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Mitogenome sequences stabilize the phylogenetics of weevils (Curculionoidea) and establish the monophyly of larval ectophagy

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ABSTRACT

The weevils and their relatives (superfamily Curculionoidea) constitute a huge radiation of beetles, but basal relationships in this group remain controversial, in particular within the largest family, Curculionidae. We used next-generation sequencing to generate mitochondrial genome data comprising 12 protein coding genes for 27 taxa. Together with two published ingroup sequences, (sub)family relationships were assessed with Bayesian and ML searches under various models and partitioning schemes. Forward and reverse strands of the mitochondrial genome differed in nucleotide skew. Consequently synonymous codon usage differed substantially on either strand for each amino acid, whereby codons ending in AT and GC were favored on the forward and reverse strands, respectively. Data partitioning by forward/reverse strand and codon position greatly improved likelihood scores and nodal support, whereas the tree topology was largely stable. The analysis generally supports the basal position of several 'orthocerus' lineages with straight antennae and male genitalia of an ancestral type; a paraphyletic mixed group of Heteromorphi exhibiting mixed ancestral and derived antennal and genitalic characters; and the derived 'gonathocerous' lineages with kinked antennae corresponding to the strongly supported Curculionidae. The latter did not include the wood boring Platypodinae that was recovered as sister to Dryophthoridae, while the Scolytinae and Cossoninae formed two independent lineages of wood borers within Curculionidae. A basal split in Curculionidae placed the Entiminae and Hyperinae as sister to all other subfamilies with high support, which provides a new ecological concept for structuring the Curculionidae according to the ectophagous larval life style in the former versus endophagous larvae in all others. This basal split is also supported by gene order rearrangements in a tRNA cluster. Recent studies supporting the monophyly of wood boring weevils may be attributable to long-branch attraction, as molecular rates in their mitochondrial genomes were found to be higher than in other lineages, but this did not confound tree searches under combined analysis of mitochondrial protein coding genes.

1. Introduction

The increased availability of mitochondrial genome sequences obtained by next-generation sequencing (Jex et al., 2010; Timmermans et al., 2010) provides the possibility to reinvestigate long-standing phylogenetic questions with expanded data sets. Among various families of beetles (Coleoptera), the weevils (superfamily Curculionoidea) have received much attention from molecular phylogeneticists, mainly because of their huge diversity of >60,000 described species (Oberprieler et al., 2007) and the possibility that this diversity is explained by co-radiation with the angiosperms (Farrell, 1998). However, key questions about their basal relationships relevant to these conclusions have not been resolved unequivocally. Phylogenetic analyses of Curculionoidea were initially based on limited taxon sampling and single genetic markers, including mitochondrial 16S rRNA (*rrnL*) (Wink et al., 1997) and nuclear 18S rRNA (Farrell, 1998; Marvaldi et al., 2002). However, the power of these data was weak, and meaningful trees were only obtained in combined analyses with morphological adult (Farrell, 1998; Wink et al., 1997) and larval (Marvaldi et al., 2002) characters. More strongly supported trees were obtained only when multiple molecular markers were combined, e.g. using *rrnL* and 18S rRNA (Hundsdoerfer et al., 2009), a six-gene data set of two mitochondrial (*cox1*, *rrnL*) and four nuclear (18S rRNA, 28S rRNA, elongation factor 1-alpha, arginine kinase) (McKenna et al., 2009), and a similar set of five genes with focus on the evolution of wood-boring lineages (Jordal et al., 2011).

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The modern concept of Curculionoidea (Crowson, 1955) splits 'Curculionidae' of earlier authors into eight families, Nemonychidae, Belidae, Oxycorynidae, Proterhinidae, Attelabidae, Brentidae, Apionidae, Anthribidae and a newly defined Curculionidae. The former set of families are known as 'orthocerous weevils' due to their straight, filiform antennae, which separates them from Curculionidae that exhibit geniculate (=elbowed) antennae (Crowson, 1955; Morimoto, 1962). The Curculionidae also show male genitalia with a fused pedon and tectum, a condition that does not occur in orthocerous groups. Hence the 'orthocerous type' and 'gonatocerous type' can be applied also to the genitalia, with some inconsistencies (Thompson, 1992). This major subdivision has been largely confirmed in the molecular trees, in particular assigning the ancient lineages (based on the fossil record) Belidae (now considered to include Oxycorynidae, Proterhinidae; Bouchard et al., 2011), Nemonychidae, Anthribidae and Attelabidae to the basal branch points of the tree (Hunt et al., 2007; Marvaldi et al., 2002; McKenna et al., 2009), even if their precise relationships differ among these studies.

However, the remaining groups do not fully fit this scheme and have created difficulties also for molecular approaches. Crowson (1955) already recognized that the form of the male genitalia associated with orthocerous weevils occurred in Nanophyinae despite their geniculate antennae of the gnathocerus type. Orthocerous-type genitalia were also recognized in the curculionid subfamily Rhynchophorinae (Morimoto, 1962) that was later promoted to Rhynchophoridae = Dryophthoridae (Alonso Zarazaga and Lyal, 1999; Bouchard et al., 2011). Careful examination of the male genitalia of the remaining subfamilies of Curculionidae (Thompson, 1992) necessitated the removal of several taxa into separate families (the so-called Heteromorphi). Although their classification is still in flux, these groups are now widely classified as Brentidae (including several subfamilies: Brentinae, Apioninae, Ithycerinae, Microcerinae, Nanophyinae) and Brachyceridae (with subfamilies Brachycerinae, Eririhininae, Cryptolarynginae, Raymondionyminae, Ocladiinae) (Bouchard et al., 2011).

The monophyly of the remaining Curculionidae (*sensu* Thompson, 1992) and their relationships are still poorly understood. The most widely debated issue has been the sister relationships of the two main wood boring groups, Platypodinae (ambrosia beetles) and Scolytinae (bark beetles). Both groups have been widely classified at the family level, and considered to have separated before the common ancestor of the advanced weevils (Morimoto and Kojima, 2003; Wood, 1973). These studies also grouped both families closely together, whereby Platypodidae were either nested within, or sister to, the Scolytidae, but this placement may be confounded by convergent adaptations to tunneling in wood (Lyal and King, 1996; Zherikhin and Gratshev, 1995). The antennae and genitalia of Platypodidae are highly reduced and not easily assigned to the plesiomorphic or apomorphic types (Thompson, 1992) although platypodid genitalia appear to have the remnant of a dorsal plate (Morimoto, 1962) and other plesiomorphic features (Jordal et al., 2011). In recent molecular studies, the issue of Platypodinae-Scolytinae affinities and their precise placement relative to other groups still remain contradictory. Whereas deep-level analyses of molecular (McKenna et al., 2009) and larval (Marvaldi, 1997; Marvaldi et al., 2002) characters place Platypodinae near Dryophthoridae, i.e. outside the main weevil clade of Curculionidae, detailed studies with focus on wood-boring lineages, including the curculionid subfamily Cossoninae, group the Platypodinae as nested within, or as sister to, the Scolytinae (Jordal et al., 2011).

The problem of the wood-boring weevils illustrates the potential effect of life style on morphological traits and the resulting difficulties for phylogenetic reconstruction. This issue has not been explored more widely with respect to other traits, specifically the internal vs. external feeding of adults and the consequences of the substrate for rostrum shape and size. Within the 16 subfamilies of Curculionidae recognized by Alonso-Zarazaga and Lyal's (1999) *World Catalogue*, the Entiminae in its current extent has been established quite recently consisting of a combination of various former 'broad-nosed' subfamilies exhibiting a broadened, shortened rostrum, such as the Brachyderinae, Otiorhynchinae, Sitoninae and several others. These groups also share a common lifestyle of ectophagy in the larval stages, feeding on roots in the soil. Other short-rostrum weevils are grouped into the Brachyceridae (Brachyderinae, Ithycerinae, etc.), while only very few lineages outside of the broad-nosed weevils show ectophagy in the larval stages (although most adult groups are ectophagous). Among these are the Hyperinae, whose larvae feed on plants above ground in a similar way as the caterpillars of Lepidoptera. The broad-nosed weevils, treated as a monophyletic group as recently as Kuschel's (1995) influential classification of the Curculionoidea, may be composed of groups from within and outside of the Curculionidae, whose common ecology has resulted in convergent external morphology.

Mitochondrial genome sequences are being used increasingly for higher-level relationships of Coleoptera (Pons et al., 2010; Sheffield et al., 2008, 2009; Timmermans et al., 2010), and have proven useful to investigate outstanding questions at the level of families and superfamilies, including the evolution of ecological transitions (Timmermans and Vogler, 2012). The mitochondrial genomics of Curculionoidea is still in its infancy. Two sequences have been reported, for *Sphenophorus striatopunctatus* (Dryophthoridae) and *Naupactus xanthographus* (Curculionidae: Entiminae). The latter show a translocation of two tRNA genes (Song et al., 2010) that may be phylogenetically informative. Mitogenomes have great potential to resolve the basal relationships in Curculionoidea if the database can be expanded to include representatives of major lineages. However, the utility of mitochondrial DNA for deep phylogenetics is considered to be poor due to high rates of molecular evolution and consequently saturation of change, which in part can be resolved by RY coding (Hassanin, 2006) or translation into amino acid sequences (Nardi et al., 2003). This is exacerbated by lineage-specific and gene-specific differences in rates, nucleotide composition and codon usage that may confound the phylogenetic relationships, in particular among protein coding genes transcribed from the forward and reverse (J and N) strands, as already shown for Coleoptera (Pons et al., 2010). The power of mitochondrial genomes over single gene analyses (Howland and Hewitt, 1995) supposedly derives from the much greater number of characters, and hence the use of all genes and nucleotide positions. However, it remains unclear to what degree the variation in rates across lineages and the non-uniformity of variation in both strands interfere with the recovery of phylogenetic signal. In particular, likelihood models may need to be partitioned to reflect the differences in rates and types of variation across genes and nucleotide positions.

This study investigates the power of mitochondrial genomes for the phylogenetic analysis of basal relationships in Curculionoidea. The density of taxon sampling exceeds that of other studies on insect groups of similar age, providing greater detail on the variation in nucleotide biases and rate changes across the tree. By including representatives of most subfamilies of Curculionidae, the analysis also addresses competing hypotheses regarding the monophyly of the xylophagous lineages, and the potential impact of ecological convergence on phylogenetic inferences. Finally, we investigate the phylogenetic utility of gene translocations, starting from the reported translocation in *Naupactus*.

2. Materials and methods

2.1. Taxon sampling, DNA extraction and sequencing

A total of 27 taxa were selected for sequencing with emphasis on the subfamily relationships within Curculionidae. Information on the taxa analyzed, including host plant usage and lifestyle, are given in Table 1. This table follows the nomenclature of Bouchard et al. (2011). Four additional mitochondrial genomes were obtained from GenBank and included two ingroup (*Sphenophorus* sp. and *Naupactus xanthographus*) and two outgroup taxa (*Clostomerus claviger*, Cerambycidae and *Zeugophora* sp., Megalopodidae).

The methodology for generating the sequenced data was described by Timmermans et al. (2010). DNA extractions were performed on a piece of animal tissue (Qiagen DNeasy Blood and Tissue Kit) followed by PCR amplification of a large 10 kb mitochondrial fragment (*cox1* to *rrnl*; Takara LA Taq polymerase). Three smaller 'bait' fragments (*cox1*, 0.9 kb; *nad3*, 0.6 kb; *cytB*, 0.4 kb) were amplified with custom designed primers that perform reliably across the Coleoptera. The large fragments were pooled and sequenced on a Roche 454GS Junior at a theoretical ~30× coverage. Library construction and sequencing were performed by the Sequencing Facility of Cambridge University (Department of Biochemisty). The three bait fragments were sequenced using Sanger technology (ABI3700) and used to establish identity and correct assembly of 454 contigs. Primers and PCR reaction conditions were as described previously (Timmermans et al., 2010), except for the use of *nad3* as bait sequences instead of *nad5*, using the primers ND3_F 5′TTATATTTGACTTCCAATC and ND3_R 5′CATTAACAGTGAT ATACCT that were newly designed for weevils.

The 454 reads were assembled in two steps using first Newbler (settings: -mi 96 -ml 60 -rip), followed by an assembly on the resulting contigs with Phrap (-forcelevel 0). Protein coding genes were delimited using the DOGMA webserver (Wyman et al., 2004) and tRNAs predictions were generated with COVE (Eddy and Durbin, 1994) using beetle specific covariance models (Timmermans and Vogler, 2012). Frame-shift sequencing errors, mostly homopolymer related artifacts, were visualized using Artemis (Rutherford et al., 2000) and corrected after translating them into amino acid sequences and aligned with corresponding functional genes from GenBank using the Genedoc software (Nicholas et al., 1997). Sanger traces were edited with Sequencher (Gene Code Corporation) and used for Blast based validation of the long 454 contigs. The annotated mitochondrial contigs were submitted to GenBank under accession numbers JN163945–JN163970.

2.2. Phylogenetic analyses

Sequences for 12 of the 13 mitochondrial protein-coding genes (full-length *cox2*, *cox3*, *atp6*, *atp8*, *nad1*, *nad3*, *nad4*, *nad4l nad5*, *nad6*, *cytb* and partial *cox1; nad2* and the 5' half of *cox1* are not amplified with the primers used) were extracted using the FeatureExtract 1.2 webserver (Wernersson, 2005), aligned using TransAlign (Bininda-Emonds, 2005) and concatenated into a single data matrix using a custom Perl script.

Intra-strand A/T and G/C skews were assessed using the formula $(R - Y)/(R + Y)$, where R stands for purines A or G and Y for pyrimidines C or T (see Pons et al., 2010). Bias in codon usage for each amino acid was explored (per gene; grouped by strand) using the relative synonymous codon usage (RSCU) metric. The RSCU expresses the proportion of the observed frequency of a particular codon relative to a uniform usage of all synonymous codons. A codon that is used less or more frequently than expected will have an RSCU below or above 1.00, respectively. The analyses were conducted with the codon_usage Perl script (http://www.genome.ou.edu/informatics.html). Saturation of the dataset was

Table 1
Taxa used in this study. The classification follows (Alonso Zarazaga and Lyal, 1999). BMNH numbers refer to voucher specimens and DNA extractions at Natural History Museum. Genbank accessions GU176342 and GU176345 are from Song et al., 2010.

Family	Subfamily	Species	BMNH	Origin	GenBank accession
Anthribidae	Anthribinae	*Platystomos albinus*	847772	France	JN163968
Nemonychidae	Cimberidinae	*Doydirhynchus austriacus*	847764	France	JN163964
Attelabidae	Attelabinae	*Apoderus coryli*	847768	France	JN163966
Rhynchitidae	Rhynchitinae	*Byctiscus populi*	847767	France	JN163965
		Deporaus betulae	832883	UK	JN163945
Apionidae	Apioninae	*Rhopalapion longirostre*	847770	France	JN163967
Nanophiydae		*Nanophyes marmoratus*	833849	France	JN163946
Dryophtoridae	Rhynchophorinae	*Sitophilus granarius*	847749	France	JN163959
		Sphenophorus sp.			GU176342
Brachyceridae	Brachycerinae	*Brachycerus muricanus*	696973	France	JN163970
Curculionidae	Entiminae	*Otiorhynchus rugosostriatus*	849828	France	JN163969
		Polydrusus marginatus	847712	France	JN039360
		Sitona lineatus	847716	France	JN163948
		Strophosoma melanogrammum	847719	France	JN163949
		Liphloeus tessulatus	847713	France	JN163947
		Barynotus obscurus	847720	France	JN163950
		Naupactus xanthographus			GU176345
	Curculioninae	*Anthonomus pomorum*	847726	France	JN163951
		Cionus olens	847732	France	JN163958
	Lixinae	*Larinus turbinatus*	847741	France	JN163952
	Hyperinae	*Hypera plantaginis*	847743	France	JN163953
	Baridinae	*Baris laticollis*	847750	France	JN163955
	Molytinae	*Hylobius abietis*	847744	France	JN163954
	Ceutorhynchinae	*Ceutorhynchus assimilis*	847753	France	JN163956
	Cryptorhynchinae	*Acalles aubei*	847756	France	JN163957
	Cossoninae	*Brachytemnus porcatus*	847759	France	JN163960
	Sclolytinae	*Ips cembrae*	847761	France	JN163961
		Scolytus mali/laevis	847762	France	JN163962
	Platypodinae	*Platypus cylindrus*	847763	France	JN163963
Chrysomelidae	Zeugophorinae	*Zeugophora* sp.	840207	S. Africa	HQ232807
Cerambycidae		*Closteromerus claviger*	840202	S. Africa	HQ232804

investigated using the Index of Substitution Saturation (Xia et al., 2003), with gaps treated as unknown nucleotides, the proportion of invariant sites estimated, and with a NJ starting tree under a GTR model. In addition, transitions and transversions were plotted against genetic distance (GTR model). Calculations were performed in DAMBE (Xia and Xie, 2001).

Phylogenetic analysis of the concatenated dataset was performed on the unpartitioned data and under three partitioning schemes of increasing complexity, including: (1) partitioned by forward and reverse strand on which genes are encoded; (2) partitioned by codon position; (3) partitioned by codon position and separating loci on the forward and reverse strands. MrAIC (Nylander, 2004) was used to obtain the most appropriate models of sequence evolution using the AIC criterion. Tree searches were performed in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003) in two independent runs with four Markov chains (three heated and one cold chain) that were run for 10 million generations and were sampled every 1000 generations. The first 10% of sampled trees was discarded (burn-in) and a majority rule consensus tree was obtained using the *sumt* command. The *sump* command was used to estimate Harmonic Mean of marginal likelihoods, which were subsequently used to compare the partitioning schemes using Bayes Factors. Likelihood searches were conducted with PhyML v. 2.4.4 (Guindon et al., 2005) and RAxML (Stamatakis, 2006). Only the latter software permits the application of different models to various partitions. RY coding was implemented by coding purines (A, G) and pyrimidines (C, T) as a single character state. Branch lengths were obtained from the MrBayes output with the *Ape* package in R (Paradis et al., 2004) and resampled using a custom Perl script.

3. Results

3.1. Nucleotide composition, strand bias and codon usage

New mitochondrial genome sequences were obtained for 27 taxa, of which 23 were complete for a ~10 kb region that covers 12 of the 13 protein coding genes, while 4 taxa lacked one or more of the gene regions (Suppl. Fig. 1). Bait sequences for *cox1*, *nad3* and *cytB* were obtained for all new taxa except for two (specimen numbers 847713 and 847719) missing *nad3*. These sequences unequivocally associated the contigs in the pool of sequences with the taxon names, i.e. various portions of the contigs were not affected by chimera formation. In addition, sequences for four additional species were obtained from GenBank, including two outgroup species. Gene boundaries were reliably obtained by reference to annotated published mitochondrial genome sequences for protein coding genes, and using gene predictions with the COVE algorithm for tRNAs (Suppl. Fig. 2). The final data matrix comprised 9612 characters. Like other mitochondrial genomes of insects, the AT content was high at 75%, increasing to 84% in third codon positions, and it was higher for genes on the reverse than forward strand (AT = 75.9% versus 71.4%).

The compositional difference resulted in a substantial skew of G over C on the reverse strand, and C over G on the forward strand (reverse G = 14.7%, C = 9.4%; forward G = 11.8%, C = 16.7%). The opposite is true for AT skew, which on the reverse strand showed a higher proportion of the pyrimidine T over the purine A, i.e. negative skew, whereas on the forward strand the skew is lower, i.e. more A than T (reverse A = 29.1%, T = 46.8%; forward A = 33.0%, T = 38.4%), in particular for 3rd position sites (Table 2). Consequently, plots of GC vs. AT skew resulted in a characteristic distribution that largely separates forward and reverse strands with regard to pyrimidine–purine 'skew', in accordance with findings of Pons et al. (2010) on a broader sample of Coleoptera. The differences in nucleotide skew were strongest for the 3rd positions, while 1st and 2nd position sites showed partial overlap in the level of skew (Suppl. Fig. 3).

This disparity in AT and GC skews between strands coincided with a striking difference in codon usage that was largely uniform among all genes on either strand. Relative codon usage as measured by the RSCU revealed great differences in the preferred codon on either strand. Examples are given in Fig. 1 (also see Suppl. Fig. 4), showing the great difference in the proportion of codons in two- and four-fold degenerate amino acids on either strand that consistently distinguished all genes according to the coding strands. For each codon we calculated the RSCU and subtracted the RSCU value of the reverse strand from the forward strand (Fig. 2). Negative values indicate relative usage of a codon is higher on the reverse strand than relative usage on the forward strand. The figure illustrates that codons ending on A and C were favored on the forward strand, whereas codons ending on T and G are favored on the reverse strand. There is only one exception in the sixfold degenerate Leucine; codon appears to be driven by the first position, specifically the first position-T (two codons) was favored on the reverse strand in accordance with the greater representation of T on this strand, whereas the four codons with first position-C are favored on the forward strand.

Given the differences in the type of variation on forward and reverse strands, model selection for phylogenetic analysis may have to consider the differences in rate of character changes. Therefore, models for ML and Bayesian analysis were compared to assess the effect of data partitioning by strand (2 partitions) and by codon position (3 partitions) and by strand and codon position (6 partitions). The selected models were GTR + I + G in all cases, except for 3rd positions under 6 partitions (HKY + I + G). Likelihood and AIC scores improved steadily with greater level of partitioning, in particular with the separation of sequences from both strands (Table 3). Mutational saturation was assessed by plotting genetic distance for transitions and transversions against each other, resulting in a near linear relationship, both for the total dataset as well as for each of the three codon positions separately (Suppl. Fig. 5). This showed that saturation was low, as also seen from the negligible level of saturation according to the Index of Substitution Saturation test statistic (Iss = 0.499; Iss.c = 0.81; $p < 0.0001$).

3.2. Phylogenetic analyses

Bayesian analysis of unpartitioned and partitioned data set under the unpartitioned and the 2-, 3- and 6-partition models produced highly similar tree topologies with minor differences only in tip level relationships within the Curculionidae (Fig. 3). Nodal support values were generally high with 26 nodes of PP $\geqslant$ 0.97, and 75% of nodes with PP = 1.00. However, several nodes showed low or non-significant nodal support under the simpler partitioning schemes, in particular when applying two partitions (forward and reverse strands) (Fig. 3). The ML searches using PhyML produced very similar topologies to those in the Bayesian analyses (Fig. 3), again with high support in particular at basal nodes, but bootstrap support was very weak within the two main clades of Curculionidae. We also performed partitioned searches in RAxML using the same partitioning scheme as those under Bayesian analysis, which produced trees very similar to those in Fig. 3, with only minor differences in the Curculionidae. RY recoding of first codon positions and removal of fast-changing third positions did not change these relationships (not shown), which supports the fact that saturation in the dataset was low or had no significant effect on estimating the tree topology.

The topology (Figs. 3 and 4) confirmed basal relationships of Curculionoidea as proposed in recent molecular studies, with the orthocerous families Nemonychidae and Anthribidae placed at

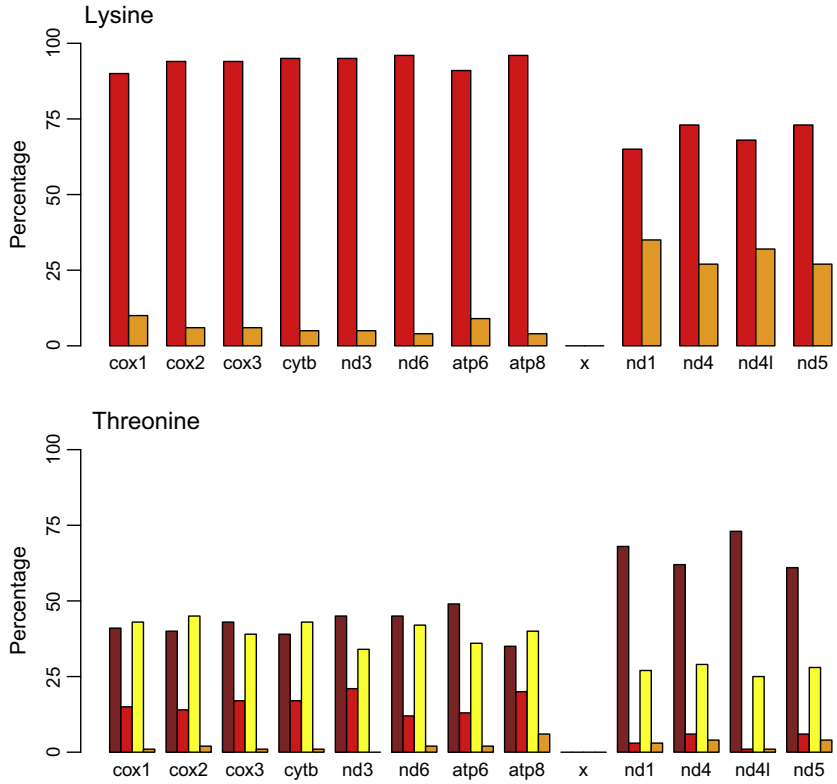

Fig. 1. Codon usage on forward and reverse strands. The Figure presents the percentage of codon usage for two amino acids, Lysin and Threonine, on the forward (8 genes) and reverse (4 genes) strands, as examples of a twofold and fourfold degenerate amino acids. For Lysin, red bars and orange bars represent AAA and AAG, respectively. For Threonine, maroon, red, yellow and orange bars represent ACT, ACA, ACC and ACG, respectively. The corresponding plots for all other amino acids are given in Suppl. Fig. 4.

Table 2
AT content on reverse and forward strands.

	Reverse	Forward
A	29.1 ± 1.63	33.0 ± 1.26
T	46.8 ± 1.65	38.4 ± 2.02
G	14.7 ± 1.59	11.8 ± 1.19
C	9.4 ± 1.33	16.7 ± 1.71
AT	75.9 ± 2.73	71.4 ± 2.74

the basal nodes of the tree, followed by a clade of Attelabidae (Attelabinae + Rhynchitinae). The Heteromorphi family Brentidae (represented by Apioninae + Nanophyinae) occupied the next basal node. The remainder of the trees revealed three major lineages, each with strong support (98% and 100% ML BS, 1.00 and 1.00 PP under the 6-partition GTR + I + G model). The first clade includes the Dryophthoridae and the subfamily Platypodinae of wood-boring beetles, the latter as sister to Dryophthoridae. This clade generally also included the Brachyceridae, but with low support. The next two major clades included all representatives of the Curculionidae, hence all gonathocerous lineages were recovered as monophyletic, but without the Platypodinae whose placement as members of Curculionidae therefore was rejected. The Curculionidae was separated into a clade of all "long-rostrum" subfamilies (Baridinae, Cryptorhynchinae, Curculioninae, Ceutorhynchinae, Lixinae and Molytinae) and the two remaining wood-boring subfamilies, Cossoninae and Scolytinae. Within this lineage, the two divergent members of Scolytinae (*Scolytus* and *Ips*) were monophyletic (69% ML BS, 1.00 PP) and sister to all other 'long-rostrum' weevils. The other major clade of Curculionidae grouped the broad-nosed subfamilies Entiminae and Hyperinae. The former was paraphyletic with respect to Hyperinae, which itself was closely related to *Sitona*. This grouping has not been proposed explicitly in any morphology-based or molecular studies, although it matched the tree topology from the six-gene analysis of McKenna (2009). This grouping combines two subfamilies largely consisting of broad-rostrum species. In addition, the monophyly of the Entiminae/Hyperinae groups also defines a critical transition from ectophagous lifestyle in the larvae, from an ancestrally endophagous larval lifestyle in all other lineages.

Visual inspection of the phylograms showed the three independent xylophagous lineages to be characterized by long terminal branches, in comparison to their respective sister taxa. We assessed the statistical significance of these length differences by resampling random sets of four taxa and comparing their root-to-tip branch length to that of the four wood boring lineages. The distribution of branch lengths was distributed uniformly around a mean, but branch lengths in the quartet of wood boring lineages far exceeded most other randomly drawn groups (Fig. 5).

3.3. Translocations of tRNA genes

The mitogenome organization in most species was identical to the widely observed gene order of Coleoptera. However, in eight species of Curculionoidea we found a gene order different from this ancestral state (Fig. 6). Rearrangements affected a cluster of six tRNA genes between the *nad3* and *nad5* genes usually arranged as Ala–Arg–Asn–Ser–Gln–Phe (ARNSEF). In all the species of Entiminae and Hyperinae the position of $tRNA^{Ala}$ is changed to be adjacent to the *nad3* gene, i.e. RANSEF. In addition, *Sitona* showed a further translocation of the $tRNA^{Ala}$ to give RNSAEF, while in *Hypera* the $tRNA^{Gln}$ was translocated to near the front of the cluster (REANSF) (Fig. 6). The monophyly of these taxa in the ML and Bayesian analyses, and high support (100% BS ML, 1.00 PP), indicate that the Arg–Ala order reversal could be interpreted as a synapomorphy of this

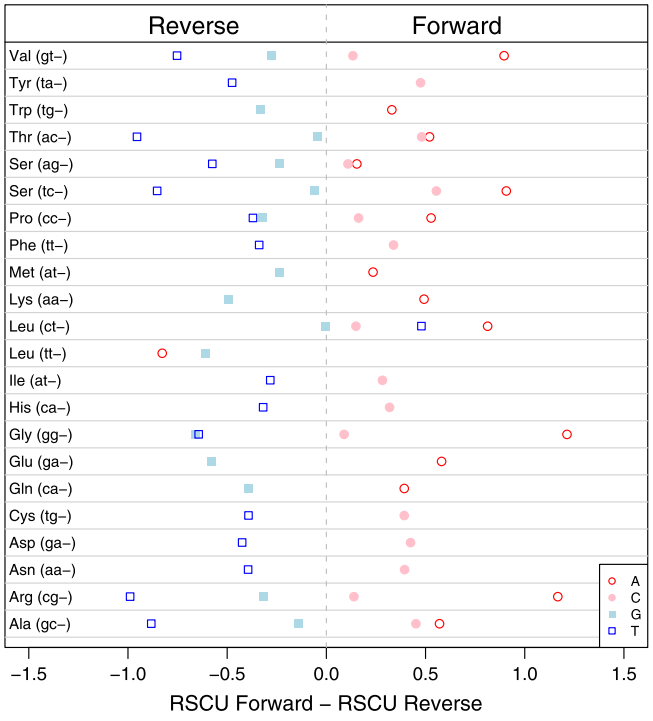

Fig. 2. Bias in codon usage for each of the 20 amino acids on forward and reverse strands. For each codon we determined the mean RSCU separately for genes on the forward and reverse strands. This measure of more or less frequent usage than expected under even codon usage (resulting in RSCU values above or below 1.00, respectively) on either strand was then used to subtract the value of the forward strand from that of the reverse strand. The panel shows if this RSCU value is greater on the forward strand (positive values of the panel) or on the reverse strand (negative values). Four different symbols represent the synonymous base of codons. Note that the reverse strand is skewed towards synonymous codons ending in G and T, while the forward strand is skewed towards codons ending in A and C.

clade, with the genomes of *Hypera* and *Sitona* showing secondary modifications. The $tRNA^{Arg}$–$tRNA^{Ala}$ order reversal therefore appears to be a suitable marker to define the limits of the Entiminae. *Sitona* and two further, unrelated species (*Acalles* and *Ceutorhynchus*) also showed a 70 base pair insertion in the tRNA cluster, but due to their different physical positions and dissimilar primary sequences these are unlikely to be synapomorphic.

4. Discussion

4.1. Information content of mitogenomes

The study showed the power of mitochondrial genome sequences for the phylogenetics of Curculionoidea. Given the difficulties of building meaningful trees from mitogenomes at higher phylogenetic levels (e.g. Nardi et al., 2003), it appears that mitogenome data in insects have greatest utility at lower hierarchical levels of families and superfamilies, and that with sufficiently dense taxon sampling it is possible to obtain highly robust trees. The critical nodes of the early radiation of Curculionidae have usually been ascribed to the mid- to late Cretaceous (Jordal et al., 2011; McKenna et al., 2009). This level of evolutionary divergence therefore may be the 'niche' of mitogenome data in insects.

Previous phylogenetic inferences of basal relationships in Coleoptera using the same 12 protein coding mitochondrial genes (Timmermans et al., 2010) gradually improved by partitioning the data according to codon positions, RY coding or removal of third positions, by recoding of the amino acid sequences, and ultimately by using complex CAT models at the amino acid level (Lartillot et al., 2009). These analyses indicated that the tree was confounded by high rates of variation and possibly other biases of sequence evolution that affected synomymous positions in particular. In the current study with focus on a lower hierarchical level of the Coleoptera, the analysis at the nucleotide level and the inclusion of all sites was perfectly adequate. Saturation at this level of hierarchy was not strongly evident, at least by standard tests of transition to transversion ratios (Suppl. Fig. 5). Applying GTR models in Bayesian and ML analyses, the topologies were largely stable under any partitioning schemes and apparently were unaffected by potential long-branch attraction of the wood-boring lineages (Fig. 3). Yet, partitioned models significantly increased the likelihood over unpartitioned models, and indicate the need for independent model specification for genes on the forward and reverse strands. The greatly increased likelihood under partitioning may result from the differences in nucleotide skew and codon usage in genes encoded by either strand, whose impact was of a similar magnitude as partitioning among the three codon positions (Table 3). Although the phylogenetic signal of the current data set is sufficiently strong to produce a similar topology under most circumstances, the broader lesson from this study is the need for partitioning along the two strands. We conducted tree searches on data from either strand separately (not shown), which produced trees that differed substantially from those obtained with all data combined. This may suggest that the full set of mitochondrial data is required for the recovery of the phylogenetic signal and therefore all data should be combined, but as demonstrated by the significantly better likelihood scores the combined analysis should be performed under model partitioning of the two coding strand (in addition to partitioning by codon positions). The Bayesian trees also showed overall greater levels of nodal support, and even under the least favorable partitioning scheme (forward and reverse strands only) the Bayesian posterior probability exceeded the ML bootstrap values (Fig. 3). While this may indicate an overestimate of confidence in the Bayesian trees, the fact that even suboptimal partitioning schemes recover the preferred topology may suggest that the Bayesian analysis provides an overall more efficient use of the underlying phylogenetic signal.

Finally, gene order rearrangements, which had been thought to be rare in Coleoptera and were first detected in a curculionid (Song et al., 2010), were here found to be an additional valuable resource for phylogenetics at the family level. Most mitogenome rearrangements in Coleoptera detected to date are confined to tip-level clades or individual sequences whose relatives have not been investigated sufficiently to determine their hierarchical extent (Timmermans and Vogler, 2012). The current study adds two new types of rearrangements in *Hypera* and *Sitona* to the seven

Table 3
Harmonic mean of likelihood (lnL), Bayes Factor (BF) and Akaike Information Criterion (AIC) values under partitioning.

No. partitions	Partitions	Model	lnL	Parameters	Δ lnL	$2*\ln \Delta BF_{10}$	RBF	AIC score	Δ AIC
1	None	GTR + I + G	−170,993	10	5020	17.042	0.37	342,006	9948
2	Strand	GTR + I + G	−168,930	20	2957	15.984	0.44	337,900	5842
3	Codon position	GTR + I + G	−168,271	30	2298	15.480	0.60	336,602	4544
6	Strand + codon position	GTR + I + G; 3f: HKY + I + G	−165,973	56	n/a	n/a	n/a	332,058	n/a

Pairwise comparisons were made between the 6-partition model (M_1) and each of the lower partition models (M_0). Values for $2*\ln \Delta BF_{10} > 10$ are usually considered to be highly significant. Relative Bayes Factor (RBF) was calculated according to Castoe et al. (2005) as $2*\ln \Delta BF10/\Delta$ parameters, to penalize greater model complexity. Note that these values do not decline in the more complex models, indicating that better likelihood of the model is not due to over-parameterization. 3f, 3rd codon forward strand.

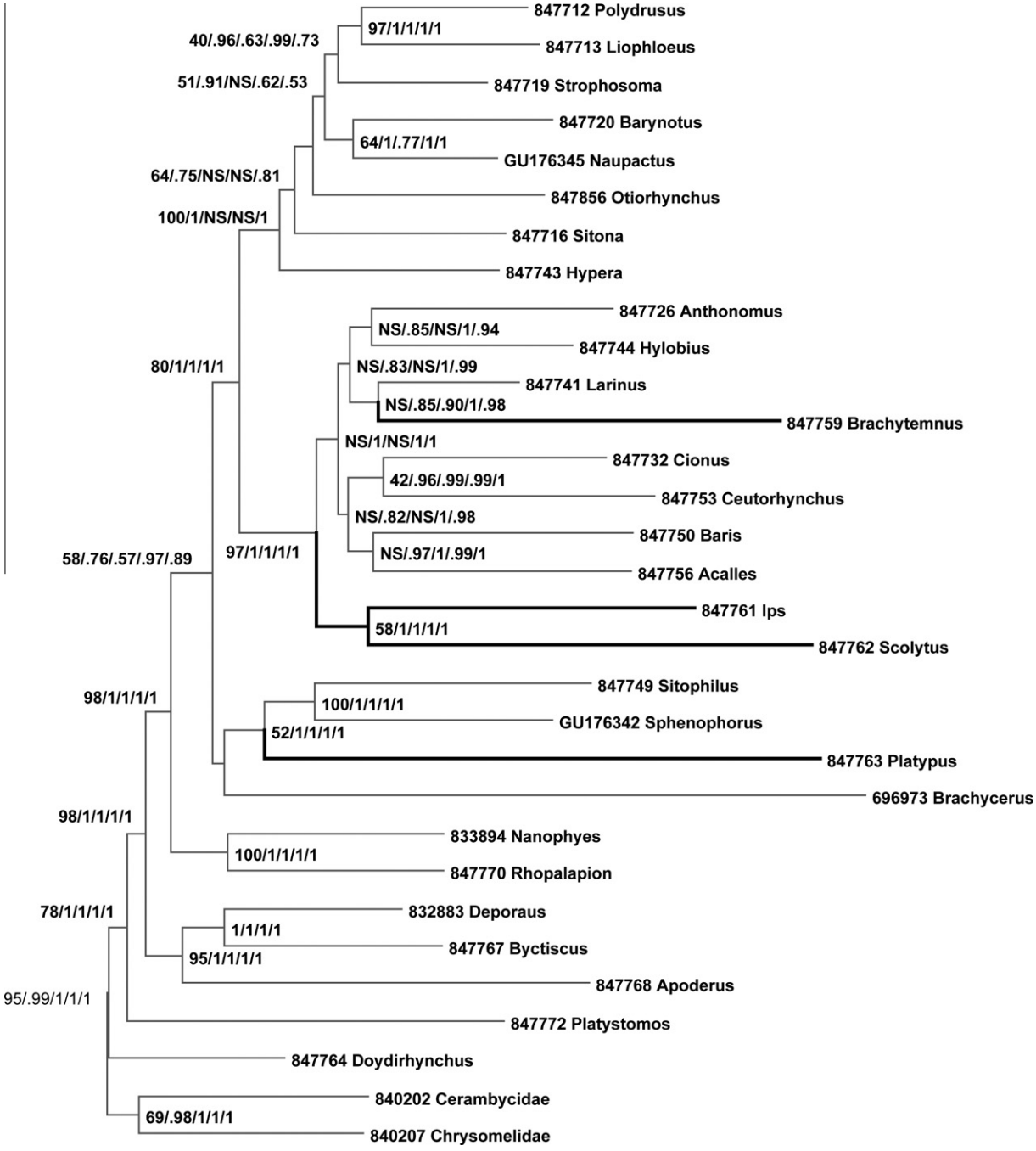

Fig. 3. Tree topology from Bayesian and ML analysis. Numbers on the branches represent bootstrap support from ML analysis/posterior probability values for the unpartitioned/twofold partitioned (forward and reverse strand)/3-fold partitioned (three codon positions)/sixfold partitions (forward and reverse strands, three codon positions. Thick branches mark the wood boring lineages.

cases reported previously (Timmermans and Vogler, 2012). These rearrangements were limited to the Entiminae/Hyperinae clade, and possibly indicate an initial $tRNA^{Ala}$–$tRNA^{Arg}$ gene order reversal, followed by two further independent changes in *Hypera* and *Sitona* which are sister groups in the nucleotide-based trees, indicating a stepwise evolution of rearranged mitogenomes. In addition, several insertions of the length of a tRNA gene and broad similarities with functional tRNAs were found in the distantly related representatives of *Acalles*, *Ceutorhynchus* and *Sitona*. These may be pseudogenes that result from gradual loss of function in supernumerary tRNA genes through replication errors (Dowton and Campbell, 2001). Pseudo-tRNA genes are widely known in insects (Shao and Barker, 2003), but these insertions are among the first to be described for the Coleoptera. They may be useful as diagnostic markers that can be easily assessed by simple tests of PCR fragment length. In addition, the mitogenome portion used in this study does not include the six tRNAs (I, Q, M, W, C, Y and V) whose organization may also contain translocations and insertions. Preliminary experiments have already shown additional modifications in these tRNAs in *Dorytomus* and *Scolytus*, and may provide further clade markers, while the $tRNA^{Ile}$ is deleted in *Naupactus* (Curculionidae) and *Sphenophorus* (Dryophthoridae), which are the only

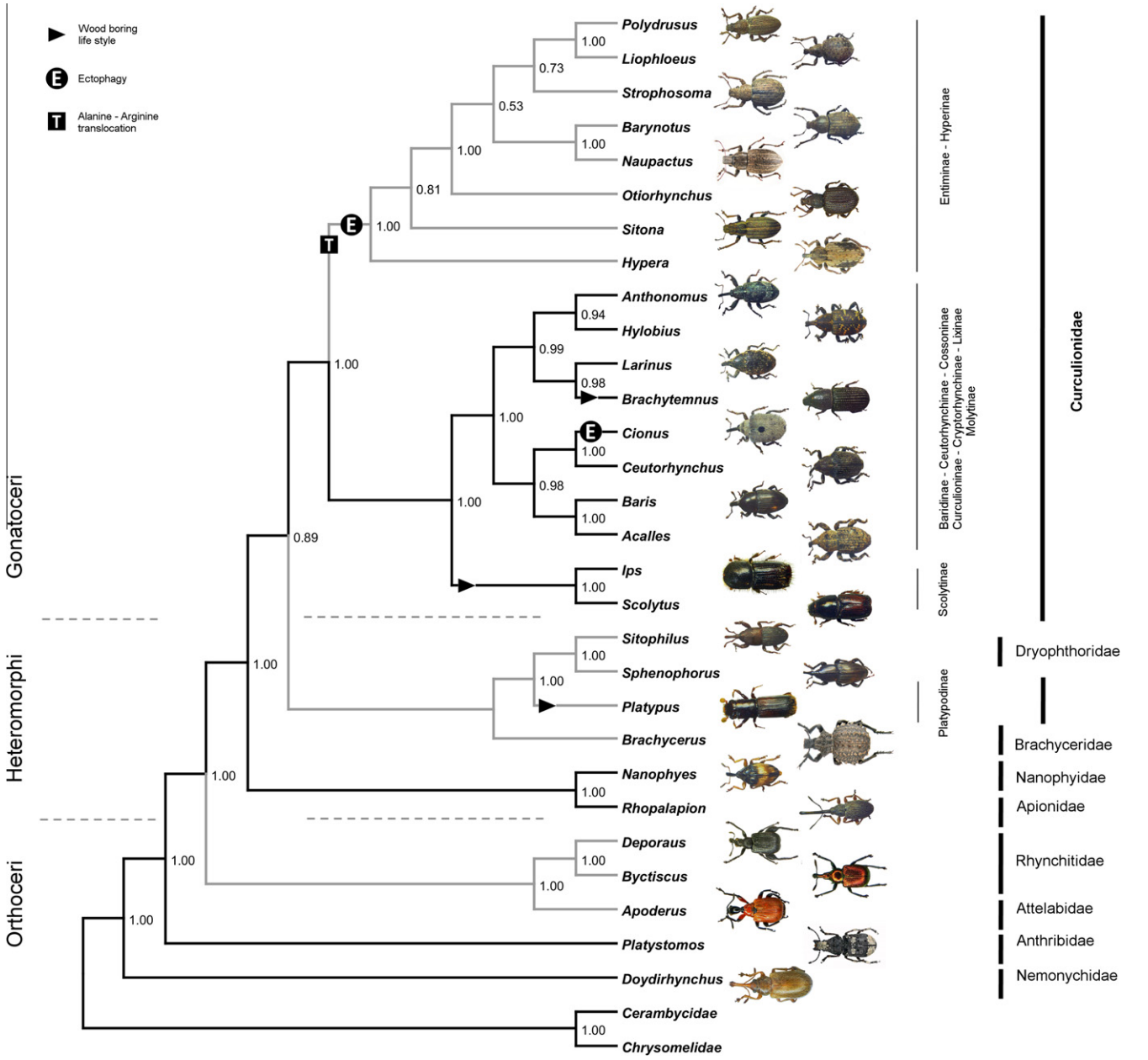

Fig. 4. Summary tree and taxonomic conclusions. The tree topology from the six-partition Bayesian analyses is shown with major taxonomic groups indicated. Key ecological traits including shifts to larval ectophagy (E) and wood boring (arrows) and the rearrangement in gene order (T) are mapped on the tree.

taxa sequenced for this region, and therefore this tRNA may be missing in weevil mitogenomes (Song et al., 2010; Timmermans and Vogler, 2012).

4.2. Weevil relationships and host plant use

The study focused on the most problematic parts of the weevil tree, the question about basal relationships in the huge family Curculionidae, and here mitogenomes made the greatest contribution by recovering two main clades that correspond to the Entiminae/Hyperinae and all other subfamilies. This leads to a major change in our understanding of the ecological-evolutionary organization of this family comprising 51,000 described species that to date has mostly been interpreted in the light of co-diversification with angiosperms (Farrell, 1998; McKenna et al., 2009). The primary phylogenetic split is correlated with the shape of the rostrum, which is short and thick in Entiminae and mainly long and thin in all others curculionids, except for the wood-tunneling Scolytinae and Cossoninae. However, the key trait of the broad-nosed clade is ectophagy of the larvae that generally feed on roots in the soil. This clade includes the long-rostrum Hyperinae whose larval feeding mode is ectophagous, as expected, but the larvae feed on leaves. *Hypera* occupied the basal node of the Entiminae, which may suggest that soil feeding has arisen in a secondary step once ectophagy had been acquired. In the other major clade, larvae are almost always endophagous, with exception of a few isolated groups such as *Cionus* or *Rhinoncus* (Ceutorhynchinae), and usually feed on the above ground parts of plants.

It is unclear how the larval feeding style is relevant to the adult rostrum morphology, but a key role of the rostrum is in egg deposition into the substrate, whereby rostrum length is directly correlated with the depth of oviposition holes (e.g. Anderson,

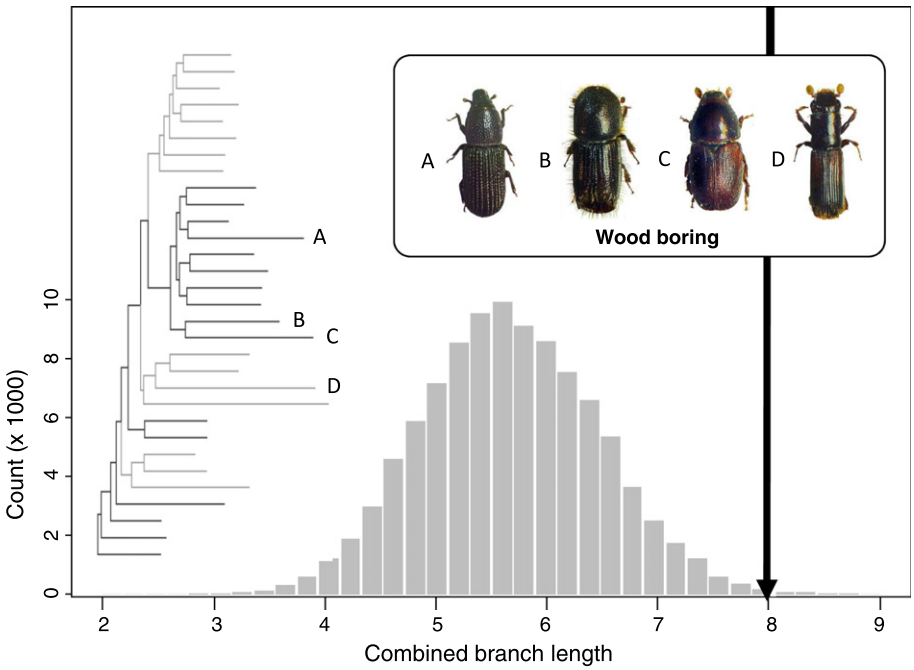

Fig. 5. Branch lengths in wood boring lineages. Root-to-tip branch lengths were summed for quartets of randomly selected terminals using the branch lengths shown in the tree on the left. The graph shows the distribution of branch lengths from 10,000 random draws, whereby values were grouped into categories of 0.2 units and the number of occurrences in each category was plotted. The value for the quartet of wood boring lineages (representatives of Platypodinae, Cossoninae and two species of Scolytinae) is given by the arrow.

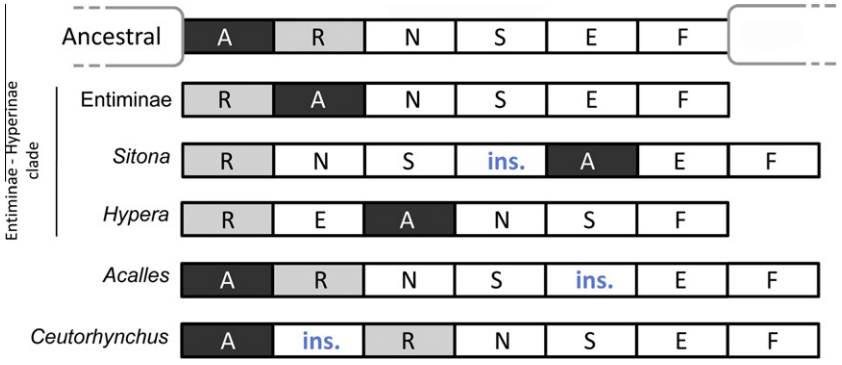

Fig. 6. tRNA arrangements found in weevil mitochondrial genomes. The ancestral order for a tRNA cluster between *nad3* and *nad5* and rearranged tRNA genes and sequence insertions are represented by red letters and blue boxes. Letters refer to the corresponding tRNA amino acids in standard one-letter codes. Identification of tRNA and extent of genes was based on model predictions with the COVE algorithm (see Suppl. Fig. 2 for details). ins, insertion of tRNA-like sequences.

1995; Hughes and Vogler, 2004). Oviposition in soil may not require this function and therefore the rostrum in these groups is comparatively stout and potentially more powerful for gnawing through soil substrate for oviposition. It is also clear that while rostrum shape is a phylogenetically conservative trait, it still shows homoplasy, e.g. in the broad-nosed Brachyceridae shown here to be placed outside of the Curculionidae clade. Likewise, wood tunneling is homoplastic according to the phylogenetic conclusions established here, as Scolytinae, Cossoninae and Platypodinae are not their closest relatives and each is derived from within a lineage that does not show the tunnel-forming habit. The recognition of these convergences is an important step in devising a natural classification, superseding earlier views that treat all xylophagous and all broad-nosed weevils as monophyletic (e.g. Kuschel, 1995).

In conclusion, the mitogenome data now stabilize the basal relationships of Curculionoidea and support a topology that is highly congruent with the six-gene tree of McKenna et al. (2009). The overall organization of Curculionoidea in the mitogenome tree agrees with the well-established basal branching of Nemonychidae, Anthribidae and Attelabidae (Hundsdoerfer et al., 2009; Hunt et al., 2007; McKenna et al., 2009), which were only superficially sampled here. The grouping of *Rhopalapion* and *Nanophyes* supports the latter as a member of Brentidae despite a long scape at the base of the antennae, which links it to the Curculionidae and has confused its position (Alonso Zarazaga and Lyal, 1999; Thompson, 1992). The next node was the only one in the backbone of the tree with poor support regarding the relationship of Brachycerinae and Dryophthoridae/Platypodidae. However, the sister relationship of the latter two groups were strongly supported by the mitochondrial genomes, which corroborates the finding from larval characters (Marvaldi, 1997) and the six-gene tree (McKenna et al., 2009), and may be supported by the partial tunneling behavior of dryophthorids in various monocots. Finally, the arrangement in the Curculionidae matches closely the topology of McKenna et al.

(2009), including the split of Entiminae/Hyperinae versus the remaining subfamilies, and the position of Scolytinae as the sister to all others in the latter clade. This leaves the question why Jordal et al. (2011) find the close association of Scolytinae and Platypodinae. That study differed from all others by the much denser taxon sampling of wood boring lineages, but lower sampling of non-boring lineages. When 'transitional' taxa such as *Mecopelmus*, *Coptonotus*, and *Schedlarius* were removed from the analyses, their study also recovered the Platypodinae/Dryophthorinae sister relationship, suggesting the finding of the current study may be due to the omission of critical taxa. Sensitivity to taxon sampling is frequently the result of long-branch attraction (e.g. Pick et al., 2010), but as we showed here (Fig. 5), the three wood-boring lineages exhibit higher molecular rates than the average of all terminals in the data set, and yet they occupy distant positions in the tree. Therefore, long-branch attraction is unlikely to have affected the topology, but ultimately this can only be established by sequencing mitochondrial genomes for additional wood boring taxa. However, if it can be confirmed that molecular rates increased independently in these lineages, this raises intriguing questions about a causal link with wood boring life style.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2012.12.022.

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