Molecular evidence of cycad seed predation by immature Aulacoscelidinae (Coleoptera: Orsodacnidae)

A L B E R T O   P R A D O[1], D U A N E   D.   M C K E N N A[2] and D O N A L D W I N D S O R[3]

[1]Department of Plant Science, McGill University, Ste Anne de Bellevue, Quebec, Canada, [2]Department of Biological Sciences, University of Memphis, Memphis, TN, U.S.A. and [3]Smithsonian Tropical Research Institute, Balboa-Ancon, Panama

Abstract. Adult beetles in the small subfamily Aulacoscelidinae (superfamily Chrysomeloidea) are known to feed on the foliage and juices of New World cycads (Order Cycadales; family Zamiaceae), but the habits of larvae have long remained a mystery. We provide the first direct evidence that Aulacoscelidinae larvae feed on and develop within the megagametophyte of the Mesoamerican cycad, *Dioon merolae* (Zamiaceae). Phylogenetic analyses based on partial DNA sequences from 3 genes recover a cycad seed-feeding larva proposed to belong to Aulacoscelidinae. These observations reveal a more intimate feeding relationship between Aulacoscelidinae and their New World cycad host plants than was previously recognized. Further, adult Aulacoscelidinae have long been noted to resemble Jurassic fossil chrysomeloids in the extinct subfamily Protoscelidinae. The molecular, morphological, ecological and fossil data reported herein are broadly compatible with an early association between Aulacoscelidinae and their gymnosperm hosts.

Introduction

The Phytophaga (1 30 000 species), comprising the Chrysomeloidea and Curculionoidea, together contain close to 50% of phytophagous insect species and nearly 80% of phytophagous beetle species (McKenna & Farrell, 2009). Most Phytophaga are associated with angiosperms, and their overwhelming taxonomic diversity may have resulted from colonization and radiation on the emerging flowering plants (angiosperms) during the Cretaceous (Crowson, 1981; Farrell, 1998; Duckett *et al.*, 2004; Gómez-Zurita *et al.*, 2007; McKenna *et al.*, 2009). During the Jurassic, before the rise of flowering plants to ecological dominance, the ancestors of Phytophaga lived in forests composed of Pteridophyta, Ginkgoales, Coniferales, Cycadales and [†]Bennettitales (Pant, 1987; Anderson *et al.*, 2007; Labandeira *et al.*, 2007). It is therefore likely that one or more of these groups were the hosts of early

Phytophaga, and that the complex plant–phytophage associations observed today may have originated well before the angiosperm radiation on one or more of these formerly important lineages of plants (Labandeira, 2000; Labandeira *et al.*, 2007). Some lineages within the Chrysomeloidea and Curculionoidea are today associated with gymnospermous plants, particularly conifers (Order Coniferales) and cycads (Order Cycadales) (Kuschel, 1983; Crowson, 1991; Farrell, 1998). These observations have led researchers to speculate that modern associations between certain Phytophaga and gymnosperms could have originated in the early Mesozoic (Kuschel, 1983; Crowson, 1991; Farrell, 1998). Although the 'early origin' hypothesis is intriguing, it has been difficult to evaluate because adult and larval host plants of certain key Phytophaga remain incompletely known. Chief among these are members of the 'pleisiomorphic' chrysomeloid subfamily Aulacoscelidinae. The interrelationships between Aulacoscelidinae (and other Orsodacnidae), Cerambycidae (s.l.), Chrysomelidae, and certain other Chrysomeloidea remain inadequately known, but their inclusion within the Chrysomeloidea is undisputed (Gómez-Zurita *et al.*, 2008). Adult Aulacoscelidinae are associated with cycads from northern Mexico to central Bolivia (Windsor *et al.*, 1999). Additionally, adults have been collected in areas of the Southwestern U.S.A. where there are no native

cycad populations (Riley *et al.*, 2003; Clark *et al.*, 2004). Angiosperm pollen-feeding by adult Aulacoscelidinae has been reported (Monrós, 1954; Santiago-Blay, 2004). Despite the angiosperm pollen-feeding habits of adults, occurrence of adult Aulacoscelidinae far from native cycad populations, the lack of trophic data for larvae, and the absence of direct fossil evidence, cycad-feeding by Aulacoscelidinae has been speculated to have possible origins in the Lower Jurassic, nearly 200 Ma ago (Medvedev, 1968; Crowson, 1981; Kuschel & May, 1990; Windsor & Jolivet, 1997; Cox & Windsor, 1999a; Santiago-Blay, 2004; Jolivet, 2005). Both the modern day association of adult Aulacoscelidinae with cycads and the position of Aulacoscelidinae within Chrysomeloidea are consistent with an early association of Aulacoscelidinae with cycads, but do not rule out other scenarios, including more recent secondary associations with gymnosperms as are known among some extant phytophagous insects, e.g. Lepidoptera and criocerine beetles on cycads (Hawkeswood, 1992; Nash *et al.*, 1992; Forster & Machin, 1994) as well as certain weevils on conifers and cycads (Oberprieler, 1999; Oberprieler *et al.*, 2007; Downie *et al.*, 2008).

Here we report the discovery of an aulacoscelidine larva associated with the megagametophyte of *Dioon meroale* De Luca, Sabato & Vázquez-Torres (Cycadales; Zamiaceae). We provide a description of the specimen and report on its phylogenetic placement among three species representing both known genera of Aulacoscelidinae and 15 additional species of Chrysomeloidea, based on analyses of DNA sequences from three genes. Our report helps fill a long-conspicuous void in our understanding of the evolution of host usage and morphology in Chrysomeloidea.

Subfamily Aulacoscelidinae

The Aulacoscelidinae comprise 28 species in two genera; *Aulacoscelis* Duponchel & Chevrolat and *Janbechynea* Monrós (Santiago-Blay, 2004), the latter being divided further into two provisional subgenera, *Janbechynea* and *Bothroscelis* Monrós. After being treated as part of the Sagrinae (Chapuis, 1874; Jacoby, 1877; Crowson, 1946), Aulacoscelidinae were elevated to subfamily rank within the Chrysomelidae (Monrós, 1953, 1954). For comprehensive reviews of the history of aulacoscelidine classification see Reid (1995) and Santiago-Blay (2004). The name Aulacoscelidinae derives from the Greek *aulax*, which refers to the furrow made by a plough on the ground, and *skelis*, which refers to the ribs of beef (Reid, 1995; Santiago-Blay, 2004). In the conjunction *Aulacoscelis* (furrowed rib), the authors probably were referring to the sulcate tibia of *Aulacoscelis melanocera,* a morphological feature shared by all Aulacoscelidinae. Features of hind wing venation, male external genitalia and the female spermatheca suggest that Aulacoscelidinae are related closely to Orsodacninae (Kuschel & May, 1990; Reid, 1995; Suzuki & Windsor, 1999; Reid, 2000). Morphology of the first instar larvae of *Orsodacne cerasi* L., *O. lineola* Panzer (Cox, 1981) and *Aulacoscelis appendiculata* Cox & Windsor (1999a, b) also suggest

close affinities of the two taxa. Similarly, molecular phylogenetic analyses recover Aulacoscelidinae and Orsodacninae as sister taxa, although without strong nodal support (Duckett *et al.*, 2004; Gómez-Zurita *et al.*, 2007, 2008).

Cycad leaf-feeding by adults has been reported in both genera of Aulacoscelidinae; *Janbechynea paradoxa* Monrós on *Zamia boliviana* (Brongn.) A. DC. (Prado *et al.,* 2011) and *A. appendiculata* on *Zamia* cf. *elegantissima* Schutzman, Vovides & Adams (Cox & Windsor, 1999a). Adults of other species of Aulacoscelidinae also have been collected on *Dioon* (Santiago-Blay, 2004; Prado *et al.*, 2011). Adults in both genera of Aulacoscelidinae appear suddenly early in the rainy season and feed mainly on the newly-produced, immature leaves of cycads, although occasionally older leaves are attacked. The beetles puncture the epidermis of cycad leaves with their sharp mandibles and ingest the oozing fluids (Windsor *et al.*, 1999) from which they sequester toxic azoxyglycosides (Prado *et al.*, 2011). These cycad-specific secondary metabolites can be exposed to potential predators through a reflex bleeding mechanism (Prado *et al.*, 2011). After feeding and mating on their cycad hosts for approximately 2 weeks, the beetles vanish until the beginning of the next year's rains. Aulacoscelidinae beetles also feed on exposed cycad root and stem tissue (A. Prado, unpublished data), as well as the fronds of ornamental plantings of Asian *Cycas* spp.. Angiosperm pollen has been identified in the gut of adult *A. melanocera* (Crowson, 1991) and also in the faeces of *A. appendiculata* (specifically, when they make their first appearance on cycads) in Central America (A. Prado, unpublished data). Adult beetles have been collected in southern North America on the flowers of *Croton* (Asteraceae) and *Hechtia* (Bromeliaceae) (Clark *et al.*, 2004; Santiago-Blay, 2004). *Aulacoscelis vogti* Monrós in northeastern Mexico has been observed feeding and mating on *Brahea dulcis* (Hum., Bonpl. & Kunth) Mart. (Arecaceae) flowers and has been observed in smaller numbers on the leaves and cones (both sexes) of *Dioon edule* Lindl. (Zamiaceae) (Prado *et al.*, 2011). *Aulacoscelis appendiculata* has been kept in the laboratory for approximately 2 months feeding on mango slices and/or palm flowers (Windsor *et al.*, 1999). These observations suggest that aulacoscelidine adults are pollen generalists but have a close association with cycads, from which they sequester toxic compounds for chemical defence (Prado *et al.*, 2011). A first instar larva of *A. appendiculata* was reared successfully from an egg deposited in the laboratory (Cox & Windsor, 1999a), but the larval host plant(s) remained a mystery (Windsor & Jolivet, 1997).

The case for an ancient association between Aulacoscelidinae and the Cycadales

The phylogenetic position of Aulacoscelidinae has long been of interest to systematists studying Chrysomeloidea due to plesiomorphic morphological features of adults (e.g. adult mandibles with a definite molar region) (Mann & Crowson, 1981; Kuschel & May, 1990; Reid, 1995; Suzuki & Windsor, 1999). The resemblance of adult Aulacoscelidinae to Jurassic

fossils assigned to the extinct subfamily Protoscelidinae has generated debate about their temporal origin and phylogenetic placement (Medvedev, 1968; Kuschel & May, 1990; Crowson, 1991; Zhang, 2005). However, Reid (1995) and later Grimaldi & Engel (2005), doubted that fossils of †*Protoscelis* Medvedev, †*Protosceloides* Medvedev, †*Pseudomegamerus* Medvedev and †*Cerambyomima* Medvedev contain sufficient character data to conclude they are close relatives of Aulacoscelidinae, and suggest that the association of Aulacoscelidinae with cycads is recent. However, the recently-discovered fossil protoscelidine, †*Tarsomegamerus mesozoicus* (Zhang, 2005) from the Jurassic Daohugou biota in present-day China – an extremely well-preserved specimen – has a tibia with a longitudinal medial carina flanked by two longitudinal furrows (Zhang, 2005). The furrows of this fossil are in the same position and orientation as in extant *Aulacoscelis* species and revision of the protoscelidine fossils could shed more light on their relationship to the aulacoscelidines (Santiago-Blay, 2004). Controversially, *T. mesozoicus* was assigned subsequently to an extinct family of Elateriformia (Kirejtshuk *et al.*, 2010), despite the apparent lack of such tibial ornamentation in elateriforms.

Whether the association of Aulacoscelidinae with cycads has a relatively recent or ancient origin is far from being resolved. Nonetheless, indirect support for the ancient association hypothesis comes from the alleged resemblance of aulacoscelidines to fossils (e.g. †*Protoscelis*, †*Tarsomegamerus*) that occur in gymnosperm-dominated strata in which cycads are common, their plesiomorphic morphological features, their phylogenetic position within Chrysomeloidea, their present-day association with cycads (as adults), their ability to sequester cycad toxins, and the unusual mode of adult feeding (Crowson, 1991; Windsor & Jolivet, 1997; Santiago-Blay, 2004; Jolivet, 2005; Prado *et al.*, 2011).

New insights, the Dioon seed

On July 2004 the Florida Department of Agriculture identified an infested *Dioon* seed (Cycadales: Zamiaceae) that had come from Mexico. The seed being shipped to the Montgomery Botanical Center contained a larva with possible resemblance to the Chrysomeloidea. The seed was collected originally in the province of Oaxaca in May 2004. From the locality information of the collection and seed morphology, the seed can be assigned with certainty to *Dioon merolae* De Luca, Sabato & Vázquez-Torres from the central valleys of Oaxaca (Fig. 1). *Dioon merolae* grows in warm humid and sub-humid climates in the Mexican States of Oaxaca and Chiapas (Cabrera-Toledo *et al.*, 2010, 2012). In the State of Oaxaca it grows on the Sierra Madre del Sur where it is separated orographically from *D. rzedowskii* De Luca, Moretti, Sabato & Vázquez-Torres, *D. purpusii* Rose, *D. califanoi* De Luca & Sabato and *D. spinulosum* Dyer which grow on the inner mountains of the Sierra del Norte (De Luca *et al.*, 1981). *Dioon merolae* grows at higher elevations (900–1700 m) than *D. holmgrenii* De Luca, Sabato & Vázquez-Torres which grows closer to the coast of Oaxaca (580–850 m) (De Luca *et al.*, 1981).

The putative chrysomeloid larva found inside the *D. merolae* seed was sent to DW at the Smithsonian Tropical Research Institute (STRI) for identification (Fig. 2). Unfortunately, the mandibles that had been dissected from the head prior to shipment, were overlooked and lost during ethanol replenishment upon arrival at STRI. The larva was photographed under a light microscope (www.discoverlife.org as *Janbechynea* sp.) and later using a SEM. After removing a sample of tissue for DNA extraction, the specimen was shipped to M.L. Cox at the Natural History Museum in London. Unfortunately the specimen was destroyed by fungus in transit. Because this is the first known chrysomeloid larva to be found in cycad tissue we consider it important to report on what we learned about its morphology and phylogenetic affinities, despite the loss of this unique specimen.

Methods

The single larva found inside a seed of *Dioon merolae* was imaged using a Jeol 5300LV SEM (Fig. 2). A modest morphological description of the larva was prepared after the specimen was lost, based on the SEM images and notes. Genomic DNA was extracted from a 4 × 4 mm section removed from
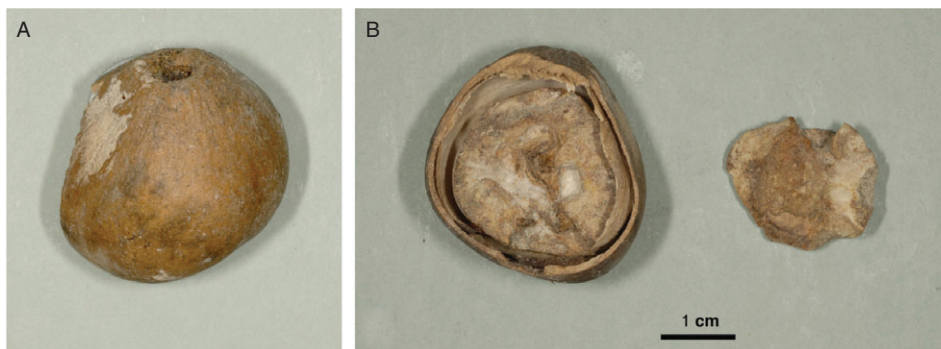

Fig. 1. Seed of *Dioon merolae* De Luca, Sabato & Vázquez-Torres showing feeding damage caused by the larva of an Aulacoscelidinae beetle: (A) hard sclerotesta; (B) interior of the seed, showing endostesta, and larval feeding damage to the embryo and endosperm.
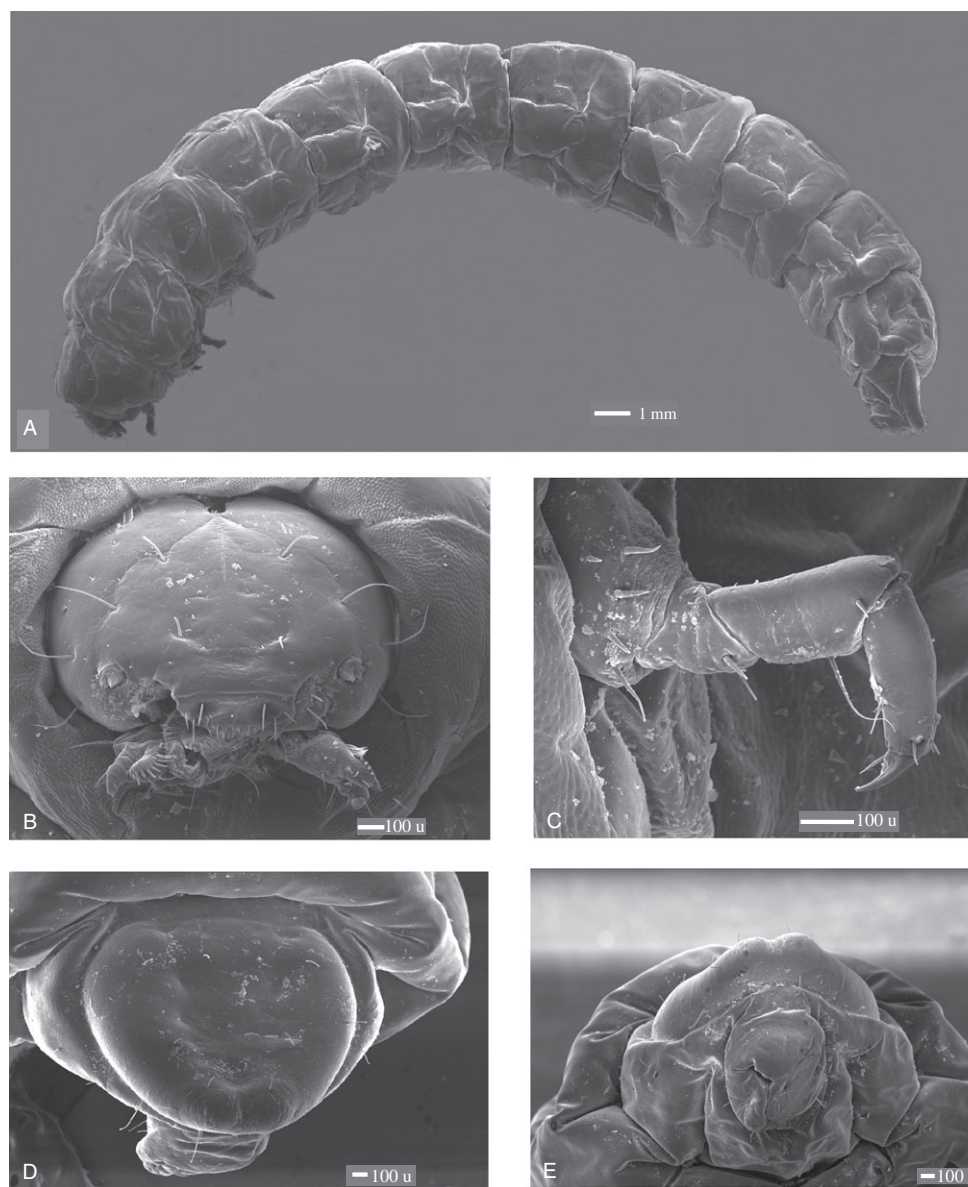
Fig. 2. SEM of Aulacoscelidinae larva: (A) General habitus, lateral view of complete larva; (B) anterior view of head; (C) lateral view of leg showing coxa, trochanter, femur and tibiotarsus; (D) dorsal view of anal plate; (E) ventral view of abdominal segment 10.

one side of the larva. Partial sequences from the *CO1* and *28S* and *18S* genes were obtained from the resulting genomic DNA using the primer sets detailed in Table S1. To identify the taxonomic affinity of the unidentified chrysomeloid larva, these DNA sequences were compared both individually and in a concatenated alignment to orthologous sequences obtained previously from 10 species of Chrysomelidae in 6 sub-families (Cassidinae, Chrysomelinae, Criocerinae, Donaciinae, Eumolpinae and Sagrinae), one Cerambycidae (subfamily Cerambycinae) and 6 other taxa representing known or presumed early divergent Chrysomeloidea (Aulacoscelidinae, Orsodacninae, Palophaginae and Oxypeltinae) (Table 1). Sequences for sixteen taxa were obtained by extracting genomic DNA from

adult specimens collected and identified by DW as indicated in Table 1. For each specimen, DNA sequences from three genes were obtained using the primer sets detailed in Table S1. DNA sequences for three other species were obtained from Genbank (accession numbers: FJ867850.1, FJ867681.1, AJ850051.1 for *Ericmodes sylvaticus*; FJ859984.1, FJ867816.1, FJ867699.1 for *Oxypeltus quadrispinosus*; FJ000508.1, FJ000433.1 for *Palophagus bunyae*). Two sequences were missing from our matrix – *CO1* for *Palophagus bunyae* and *18SA* for *Lilioceris nigripes*.

All 20 sequences were aligned using Clustal W (Thompson *et al.*, 1994) and the alignment then refined manually. Substitution models were selected for each gene using Modeltest v3.7

Table 1. Species list.

ID number	Species	Family/Subfamily
BTOLDDM0176	*Ericmodes sylvaticus*	Protocucujidae[a,b]
695	*Aulacoscelis appendiculata*	Aulacoscelidinae
7721	*Aulacoscelis vogti*	Aulacoscelidinae
7598	*Janbechynea paradoxa*	Aulacoscelidinae
761/1153	*Acromis sparsa*	Cassidinae
1062	*Spaethiella marginata*	Cassidinae
1038	*Xenochalepus hespenheidi*	Cassidinae
1355	Unidentified Cerambycinae	Cerambycinae
1145	*Platyphora flavoannulata*	Chrysomelinae
1131	*Lilioceris nigripes*	Criocerinae
1132	*Lilioceris* sp.	Criocerinae
845	*Donacia* sp.	Donaciinae
JJG321	*Callisina quadripustulata*	Eumolpinae
696	*Megascelis puella*	Eumolpinae
7692	*Orsodacne cerasi*	Orsodacninae
7691	*Orsodacne humeralis*	Orsodacninae
BTOLDDM0168	*Oxypeltus quadrispinosus*	Oxypeltinae[b]
FJ000433	*Palophagus bunyae*	Palophaginae[b]
9007	*Mecynodera coxalgica*	Sagrinae
1118	Putative Aulacoscelidinae	Orsodacnidae

[a] Outgroup.
[b] Obtained from Genbank.

(Posada, 2005) for maximum-likelihood (ML) analyses and MrModeltest v2.3 (Nylander, 2004) for Bayesian analyses.

Phylogenetic analysis was performed via parsimony inference using a heuristic search with and without coding gaps in the alignments, via ML inference, and via Bayesian inference. Each analysis was performed for the three individual genes separately and for a concatenated matrix that included data from all three genes (2145 total bp). *Ericmodes sylvaticus* (Protocucujidae) was used as an outgroup in all analyses (Table 1) because it is a member of the superfamily Cucujoidea (close relatives of Phytophaga) and because DNA sequences were available for several genes from a previous study (McKenna *et al.*, 2009).

A parsimony analysis was implemented in PAUP v4.0 beta (Swofford, 1998) and a ML analysis was implemented in RAxML v7.2.8 (Stamatakis *et al.*, 2008). For the concatenated ML analysis the model of evolution chosen was GTR + I + G. Bayesian inference was implemented in MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001) using two separate runs, each of 10 000 000 generations, with four chains and parameters according to the models specified in Table S1. For analyses of the combined dataset, partitions were set to allow analytical parameters to vary within each partition. The CIPRES Science Gateway was used to perform the Bayesian analyses (Miller *et al.*, 2010). We assessed convergence and stationarity under Bayesian inference using the standard deviation of split frequencies between runs, trace plots of log-likelihood scores, and using graphical and statistical analyses implemented in Tracer v1.4 (Rambaut & Drummond, 2007) and AWTY (Nylander *et al.*, 2008). Post-burn in trees were pooled and used to generate a 50% majority-rule consensus tree.

Results

Larval morphology

Final instar larva. Head width: 1.37 mm; Body length: 33.2 mm; Body width: 3.17 mm.

General habitus. Elongate and cylindrical, approximately same width along entire length, greatest width at meso and metathorax; head and anal plate darker than the rest of body; mostly glabrous with well-developed setae on the head, prothorax, abdominal segments 9–10.

Head. Small, pro- to hypognathous, protracted, oval (Fig. 3A); epicranial suture with stem absent or very short; frontal sutures narrow and reaching antennae; endocarina reaching mid-frons; frons bearing two large setae and two sensilla along the frontal sutures, mid-frons bearing two sensilla and lower frons bearing one large seta; vertex bearing five sensilla; gena bearing three large setae; stemmata apparently absent (there is a slight possibility that a single highly reduced stemma is present posterodorsal to the antenna, but higher resolution scans will be needed to verify its presence); labrum bearing six moderately long setae dorsally (Fig. 3F), more than eight curved setae along anterior margin on each side, anteromedially bearing four setae and numerous elongate spicules; antennae very short and embedded in antennal fossa (Fig. 3D), 3-segmented, anteriorly-directed, First segment at least three times broader than long, second segment with apex truncate bearing two small setae dorsally, one larger seta ventrally, a basiconic sensillum twice as long as segment two and segment three, segment three cigar-shaped bearing three small setae; maxillary palpi 3-segmented (Fig. 3C), basal two segments sub-equal in length, apical segment conical, apex truncated with ring of small setae apically; galea club shaped (Fig. 3C), bearing two setae ventrally and more than 14 thick setae on its lateral margin extending to apical margin.

Legs. Three pairs well developed, chaetotaxy similar in each pair (Fig. 3B); coxa bearing 6 large and 2 very small setae ventrally plus 2 small setae posteriorly; trochanter bearing 2 large setae ventrally and 1 short seta dorsally plus 1 short seta posteriorly; femur bearing 1 very long seta ventrally, plus 2 long and 3 short setae apically plus 1 short seta dorsally; tibiotarsi bearing 1 long seta ventrally and 3 setae apically; pretarsal claw elongate, slightly curved and bearing a long seta on inner margin.

Abdomen. Abdominal segments 1–8 mostly glabrous with annular spiracles disposed laterally, abdominal segments 1–6 bearing two small setae, one dorsal and one posterior to the spiracle (Fig. 3E); abdominal segments 7–8 bearing one setae dorsal to the spiracle; abdominal segment 9 bearing a smooth anal shield slightly bifid in its posterior margin, bearing 8 long setae dorsally plus 4 long setae ventrally (Fig. 3G); abdominal
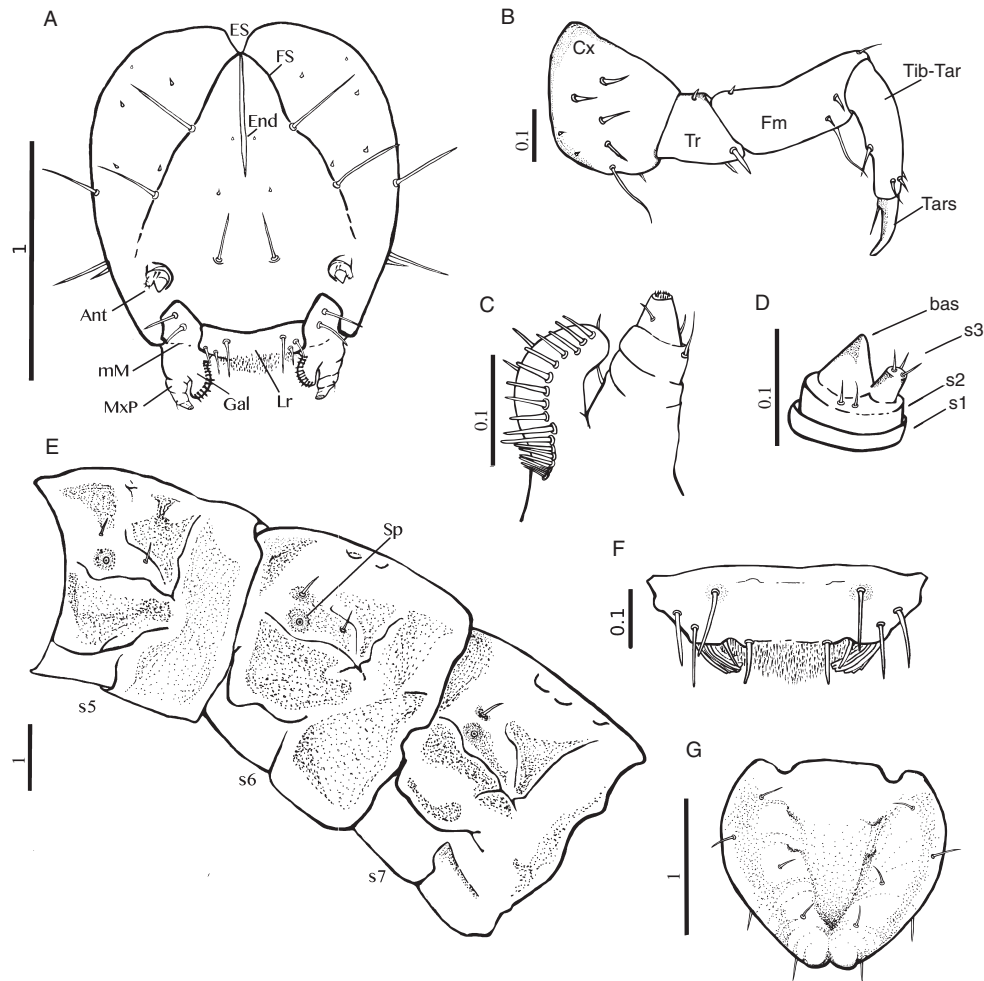
Fig. 3. Head and body of Aulacoscelidinae final instar larva: (A) frontal view of head; (B) ventral view of leg, (C) dorsal view of right galea and maxillary palpus; (D) dorsal view of right antenna; (E) left lateral view of abdominal segments 5–7; (F) frontal view of labrum; (G) dorsal view of anal plate. Abbreviations: Ant, antenna; bas, basiconic sensillum; Cx, coxa; End, endocarina; ES, epicranial suture; Fm, femur; FS, frontal suture; Gal, galea; Lr, labrum; mM, missing mandibles; MxP, maxillary palpus; Sp, spiracle; s1, s2, s3, antennal segments 1-3; s5, s6, s7, abdominal segments 5-7; Tars, pretarsal claw; Tib-Tar, tibiotarsus; Tr, trochanter.

segment 10 ventrally situated, circular, bearing 6 long setae along its margin.

DNA sequences

The final *COI* alignment was 527 characters long, of which 216 characters were parsimony informative (Table S1). No informative gaps were obtained from the *COI* alignment. The *28S* alignment was 548 characters long, of which 129 characters were parsimony informative, 26 parsimony informative gaps were identified. The *18S* alignment was 516 characters long, of which 46 characters were parsimony informative. There were seven informative gaps. Concatenation of the three alignments produced a matrix 2145 characters long that contained 391 parsimony informative characters and 33 parsimony informative gaps.

Phylogenetic analyses

The intention of our phylogenetic reconstructions was not to resolve the phylogenetic relationships of the subfamilies of the large superfamily Chrysomeloidea, but instead to evaluate the phylogenetic affinity of the putative aulacoscelidine larva. Therefore, our taxon sample was focused on Aulacoscelidinae, known and putative relatives, and selected outgroups. We analysed the concatenated 3-gene matrix under ML and Bayesian inference (Figs 4, 5). Based on our evaluation (see Methods) the Bayesian analyses reached convergence quickly, at approximately 12 000 generations.

Interrelationships among the early diverging branches of Chrysomeloidea were mostly unresolved, consistent with previous molecular phylogenetic studies involving Chrysomeloidea (Gómez-Zurita *et al.*, 2007). The resulting 50% majority-rule ML consensus tree recovers the putative aulacoscelidine
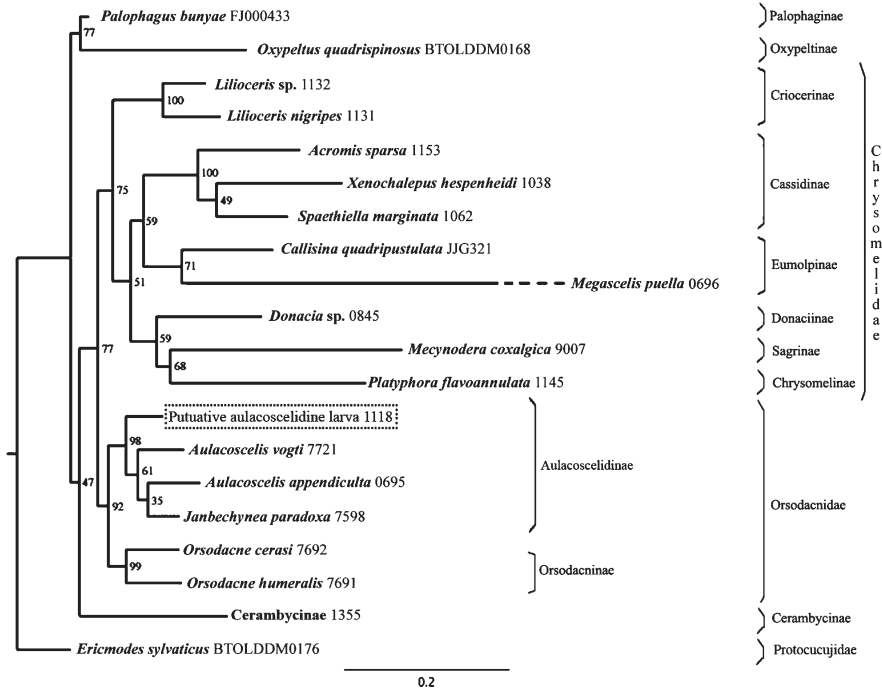
Fig. 4. Maximum likelihood phylogenetic tree (best tree logL = −10904.57) based on a combined matrix of *CO1*, *28S* and *18S* DNA sequences from 19 Chrysomeloidea and one cucujoid outgroup. Numbers alongside each node are estimates of ML bootstrap support.
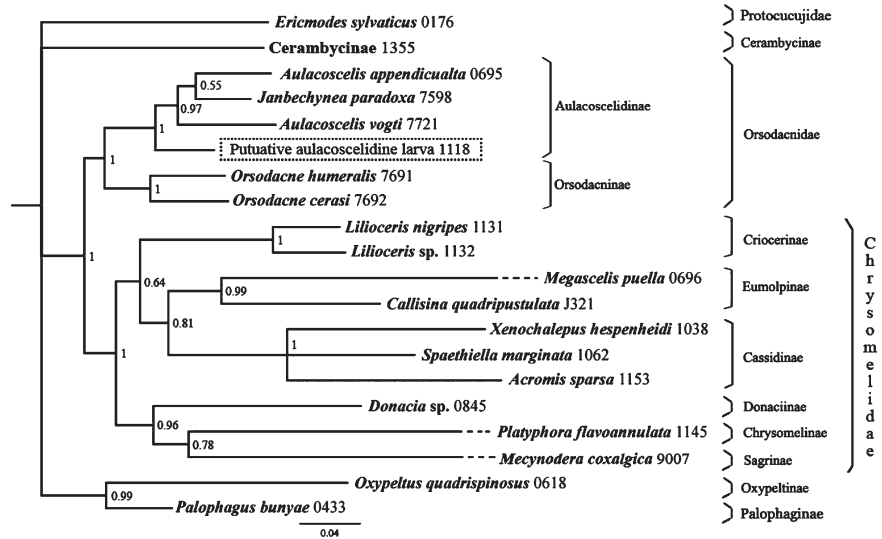

Fig. 5. Bayesian 50% majority rule consensus tree resulting from analysis of the combined data (*CO1*, *28S* and *18S*). Numbers alongside each node are posterior probabilities.

larva in a position sister to the other three sampled Aulacoscelidinae, with strong nodal support (Figs 4, 5; 98% BS, 1.0 PP), consistent with its identification as a member of the subfamily Aulacoscelidinae. Additionally, *Aulacoscelis appendiculata* was found to be more closely related to *Janbechynea paradoxa* than to *A. vogti*, but with weak support. Aulacoscelidinae was recovered as a sister clade to Orsodacninae (Table 2).

Discussion

Larval morphology

The cycad-feeding larva described herein differs from the larvae of Chrysomelinae (Reid, 1991) by lacking a paronychial appendix in the tibio-tarsi. It differs from the shape of Cassidinae larvae (Lawrence, 1991) by being cylindrical and

Table 2. Graphical summary of the phylogenetic affinity of the putative chrysomeloid larva, and the phylogenetic position of the Aulacoscelidinae, based on analysis of partial sequences of 18S, 28S and CO1.

Tree building method	Markers			
	18S	28S	CO1	All
Parsimony best tree		A	O	A
Parsimony best tree with gaps coded		A	*a*	A
Maximum Likelihood Majority Rule		A	O	A
Maximum Likelihood best tree		A	A	A
Bayesian Majority Rule		A	O	A
Bayesian best tree	O	A	O	A

[a]No informative gaps were obtained from the CO1 sequences.
An '**O**' or an '**A**' indicates that the sequences from the larva in the *Dioon merolae* seed were recovered within a monophyletic Orsodacnidae (Orsodacninae & Aulacoscelidinae sequences mixed) or in a monophyletic Aulacoscelidinae, respectively. Cross-hatching indicates a monophyletic Aulacoscelidinae not sister to Orsodacninae, a light gray square represents a polyphyletic Orsodacnidae (Aulacoscelidinae + Orsodacninae); an intermediate grey represents a monophyletic Orsodacnidae with Aulacoscelidinae and Orsodacninae paraphyletic, and a dark grey square represents a monophyletic Orsodacnidae with Aulacoscelidinae and Orsodacninae as sister groups.

elongate, and from the larvae of the Eumolpinae (Reid & Storey, 1993) by the reduced length of the stem of the epicranial suture. The larvae of Palophaginae differ from the cycad-feeding larva by having six stemmata and the anal shield on abdominal segment nine not sclerotized (Kuschel & May, 1990).

All known larvae of Orsodacnidae (*Orsodacne* species and *Aulacoscelis appendiculata*) are first instars. The size of our larva indicates it is final instar. However, according to Cox (1981) the fundamental morphology of chrysomeloid first-instar larvae changes little during development, permitting some comparisons to be made between instars. The Aulacoscelidinae larva found in the *Dioon merolae* seed strongly resembles the first-instar larva of *A. appendiculata* described by Cox & Windsor (1999a), as well as the *Orsodacne* larvae described by Cox (1981) and Mann & Crowson (1981). All four descriptions (including ours) indicate larvae have an epicranial suture with a very short stem, a reduced number of stemmata and an anal plate on abdominal segment nine. The aulacoscelidine larva described herein bears three pairs of large setae in the frons, as does the first-instar larva of *A. appendiculata* (Cox & Windsor, 1999a), but not the *Orsodacne* species (which have five pairs of setae in the frons) (Cox, 1981; Mann & Crowson, 1981). The shape of the anal shield is distinct in the larvae of all three orsodacnids, with *A. appendiculata* having a smooth posterior margin, the *Orsodacne* species bearing two urogomphi, and the aulacoscelidine from the *Dioon* seed having a slightly bifid margin. The anal shield differs from *A. appendiculata* and the *Orsodacne* spp. by having a smooth surface. The larva described herein bears little resemblance to the *Orsodacne* sp. larva described by Böving & Craighead (1931) in

the prothorax, presence of legs, shape of head, epicranial suture stem, stemmata and shape of second antennal segment.

Based on the reduced number of stemmata, Cox & Windsor (1999a) speculated an internal feeding behavior for *A. appendiculata* in Panama. This is supported by the apparent absence or extreme reduction of stemmata in the SEM of the Mexican specimen and its habit of internal feeding on cycad seeds. Whereas one pair of stemmata has been found in other orsodacnids, our evidence is insufficient to distinguish if there is one extremely reduced stemma posterodorsal to the antenna, or if it is completely absent.

The Aulacoscelidinae and the Cycadophyta

Cycads have been regarded as the most ancient extant lineage of seed-bearing land plants. Although evidence of recent radiations in cycads (e.g. Zamiaceae) show that many cycad species are relatively young (Nagalingum *et al.*, 2011), extant cycad lineages (including several genera) were well established by the Late Cretaceous (Pant, 1987; Norstog & Nicholls, 1997; Anderson *et al.*, 2007; Nagalingum *et al.*, 2011). The absence of cycadophilous insect taxa common to both old and new world cycads (Crowson, 1991), could result from early divergence of cycad genera. Herbivory of aulacoscelidines on cycads might well be as ancient as speculated by Crowson (1981) and Medvedev (1968), but a proper evaluation of this idea requires a better understanding of the Mesozoic fauna as well as documentation of current associations. If the Aulacoscelidinae have been associated with cycads for the last 160 Ma, their restriction to the Neotropics might represent an ancestral relationship to one particular lineage of Neotropical Zamiaceae (i.e. *Ceratozamia*, *Dioon* or *Zamia*) and a later expansion to other cycad genera/species. *Aulacoscelis appendiculata* in Panama and *Janbechynea paradoxa* in Bolivia are strongly attracted to the leaves of the exotic *Cycas revoluta* (Cycadaceae) and will feed on these introduced plants [L. Werding (Bolivia), A. Prado, personal observations]. This behaviour demonstrates an ability to feed on the leaves of distantly-related cycad species.

A majority of known cycad–insect associations appear to be secondary. For example, all weevil genera associated with cycad gametophytes in Africa and Australia have close relatives in genera associated with angiosperms (Oberprieler, 1999; Oberprieler *et al.*, 2007; Downie *et al.*, 2008). Therefore in the majority of cases the colonization of cycad gametophytes by weevils may be a relatively recent phenomenon. Studies on the African cycad-weevils (Amorphocerini) (Downie *et al.*, 2008) support a recent colonization of *Encephalartos* (Zamiaceae) species, which radiated during the Pliocene/Pleistocene, according to Treutlein *et al.* (2005) or the Miocene, according to Nagalingum *et al.* (2011). Reid (1995) suggested that the association of certain Criocerinae with Cycadaceae (e.g. *Lilioceris nigripes* in Australia) is likely to be of recent origin because only two species feed on cycads and most criocerines are associated with monocotyledonous angiosperms.

We present molecular and morphological evidence that the immature chrysomeloid found feeding on a Mexican *Dioon* cycad seed belongs to Aulacoscelidinae. This is the first account of a chrysomeloid larva developing in a cycad gametophyte. The species of Aulacoscelidinae involved remains unknown until a greater number of species of adult Mexican Aulacoscelidinae are sequenced. A phylogenetic analysis including more members of the subfamily will improve our ability to attribute the larva to a genus. Based on collecting locality information from specimens of Aulacoscelidinae in collections, the larva could belong to either *Aulacoscelis candezi* Chapuis, *A. confusa* Monrós or *A. melanocera* Duponchel & Chevrolat which have been collected in the Tehuantepec region of Mexico or in the central valleys of Oaxaca (Jacoby, 1891; Monrós, 1954; Santiago-Blay, 2004).

The rarity of these beetles and their brief period of activity in nature may explain why the feeding habit has been observed so seldom. During mating congregations, female Aulacoscelidinae may oviposit on only a small number of individual cycad plants, making observation unlikely. Eggs might be deposited near the developing seeds, possibly inside the female cone with the young larvae borrowing their way into the seed, eventually being well protected by a toxic sarcotesta and a hard scelerotesta (Norstog & Nicholls, 1997). Larval behaviour might be similar to that of some bruchid beetles that oviposit on the outside of the pod or seed of their hosts (Southgate, 1979). Whether pupation of the aulacoscelidine described herein occurs inside the seed (which is most likely based on the advanced state of the larva) or whether the larva exits the seed to pupate in the soil remains unknown. The phenology of its host, *D. merolae* is poorly known, but from a study carried out on *D. edule* by Vovides (1990) we can expect the female coning intervals for *D. merolae* to be at least 5–10 years. This sparse coning behaviour could also account for apparent beetle rarity and for concentration of overposition on a few individual plants.

Despite their occasional visits to cycad cones, it is unlikely that aulacoscelidines are effective pollinators, as no pollen-bearing structures have been described for the Aulacoscelidinae. Even though *Aulacoscelis vogti* visits both female and male cones of *D. edule*, a weevil in the genus *Rhopalotria* (Belidae: Curculionoidea), is considered the main pollinator (Vovides, 1991). When visiting *D. edule* cones, *A. vogti* probably feeds on pollen and pollination drops.

Our analyses support the placement by Kuschel & May (1990), Reid (1995) and Gómez-Zurita *et al.* (2007) of the Aulacoscelidinae as a clade within the Orsodacnidae and as a sister clade to the Orsodacninae. The Orsodacninae have been collected mainly on flowers where they can damage floral tissues (Cox, 1981; Mann & Crowson, 1981). *Orsodacne atra* (Ahrens) has been collected in North America on a variety of different plant families (more than 12), mostly angiosperms, but also on two genera of the Pinaceae (Clark *et al.*, 2004). This could be indicative of a pollen generalist habit, as proposed for the Aulacoscelidinae.

Multiple lines of natural history evidence point toward a deep association between aulacoscelidines and cycads (i.e. adult feeding, cycad-toxin sequestration, and larval feeding habits). However, the recent association scenario cannot be ruled out. The ancient association hypothesis for the aulacoscelidines can be tested further only by documenting the feeding relationships for more species in the subfamily, including both immature stages and adults.

Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-3113.2012.00639.x

Table S1. Genes, primers, and substitution models used in phylogeny reconstruction.

Please note: Neither the Editors nor Wiley-Blackwell are responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

Acknowledgements

References

Anderson, J.M., Anderson, H.M. & Cleal, C.J. (2007) *Brief History of the Gymnosperms: Classification, Biodiversity, Phytogeography and Ecology.* South African National Biodiversity Institute, Pretoria.

Böving, A.G. & Craighead, F.C. (1931) *An Illustrated Synopsis of the Principal Larval Forms of the Order Coleoptera.* The Brooklyn Entomological Society, Brooklyn, New York.

Cabrera-Toledo, D., González-Astorga, J., Nicolalde-Morejón, F., Vergara-Silva, F. & Vovides, A.P. (2010) Allozyme diversity levels in two congeneric *Dioon* spp. (Zamiaceae, Cycadales) with contrasting rarities. *Plant Systematics and Evolution*, **290**, 115–125.

Cabrera-Toledo, D., González-Astorga, J. & Flores-Vázquez, J.C. (2012) Fine-scale spatial genetic structure in two mexican cycad species *Dioon caputoi* and *Dioon merolae* (Zamiaceae, Cycadales):

implications for conservation. *Biochemical Systematics and Ecology*, **40**, 43–48.

Chapuis, F. (1874) Famille des phytophages. *Historie naturelle des insectes. Genera des coleopteres; ou expose methodique et critique de tous les generes proposes jusqu' ici dans cet orde d' insects* (ed. by J.T. Lacordaire and F. Chapuis), p. 455. Librairie Encyclopédique de Roret, Paris.

Clark, S., LeDoux, D., Seeno, T., Riley, E., Gilbert, A. & Sullivan, J. (2004) *Host Plants of Leaf Beetle Species Occurring in the United States and Canada (Coleoptera: Megalopodidae, Orsodacnidae, Chrysomelidae, Excluding Bruchinae)*. The Coleopterists Society, Sacramento, CA.

Cox, M.L. (1981) Notes on the biology of *Orsodacne* Latreille with a subfamily key to the larvae of the british Chrysomelidae (Coleoptera). *Entomologist's Gazette*, **32**, 123–135.

Cox, M.L. & Windsor, D.M. (1999a) The first instar larva of *Aulacoscelis appendiculata* n. sp. (Coleoptera: Chrysomelidae: Aulacoscelinae) and its value in the placement of the Aulacoscelinae. *Journal of Natural History*, **33**, 1049–1087.

Cox, M.L. & Windsor, D.M. (1999b) The first instar larva of *Aulacoscelis* sp. and *Megascelis puella* Lacordaire (Coleoptera: Chrysomelidae: Aulacoscelinae, Megascelinae) and their value in the placement of the Aulacoscelinae and Megascelinae. *Advances in Chrysomelidae Biology*, **1**, 51–70.

Crowson, R.A. (1946) A revision of the genera of the chrysomelid group Sagrinae (Coleoptera). *Transactions of the Royal Entomological Society of London*, **97**, 75–115.

Crowson, R.A. (1981) *The Biology of the Coleoptera*. Academic Press, London.

Crowson, R.A. (1991) The relations of Coleoptera to Cycadales. *Advances in Coleopterology* (ed. by M. Zunino, X. Belles and M. Blas), pp. 13–28. AEC, Barcelona.

De Luca, P., Sabato, S. & Torres, M. (1981) *Dioon holmgrenii* (Zamiaceae), a new species from Mexico. *Brittonia*, **33**, 552–555.

Downie, D.A., Donaldson, J.S. & Oberprieler, R.G. (2008) Molecular systematics and evolution in an african cycad-weevil interaction: Amorphocerini (Coleoptera: Curculionidae: Molytinae) weevils on *Encephalartos*. *Molecular Phylogenetics and Evolution*, **47**, 102–116.

Duckett, C., Gillespie, J., Kjer, K., Jolivet, P., Santiago-Blay, J. & Schmitt, M. (2004) Relationships among the subfamilies of Chrysomelidae inferred from small subunit ribosomal DNA and morphology, with special emphasis on the relationship among the flea beetles and the Galerucinae. *New Developments in the Biology of Chrysomelidae* (ed. by P. Jolivet, J.A. Santiago-Blay and M. Schmitt), pp. 3–18. SPB Academic Publishing, Amsterdam.

Farrell, B.D. (1998) "Inordinate fondness" explained: why are there so many beetles? *Science*, **281**, 555–559.

Forster, P. & Machin, P. (1994) Cycad host plants for *Lilioceris nigripes* (Fabricius)(Coleoptera: Chrysomelidae) and *Theclinesthes onycha* (Hewitson)(Lepidoptera: Lycaenidae). *Australian Entomologist*, **21**, 99–102.

Gómez-Zurita, J., Hunt, T., Kopliku, F. & Vogler, A.P. (2007) Recalibrated tree of leaf beetles (Chrysomelidae) indicates independent diversification of angiosperms and their insect herbivores. *PLoS ONE*, **2**, e360.

Gómez-Zurita, J., Hunt, T. & Vogler, A.P. (2008) Multilocus ribosomal RNA phylogeny of the leaf beetles (Chrysomelidae). *Cladistics*, **24**, 34–50.

Grimaldi, D. & Engel, M.S. (2005) *Evolution of the Insects*. Cambridge University Press, Cambridge, U.K.

Hawkeswood, T.J. (1992) Notes on the biology and host plants of the Australian leaf beetle *Lilioceris (Crioceris) nigripes* (Fabricius) (Coleoptera: Chrysomelidae). *The Entomologist*, **111**, 210–212.

Huelsenbeck, J.P. & Ronquist, F. (2001) Mrbayes: bayesian inference of phylogeny. *Bioinformatics*, **17**, 754–755.

Jacoby, M. (1877) Descriptions of new species of phytophagous Coleoptera. *Proceedings of the Zoological Society of London*, **1877**, 510–520.

Jacoby, M. (1891) Insecta, Coleoptera, supplement to Phytophaga. *Biologia Centrali-Americana*, **6**, 233–312.

Jolivet, P. (2005) Cycads and beetles. Recent views on pollination. *The Cycad Newsletter*, **28**, 3–7.

Kirejtshuk, A.G., Chang, H., Ren, D. & Kun, S.C. (2010) Family Lasiosynidae n.fam., new palaeoendemic Mesozoic family from the infraorder Elateriformia (Coleoptera: Polyphaga). *Annales de la Société Entomologique de France*, **46**, 67–86.

Kuschel, G. (1983) Past and present of the relict family Nemonychidae (Coleoptera, Curculionoidea). *GeoJournal*, **7**, 499–504.

Kuschel, G. & May, B.M. (1990) Palophaginae, a new subfamily for leaf-beetles, feeeding as adult and larva on araucarian pollen in Australia (Coleoptera: Magalopodidae). *Invertebrate Taxonomy*, **3**, 697–719.

Labandeira, C.C. (2000) The Paleobiology of Pollination and its Precursors. *Paleontological Society Papers*, **6**, 233–269.

Labandeira, C.C., Kvacek, J. & Mostovski, M.B. (2007) Pollination drops, pollen, and insect pollination of mesozoic gymnosperms. *Taxon*, **56**, 663–695.

Lawrence, J.F. (1991) Order Coleoptera. *Immature Insects* (ed. by F.W. Sterh), pp. 144–150. Kendall/Hunt Publishing Co., Dubuque, IA.

Mann, J.S. & Crowson, R.A. (1981) The systematic positions of *Orsodacne* Latr. and *Syneta* Lac. (Coleoptera Chrysomelidae), in relation to chararcters of larvae, internal anantomy and tarsal vesiture. *Journal of Natural History*, **15**, 727–749.

McKenna, D.D. & Farrell, B.D. (2009) Beetles (Coleoptera). *The Timetree of Life* (ed. by S.B. Hedges and S. Kumar), pp. 278. Oxford University Press, New York, NY.

McKenna, D.D., Sequeira, A.S., Marvaldi, A.E. & Farrell, B.D. (2009) Temporal lags and overlap in the diversification of weevils and flowering plants. *Proceedings of the National Academy of Sciences*, **106**, 7083–7088.

Medvedev, L.N. (1968) Leaf beetles of Kara Tau (in russian). *Zhurskie Nasekhomie Katatau* (ed. by B.B. Rohdendorf), pp. 155–165. Nauka, Moscow.

Miller, M.A., Pfeiffer, W. & Schwartz, T. (2010) Creating the CIPRES science gateway for inference of large phylogenetic trees. *Proceedings of the Gateway Computing Environments Workshop*. New Orleans, LA.

Monrós, F. (1953) Aulacoscelinae, eine neue chrysomeliden-unterfamilie, mit beschreinbung einer neuen bolivianischen gattung (Col.). *Entomologische Arbeiten aus dem Museum G. Frey*, **4**, 19–25.

Monrós, F. (1954) Revision of the Chrysomelid subfamily Aulacoscelinae. *Bulletin of the Museum of Comparative Zoology*, **112**, 320–360.

Nagalingum, N.S., Marshall, C.R., Quental, T.B., Rai, H.S., Little, D.P. & Mathews, S. (2011) Recent synchronous radiation of a living fossil. *Science*, **334**, 796–799.

Nash, R.J., Arthur Bell, E. & Ackery, P.R. (1992) The protective role of cycasin in cycad-feeding lepidoptera. *Phytochemistry*, **31**, 1955–1957.

Norstog, K.J. & Nicholls, T.J. (1997) *The Biology of the Cycads*. Cornell University Press, New York, NY.

Nylander, J.A.A. (2004) *MrModelTest v2*. Evolutionary Biology Center, Uppsala University, Uppsala.

Nylander, J.A.A., Wilgenbusch, J.C., Warren, D.L. & Swofford, D.L. (2008) AWTY (Are we there yet?): A system for graphical

exploration of MCMC convergence in bayesian phylogenetics. *Bioinformatics*, **24**, 581–583.

Oberprieler, R. G. (1999) Systematics and evolution of the cycad-associated weevil genus *Apinotropis jordan* (Coleoptera : Anthribidae). *African Entomology*, **7**, 1–33.

Oberprieler, R.G., Marvaldi, A.E. & Anderson, R.S. (2007) Weevils, weevils, weevils everywhere. *Zootaxa*, **1668**, 491–520.

Pant, D.D. (1987) The fossil history of the Cycadales. *Geophytology*, **17**, 125–162.

Posada, D. (2005) *Modeltest v3.7*. Facultad de Biología, Universidad de Vigo, Vigo.

Prado, A., Ledezma, J., Cubilla-Rios, L., Bede, J. & Windsor, D. (2011) Two genera of Aulacoscelinae beetles reflexively bleed azoxyglycosides found in their host cycads. *Journal of Chemical Ecology*, **37**, 736–740.

Rambaut, A. & Drummond, A. (2007) *TRACER, Version 1.4* [WWW document]. URL http://beast.bio.ed.ac.uk/Tracer [accessed on 9 August 2011].

Reid, C.A.M. (1991) Immature stages of the genera *Johannica blackburn*, *Lamprolina baly* and *Chalcolampra blanchard* (Coleoptera, Chrysomelidae, Chrysomelinae). *Journal of Natural History*, **25**, 341–357.

Reid, C.A.M. (1995) A cladistic analysis of subfamilial relationships in the Chrysomelidae sensu lato (Chrysomeloidea). *Biology, Phylogeny and Classification of Coleoptera: Papers Celebrating the 80th Birthday of Roy A. Crowson* (ed. by J. Pakaluk and S.A. Slipinski), pp. 559–632. Muzeum i Instytut Zoologii PAN, Warszawa.

Reid, C.A.M. (2000) Spilopyrinae Chapuis: a new subfamily in the Chrysomelidae and its systematic placement (Coleoptera). *Invertebrate Systematics*, **14**, 837–862.

Reid, C.A.M. & Storey, R. (1993) Redescription of adult and larva of *Colasposoma sellatum* Baly (Coleoptera: Chrysomelidae: Eumolpinae): a pest of sweet potato in Australia. *Journal of Natural History*, **27**, 669–681.

Riley, E.G., Clark, S.M. & Seeno, T.N. (2003) *Catalog of the Leaf Beetles of America North of Mexico (Coleoptera: Megalopididae, Orsodacnidae and Chrysomelidae, excluding Bruchinae)*. Coleopterists Society, Sacramento, CA.

Santiago-Blay, J.A. (2004) Some aspects of the biology of the Aulacoscelinae Chapuis 1874 (Orsodacnidae or Chrysomelidae, sensu lato), with a description of new species of *Janbechynea*. *New Developments in the Biology of Chrysomelidae* (ed. by P.H. Jolivet, J.A. Santiago-Blay and M. Schmitt), pp. 1–65. SPB Academic Publishing, The Hague.

Southgate, B. (1979) Biology of the Bruchidae. *Annual Review of Entomology*, **24**, 449–473.

Stamatakis, A., Hoover, P. & Rougemont, J. (2008) A rapid bootstrap algorithm for RaxML web-servers. *Systematic Biology*, **75**, 758–771.

Suzuki, K. & Windsor, D.M. (1999) The internal reproductive system of panamanian *Aulacoscelis* sp. (Coleoptera: Chrysomelidae, Aulacoscelinae) and comments on the systematic position of the subfamily. *Entomological Science*, **2**, 391–398.

Swofford, D.L. (1998) *PAUP, Phylogentic Analysis Using Parsimony*. Sinauer Associates, Sunderland, Massachusetts.

Thompson, J.D., Higgins, D.S. & Gibson, T.J. (1994) Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-sequence penalties and weighting matrix choice. *Nucleic Acids Research*, **22**, 4673–4680.

Treutlein, J., Vorster, P. & Wink, M. (2005) Molecular relationships in *Encephalartos* (Zamiaceae, Cycadales) based on nucleotide sequences of nuclear ITS 1&2, rbcl, and genomic ISSR fingerprinting. *Plant Biology*, **7**, 79–90.

Vovides, A.P. (1990) Spatial distribution, survival, and fecundity of *Dioon edule* (Zamiaceae) in a tropical deciduous forest in Veracruz, Mexico, with notes on its habitat. *American Journal of Botany*, **77**, 1532–1543.

Vovides, A.P. (1991) Insect symbionts of some mexican cycads in their natural habitat. *Biotropica*, **23**, 102–104.

Windsor, D. & Jolivet, P.H. (1997) Cycad-feeding by ancient beetle, *Aulacoscelis* in Panama. *Chrysomela*, **34**, 5.

Windsor, D., Ness, J., Gomez, L.D. & Jolivet, P.H. (1999) Species of *Aulacoscelis* Duponchel and Chevrolat (Chrysomelidae) and *Nomotus* Gorham (Languriidae) feed on fronds of central american cycads. *The Coleopterists Bulletin*, **53**, 217–231.

Zhang, J.-F. (2005) The first find of chrysomelids (Insecta: Coleopetra: Chrysomeloidea) from Callovian-Oxfordian Daohugou biota of China. *Geobios*, **38**, 865–871.