Phylogenomic Data Yield New and Robust Insights into the Phylogeny and Evolution of Weevils

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Abstract

The phylogeny and evolution of weevils (the beetle superfamily Curculionoidea) has been extensively studied, but many relationships, especially in the large family Curculionidae (true weevils; > 50,000 species), remain uncertain. We used phylogenomic methods to obtain DNA sequences from 522 protein-coding genes for representatives of all families of weevils and all subfamilies of Curculionidae. Most of our phylogenomic results had strong statistical support, and the inferred relationships were generally congruent with those reported in previous studies, but with some interesting exceptions. Notably, the backbone relationships of the weevil phylogeny were consistently strongly supported, and the former Nemonychidae (pine flower snout beetles) were polyphyletic, with the subfamily Cimberidinae (here elevated to Cimberididae) placed as sister group of all other weevils. The clade comprising the sister families Brentidae (straight-snouted weevils) and Curculionidae was maximally supported and the composition of both families was firmly established. The contributions of substitution modeling, codon usage and/or mutational bias to differences between trees reconstructed from amino acid and nucleotide sequences were explored. A reconstructed timetree for weevils is consistent with a Mesozoic radiation of gymnosperm-associated taxa to form most extant families and diversification of Curculionidae alongside flowering plants—first monocots, then other groups—beginning in the Cretaceous.

Key words: **Curculionoidea, Curculionidae, chronogram, exon, hybrid enrichment, phylogenetics.**

Introduction

The beetle superfamily Curculionoidea Latreille (weevils) contains approximately 62,000 described extant species in more than 5,800 genera (Kuschel 1995; Oberprieler et al. 2007), making it one of the most species-rich radiations of metazoans. The apparent success of weevils has been ascribed to coevolution with plants, especially flowering plants (e.g., Farrell 1998; McKenna et al. 2009), and the development of a specialized "oviposition rostrum" (e.g., Anderson 1993, 1995)—a purported key innovation in which the female uses her rostrum/mouthparts to drill an oviposition site deep inside the host plant. However, weevil diversity is likely attributable to a "cascade of evolutionary innovations" (Oberprieler et al. 2007), at least some of which facilitated specialized trophic interactions with plants, and also with fungi, as several weevil lineages are mycetophagous or feed on plant substrates modified by fungi (Holloway 1982; Zimmerman 1994; Marvaldi et al. 2002). Together, these innovations are proposed to have enhanced weevil speciation rates and/or reduced extinction rates, promoting lineage accumulation (e.g., McKenna et al. 2015). Achieving a stable higher-level classification and a robust phylogenetic backbone for the superfamily Curculionoidea promises to facilitate both the exploration of factors underlying the apparent evolutionary success of weevils and the evolutionary dynamics of their intimate associations with plants (e.g. Farrell 1998; Oberprieler et al. 2007; McKenna et al. 2009). It will also facilitate the predictive power expected of classifications rooted in robust phylogenetics and thereby provide the much needed resources for biosecurity, conservation and pest control applications that is currently lacking for this largest of phytophagous insect radiations.

Most recent classifications recognize seven major lineages of weevils (Anthribidae, Attelabidae, Belidae, Brentidae, Caridae, Curculionidae, and Nemonychidae: e.g., Oberprieler et al. 2007; Oberprieler, Anderson, et al. 2014). These have been variously sampled and supported as monophyletic groups in recent morphological and molecular phylogenetic studies (e.g., Marvaldi and Morrone 2000; Marvaldi et al. 2002; Hunt et al. 2007; McKenna et al. 2009, 2015; McKenna 2011;

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Haran et al. 2013; Bocak et al. 2014; Gillett et al. 2014; Gunter et al. 2016). Curculionidae, with approximately 51,000 described species in more than 4,600 genera (Oberprieler 2014c), is the largest family of weevils and the second-largest family of metazoans. Subfamily concepts in Curculionidae remain highly tentative and controversial, though some natural groupings have been suggested by studies of adult morphology (Morimoto 1962; Kuschel 1971; Thompson 1992; Alonso-Zarazaga 2007) and phylogenetic studies employing morphological and/or molecular data (Kuschel 1995; Marvaldi and Morrone 2000; Marvaldi et al. 2002, 2009; Morimoto and Kojima 2006; Hundsdörfer et al. 2009; McKenna et al. 2009, 2015; Jordal et al. 2011; Haran et al. 2013; Gillett et al. 2014; Gunter et al. 2016).

The phylogeny of the superfamily Curculionoidea has been reconstructed using molecular data from one or a small number of genes obtained via traditional polymerase chain reaction and Sanger sequencing (e.g., Wink et al. 1997; Marvaldi et al. 2002, 2009; Hundsdörfer et al. 2009; McKenna et al. 2009, 2015; Jordal et al. 2011; Haran et al. 2013; Bocak et al. 2014; Gillett et al. 2014; Gunter et al. 2016) and using next-generation-sequencing methods to generate data from mitochondrial genomes (Haran et al. 2013; Gillett et al. 2014). Nonetheless, several family-level relationships and most subfamily-level relationships in Curculionidae remain uncertain due to weak statistical support and/or limited taxon sampling in all studies to date. McKenna et al. (2009) undertook the only molecular phylogenetic study that has sampled all families and subfamilies of weevils. However, in common with most other studies, nodal support values were moderate to low or lacking for nearly all relationships resolved by McKenna et al. (2009), thus leaving the phylogenetic placement, monophyly and morphological definition of most higher weevil taxa uncertain.

One traditional means of increasing resolving power in molecular phylogenetic data sets is to sample more loci (e.g., Niehuis et al. 2012; Misof et al. 2014; Peters et al. 2017). Leache and Rannala (2011) demonstrated that hundreds of nuclear loci measuring ~1 kb (per locus) in length may be needed to resolve difficult nodes resulting from presumably rapid radiations and/or recent divergences (Leache and Rannala 2011; Prum et al. 2015). However, until recently there were no widely available cost- or time-efficient approaches for increasing the number of loci included in molecular phylogenetic studies of nonmodel taxa (Lemmon EM and Lemmon AR 2013). Phylogenomic approaches have now become available that allow for the generation of DNA sequence data from large numbers of known/targeted loci from nonmodel taxa (e.g., see Lemmon EM and Lemmon AR 2013; Misof et al. 2014). These approaches have been widely used in the studies of vertebrates but have only recently been employed in the studies of insects (Young et al. 2016; Breinholt et al. 2018; Haddad et al. 2018).

This study was designed to reconstruct family-level relationships across Curculionoidea and subfamily-level relationships in Curculionidae using phylogenomic data comprised of DNA sequences (separate analyses of nucleotide [NT] and amino acid [AA] data) from > 500 1:1 orthologous nuclear genes (see Materials and Methods) obtained via anchored hybrid enrichment (AHE) from an exemplar set of weevil species and outgroups. AHE is a highly efficient and scalable method for generating high-throughput DNA sequence data. It allows for the capture and amplification of target DNA using specially designed probes (Lemmon et al. 2012) and can efficiently and selectively harvest specific desired parts of the genome (here 941 exons). We used the results of our phylogenetic analyses along with information from the fossil record to estimate chronograms for weevils using several different calibration schemes and compared and contrasted the results with those from previous studies.

New Approaches

In this study we report results from use of the first AHE probe set for Coleoptera (beetles), the most species-rich order of metazoans. The AHE probes were designed for broad utility across Coleoptera as well as the other neuropteroid insect orders (Strepsiptera, Megaloptera, Neuroptera and Raphidioptera [McKenna 2014, 2016]) (Haddad et al. 2018; Materials and Methods). Probe sequences are available via Dryad (accession number doi: 10.5061/dryad.v0b7v). It is difficult to translate AHE sequence data using existing analytical pipelines for hybrid enrichment data because many of the assembled targeted exons include flanking intron sequences of varying lengths (Breinholt et al. 2018). Our AHE analytical pipeline uses protein-based orthology searches to identify coding regions in the AHE data and translate these regions into AA sequences for orthology assessment (see Materials and Methods).

Results and Discussion

New Insights into the Phylogeny and Evolution of Weevils

Most of the nodes in our phylogenies had maximal statistical support (100% maximum likelihood [ML] bootstrap support [MLBS], 1.0 Bayesian posterior probability [PP]), demonstrating the utility of our probes for generating nuclear DNA sequence data useful in resolving both deep and shallow divergences. Furthermore, most major weevil groupings were consistently supported regardless of data set (AA or NT) or method of phylogenetic inference (ML or Bayesian [BI]), and under various modeling and partitioning schemes (fig. 1; supplementary table S2, Supplementary Material online). Phylogenetic trees resulting from separate concatenated analyses of each codon position (C1, C2, C3: supplementary figs. S7, S8, and S9, respectively, Supplementary Material online), a concatenated analysis of combined first and second codons (C12: supplementary fig. S10, Supplementary Material online), and both AA- and NT-based binned and weighted coalescent species tree analyses were also largely congruent (supplementary table S2, Supplementary Material online). One exception was the discordant placement of Cimberididae as sister group of the clade (Nemonychidae + Anthribidae) in the ASTRAL NT analysis (with 91% support). However, this relationship was otherwise found and only weakly supported in analyses of third-codon

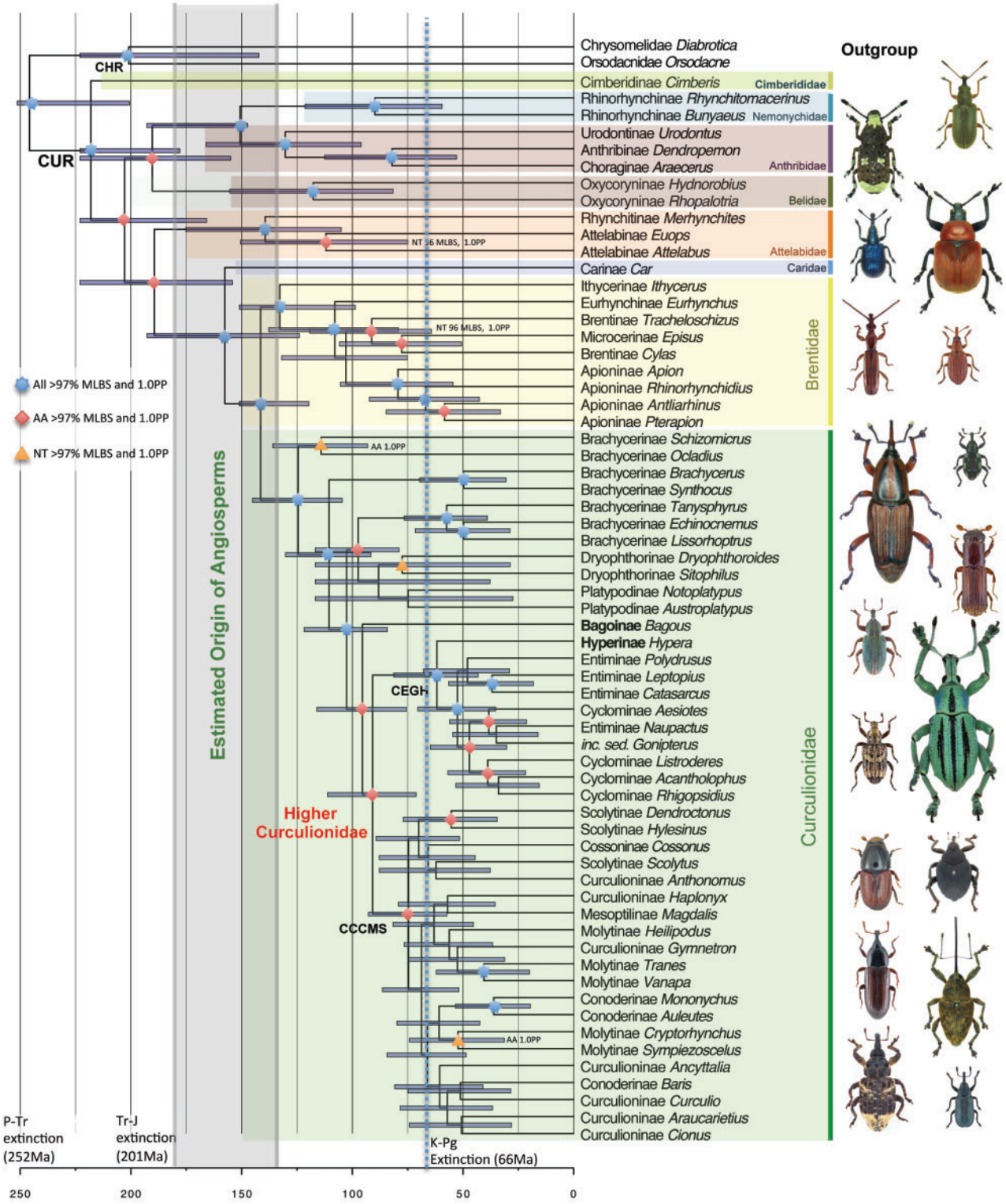

Fig. 1. Chronogram for weevils (superfamily Curculionoidea) showing RelTime estimates of lineage divergence times based on the partitioned ML analysis of amino acid data (supplementary fig. S6, Supplementary Material online). Subfamily (family for chrysomeloid outgroups) and genus names are given for all ingroup (weevil) terminals. The three taxon names in bold indicate (elevated) ranks different from the classification of Oberprieler, Anderson et al. (2014) in Leschen and Beutel (2014). The outgroups *Aethina* and *Cucujus* (both Cucujoidea) are not shown (following Kumar et al. 2016; Mello et al. 2017). Stars indicate nodes for which all trees from primary analyses (supplementary figs. S1–S6, Supplementary Material online) have support values greater than 97% MLBS and 1.0 PP; diamonds indicate nodes with support values greater than 97% MLBS and 1.0 PP in trees resulting from the primary AA analyses (supplementary figs. S2, S4, and S6, Supplementary Material online); triangles indicate nodes with support values greater than 97% MLBS and 1.0 PP in trees resulting from the primary NT analyses (supplementary figs. S1, S3, and S5, Supplementary Material online). The origin of angiosperms is indicated, as estimated from fossils (132–141 Ma [Brenner 1996]) and molecules (140–180 Ma [Bell et al. 2005]). CUR, Curculionoidea; CHR, Chrysomeloidea; *inc. sed.*, *incertae sedis* (where a taxon's broader relationships are

positions (C3; supplementary fig. S9, 40% MLBS; supplementary table S2, Supplementary Material online), which also supported a relationship between Belidae and Attelabidae (same as in the partitioned NT analysis; supplementary fig. S5, Supplementary Material online). Specifically, our analyses robustly supported a "phylogenetic backbone" for weevils—a series of early-diverging relationships among families. Curculionoidea and five of its seven family-level lineages were maximally supported monophyletic groups across all of our analyses. Notably, Cimberididae (formerly a subfamily of Nemonychidae; pine flower snout beetles) were placed as the sister group of all other weevils, a relationship previously suggested by Haran et al. (2013) based on data from mitochondrial genomes (Supplementary Material online). The morphologically plesiomorphic and largely conifer-feeding pine flower snout beetles therefore do not form a natural group as currently defined, warranting a narrowing of the limits of the family and recognition of Cimberididae as a distinct family-level lineage (Crowson, 1985 previously suggested the family-group name Cimberidae [sic] for *Cimberis* and Rhinorhynchinae). As Nemonychidae are widely regarded as retaining many morphologically "ancestral" character states and have the oldest fossil history among extant weevils (Kuschel 1983, 2003; Oberprieler RG and Oberprieler SK 2012; Kuschel and Leschen 2011), our results suggest a more complex evolutionary history for the many fossil nemonychids. Similar to some other recent molecular phylogenetic studies (e.g., Marvaldi et al. 2002, 2009; McKenna et al. 2009), our analyses maximally support a monophyletic group comprising fungus weevils (family Anthribidae) and its placement as sister group of the other Nemonychidae sampled.

Our analyses robustly support placement of the conifer-associated family Caridae as sister group of the predominantly angiosperm-feeding clade comprising straight-snouted and true weevils (Brentidae and Curculionidae, respectively). Previous studies, with the exception of McKenna et al. (2015), have either placed this isolated family in different positions (Gunter et al. 2016) or found only weak to moderate support for it among analyses (Supplementary Material online). The placement of Caridae in our trees is also consistent with results from analyses of morphological data (e.g., Marvaldi and Morrone 2000; Marvaldi et al. 2002; Supplementary Material online).

Monophyly of the species-rich clade comprising the sister families Brentidae and Curculionidae was maximally (and ubiquitously) supported (fig. 1; supplementary figs. S1–S6 and table S2, Supplementary Material online); support for this relationship in previous molecular studies was inconsistent and/or those studies were inconclusive about the definition and limits of the two families, especially Brentidae. The true weevils (Curculionidae) were strongly supported as a monophyletic group in our study regardless of data set, partitioning scheme or analysis method. This therefore firmly establishes the limits of the family by demonstrating, for example, inclusion of the palm and pinhole borer weevils (Dryophthorinae and Platypodinae), while definitively excluding other groups. Notable among the latter are the morphologically enigmatic subfamilies Microcerinae and Ithycerinae (monotypic; New York weevil), which were here placed among the straight-snouted weevils. Brentidae in the present sense (Oberprieler 2000, 2014a; Oberprieler et al. 2007) were maximally supported as a monophyletic group in all of our analyses. This is notable because there is currently no strong morphological evidence for its monophyly (Oberprieler 2014a) and because Ithycerinae and Microcerinae were both placed in Curculionidae by McKenna et al. (2009) and have had different systematic placements in the past (reviewed by Oberprieler 2014b).

Most lower-level relationships found within the five broadly sampled weevil families were maximally supported (with the exception of relationships in the "CCCMS clade" of Curculionidae [Conoderinae, Cossoninae, Curculioninae, Molytinae, Scolytinae]; fig. 1, supplementary figs. S1–S6 and table S2, Supplementary Material online). Lower-level relationships found by ASTRAL analyses (binned and weighted), such as those within the "CEGH clade" of Curculionidae (Cyclominae, Entiminae, Gonipterini, Hyperinae) and the CCCMS clade, were either insufficiently or no better resolved than those found by analyses of concatenated data. This is not a surprising result given the deep time scale (Jurassic origin) over which weevils have diversified (fig. 1, supplementary table S4, Supplementary Material online; and see below) and also given that individual genes in AHE data sets may have relatively low phylogenetic signal (e.g., Prum et al. 2015). Our results therefore establish a robust phylogenetic "backbone" for Curculionidae, and both the relationships and divergence times we found are consistent with the proposals by Marvaldi et al. (2002), Oberprieler et al. (2007) and McKenna et al. (2009) (see also Farrell 1998) that the ancestral diversification of Curculionidae occurred in association with monocotyledonous angiosperm host plants and that the family subsequently diversified onto other plant groups, including other angiosperms (fig. 1; supplementary figs. S18–S25, Supplementary Material online).

Consistent with other phylogenetic studies (Marvaldi 1997; McKenna et al. 2009; Haran et al. 2013; Gillett et al. 2014; Gunter et al. 2016), the hypothesized ancestrally monocot-associated subfamilies Brachycerinae and Dryophthorinae (palm weevils) and the wood-boring Platypodinae (pinhole borers) form early-diverging groups of true weevils, the first of these subfamilies a polyphyletic grade but the others monophyletic clades, subtending a major lineage within the family, here informally called the "higher Curculionidae" (Curculionidae *sensu* Thompson, 1992; "higher weevils"). The

Fig. 1. Continued

unknown or undefined). Weevil photos are courtesy of Udo Schmidt (used with permission). Left column, top to bottom: *Anthribus albinus*, *Involvulus caeruleus*, *Cerobates (Cerobates) sexsulcatus*, *Odoiporus longicollis*, *Hypera nigrirostris*, *Gronops lunatus*, *Scolytus scolytus*, *Cossonus parallelepipedus*, *Cryptorhynchus lapathi*. Right column, top to bottom: *Cimberis attelaboides*, *Attelabus nitens*, *Apion rubens*, *Tanysphyrus lemnae*, *Platypus cylindrus*, *Eupholus cuvieri*, *Mononychus punctumalbum*, *Curculio glandium*, *Magdalis duplicata*.

relationships among subgroups of Brachycerinae and the nested placement of palm and pinhole borer weevils were strongly supported (fig. 1; supplementary table S2, Supplementary Material online). This has important implications for the classification and interpretation of morphological evolution of weevils because although the majority of species in the subfamilies Brachycerinae, Dryophthorinae and Platypodinae retain the hypothesized "ancestral" male genital structure (pedotectal aedeagus) characteristic of other weevil families (Supplementary Material online), they are each also morphologically derived groups. The remaining true weevils—the higher Curculionidae—possess a more derived male genital structure (pedal aedeagus) and are robustly established here as a monophyletic group (congruent with Thompson's [1992] more restricted concept of Curculionidae) and thus provide an important point of congruence between conclusions based on morphological and molecular data. Among other things, these results highlight the potential value of phylogenomic data for advancing the classification of diverse groups such as weevils, in which morphology-based classifications have historically been unstable. For example, our results suggest that a change in the classification of the subfamily Brachycerinae is warranted (Supplementary Material online), and we found that the small aquatic subfamily Bagoinae constitutes an isolated lineage forming the sister group of the higher curculionids (fig. 1, Supplementary Material online), raising questions about the botanical and ecological associations of the stem lineage of this more inclusive clade. Although our results concur with those of Gillett et al. (2014) and support the conclusion that the former tribe Bagoini be re-elevated to subfamily rank (previously its placement in Curculionidae was uncertain; Oberprieler, Caldara et al. 2014), others have found *Bagous* to be more closely related to taxa with the pedotectal type of male genitalia (Oberprieler, Anderson et al. 2014; Gunter et al. 2016), so our results also suggest a more complex interpretation of evolutionary changes in the genital structure of weevils (Supplementary Material online).

The pinhole borer and palm weevils (Platypodinae and Dryophthorinae, respectively) form a monophyletic clade as sister group of a subset of Brachycerinae (the erirhinines), with moderate to low support (fig. 1, supplementary figs. S1, S2 and S4–S6, Supplementary Material online). This result is unsurprising given the close relationship between these two groups found by McKenna et al. (2009, 2015), Haran et al. (2013), and Gillett et al. (2014) and also the support for this relationship from larval characters (Marvaldi 1997). However, it is also surprising given their very divergent anatomy and the morphological similarities between Platypodinae and Scolytinae (bark beetles). The systematic position and rank of Platypodinae has long been the subject of considerable debate. Sharing many morphological features with the similarly wood-boring/tunneling bark beetles (Kuschel et al. 2000; Marvaldi et al. 2002; Jordal et al. 2011; Hulcr et al. 2014), both groups have been considered either as distinct families (e.g., Morimoto and Kojima 2006) or as closely or distantly related groups within the true weevils, and it has even been suggested that pinhole borers are nested inside the bark beetles (rejected family and subfamily status: Kuschel et al. 2000). Interestingly, in our NT Bayesian analysis, *Austroplatypus* (Platypodinae) formed the sister group of a monophyletic Dryophthorinae and *Notoplatypus* (Platypodinae) the sister group of a subset of Scolytinae (supplementary fig. S3, Supplementary Material online, 0.62 PP). Patterns of codon usage and data from 4-fold degenerate sites (FDS) for third-codon positions may help explain this incongruent result. Mean FDS GC content was 38.9% (61.1% AT), indicating that our data are generally AT-rich (supplementary table S6, Supplementary Material online). Notably, the FDS GC content of *Austroplatypus* reflects substantial AT bias (82% AT), whereas that of *Notoplatypus* is much less AT-rich (59% AT; slightly less than the study mean) (supplementary fig. S16, Supplementary Material online). Thus, differences in mutational bias (resulting in bias of synonymous codon usage) in Platypodinae may contribute to the observed incongruence.

Our results support a deep phylogenetic split dividing the higher weevils into two main clades (fig. 1). These clades, called the "CCCMS clade" and "CEGH clade" (Gunter et al. 2016), have been variously found and supported in other studies (Marvaldi et al. 2002, in part; McKenna et al. 2009; Haran et al. 2013; Gillett et al. 2014; Gunter et al. 2016; Supplementary Material online). The CCCMS clade, comprising seven current curculionid subfamilies and constituting one of the most diverse plant-feeding groups of beetles (> 34,000 described species), was monophyletic in all analyses but maximally supported only in AA-based analyses (fig. 1; supplementary figs. S1–S6, Supplementary Material online). In the CCCMS clade, the hugely diverse subfamilies Curculioninae and Molytinae are clearly not monophyletic, and the subfamily Conoderinae was polyphyletic in all analyses (fig. 1). Scolytinae, while also polyphyletic in all our analyses (itself an interesting though not entirely unexpected result given some previous analyses, e.g., Kuschel et al. 2000; Gillett et al. 2014), were more closely related to other groups in the CCCMS clade than to Platypodinae. The CEGH clade, comprising over 13,000 species of the traditionally defined "broad-nosed" weevils (e.g., Kuschel 1995; Marvaldi 1997), was maximally supported in all analyses (fig. 1; supplementary table S2, Supplementary Material online), but neither of the two large subfamilies in this clade (Entiminae and Cyclominae) was strictly monophyletic (fig. 1, supplementary figs. S1–S6, Supplementary Material online; but see below). This is notable because Entiminae are relatively well characterized morphologically, and support for their monophyly has been found in some previous analyses of molecular (Haran et al. 2013; Gillett et al. 2014) and morphological data (Marvaldi et al. 2014). Cyclominae are maximally supported as a monophyletic group if the tribe Gonipterini is included (fig. 1; supplementary figs. S1–S6, see also Supplementary Material online), though analyses of AA data also placed the entimine genus *Naupactus* in this group (but also in several other positions depending on analysis and data; supplementary figs. S1–S15, Supplementary Material online). The nested and maximally supported position of Gonipterini in the CEGH clade in all our analyses confirms its relationship to the "broad-nosed" weevils, although further studies are

required to clarify its exact position in this clade (Haran et al. 2013; Gillett et al. 2014) and to resolve the higher-level classification of the "broad-nosed" weevils more generally. This is an interesting result both because of the lack of morphological support for the monophyly of Cyclominae (Oberprieler 2010) and because of the ecological implications of our results. The Australo-Pacific tribe Gonipterini, likely monophyletic based on morphological characters (Oberprieler, Caldara et al. 2014) and with ectophytic larvae (feeding externally on plants) as far as known, has been variously allied with taxa in both the CEGH and CCCMS clades (Kuschel 1995; Marvaldi 1997; Marvaldi et al. 2002; Hundsdörfer et al. 2009; McKenna et al. 2009). With the placement of Hyperinae (also with ectophytic larvae, see Supplementary Material online) in the CEGH clade in all our analyses, and nearly uniformly as the sister group of the rest of the CEGH clade (supplementary figs. S1–S15, Supplementary Material online), our results firmly refute the hypothesis that this group may belong in the CCCMS clade (e.g., Oberprieler et al. 2007, in Curculioninae). These results also build on and reinforce with nuclear phylogenomic data the hypothesis presented by Haran et al. (2013) that the major phylogenetic split within the higher weevils may also correspond to an important ecological division within this clade, wherein the CEGH clade mainly comprises groups with larvae that live (and hence feed) external to the plant (ectophytic) whereas the majority of taxa in the CCCMS clade seemingly retain the putative ancestral larval habit of living (and hence also feeding) inside plant tissues (endophytic) (Haran et al. 2013; Oberprieler 2014c, 2014d; Oberprieler, Anderson et al. 2014; Oberprieler, Caldara et al. 2014). Exceptions to this pattern (e.g., CCCMS clade: *Cionus* is ectophytic) combined with future more broadly sampled studies of particularly the CCCMS clade may allow for tests of diversification in relation to the larval feeding habit within weevils. For example, by robustly supporting the placement of Hyperinae and Gonipterini in different positions in the CEGH clade (fig. 1), our data indicate that changes in larval ecology may be associated with taxonomic diversification. Both of these tribes differ from the majority of "broad-nosed" weevils with ectophytic but generally subterranean larvae (Oberprieler, Anderson et al. 2014) in having subaerial larvae. These larvae are instead active on exposed plant surfaces, and several of their taxa, like those of other ectophytic groups, have developed morphological and behavioral traits likely connected with their subaerial or ectophytic lifestyles (e.g., Costa et al. 2004; Oberprieler, Caldara et al. 2014; Skuhrovec and Bogusch 2016).

Some relationships deep in our trees (e.g., involving the placement of Belidae) were not strongly supported or consistently found in analyses of NT data (supplementary figs. S1, S3, and S5; supplementary table S2, Supplementary Material online). In contrast, we consistently found strong support for all the deep splits in trees reconstructed from AA data (all $>$ 97% MLBS, 1.0 PP; supplementary figs. S2, S4, and S6 and, table S2, Supplementary Material online). Several of the nodes that were strongly supported in our AA-based analyses (fig. 1) but poorly supported in our NT-based ones, such as the higher Curculionidae, the placement of the subfamily Bagoinae and the CCCMS clade, have also been found in other phylogenetic studies (Marvaldi 1997; Marvaldi et al. 2002; McKenna et al. 2009; Haran et al. 2013; Gillett et al. 2014), consistent with the observation that deep splits are often more accurately reconstructed from analyses of AA data (Rota-Stabelli et al. 2013; Cox et al. 2014). The observed incongruence and differences in nodal support between our NT- and AA-based analyses may relate to substitution modeling problems among large numbers of genes, codon usage bias and/or mutational bias (e.g., Inagaki et al. 2004; Inagaki and Roger, 2006; Rota-Stabelli et al. 2013; Cox et al. 2014) or even patterns of missing data (e.g., Xi et al. 2016), all of which may have contributed to the instability in the placement of Platypodinae and a few other taxa. Holder et al. (2008) proposed that the application of the CAT substitution model in Bayesian analyses (in PhyloBayes) should offer a solution to the AA–NT incongruence problem. As already noted, higher-level (backbone) relationships were largely congruent and strongly supported by both these data sets in our analyses. This was true also for trees derived from analyses of AA and NT data using the CAT–GTR models in PhyloBayes v4.1 (Lartillot et al. 2009), but these trees showed some incongruence in lower-level relationships, for example, in Curculionidae (supplementary figs. S12 and S13, Supplementary Material online). These trees also placed the early-diverging family Belidae in a different phylogenetic position than in otherwise identical AA and NT analyses employing the GTR model. The position of Belidae in both of the CAT–GTR trees is the same as in the tree based on the "dayhoff6"-recoded AA data (where the 20-AA data set was recoded to represent only six Dayhoff categories; supplementary material and fig. S11, Supplementary Material online). Trees generated from dayhoff6-recoded analyses of the AA data (supplementary fig. S11, Supplementary Material online) differed in lower-level relationships from trees found by both the GTR and the CAT–GTR-based NT analyses (supplementary figs. S1, S3, S5, and S13, Supplementary Material online). Analyzing our data sets under different models therefore did not completely resolve the observed incongruence between the NT and AA phylogenies. Based on these CAT–GTR and dayhoff6-recoded analyses, some of the groupings present only in the trees derived from GTR-based ML analyses of NT data consist of clades characterized by similar compositional issues (see also supplementary figs. S16 and S17, Supplementary Material online). We suggest, then, that the GTR-based ML NT trees are probably biased and affected by systematic errors related to the uneven distribution of base composition across the tree.

In NT-based partitioned ML analyses, the placement of Belidae lacks strong nodal support ($<$65% MLBS, supplementary fig. S5, Supplementary Material online). In results of the NT-based analyses using the GTR model, Belidae are recovered either as the sister group of Attelabidae (supplementary fig. S5, Supplementary Material online, 63% MLBS) or of the clade (Attelabidae (Caridae (Curculionidae + Brentidae))) (supplementary fig. S3, Supplementary Material online, 0.5 PP). But in all AA-based analyses using the GTR model, Belidae are included in a strongly supported clade along

with Nemonychidae: Rhinorhynchinae and Anthribidae (supplementary fig. S2, 97% MLBS; supplementary fig. S6, 98% MLBS; supplementary fig. S4, 1.0 PP)—a relationship not found by previous studies, and Attelabidae are the sister group of the clade (Caridae (Brentidae + Curculionidae)), with strong support (supplementary figs. S2, S6, both 99% MLBS; supplementary fig. S4, 1.0 PP; Supplementary Material online). To explore this incongruence further, codon-usage bias in the NT data was also investigated using skew (a measure of deviation from expected base frequencies) for arginine, leucine and serine (following Rota-Stabelli et al. 2013) (supplementary fig. S17, Supplementary Material online). Skew values range from +1 to −1 (e.g., for the arginine codon sets CGN/AGR: 1 = only CGN, −1 = only AGR, 0 = CGN and AGR used equally). Looking specifically at arginine, skew values ranged from −0.37 to 0.45 and were low (AGR-rich) in clades containing taxa whose phylogenetic placement was different in NT versus AA analyses and/or poorly supported, for example, Attelabidae, Belidae and Nemonychidae: Rhinorhynchinae (supplementary fig. S17, Supplementary Material online). Interestingly, in NT-based analyses these taxa tended to group together with other taxa showing similar (low) skew values for arginine. Although the CGN/AGR arginine skew values for both species of Belidae in our analysis range from −0.03 to -0.07 (mean: −0.05), these represent only a slight bias compared with other taxa in the analysis and the values for Nemonychidae and Anthribidae, while similarly biased, are actually much higher (e.g., Anthribidae mean: −0.29; Rhinorhynchinae mean: −0.21; supplementary fig. S17, Supplementary Material online). Moreover, the mean of arginine skew values for Attelabidae is 0.13, indicating a bias instead toward codons in the CGN set. This is interesting because in all trees except the C3-only tree and the partitioned NT tree (in which Belidae were the sister-group of Attelabidae), Belidae were the sister-group of the clade Anthribidae + Rhinorhynchinae with varying MLBS support (particularly high in AA analyses), suggesting a possible but unclear effect of codon usage bias on the NT-based analyses. Synonymous codon-usage bias has been shown to produce incongruence between NT- and AA-based phylogenies (Inagaki et al. 2004; Rota-Stabelli et al. 2013; Cox et al. 2014), and the alternative placements of the pinhole-borer weevils *Austroplatypus* and *Notoplatypus* may be the clearest example of incongruence explained by codon-usage bias (and related factors) in our study. These two genera, while exhibiting similar bias for serine codon sets, demonstrate bias in opposite directions for both arginine and leucine codon sets (supplementary fig. S17, Supplementary Material online), which may both explain their alternate placements (and low support for Platypodinae monophyly) among our analyses (supplementary figs. S1–S15 and table S2, Supplementary Material online) and reinforce our conclusion that mutational bias may have impacted the placement of these genera in our NT-based trees.

Reconstructing the Temporal Framework of Weevil Evolution

The radiation of weevils in association with plants is—on account of the large number of species involved—one of the most notable plant–insect coevolutionary stories (e.g., Oberprieler et al. 2007, McKenna et al. 2009). We leveraged our robust AHE phylogeny to investigate the temporal origins of major weevil groups via divergence time analyses implemented in RelTime (Tamura et al. 2012, 2013; Kumar et al. 2016). Our divergence time analyses support an early Triassic origin of the clade Phytophaga (weevils plus their sister group, the superfamily Chrysomeloidea [leaf and longhorned beetles]; Haddad and McKenna 2016) and a late Triassic (Norian 208.5–228 Ma) to early Jurassic (Toarcian 174.1–182.7 Ma) origin for the divergence of early weevil lineages (fig. 1, supplementary figs. S18–S25, Supplementary Material online). This latter result implies an age of origin for the weevils 40–50 Ma older than the oldest known weevil fossils (Nemonychidae; Arnoldi 1977; supplementary table S4, Supplementary Material online). Consistent with McKenna et al. (2009) and Gunter et al. (2016), our results further suggest that the diversification of weevils into families (except Brentidae and Curculionidae) occurred on gymnosperms in the Mesozoic and that the early branching events occurred well before the origin of angiosperms. Age estimates for family and other higher-level nodes in our study are generally higher than in other studies of weevils (McKenna et el. 2009; Misof el al. 2014; Gunter et al. 2016) and higher than estimates in higher-level studies of Coleoptera (beetles) that include weevils (Hunt et al. 2007; McKenna and Farrell 2009; McKenna et al. 2015), with the exception of the Coleoptera-wide study of Toussaint et al. (2017), which found similar estimated ages for the deep splits in the clade Phytophaga. Unlike previous divergence time studies that suggested an age of origin and diversification largely contemporaneous with the evolution of flowering plants, the older age estimates from our RelTime analysis (fig. 1) effectively exclude the possibility that the earliest ancestral weevils fed on angiosperms but instead suggest that these beetles exclusively fed on and diversified in association with nonangiosperm (likely gymnosperm) hosts, lending further support to the hypothesized ancestral association of weevils with conifers (Oberprieler et al. 2007).

Our new timetree indicates that most of the extant family/subfamily-level lineages/splits occurred after the origin of the flowering plants (fig. 1). The fungus-feeding habits of most Anthribidae (most are associated with fungi growing in/on angiosperm wood) may have evolved from feeding on the decaying cones of conifers or cycads (Oberprieler 1999; Oberprieler et al. 2007), and a similar trajectory may have occurred in the Attelabidae (also largely associated with fungi on angiosperms but with a few ancient lineages on conifers; Oberprieler et al. 2007). The angiosperm-associated sister groups Brentidae and Curculionidae diverged during the Late Jurassic (Tithonian, 145.0–152.1 Ma) to early–middle Cretaceous (Aptian, 112.0–125.0 Ma). This overlaps with the estimated timing of first appearance of angiosperms (fossils 132–141 Ma [Brenner 1996], molecular dating

140–180 Ma [Bell et al. 2005]) and monocots (stem-group origin 140–150 Ma [Chaw et al. 2004]; crown-group origin 112 Ma, from fossil pollen [Friis et al. 2004], 130 Ma molecular dating [Bremer 2000]) and includes the interval over which angiosperms rose to ecological dominance (~70–120 Ma; Lidgard and Crane 1988, 1990).

Our new timetree also suggests that the K–Pg extinction event played a larger role in the diversification of the two major clades of higher weevils than previously thought. The estimated dates for the diversification of the CCCMS (> 34,000 species) and CEGH (> 13,000 species) clades in our timetree are very close to the K–Pg boundary and not clearly pre-K–Pg, as reported in previous studies (McKenna et al. 2009; Gunter et al. 2016)—though the estimated age of origin for these groups is similar to that of previous studies (~85–90 Ma; fig. 1). Therefore, diversification of these two clades may actually be more closely correlated with post-K–Pg (early-to-mid-Cenozoic) evolutionary processes and was perhaps initiated by the K–Pg event, but then facilitated by early-to-middle Paleogene warming and associated plant diversification (especially of core eudicots) (McKenna et al. 2009). The extraordinarily ecologically diverse CCCMS and CEGH clades also contain most weevil species and are among the most species-rich clades of plant-feeding animals on Earth.

We explored the effects of using alternative fossils as age constraints in our timetree analysis (supplementary figs. S18–S25 and supplementary table S4, Supplementary Material online). Among other alternatives (see supplementary material and supplementary tables S4 and S5, Supplementary Material online), we explored two alternate minimum ages for each of the CEGH and CCCMS clades representing significantly different interpretations for the minimum ages of the relevant taxa (Entiminae for CEGH constraint, Scolytinae for CCCMS constraint, Eocene vs. Cretaceous minimum age alternatives for each clade). Ages of the early-divergent nodes are not significantly affected by the minimum age calibration placed on the CCCMS and CEGH clades (fig. 1, supplementary table S4, Supplementary Material online). For example, the age of the divergence between Brentidae and Curculionidae remains near the Jurassic–Cretaceous boundary, which is approximately 40 Ma older than any known fossil assignable to this clade (Gunter et al. 2016). When a less conservative (older) fossil calibration (the controversial fossil *Microborus inertus* (Scolytinae) [Cognato and Grimaldi, 2009]) is applied as a minimum age for the CCCMS clade (supplementary figs. S20, S21, S24, and S25, Supplementary Material online), it causes the crown age of the higher Curculionidae to be very near that of the crowns of the CEGH and CCCMS descendent nodes (supplementary figs. S21 and S24, Supplementary Material online). This is quite unlike the results from our other analyses, which use a more conservative (younger) Eocene Baltic amber fossil to constrain the age of the CCCMS clade (supplementary figs. S18, S19, S22, and S23, Supplementary Material online). Therefore, although it is possible that these internodes are very short, it may also be worth further investigating whether the *Microborus* fossil truly belongs to the 98+-My-old Burmese amber (see Gunter et al. 2016). Another major and consistent difference between the results of using a younger or older calibration age for Scolytinae is a dramatic compression in the time intervals (internodes) between the Brentidae–Curculionidae clade and the CCCMS–CEGH clade (especially different in supplementary figs. S21 and S25, Supplementary Material online).

Dorotheus guidensis, a fossilized elytron from the late Cretaceous of southern Chile (Kuschel 1959), is considered to be the oldest fossil evidence of Entiminae and was therefore used to constrain the age of the CEGH clade (supplementary figs. S18–S21, Supplementary Material online). However, due to uncertainty concerning its identification (characters of the elytra observed in *Dorotheus* are possibly plesiomorphic, occurring also in the CCCMS clade), we separately applied a minimum age for the CEGH clade using more reliably identified entimines from Baltic amber (*Polydrusus* spp.: Yunakov and Kirejtshuk 2011). However, we found no significant difference in the results for the age of the CEGH clade in analyses using either the older (*Dorotheus*) or younger (*Polydrusus*) constraint (supplementary figs. S18–S25, Supplementary Material online).

Conclusions

This work establishes a uniquely robust phylogenetic and temporal framework for evaluating patterns of ecological and taxonomic diversification in weevils, something that has not yet been possible due, in part, to the lack of a robust phylogenetic framework (e.g., Oberprieler et al. 2007). Further, it provides molecular support for several groups morphologically defined in Curculionoidea, indicates strong support for other relationships and suggests the need for future changes to the classification of weevils. Most notably, it solidifies the phylogenetic relationships and classificatory position of a number of taxa of controversial placement, such as Ithycerinae, Microcerinae, Platypodinae and Bagoinae. Further, our results demonstrate the utility of AHE for reconstructing the phylogeny of weevils—especially regions of the tree that have long been recalcitrant to resolution—and suggest several ways in which our AHE probe set (the first for beetles) might be improved, and taxon sampling expanded, to help further reconstruct the phylogeny and evolution of weevils. The probes we use in this article were designed to be informative for analyses spanning all beetles and will likely also be useful for analyses of insect groups closely related to beetles, such as lacewings, dobsonflies, snakeflies, scorpionflies and twisted-wing parasites. Our AHE probes proved highly effective at robustly resolving many critical nodes within weevils, including nodes spanning the backbone and terminal nodes of the tree. Our timetree suggests that some critical early events in the coevolution of weevils and plants may have occurred 40–50 My earlier than previously thought and that major transitions in the evolutionary history and diversification of both groups are closely correlated. Our divergence time analyses suggest that changes in the floristically dominant plant group (gymnosperms to angiosperms) were tracked by the evolution of new weevil taxa radiating first in association with angiosperms and again later, after the K–Pg mass extinction event. The latter may have promoted the

radiation of the CEGH and CCCMS clades, the two most speciose groups of higher weevils (fig. 1). With a robust backbone phylogeny in place, including a clear understanding of the composition of the family Curculionidae, we are now well positioned to further sample/resolve relationships within this extraordinarily diverse family and further explore the temporal dimension of evolution at the beetle–plant interface.

Materials and Methods

Probe Design and Locus Selection

For probe design, we chose 26 species (here called "models") whose genomes and/or transcriptomes had been sequenced (supplementary table S1, Supplementary Material online, also see Haddad et al. 2018). These comprised six species of Curculionoidea: *Arrhenodes minutus* (Transcriptome, 1KITE unpublished data), *Dendroctonus ponderosae* (Transcriptome, PRJNA178770), *Ips typographus* (Transcriptome, 1KITE unpublished data), *Pissodes strobi* (Transcriptome, PRJNA186387), *Rhynchophorus ferrugineus* (Transcriptome, PRJNA79205), *Sitophilus oryzae* (Transcriptome, PRJNA79583, Pauchet et al. 2010), four of Cerambycidae: *Anoplophora glabripennis* (Genome, PRJNA274806, McKenna et al. 2016), *Phymatodes amoenus* (Genome, Mitchell R, unpublished data), *Rhamnusium bicolor* (Transcriptome, 1KITE unpublished data), *Xylotrechus colonus* (Genome, Mitchell R, unpublished data), six of Chrysomelidae: *Callosobruchus maculatus* (Genome, PRJNA308906), *Diabrotica undecimpunctata* (Genome, Robertson H, unpublished data), *Gastrophysa viridula* (Transcriptome, PRJNA79577, Pauchet et al. 2010), *Leptinotarsa decemlineata* (Transcriptome, PRJNA79581, Pauchet et al. 2010), *Donacia marginata* (Transcriptome, 1KITE unpublished data), *Chrysomela tremulae* (Transcriptome, PRJNA79423, Pauchet et al. 2010), one of Cryptophagidae: *Atomaria fuscata* (Transcriptome, 1KITE unpublished data), one of Tenebrionidae: *Tribolium castaneum* (TCAST) (Genome, PRJNA12540, Richards et al. 2008), one of Carabidae: *Calosoma scrutator* (Genome, McKenna D, unpublished data), one of Coccinellidae: *Harmonia axyridis* (Genome, McKenna D, unpublished data), one of Hydroscaphidae: *Hydroscapha redfordi* (Genome, McKenna D, unpublished data), one of Cupedidae: *Priacma serrata* (Genome, McKenna D, unpublished data), one of Byturidae: *Byturus ochraceus* (Transcriptome, 1KITE unpublished data), one of Cleridae: *Thanasimus formicarius* (Transcriptome, 1KITE unpublished data), one of Strepsiptera: Mengenillidae: *Mengenilla moldryzki* (Genome, PRJNA181027), and one of Megaloptera: Corydalidae: *Chauliodes pectinicornis* (Genome, McKenna D, unpublished data).

Anchored hybrid enrichment probes were developed targeting 941 orthologous nuclear loci of known utility for phylogenetic analysis (e.g., Niehuis et al. 2012; Misof et al. 2014; also see Haddad et al. 2018). These loci were located in conserved "anchor regions" of the genomes and/or transcriptomes of the model species, were flanked by less conserved regions and were selected based on their presence as 1:1 orthologs across the model species used in this study. Loci were therefore included only if they were single-copy orthologs. The probes were designed to target loci suitable for use across all insects and to ensure utility across all Neuropteroidea (Coleoptera, Neuropterida, Strepsiptera). The 941 target loci were selected from a pool of ~1,200 candidate loci that were identified by seeking the intersection of a genome-based data set [4,485 1:1 orthologs from Holometabola; Niehuis et al. (2012)] with a transcriptome-based data set [1,478 1:1 orthologs from Misof et al. (2014)]. Those resulting candidate loci were then sought in the aforementioned genomes and transcriptomes from the 26 model species, to confirm their presence and assess their phylogenetic utility using criteria enumerated by Lemmon et al. (2012: 728–729). Accordingly, we filtered the candidate loci for unique (single-copy), widely dispersed genomic loci lacking any indels or repetitive elements and with a ~240-bp conserved center of the probe region but variable sites in at least one of the flanks. A core set of 236 loci with variation and phylogenetic utility across Arthropoda, but primarily in Insecta, was chosen for inclusion in the probe set, along with 705 loci chosen because they were phylogenetically informative across Neuropteroidea (Coleoptera, Strepsiptera and Neuropterida; McKenna and Farrell 2010; Beutel and McKenna 2016; McKenna 2016). Probes were tiled approximately every 50 bp for each of the 26 model species (2.4× coverage per species), starting at the beginning of the alignment. Alignments for the 941 target loci containing the 26 model species were used to identify enrichment probes. Final alignments and probe sequences are available from Dryad (accession numbers doi: 10.5061/dryad.v0b7v and doi:10.5061/dryad.42d3b).

Taxon Sampling and DNA Extraction

Our taxon sample included 63 weevil species representing all extant families of Curculionoidea and all subfamilies of Curculionidae following the classification of the *Handbook of Zoology, Coleoptera Vol. III* (Leschen and Beutel 2014) (supplementary table S3, see details in Supplementary Material online; see details in supplementary material). Genomic DNA was extracted from one to six legs, thoracic muscle or the whole body of specimens preserved in 80–100% ethanol, depending on their size and state of preservation. Total genomic DNA (~100–500 ng was ultimately used) was extracted from air-dried specimens using the OmniPrep kit (G Biosciences, St. Louis, MO) and was treated with RNase A. DNA amount and concentration was estimated using a Qubit fluorometer, and DNA quality (degree of fragmentation/degradation and contamination with RNA) was assessed via gel electrophoresis. Voucher specimens and specimen parts are preserved in 100% ethanol in the McKenna Laboratory at the University of Memphis, at the Harvard University Museum of Comparative Zoology or at the Commonwealth Scientific and Industrial Research Organization.

Library Preparation, Enrichment, and Sequencing

Genomic DNAs were sent to the Center for Anchored Phylogenomics (www.anchoredphylogeny.com) at Florida State University (FSU) for library preparation, enrichment

and high-throughput sequencing. Two rounds of library preparation and sequencing were performed. Data were collected following the general methods of Lemmon et al. (2012). Each genomic DNA sample (ranging from ~80 to 1,000 ng) was sonicated to a fragment size of 150–350 bp using an E220 Focused-ultrasonicator (Covaris Inc., www.covarisinc.com, Woburn, MA). Library preparation and indexing was performed on a Beckman-Coulter Biomek FXp liquid-handling robot following a protocol modified from Meyer and Kircher (2010). One modification to this protocol was a size-selection step after blunt-end repair using SPRIselect beads (Beckman-Coulter Inc., www.beckman.com, Brea, CA; 0.9× ratio of bead to sample volume). Indexed samples were then pooled at equal quantities (12–16 samples per pool), and enrichment was performed on each multisample pool using an Agilent Custom SureSelect kit (Agilent Technologies Inc., www.agilent.com, Santa Clara, CA) containing probes designed to anneal to the targeted loci. After enrichment, each set of six (for the first round) or three (for the second round) enrichment reactions was pooled in equal quantities for PE150 sequencing at the Translational Science Laboratory in the College of Medicine at FSU on three lanes of an Illumina HiSeq 2000 sequencer (Illumina Corp., www.illumina.com, San Diego, CA), shared with samples from other projects (~77 Gb raw DNA sequence data were used for this study).

Read Processing and Assembly

Quality control and assembly for sequenced raw reads was performed for each species using the AHE bioinformatics pipeline described in Prum et al. (2015). Prior to assembly, overlapping reads were identified and merged following the methods of Rokyta et al. (2012). Most (50–75%) of the sequenced library fragments had an insert size of 150–300 bp. Therefore, the majority of the paired reads were overlapping such that they could be merged before assembly. For each read, we computed the probability of obtaining the observed number of matches by chance for each degree of overlap and selected the degree of overlap that produced the lowest probability. A *P*-value of less than 10^{-10} was used to determine when to merge reads. Mismatched reads were reconciled using base-specific quality scores after merging, which were combined to form the new quality scores for the merged read (see Rokyta et al. [2012] for details). We kept separate the reads that failed to meet the probability threshold but still used them in the final assembly. The merging process generated fastq files; one containing merged reads and two containing the unmerged reads. Merged reads were assembled (using the script Assembler.class) by mapping reads to the probe regions for each locus (see taxa used as references below) and by extending the flanking regions using a de novo approach. Orthology-matched NT probe regions from the following genomes and transcriptomes were used as references for locus assembly: *A. minutus* (Brentidae, transcriptome, 768 loci), *P. strobi* (Curculionidae, partial transcriptome, 220 loci), *R. ferrugineus* (Curculionidae, transcriptome, 789 loci), *S. oryzae* (Curculionidae, transcriptome, 746 loci), *D. ponderosae* (Curculionidae, transcriptome, 780 loci) (Andersson et al. 2013), and *T. castaneum* (Tenebrionidae, genome, 941 loci) (Richards et al. 2008). The TCAST reference data covered 100% of the targeted loci, so we used it as the main reference gene set. Assembly parameters and scripts were obtained from Prum et al. (2015). After the reads were assembled, we checked for possible cross-contamination using (for each taxon) an all-versus-all Blast search (following Camacho et al. 2009).

AHE Data Harvesting

Although the assembled AHE fasta files were mapped in a previous step, we also employed the protein-based orthology search pipeline Orthograph for strict orthology assessment (Petersen et al. 2017). Orthograph removes possible paralogous genes using HMM (hidden Markov model)-based orthology searches of protein-translated sequences. For this pipeline, the official gene sets (OGSs) from three insect taxa selected from among the holometabolous insects included in OrthoDB 7 (Waterhouse et al. 2013; Kriventseva et al. 2015) were used as a reference for orthology prediction. These included one beetle (TCAST: Richards et al. 2008; the only beetle genome available at the time in OrthoDB) and two other holometabolous insects: *Danaus plexippus* (DPLEX; Lepidoptera: Danaidae, Zhan et al. 2011) and *Nasonia vitripennis* (NVITR; Hymenoptera: Pteromalidae; Werren et al. 2010). We used the database tool OrthoDB 7 (Waterhouse et al. 2013; Kriventseva et al. 2015) to generate a table of clusters of orthologous genes (COGs) for the three selected OGSs. In this step, the TCAST 941 AHE reference locus set was remapped by BlastX ($E < 1e{-}6$) against the reference OGS for TCAST (OGS 3.0; Richards et al. 2008). This exercise recovered 663 genes (each comprised sequences from one or more of the targeted loci). AHE reference assemblies can generate spurious duplicates of single-copy orthologs if the flanking regions of different targeted loci (here, different exons from the same gene) overlap between loci. This occurs when the assembled flanking regions extend beyond short introns flanking a target exon, resulting in the recovery of neighboring exons that may also be targets. Based on the BlastX results, we ultimately settled on a total of 522 COGs that matched with all single-copy COGs of the aforementioned three OGSs in the Orthograph pipeline (we excluded 141 of the 663 total genes represented by our 941 target loci because they had multiple copies in at least one of the three OGSs).

The COG tables and OGS sequences selected from the previous step were loaded into Orthograph as the reference database for subsequent strict protein-based orthology searches. All 63 fasta files from the assembly steps (one for each terminal taxon) were used as Orthograph input. In the first step of the Orthograph pipeline, all DNA sequences were translated into the six possible reading frames (finding a correct AA translation point for each locus), and then the resulting library of AA sequences was searched using profile HMMs that were trained by the three OGSs we selected from OrthoDB 7. For each taxon-based result from the Orthograph pipeline, the orthology of our targeted (522) genes was assessed using a reciprocal Blast search and the results were stored in both AA and NT format, following Petersen et al. (2017). The resulting fasta-formatted NT files

for each species were screened for vector contamination using UniVec (Cochrane and Galperin 2010). We used an all-versus-all Blast to further assess our data set for apparent cross-contamination.

Phylogenomic Pipeline for the AHE Analysis

Orthology search pipelines generate fasta files using OrthoDB 7 identifiers (e.g., EOG7B3CRG.nt.fas) for every species and gene. Thus, a custom bioinformatics pipeline was required to process the files. Specifically, reference taxa had to be removed and headers on each fasta file had to be modified for later phylogenetic analysis. The AA and NT fasta files from the Orthograph pipeline (these lack introns) included information from the results in their headers, in addition to three OGS sequences for each gene. The headers on each sequence were modified to Hamstrad format (Orthograph package by Petersen et al. [2017]). Then, the reference genes from the OGSs were removed for each fasta file. As such, each fasta file then had only one target sequence for each gene and a taxon name that includes an OrthoDB 7 ID. After this trimming step, and prior to alignment, we used a script "summarize_orthograph_results.pl" from Orthograph by Petersen et al. (2017) to combine the fasta files for each gene based on the OrthoDB 7 IDs. Next, each AA sequence was aligned in MAFFT 7 with L-INS-i and all default options. After AA sequences were aligned each NT sequence was aligned by PAL2NAL using the corresponding codon alignments (Suyama et al. 2006). Before concatenation, we changed the Hamstrad header in all resulting files to a simple taxon code using a custom script included in the pipeline. Finally, the final aligned files were concatenated with AMAS 0.97 (Borowiec 2016). All scripts and other details of our pipeline are available from Dryad (accession number doi:10.5061/dryad.42d3b).

Phylogenetic Analysis

Most analyses were run on the HPC (high-performance computing) cluster at the University of Memphis. Model selection and partitioning for both the AA and NT data sets was performed using PartitionFinder 1.1.1 (Lanfear et al. 2012). The AA and NT matrices were analyzed separately in RAxML (Stamatakis 2014) (10 replicate ML searches; 1,000 rapid bootstrap replicates). Results from the bootstrap analyses were mapped onto the resulting ML trees (AA and NT). Trees based on nonpartitioned and partitioned AA and NT data sets are provided (supplementary figs. S1–S2 and S5–S6, respectively, Supplementary Material online). We used the command line version of MEGA7 (Kumar et al. 2016) for codon usage bias analyses (e.g., Inagaki et al. 2004; Inagaki and Roger 2006; Rota-Stabelli et al. 2013; Cox et al. 2014) based on the partitioned NT ML tree. We also analyzed the AA and NT data using BI implemented in MrBayes 3.2.5 (Ronquist et al. 2012) (supplementary figs. S3-S4) and PhyloBayes-MPI v1.7a (Lartillot et al. 2013) (supplementary figs. S12-S13, Supplementary Material online). Bayesian analyses were only conducted on the nonpartitioned (concatenated) data set (see Supplementary Material online). The coalescent species tree analysis was performed on our AA and NT data sets using ASTRAL 4.11.1 (Mirarab, Bayzid, et al. 2014) with the weighted statistical binning scripts from Mirarab, Reaz et al. (2014) and Bayzid et al. (2015) (Supplementary Material online; results are presented in supplementary figs. S14-S15, Supplementary Material online). Several papers have considered a possible preference for results of AA- over NT-based phylogenetic analyses, especially for resolving deep divergences (e.g., Inagaki et al. 2004; Cox et al. 2014). Although the major weevil lineages diverged much later than groups studied by those authors, our analyses of codon usage and base composition suggested that similar compositional biases may also be present in our weevil NT data set (see Results and Discussion). Our phylogenetic analyses also strongly indicated saturation at (at least) the third-codon positions. We therefore used our AA-based results for summarizing our results and conducting divergence time analyses.

Divergence Time Analyses

We calculated divergence times using the program RelTime (Tamura et al. 2012, 2013; Kumar et al. 2016). Local clocks were used for each lineage, with no clock rates merged (see Bond et al. 2014). The LG substitution model was employed with a gamma distributed model and five discrete gamma categories. The "Use all site" option was used for all analyses. In our analyses, we used dated fossils to apply constraints to up to ten nodes in the preferred phylogeny (Fig. 1; partitioned ML, AA data set) and tested different combinations of minimum/maximum ages to gauge the effect of including/excluding some key fossils and min–max combinations (see details in Supplementary Material online).

Supplementary Material

Supplementary data are available at *Molecular Biology and Evolution* online.

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