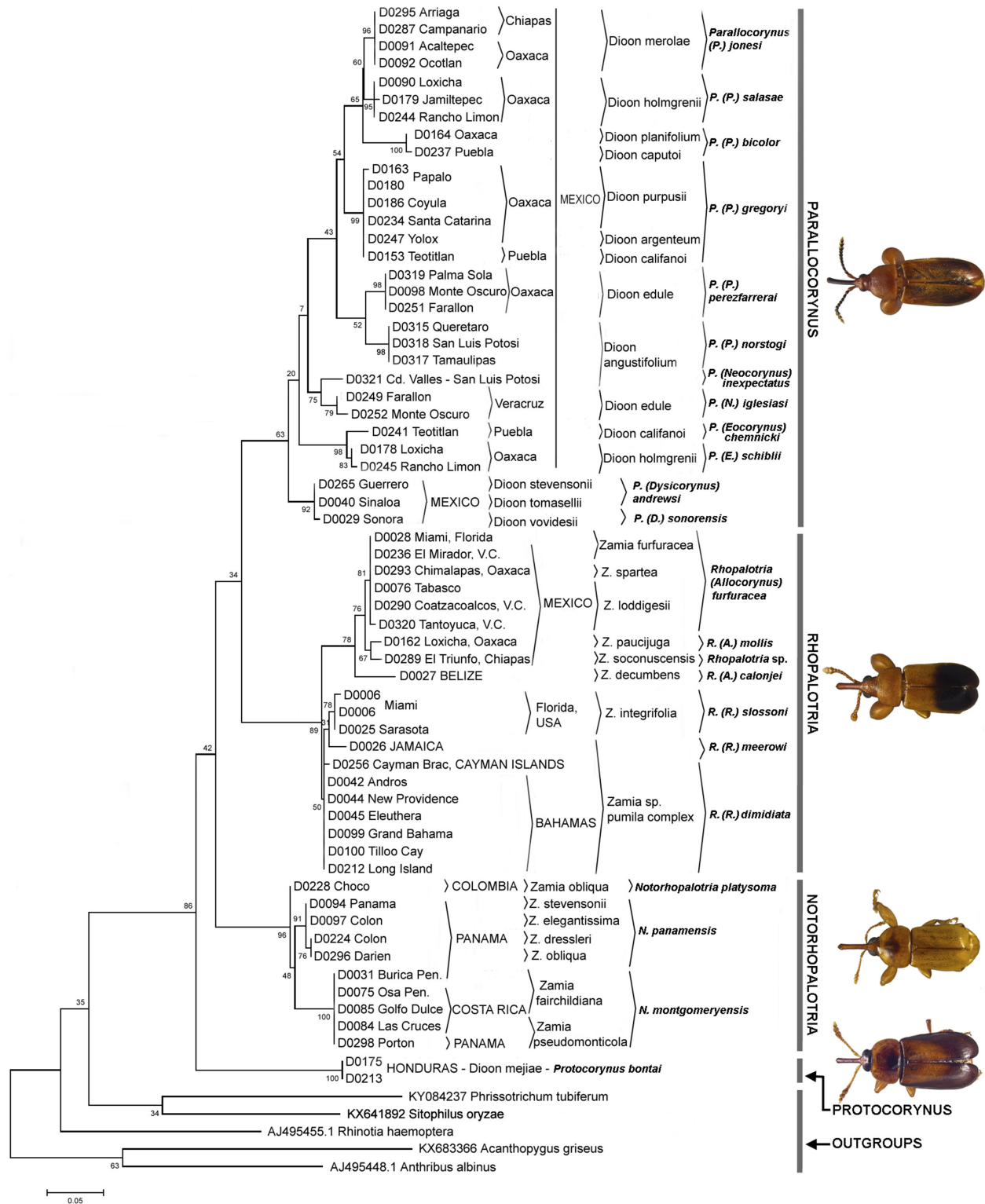


Figure 1. Phylogenetic tree for New World Allocorynina weevils based on maximum likelihood analysis of 16S rRNA mitochondrial gene sequences, scale bar indicates base pair substitution per nucleotides position, numbers on branches are bootstrap values.

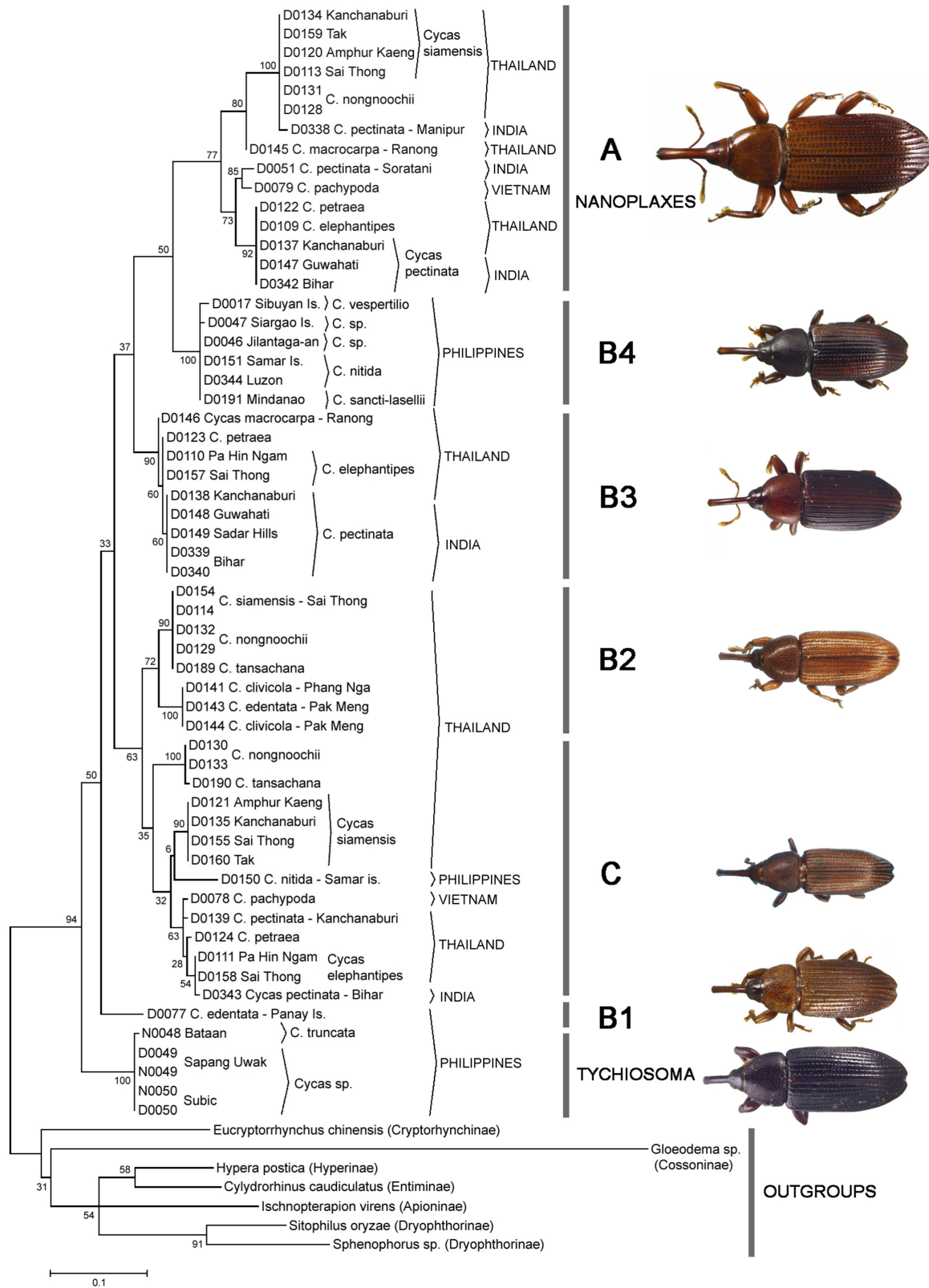


Figure 2. Phylogenetic tree for Asian weevils of the *Nanoplaxes* group based on maximum likelihood analysis of 16S rRNA mitochondrial gene sequences, scale bar indicates base pair substitution per nucleotides position, numbers on branches are bootstrap values.

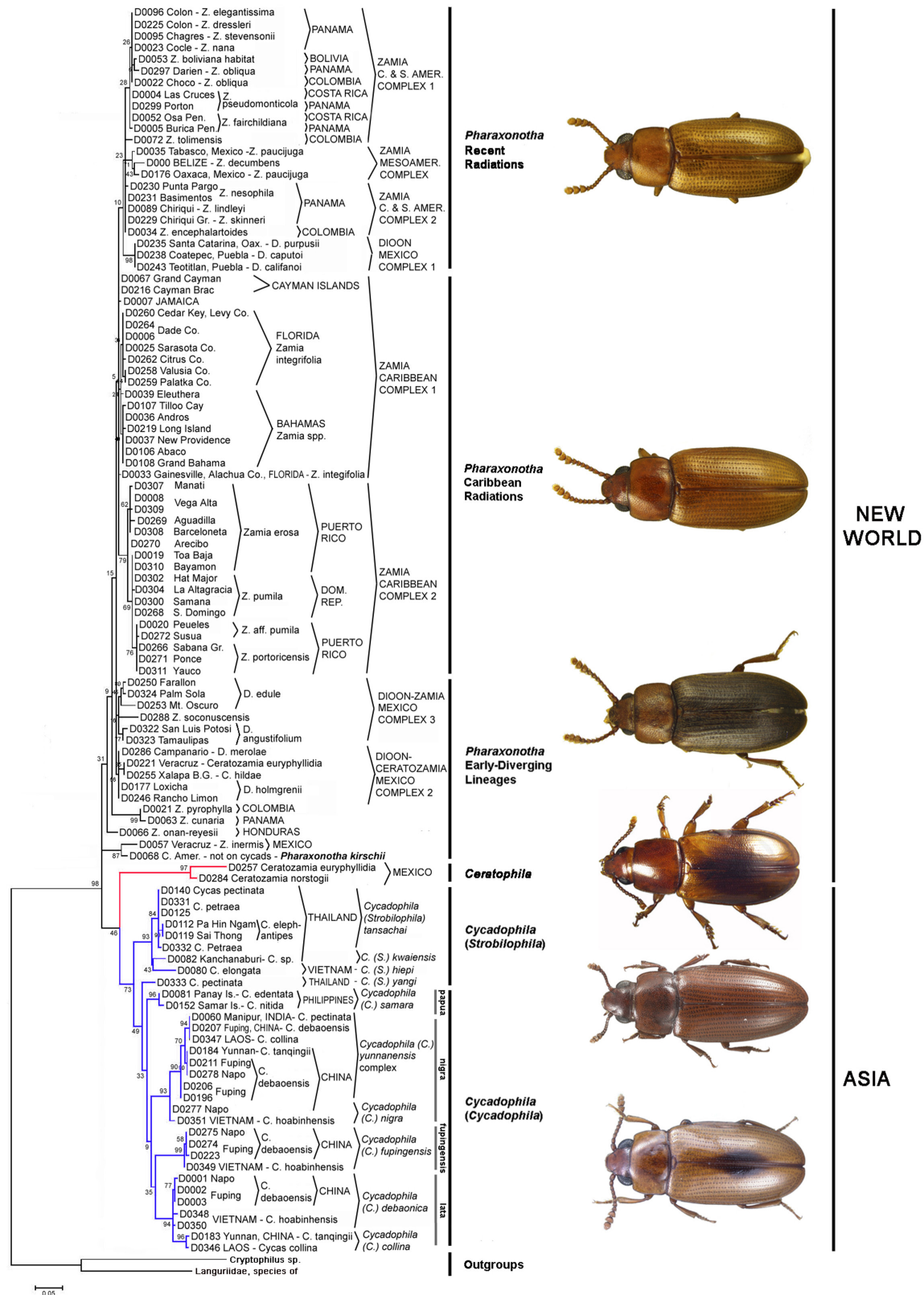


Figure 3. Phylogenetic tree for northern hemisphere Pharaxonothinae beetles associated with cycads based on maximum likelihood analysis of 16S rRNA mitochondrial gene sequences, scale bar indicates base pair substitution per nucleotides position, numbers on branches are bootstrap values.

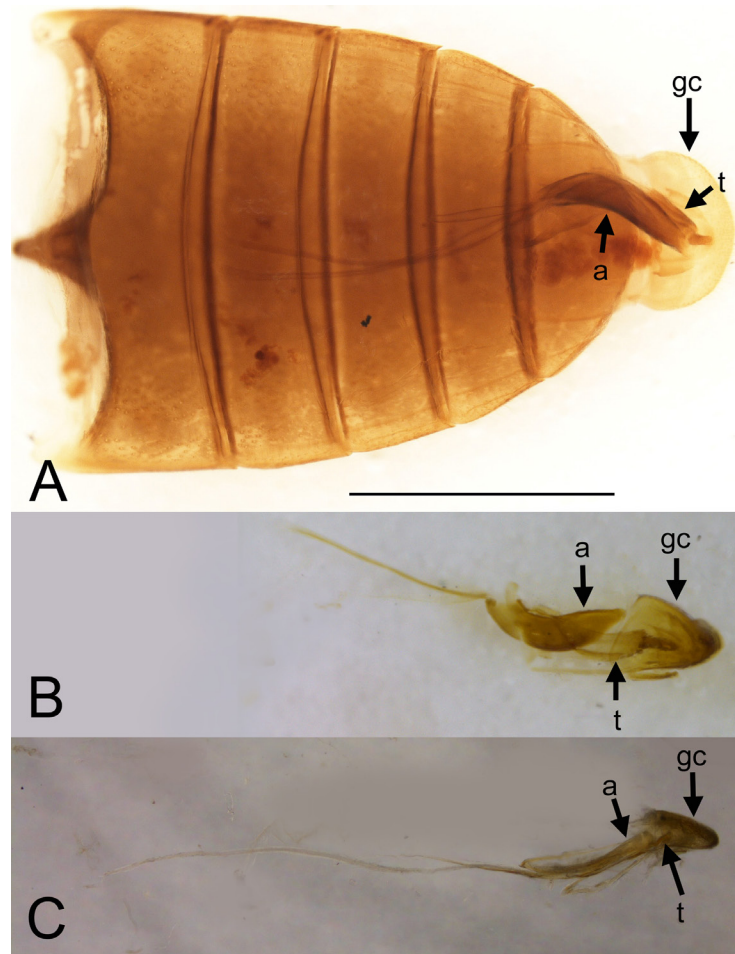


Figure 4. Photomicrographs of male genitalia of three Pharaonothinae genera. **A)** *Cycadophila debonica*, dorsal view, intact in the abdomen, showing lateral orientation with apex of aedeagus pointing toward the left side of the body. **B)** *Ceratophila* sp., lateral view, removed from the abdomen, tegmen is ventrad of the aedeagus instead of the typical dorsad position. **C)** *Pharaonotha kirschii*, lateral view, displaying pronounced dorsoventral compression compared to the other two genera; tegmen is located ventrad of the aedeagus. a = aedeagus; gc = genital capsule; t = tegmen; scale = 1 mm.

