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The phylogeny of leaf beetles (Chrysomelidae) inferred from mitochondrial genomes

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Abstract. The high-level classification of Chrysomelidae (leaf beetles) currently recognizes 12 or 13 well-established subfamilies, but the phylogenetic relationships among them remain ambiguous. Full mitochondrial genomes were newly generated for 27 taxa and combined with existing GenBank data to provide a dataset of 108 mitochondrial genomes covering all subfamilies. Phylogenetic analysis under maximum likelihood and Bayesian inference recovered the monophyly of all subfamilies, except that *Timarcha* was split from Chrysomelinae in some analyses. Three previously recognized major clades of Chrysomelidae were broadly supported: the 'chrysomeline' clade consisting of (Chrysomelinae (Galerucinae + Alticinae)); the 'sagrine' clade with internal relationships of ((Bruchinae + Sagrinae) + (Criocerinae + Donaciinae)), and the 'eumolpine' clade comprising (Spilopyrinae (Cassidinae (Eumolpinae (Cryptocephalinae + Lamprosomatinae)))). Relationships among these clades differed between data treatments and phylogenetic algorithms, and were complicated by two additional deep lineages, *Timarcha* and Synetinae. Various topological tests favoured the PhyloBayes software as the preferred inference method, resulting in the arrangement of (chrysomelines (eumolpines + sagrines)), with *Timarcha* placed as sister to the chrysomeline clade and Synetinae as a deep lineage splitting near the base. Whereas mitogenomes provide a solid framework for the phylogeny of Chrysomelidae, the basal relationships do not agree with the topology of existing molecular studies and remain one of the most difficult problems of Chrysomelidae phylogenetics.

Introduction

The Chrysomelidae (leaf beetles) is one of the largest families of Coleoptera and includes nearly 40 000 species according to a recent count (Leschen & Beutel, 2014). Leaf beetles are of considerable ecological and economic significance (Reid, 1995, 2000). They also have been the subject of evolutionary studies, in particular because of their hypothesized co-radiation with the angiosperms (flowering plants) that presumably promoted their great species diversity (Farrell, 1998). The Chrysomelidae feed on virtually any species of angiosperm, drawn from all major branches including dicotyledons, magnoliids and monocotyledons. These associations may have existed since the origin of

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angiosperms in the Triassic (Zeng *et al.*, 2014; Li *et al*., 2019), which leads to the expectation that the phylogeny of leaf beetles broadly matches the evolution of their host plants, including the split of monocotyledons from other lineages (Mitter & Farrell, 1991; Farrell, 1998). However, basal relationships of Chrysomelidae and the evolution of monocot feeding remain partly unclear, despite the long history of molecular and morphological phylogenetic analyses in this group (Farrell, 1998; Gómez-Zurita *et al*., 2007, 2008; Hunt *et al*., 2007; Matsumura *et al*., 2014; McKenna *et al*., 2015; Timmermans *et al*., 2015).

The current classification of Chrysomelidae involved the gradual recognition of major clades. Starting from Latreille (1802), who first proposed the leaf beetles, Chrysomelines, and several subgroups (Cryptocephalinae, Chlamysinae, Clytrinae, Lamprosomatinae, Eumolpinae, Alticinae, Galerucinae and Chrysomelinae), many modifications to the classification have been suggested. Chapuis (1874) developed the first comprehensive system that is the foundation of the modern classification in use today and included four sections and 15 tribes. Jacoby (1908) modified Chapuis' system to include five sections and 16 subfamilies, which were then used by Chen (1940, 1964) to propose a new classification of all Chrysomeloidea separated into six divisions, each of which were elevated to family rank. Several elements of this system match the contemporary understanding of the major subdivisions (see Discussion). The more recent consensus classification, that provided by Löbl & Smetana (2010), uses 13 subfamilies based on the system of Lawrence & Newton (1995) and is widely used today.

In addition to efforts to establish an evolutionary classification, numerous studies since the 1950s have attempted to determine the phylogeny of Chrysomelidae (Crowson, 1955, 1960; Furth, 1988; Suzuki, 1988, 1994; Konstantinov, 1994; Reid, 1995, 2000; Lawrence *et al*., 2011). Schmitt (1996) gave a comprehensive review on the phylogenetic system of the Chrysomelidae based on 18 source papers, but these generally demonstrated little agreement with one another and no summary cladogram is available. Reid's (2000) reanalysis of subfamily relationships in the Chrysomeloidea based on existing morphological (38 characters) and newly added larval (18 characters) data produced a widely cited hypothesis of the relationships of subfamilies and found several cases of close affinity such as a sister group relationship between subfamilies Lamprosomatinae and Cryptocephalinae, Galerucinae and Chrysomelinae, Bruchinae and Sagrinae, and Criocerinae and Hispinae (Cassidinae). The study also recovered Megascelidini within Eumolpinae, and elevated Spilopyrini to subfamily Spilopyrinae. Molecular studies of Chrysomelidae were initially conducted in combination with morphological data (Farrell, 1998; Kim *et al*., 2003; Duckett *et al*., 2004; Farrell & Sequeira, 2004; Gómez-Zurita *et al*., 2005; Kergoat *et al*., 2008) or focused on subgroups within Chrysomelidae (Kölsch & Pedersen, 2008; Ge *et al*., 2011, 2012; Chaboo *et al*., 2014; Matsumura *et al*., 2014; Wang *et al*., 2014; Nie *et al*., 2018), or were part of wider analyses of all Coleoptera (Hunt *et al*., 2007; Bocak *et al*., 2014; Zhang *et al*., 2018). Only the studies of Gómez-Zurita *et al*. (2007, 2008) focused specifically on the basal relationships of Chrysomelidae with the aim of comprehensively covering all major groups using three markers, including mitochondrial *rrnL* (16S) and nuclear 18S and 28S rRNA genes. This study defined three major sublineages, the 'chrysomelines', 'eumolpines' and 'sagrines'. The composition of these clades and the remaining problematic issues in their internal relationships are discussed in the following.

1. The 'chrysomeline' clade includes Chrysomelinae, Galerucinae and Alticinae. The latter two are easily recognized as a monophylum based on morphological and molecular data (Lee, 1993; Daccordi, 1994; Furth & Suzuki, 1994; Farrell, 1998; Gómez-Zurita *et al*., 1999, 2007, 2008; Lingafelter & Konstantinov, 1999; Duckett *et al*., 2004; Farrell & Sequeira, 2004; Hunt *et al*., 2007; Bocak *et al*., 2014; McKenna *et al*., 2015; Nie *et al*., 2018) and can be separated into two reciprocally monophyletic groups after correctly placing some so-called 'problematic genera' [see Ge *et al*. (2012) for a list] that exhibit conflicting morphological character combinations (Farrell & Sequeira, 2004; Ge *et al*., 2011, 2012; Nie *et al*., 2018). Reid (1995) proposed that Alticinae should be subsumed within Galerucinae. By contrast, the inclusion of *Timarcha*, an isolated genus consisting of more than 100 species (Gómez-Zurita *et al*., 2000; Gómez-Zurita, 2004), within Chrysomelinae remains unclear. The group exhibits several plesiomorphic characters such as genitalia with a ring-like tegmen, covered with a setose parameral cap, together with apomorphic characters, including apterism and fused elytra (Jolivet *et al*., 2013). *Timarcha* has been proposed as the sister group to Chrysomelini (Reid, 1995, 2000), nested within Chrysomelini (Duckett *et al*., 2004), or as at the root of the chrysomeline clade (Gómez-Zurita *et al*., 2007, 2008; Wang *et al*., 2014).
2. The 'sagrine' clade includes Bruchinae, Donaciinae, Criocerinae and Sagrinae and possibly Synetinae. Bruchinae are unique among chrysomelids for feeding on seeds and were initially considered to represent a separate family within Chrysomeloidea (Crowson, 1955; Kingsolver, 1995; Reid, 1996; Duckett, 1997; Verma, 1998), but now are firmly established as a subfamily within Chrysomelidae (Reid, 1995, 2000; Farrell & Sequeira, 2004; Gómez-Zurita *et al*., 2008; Bocak *et al*., 2014). Donaciinae and Criocerinae, as well as some bruchids associated with palms, predominantly feed on monocots, which in the case of Donaciinae are mostly associated with aquatic habitats. The relationship of ((Bruchinae + Donaciinae) Criocerinae) was strongly supported (Farrell, 1998; Gómez-Zurita *et al*., 2007, 2008; Wang *et al*., 2014), with the likely inclusion of Sagrinae, omitted from these earlier studies, in this clade.
3. The 'eumolpine' clade includes several subfamilies: Cryptocephalinae sensu lato (after the inclusion of Clytrinae and Chlamysinae, as proposed by Reid (1995), Lamprosomatinae [including Sphaerocharitinae (Reid, 1995)], Eumolpinae and Cassidinae [including Hispinae (Reid, 1995)]. Within the eumolpine clade, the close association of Cryptocephalinae s.l., Lamprosomatinae and Eumolpinae has been widely supported (Farrell, 1998; Hunt *et al*., 2007), but the position of the partly monocot-feeding Cassidinae is less clear

and has been considered either as the sister group of Cryptocephalinae s.l. (Duckett *et al*., 2004; Gómez-Zurita *et al*., 2007), as the sister group of the other three subfamilies (Hunt *et al*., 2007), or even outside the eumolpines as a sister group of Galerucinae (Reid, 1995; Chaboo, 2007). The subfamily Hispinae is now treated within Cassidinae s.l. (Chaboo, 2007; Borowiec & Świetojańska, 2014).

Two small subfamilies, Spilopyrinae and Synetinae, remain difficult to place but generally have been associated with the eumolpines. The Spilopyrinae is a small group with disparate distribution in the southern hemisphere and was elevated to subfamily status by Reid (2000), based on larval and adult characters indicating that Spilopyrinae formed an independent clade or was sister to Lamprosomatinae + Cryptocephalinae + Eumolpinae + Synetinae. Molecular data similarly place it in the vicinity of Eumolpinae (Gómez-Zurita *et al*., 2005), Lamprosomatinae (Farrell & Sequeira, 2004) or as sister to all other groups in the eumolpine clade (Gómez-Zurita *et al*., 2005). Similarly, the position of the species-poor [11 species (Lawrence & Ślipiński, 2014)] subfamily Synetinae remains unresolved; it was placed into Eumolpinae in some studies (Reid, 1995, 2000; Farrell & Sequeira, 2004), as a sister group to Eumolpinae (Farrell, 1998; Reid, 2000; Gómez-Zurita *et al*., 2007, 2008), related to Chlamysini (Marvaldi *et al*., 2009), or treated as an early-branching lineage at subfamily rank (Farrell, 1998; Verma & Jolivet, 2000; Gómez-Zurita *et al*., 2005, 2007; Jolivet & Verma, 2008) or at the root of the chrysomeline clade (Duckett *et al*., 2004).

Although the existing work has resulted in a fairly satisfactory classification of the major groupings of Chrysomelidae, the relationships among the three main lineages and some relationships among the widely recognized subfamilies are not well established (Haddad & McKenna, 2016). Some of the smaller groups, such as Spilopyrinae, Synetinae, Sagrinae and Lamprosomatinae, have been omitted from various analyses, whereas other groups, in particular the subfamily Cassidinae and the enigmatic *Timarcha*, have been placed inconsistently. The uncertainty is likely to reflect the rather limited gene and taxon sampling, most of which has consisted of small fragments such as 18S and 28S rRNA genes and a few mitochondrial genes.

Mitochondrial genomes can be obtained readily by shotgun sequencing from total genomic DNA. They have proven to be powerful markers to resolve relationships among deep Coleoptera lineages (Sheffield *et al*., 2008, 2009; Pons *et al*., 2010; Timmermans *et al*., 2016) and within Chrysomelidae, such as to address the issue of Galerucinae–Alticinae relationships (Nie *et al*., 2018). In addition to the nucleotide data themselves, mitogenome rearrangements and changes in anticodon sequences are useful clade markers (Dowton *et al*., 2003, 2009; Timmermans & Vogler, 2012; Cameron, 2014). In the present work, we use mitochondrial genomes to address the subfamily-level relationships of Chrysomelidae, with particular focus on the relationships among the three main clades of Chrysomelidae and the positions of several other lineages whose affiliations remain problematic.

Materials and methods

Taxon sampling and sequencing

Genomic DNA was extracted from 27 species representing 10 subfamilies with a DNeasy Blood and Tissue Kit (Qiagen Sciences, Inc., Germantown, MD, U.S.A.). Mitochondrial genomes were sequenced using shotgun sequencing of total genomic DNA on the HiSeq2000 platform (Illumina, Inc., San Diego, CA, U.S.A.) using libraries with an insert size of 200 bp and paired-end sequencing of 100 bp. Distantly related species were sequenced in pooled libraries of maximally 10 samples. The sequence reads were first filtered following Zhou *et al*. (2013) and high-quality reads were assembled using SOAPdenovo-Trans (Xie *et al*., 2014). Contigs from mixed libraries were linked to a particular species by matches to Sanger sequenced *cox1* and *rrnL* gene fragments used as baits (Timmermans *et al*., 2010) obtained with primers given in Appendix S1. A minimum of 98% identity in the BLAST alignment was required for a positive identification. In all cases the two baits obtained from a single specimen matched the same contig, indicating the absence of chimeras in the assembly from the mixed shotgun reads. Annotations of genes were performed in GENEIOUS 8.0.5 (Kearse *et al*., 2012) and tRNAscan-SE 1.21 (Schattner *et al*., 2005), using *Diabrotica barberi* (GenBank: NC_022935) as a reference. Finally, each protein-coding and rRNA gene were exported separately for phylogenetic analyses. All new specimen identifications were made by co-author XKY and Drs Hongbin Liang and Fengyan Wang (Beijing). Voucher specimens for all newly sampled taxa are kept at the Institute of Zoology, Chinese Academy of Sciences. Mitogenomes from 81 additional species were obtained from the National Center for Biotechnology Information (NCBI) (Bethesda, MD, U.S.A.) or from various published studies (Gómez-Rodríguez *et al*., 2015; Nie *et al*., 2018; Zhang *et al*., 2018), which included five species of Cerambycidae and Vesperidae as outgroups (Appendix S2).

Measures of nucleotide variation

Base composition was calculated in MEGA Version 6.06 (Tamura *et al*., 2013). The heterogeneity of nucleotide variation among sequences was analysed separately for different datasets with AliGROOVE (Kück *et al*., 2014). This method establishes non-random similarity between any two sequences at each site in a matrix of pairwise comparisons, relative to the variation in the entire set of sequences. The sliding window size was applied under default settings and indels were treated as ambiguity. The BLOSUM62 matrix was used as a substitution model when assessing heterogeneity in the translated amino acid sequences.

Phylogenetic and topology test analysis

Protein coding genes (PCGs) were aligned with TransAlign (Bininda-Emonds, 2005) and rRNA gene sequences were aligned with MUSCLE Version 3.8.31 (Edgar, 2004), under

default parameters. The aligned data from each locus were concatenated with SequenceMatrix Version 1.7.8 (Vaidya *et al*., 2011). Phylogenetic relationships were inferred from various combinations and partitioning schemes of rRNA genes and 13 protein coding genes (PCGs) based on 108 taxa, as follows: (i) 13 PCGs (*13PCGs*), (ii) 13 PCGs partitioned by the first and second codon positions with the third position removed (*13PCGs-codon12*), (iii) amino acids of 13 mitochondrial PCGs (*13PCGs-AA*), and (iv) the combination of two rRNA genes and 13 PCGs (*15-genes*).

Phylogenetic inferences were performed using PhyloBayes MPI Version 1.5a (Lartillot *et al*., 2013), MrBayes Version 3.2 (Ronquist & Huelsenbeck, 2003) and RAxML (Stamatakis, 2006; Stamatakis *et al*., 2008). In the PhyloBayes analysis (all four datasets above) the CAT-GTR model was used. Two chains were run until the likelihood had satisfactorily converged (maxdiff < 0.1).

In the MrBayes analysis, the following datasets and partition schemes were used: (i) *13PCGs* partitioned using PartitionFinder2 (Lanfear *et al*., 2016) offering partitions by gene and codon, (ii) *13PCGs-codon12* by codon, and (iii) *15-genes* using PartitionFinder2 offering partitions by gene and codon. The most appropriate nucleotide substitution model was selected in jModelTest 0.1.1 using the Akaike information criterion (AIC) (Posada, 2008), which was determined for each *15gene* partition by gene. The Markov Chain Monte Carlo (MCMC) search was conducted for a minimum of 100 000 000 generations, and sampling was done every 1000 generations until the average standard deviation of split frequencies was less than 0.01. The first 25% of trees were discarded as burn-in and posterior probabilities were estimated for each node. The effects of different model complexities were tested by implementing: (i) a Hasegawa–Kishino–Yano (HKY) model applied to the setting partitions by gene and by codon, which was partitioned by PartitionFinder2, with the parameters set as Lset applyto = (all) nst = 2, rates = invgamma; prset applyto = (all) ratepr = variable, and (ii) a general time reversible (GTR) model, with the settings Lset applyto = (all) nst = 6 rates = invgamma, Pset parameter with four base frequency parameters and six substitution rate and shape value parameters determined by jModelTest (AIC criterion).

The RAxML search (all four datasets above) was performed in CIPRES (Miller *et al*., 2010) and node support was assessed by performing 1000 rapid bootstrap replicates. The tree was rooted post hoc using *Spiniphilus spinicornis* (Vesperidae) (GenBank accession no. NC_029515). The GTR-CAT model was chosen for the bootstrapping phase. All analyses were performed on the *15-genes* dataset, and the ways in which different partitioning schemes affect the topology and likelihood values were tested. Partitions analysed were as follows: un-partitioned ($n = 1$); partitions determined by PartitionFinder2 allowing partitions by gene ($n = 14$); partitions by gene ($n = 15$); partitions selected by PartitionFinder2 offering partitions by gene and codon position ($n = 36$), and partitions by gene and codon position ($n = 41$). IQtree (Nguyen *et al*., 2015) was used to build majority rule consensus trees from primary trees obtained under various parameters.

Substitution rates of combined data of *15genes* from 108 species were calculated using phyloFit (Siepel & Haussler, 2004) in PHAST Version 1.4 under the generalized HKY85 substitution model and fitted onto the phylogenetic tree (Appendix S7). The GC content of each gene was calculated for each species using iTOL tools and results were presented as a heat map (Letunic & Bork, 2016). Substitution rates for all *15genes* combined were calculated for each subfamily. Differences in these values between subfamilies were assessed using analyses of variance (ANOVAS) and a *t*-test in a comparison against the subfamily Cryptocephalinae, which showed the highest rate.

The IQtree software was also used to assess the significance of differences in tree topologies by determining the likelihood of the data under topological constraints according to alternative tree searches, using various statistical tests, including the Kishino–Hasegawa (KH), Shimodaira–Hasegawa (SH) and approximately unbiased (AU) tests.

Critical nodes defining basal relationships in Chrysomelidae were analysed by four-cluster likelihood mapping (FcLM) in TREE-PUZZLE Version 5.2 (Schmidt *et al*., 2002; Schmidt & Von Haeseler, 2007). The method assesses the support for alternative quartet topologies among terminals drawn from four *a priori* specified monophyletic groups. The likelihood of each quartet is presented as a triangle, the corners of which each represent one of the three possible alternative topologies. The concatenated matrix *13PCGs* and *15-genes* was used for these tests, as either a nucleotide sequence or an amino acid translation. The GTR + Γ + I model was applied (four gamma rates and invariable sites), with rate heterogeneity set as mixed.

Results

Generation of sequence data

The phylogenetic matrix included a total of 108 mitogenomes representing 13 subfamilies of Chrysomelidae and outgroups (Appendix S2). All 27 newly sequenced mitogenomes contained the entire set of 37 genes (13 PCGs, 22 tRNA genes and two rRNA genes) usually present in insect mitogenomes, and a large non-coding region (control region). The average GC composition was 25.3%. Among the 108 mitogenomes, 93 were complete and were at least 15 kb in length (range: 15.0–17.5 kb) and the remaining 15 mitogenomes ranged between 9.6 kb and 14.0 kb. The concatenated supermatrix included the complete set of 13 PCGs, with four exceptions (Appendix S2). All 108 sampled mitogenomes showed the derived UUU anticodon in tRNA-Lys unique to Chrysomelidae (Timmermans *et al*., 2016; Nie *et al*., 2018). The gene order followed the presumed ancestral arrangement of the insect mitogenome, except in Cryptocephalinae s.l., Lamprosomatinae and Eumolpinae, which were characterized by an inverted order of the tRNA-Arg and tRNA-Ala genes (indicated on the phylogenetic tree; Figs 1,2).

Heterogeneity of sequence variation was assessed with AliGROOVE, separately for different datasets. In general, the mitogenomes had low heterogeneity of sequence

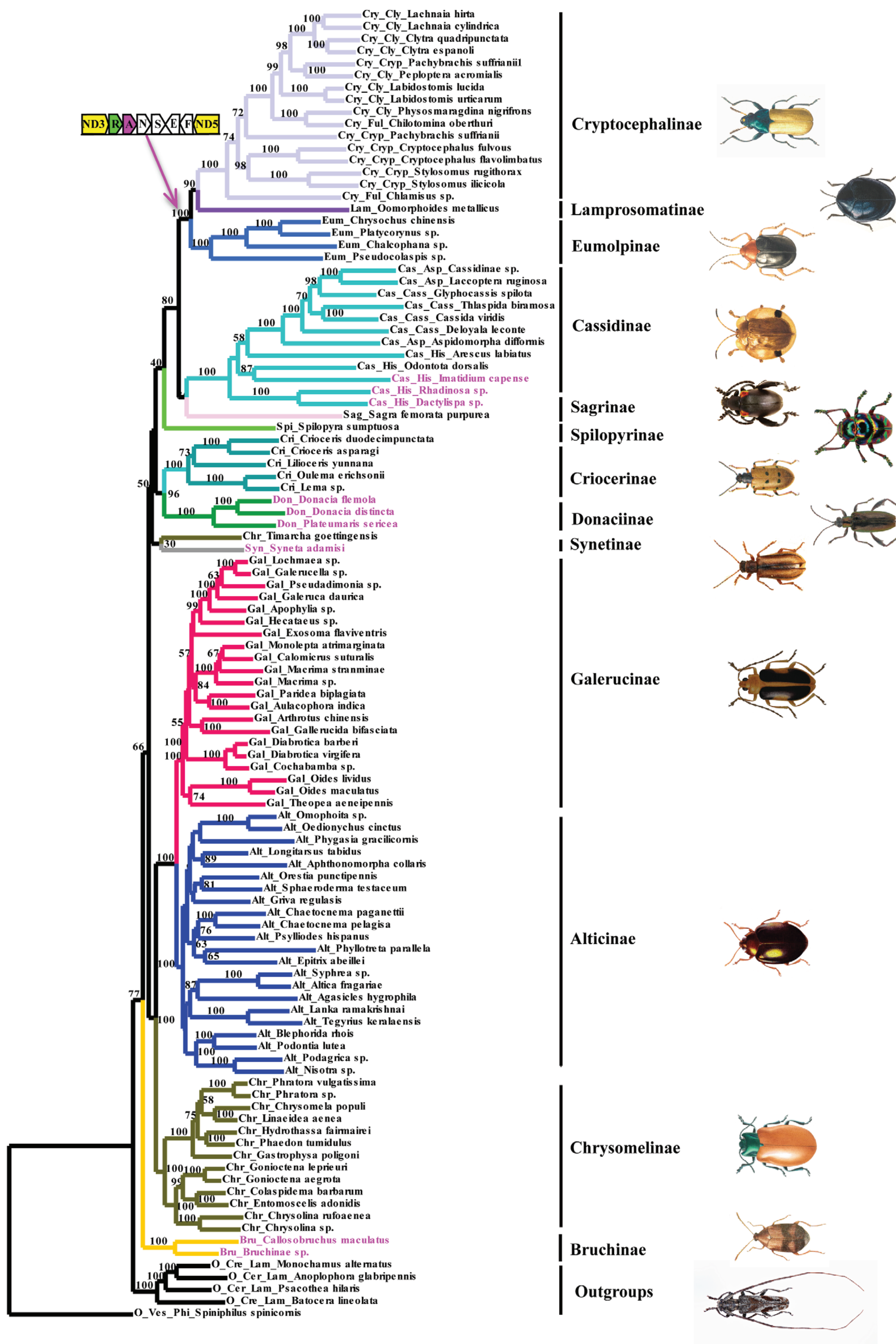

Fig. 1. RAxML tree based on combined data of *15genes* and partitioning by gene and codon positions. Numbers on each node are bootstrap support values. Taxon names in pink lettering indicate monocot feeding. Rearrangements of tRNA genes are indicated by the arrow. The subfamily habitus photographs are from Tan *et al.*, 1980, Yu *et al.*, 1996 and the Natural History Museum (BMNH), London. [Colour figure can be viewed at wileyonlinelibrary.com].

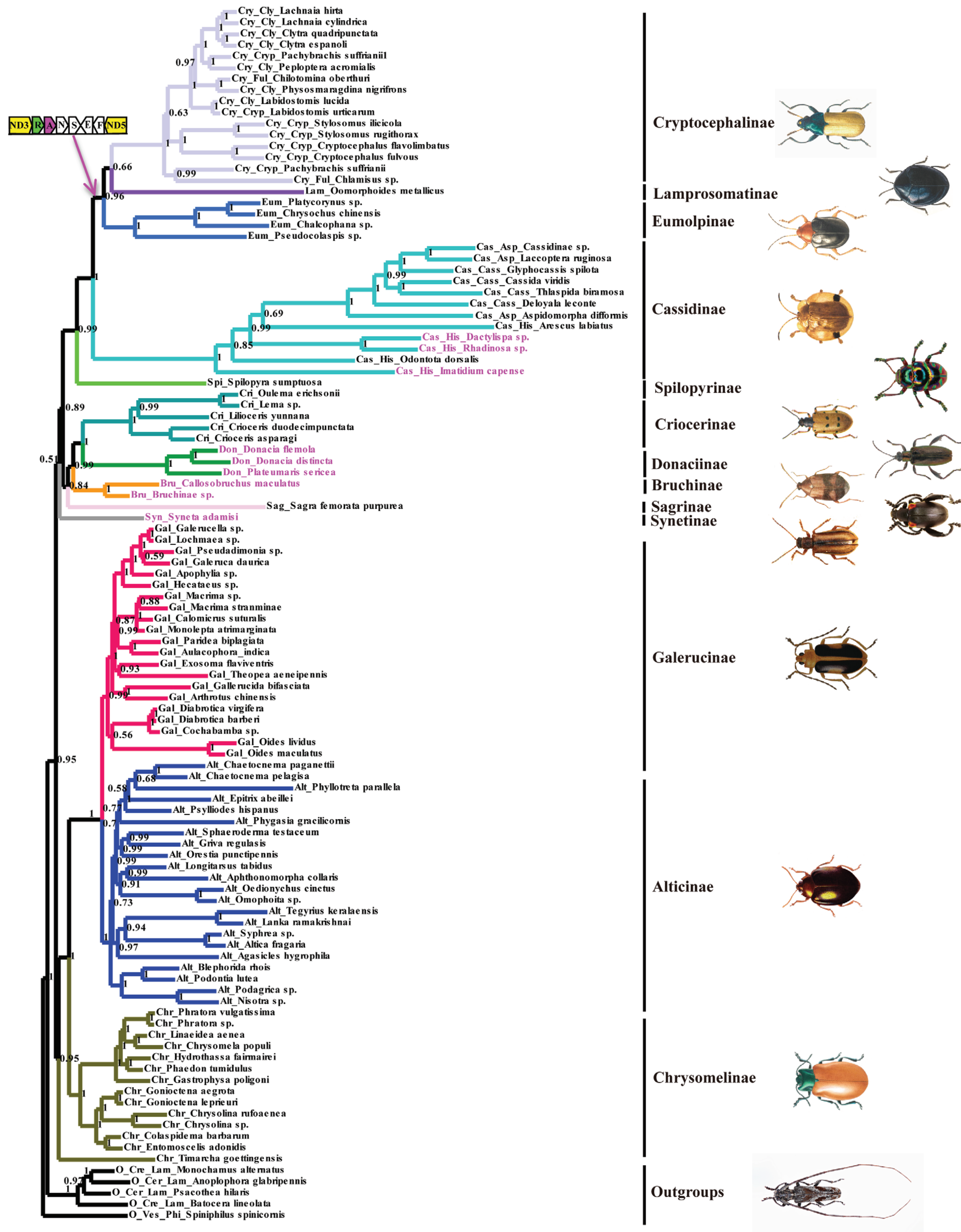

Fig. 2. PhyloBayes tree based on the combined amino acid dataset (*13PCGs-AA*). All legends as in Figure 1. [Colour figure can be viewed at wileyonlinelibrary.com].

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composition for most pairwise comparisons between the sequences. Heterogeneity according to this test was lowest for the chrysomeline clade (Chrysomelinae, Galerucinae and Alticinae) (Fig. 3), whereas heterogeneity in pairwise comparisons with members of other major lineages was higher. Four taxa (JX220988, JX220993, JX412789, JX220999), whose mitogenome sequences were nearly complete and did not show any obvious alignment errors, deviated from all others, but removing these taxa had no effect on the tree topologies.

The substitution rate for PCGs differed greatly among subfamilies ($P < 0.001$, ANOVA), and rates in the subfamily Cryptocephalinae in particular were significantly higher than those in most other subfamilies (Bruchinae, $P < 0.01$; other seven subfamilies, $P < 0.001$, *t*-tests) except Cassidinae ($P > 0.05$, *t*-test) (Fig. 4a, b, Appendices S4, S5). The high mutation rate correlated with elevated GC content (Fig. 4c), which makes Cryptocephalinae stand out from other clades. The subfamily Cassidinae also showed a high substitution rate (and was the only subfamily not to show a significantly different rate from that in Cryptocephalinae), whereas Synetinae had the lowest. GC content was also high (and not significantly different from that in Cryptocephalinae) in Synetinae and Lamprosomatinae, which were represented by a single sequence each. GC content for each gene was broadly correlated across taxa, and the *cox* genes and *cob* generally varied at higher rates than the *nad* and *rrnL* genes (Fig. 4a).

Tree topologies and phylogenetic relationships

We chose key groups to assess the tree topologies obtained under different data treatments (*13PCGs*, *13PCGs-condon12*, *13PCGs-AA*, *15-genes*) and with different tree construction methods (RAxML, MrBayes, PhyloBayes). Specifically, we recorded the monophyly of the three chrysomelid main clades (chyrsomelines, sagrines, eumolpines), the monophyly of the large subfamilies, the placement of the problematic small lineages *Timarcha* and Synetinae with each other and with other potential sister groups, and several nodes determining the internal relationships within the three main clades (Table 1). The overall structure of tree topologies was similar under all treatments, although with several differences at all hierarchical levels. Support levels were generally lower in the MrBayes and RAxML trees than in the PhyloBayes trees, and the MrBayes- and RAxML-based topologies were more comb-shaped and were affected by 'rogue taxa' whose positions varied between data treatments. We present the trees for RAxML analyses based on nucleotide data (Fig. 1), the MrBayes tree from nucleotide data (Appendix S10), and the PhyloBayes analysis of amino acid coded data (Fig. 2), and further trees using different coding and partitioning approaches in Appendices S7–13 and S15–17.

In general, all trees recovered the eight subfamilies represented by more than one member as monophyletic, except that *Timarcha* was separated from other Chrysomelinae in some cases. Most analyses also recovered the chrysomeline (with or without *Timarcha*), sagrine and eumolpine clades, reflecting the three deep clades of the Chrysomelidae. In addition, *Timarcha* and Synetinae (*Syneta*) added further deep branches (and in some cases were each other's sister group). The internal relationships within the three clades were largely uniform across the trees. In the chrysomeline clade, Chrysomelinae was sister to Galerucinae + Alticinae in all analyses. Similarly, the internal relationships in the eumolpine clade were largely uniform across all analyses. The basal split separated the Spilopyrinae from all others, followed by the Cassidinae, then Eumolpinae and finally a clade of Lamprosomatinae + Cryptocephalinae s.l., which was supported by the tRNA-Arg and tRNA-Ala translocation (Figs 1,2). Cryptocephalinae and Cassidinae were never seen as sister taxa, unlike in the findings of Gómez-Zurita *et al*. (2007).

Finally, the sagrine clade included ((Bruchinae + Sagrinae) + (Criocerinae + Donaciinae)) in the PhyloBayes trees (Fig. 2), whereas in the RAxML and MrBayes trees the Sagrinae was missing from the sagrine clade and instead was associated with the eumolpine clade (Fig. 1, Appendix S10). Similarly, the MrBayes tree removed Bruchinae from the remaining sagrines and placed it as sister to all other Chrysomelidae (Appendix S10), consistent with the traditional taxonomic status of the seed beetle family Bruchidae.

The relationships of the three main clades and the positions of *Timarcha* and *Syneta* differed among the tree construction methods. The PhyloBayes trees, as the only trees not to be affected by paraphyly of the three main clades, showed similar topologies for nucleotide- and amino acid-based analyses, which mainly differed in the placements of the root and of *Syneta* and *Timarcha*. The nucleotide-based tree split *Timarcha* + *Syneta* first, after which chrysomelines + sagrines formed the sister clade to eumolpines (Appendices S7–S9). Amino acid recoding defined a basal split of the chrysomelines including *Timarcha* from the sagrines + eumolpines with *Syneta* as sister to all of them (Fig. 2, Appendix S17). All other reconstructions of basal relationships were further complicated by the non-monophyly of sagrines and eumolpines, but principally also supported the sagrines + eumolpines versus chrysomelines, as was clearly evident in the RAxML tree (Fig. 1). This tree also supported the unexpected *Syneta* + *Timarcha* sister relationships, as seen in other nucleotide-based reconstructions.

Selection of the preferred topology

The effects of different data coding, model choice, partitioning strategy and phylogenetic inference method were investigated in order to establish the preferred tree topology. We first assessed how the models and partitioning schemes affected the topology and likelihood values in RAxML and MrBayes analyses. For RAxML based on the *15-genes* dataset, the different partitioning schemes (1, 14, 15, 36, 41 partitions) produced trees almost identical to that described above (Fig. 1; see Appendix S14 for a consensus tree), although the likelihood of the trees increased significantly with the increasing number of partitions (Appendix S3). For MrBayes analyses on the *15-genes* dataset, we tested different models under the partitioning scheme determined by PartitionFinder2 allowing partitions: (i) by gene ($n = 14$), and (ii) by gene and codon position ($n = 36$). We changed the complexity

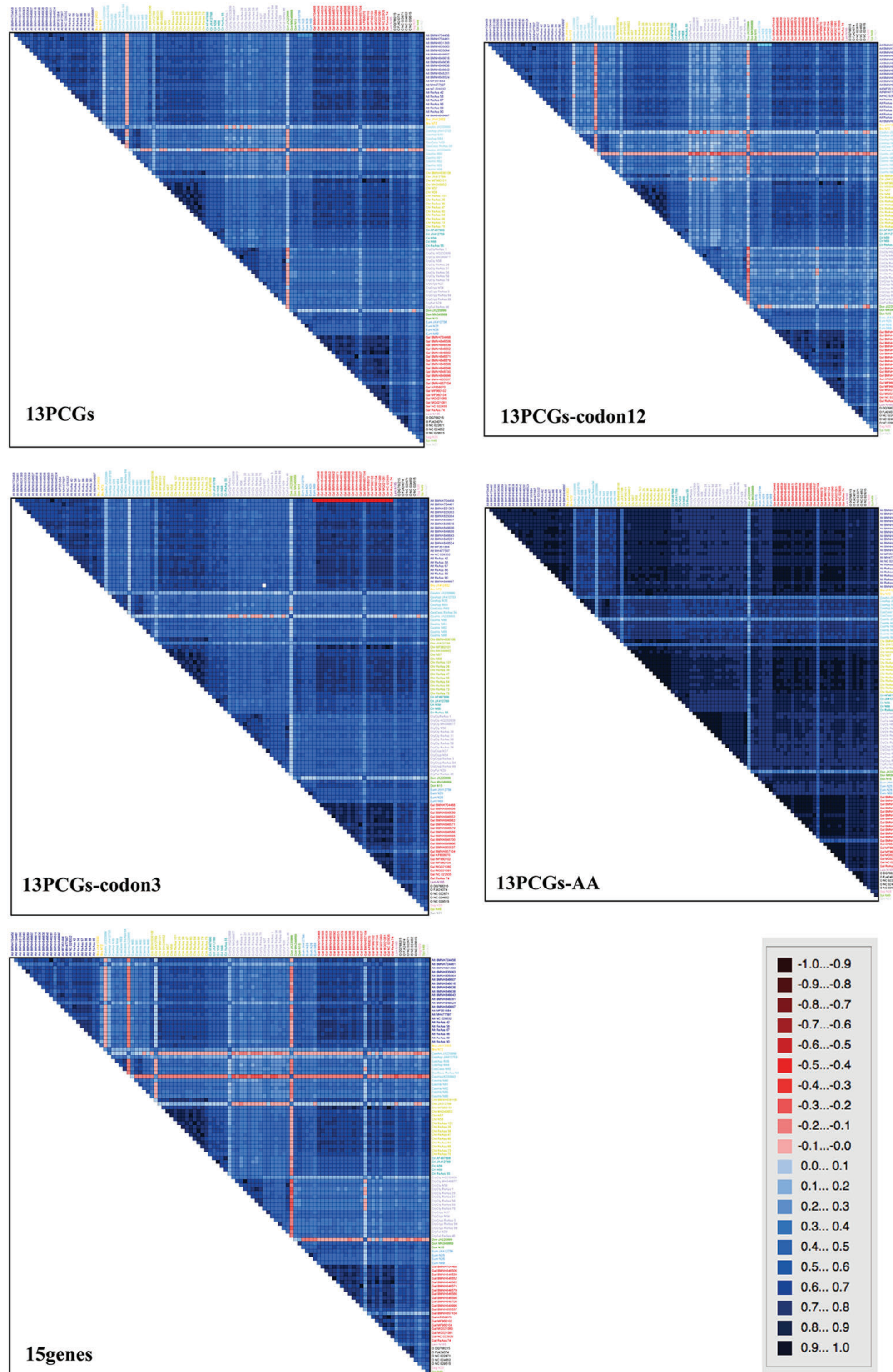
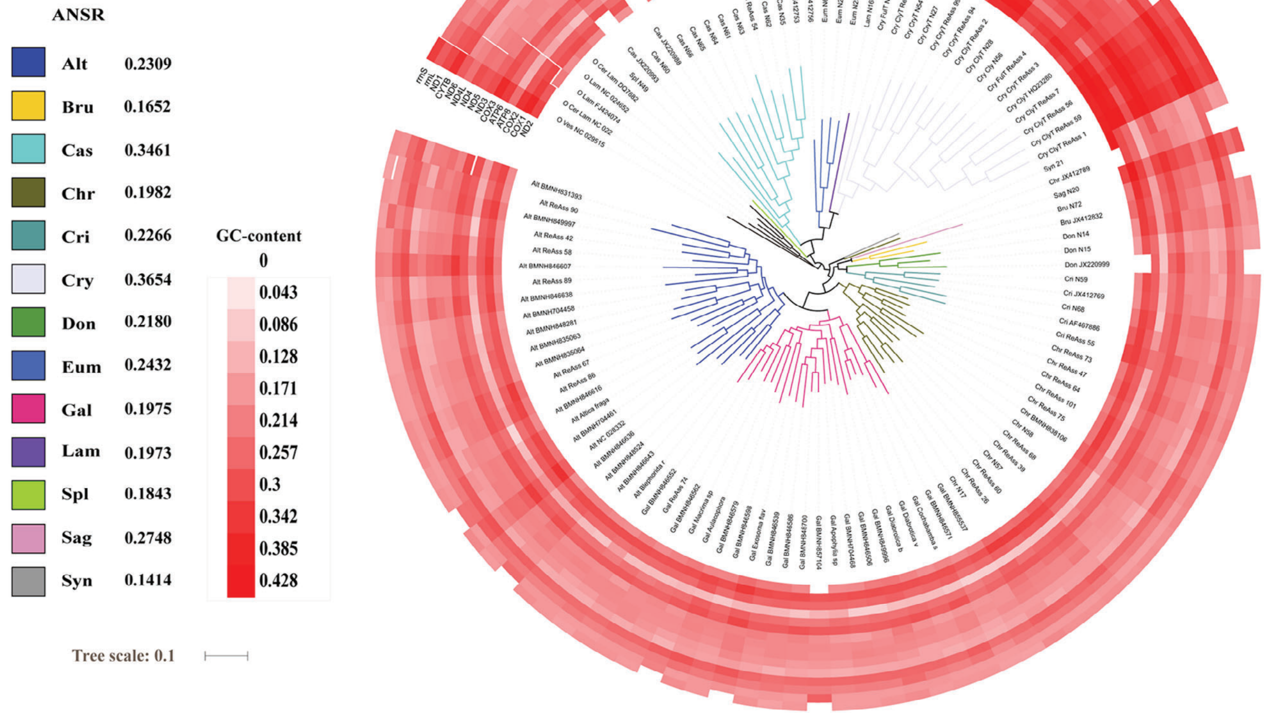
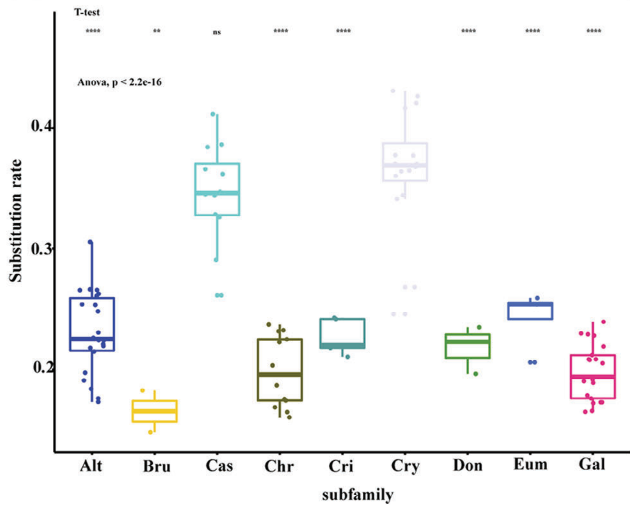


Fig. 3. Heterogeneity of sequence composition of mitochondrial genomes for different data sets. The pairwise Aliscore values are represented by coloured squares. The scores range from −1 indicating full random similarity (dark blue), to +1 indicating non-random similarity (bright orange). All taxon names of different subfamilies listed on top and on the right hand side of the matrix are colour-coded. [Colour figure can be viewed at wileyonlinelibrary.com].

(A)



(B)



(C)

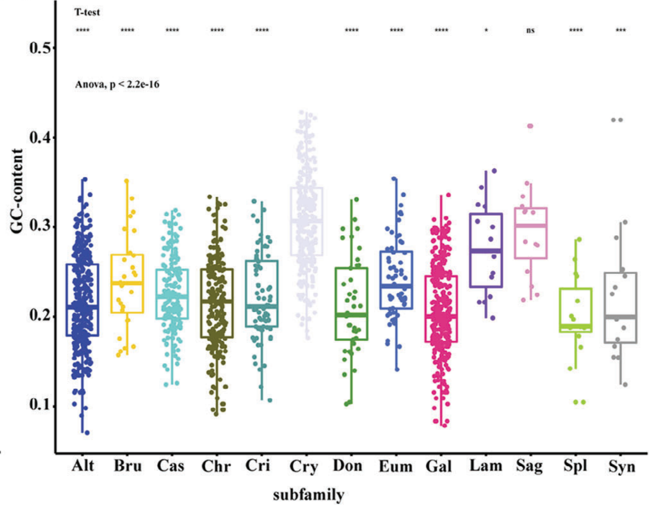


Fig. 4. Average nucleotide substitution rate (ANSR) and GC content of mitochondrial protein coding genes and rRNA genes in each subfamily. (a) Tree of Chrysomelidae based on the preferred topology with branch lengths shown at the centre, and heat map representing GC content for each gene corresponding to the respective terminals. Subfamilies were differentiated with coloured branches whose average nucleotide substitution rate are presented at the left. (b) ANOVA and *t*-test comparison of substitutions rate between subfamily Cry and other subfamilies. Only subfamilies with more than one terminal are presented. (c) ANOVA and *t*-test comparison of GC contents between subfamily Cry and other subfamilies. (*t*-test results between two subfamilies are shown on the top: ns, $P > 0.05$; *$P \leq 0.05$; †$P \leq 0.01$; ‡$P \leq 0.001$; §$P \leq 0.0001$). [Colour figure can be viewed at wileyonlinelibrary.com].

Table 1. Topological comparisons of PhyloBayes, Bayes and RAxML trees based on different datasets

		13PCGs (partitioned by gene)			*13PCGs-codon12*			*13PCGs-AA*		*15genes*			
Clades	Clades notes	PB AppS8	B nst = 6 AppS12	ML AppS15	PB AppS9	B nst = 6 AppS13	ML AppS16	PB Fig. 2	ML AppS17	PB AppS7	B nst = 2 AppS10	B nst = 6 AppS11	ML Fig. 1
1Cry		0.99	0.68	1	1	1	1	1	1	1	1	1	1
2Eum		0.99	0.87	1	1	1	1	1	1	1	1	1	1
3Cas		0.99	0.82	1	1	1	1	1	1	1	1	1	1
4Alt		0.99	1	1	1	1	1	1	0.98	1	1	1	0.99
5Gal		0.99	0.91	0.97	1	1	0.98	0.99	0.77	1	1	1	0.99
6aChr	without *Timarcha*	1	1	1	1	1	1	1	1	1	1	1	1
6bChr	with *Timarcha*	P	P	P	P	P	P	P	P	P	P	P	P
7Bru		0.99	0.96	1	1	1	1	1	1	1	1	1	1
8Cri		0.67	0.68	1	0.88	1	1	1	1	0.74	1	1	1
9Don		0.99	1	1	1	1	1	1	1	1	1	1	1
10CHR		0.99	0.58	1	1	1	1	1	1	1	1	1	0.9
11SAG		0.99	x	x	1	x	x	0.84	x	1	1	x	x
12EUM		0.99	0.53[a]	0.98	0.99	x	0.59	0.99	0.41	1	1[a]	1	0.18
13CHR	(Chry (Alt + Gal))	1	1	1	1	1	1	1	1	1	1	1	1
14a	SAG: ((Sag + Bru) (Crio + Dona))	0.99	x	x	0.96	x	x	x	x	1	x	x	x
14b	SAG: (Sag (Bru((Crio + Dona)))	x	x	x	x	x	x	0.84	x	x	x	x	x
15a	EUM: (Cass (Eum (Lamp + Cryp)))	1	1	1	0.99	1	1	1	1	1	1	1	1
15b	EUM: (Spi (Cass (Eum (Lamp + Cryp))))	0.99	1[a]	0.44	0.99	0.50[c]	0.50	0.99	0.41	1	0.5[a]	0.86[a]	0.40
16	SYN/TIM: (Syn + Tim)	0.93	1	0.8	0.79	x	x	x	x	0.98	x	0.86	0.30
17	TIM: (Tim (Chry ((Alt + Gal)))	x	x	x	x	x	x	0.95	0.27	x	x	x	x
18	SYN_basal (Syn + EUM)	x	x	x	x	x	x	0.51	x	x	x	x	x
19	Bruchinae vs. all other Chrysomelidae	x	x	0.66	x	0.49	0.98	x	0.96	x	0.81	x	0.77
20a	CHR (EUM + SAG)	x	x	0.43[b]	x	x	x	0.95	0.96	x	x	x	0.67
20b	EUM (CHR + SAG)	0.89	1[a]	x	0.59	x	x	x	x	0.94	x	0.86[b]	x

PB, PhyloBayes tree; B, MrBayes tree; RAxmL, RAxML tree; P, Paraphyly; x, a feature is not present.
1Cry, Cryptocephalinae; 2Eum, Eumolpinae; 3Cas, Cassidinae; 4Alt, Alticinae; 5Gal, Galerucinae; 6aChr, Chrysomelinae without *Timarcha*; 6bChr, Chrysomelinae with *Timarcha*; 7Bru, Bruchinae; 8Cri, Criocerinae; 9Don, Donaciinae; 10CHR, ‘chrysomeline’ clade (Alticinae, Galerucinae and Chrysomelinae);
11SAG, ‘sagrine’ clade (Bruchinae, Criocerinae, Sagrinae and Donaciinae);
12EUM, ‘eumolpine’ clade (Cryptocephalinae, Eumolpinae, Lamprosomatinae, Cassidinae and Spilopyrinae); [a]includes Sag;
13CHR, internal topology of chrysomelines (Chry (Alt + Gal));
14, internal topology of sagrines; 14a,((Sag + Bru) (Crio + Dona)); 14b, Clade 15a: (Sag (Bru (Crio + Dona)));
15, monophyly and internal topology of eumolpines; 15a, EUM: (Cass (Eum (Lamp + Cryp))); 15b, EUM: (Spi (Cass (Eum (Lamp + Cryp]))); [a]includes Sagra;
16, (Syn + Tim);
17, position of *Timarcha*: (Tim (Chry (Alt + Gal)));
18, position of Synetinae: SYN_basal (Syn (EUM + SAG));
19, position of Bruchinae: sister to all other Chrysomelidae;
20, the relationship of three clades; 20a, CHR (EUM + SAG); 20b, (CHR + SAG) + EUM; [b]Sagra not present.
Not listed: Lamprosomatinae, Spilopyrinae, Sagrinae and Synetinae.

Table 2. Test of conflicting tree topologies using the Kishino–Hasegawa (KH), Shimodaira–Hasegawa (SH) and approximately unbiased (AU) tests

Tree	logL	deltaL	bp-RELL	*P*-KH	*P*-SH	*P*-WKH	*P*-WSH	c-ELW	*P*-AU
1	−662 848.9926	190.56	0.003−	0.002−	0.002−	0−	0.001−	0.00257−	0.0341−
2	−662 660.9641	2.5269	0.489+	0.653+	0.653+	0.475+	0.646+	0.489+	0.533+
3	−662 658.4372	0	0.508+	1+	1+	0.525+	0.726+	0.508+	0.477+

Topologies from PhyloBayes (Tree1), MrBayes (Tree2) and RaxML (Tree3, nst = 6) based on *15genes* were subjected to the test of significance in the IQ-tree, which uses a general time reversible (GTR) model identical to that in RAxML.
deltaL, logL difference from the maximal logl in the set.
bp-RELL, bootstrap proportion using RELL method (Kishino *et al*., 1990).
P-KH, *P*-value of one-sided KH test (1989).
P-SH, *P*-value of SH test (2000).
P-WKH, *P*-value of weighted KH test.
P-WSH, *P*-value of weighted SH test.
c-ELW, expected likelihood weight (Strimmer & Rambaut 2002).
P-AU, *P*-value of AU test (Shimodaira, 2002).
+, values within the 95% confidence sets.
−, significant exclusion.
All tests performed 1000 resamplings using the RELL method.

of the model by moving from the HKY model, which allows for differences in base frequencies and two different substitution rates (for transitions and transversions) (nst = 2) to a full GTR model with separate rate estimates for the six substitution types (nst = 6). The use of the more complex model shifted the tree towards the PhyloBayes topology (nucleotide-based reconstruction) in that there was no basal split of Bruchinae and the relationship of three major clades matched. However, only the PhyloBayes analyses, especially *13PCGs-AA* (Fig. 2), avoided the presumed problematic association of Sagrinae with the eumolpine clade observed in RAxML and the attraction of Bruchinae to the outgroup.

Next, we tested the strength of discrimination among alternative topologies obtained under the different models in the Bayesian analysis, against the RAxML analysis under the standard GTR model. We conducted pairwise tests on the topologies from MrBayes and PhyloBayes and assessed their likelihood under the GTR model employed by RAxML, using the IQtree software and various statistical tests, including the KH, SH and AU tests. These analyses showed that the topology obtained with PhyloBayes was strongly rejected under the GTR model (Table 2), and thus this topology is not defensible under a broader range of models other than the site-heterogeneous model implemented in PhyloBayes. This analysis showed the critical effect of model choice for the selection of the preferred tree, and indicated that the PhyloBayes topology differed significantly from those obtainable with likelihood and Bayesian analysis employing a standard GTR model.

Finally, we applied topological tests on the nodes defining the three major clades of Chrysomelidae using the FcLM approach to investigate support for the preferred tree and model choice. This analysis, which relies on the number of quartets drawn from predefined groups, was not straightforward as a result of the non-monophyly of the three clades in some of the analyses (see above). Various exploratory FcML tests favoured the placement of *Timarcha* (+ *Syneta*) as sister to the chrysomeline clade (see Appendix S6, Partition i for details) and the grouping of Bruchinae with sagrines (Appendix S6, Partition ii). The FcML analysis carried out on these three major clades (chrysomelines including *Timarcha*, sagrines including Bruchinae, and eumolpines) and a non-chrysomelid outgroup as the fourth group, using both nucleotide and amino acid data, supported the basal split of the chrysomelines linked to the outgroup, as sister to the eumolpines + sagrines [topology (a, d) (b, c) in Fig. 5], and thus matched the PhyloBayes amino acid tree (although it was based on a GTR model).

Discussion

Mitochondrial genome sequences are a rapidly growing source of phylogenetic information for increasingly densely sampled trees. Our study contributes 27 new mitogenomes and includes a total of 103 mostly complete sequences representing all subfamilies and major lineages of Chrysomelidae. Our work refines the findings of previous studies mainly obtained with nuclear rRNA genes and is the first independent confirmation of the three major clades within Chrysomelidae established initially by Gómez-Zurita *et al*. (2007). However, we also demonstrate the difficulties of determining the relationships of these three lineages relative to one another, and the problems of placing several small divergent lineages, the positions of which remain poorly supported. These problems required a careful investigation of spurious groupings from those supported by phylogenetic signal.

Mitochondrial markers are affected by shifts in both evolutionary rates and nucleotide composition, and given the overall high level of substitution, these effects may lead to long-branch attraction, particularly affecting deep branches. All our phylogenetic trees showed the eumolpine clade with increased branch length, especially in Cassidinae and Cryptocephalinae s.l. However, the latter two were not grouped together, despite the earlier suggestions from nuclear rRNA markers that they are sister taxa (Gómez-Zurita *et al*., 2007, 2008). They differ in GC content, which may counteract the potential long-branch attraction (Fig. 4c) and in fact can lead to the opposite effect

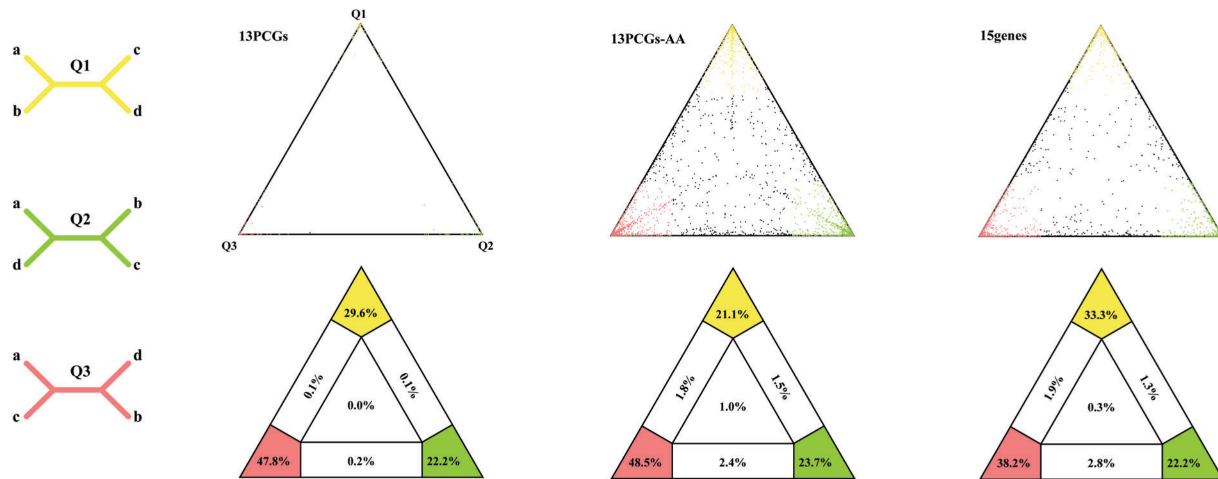


Fig. 5. Four-cluster likelihood mapping (FcLM) of major clades of Chrysomelidae. *A priori* groups in the analysis were: a, chrysomeline clade (Chrysomelinae with Timarchini, Galerucinae and Alticinae); b, sagrine clade: Sagrinae, Criocerinae, Donaciinae and Synetinae, Bruchinae; c, eumolpine clade: Cryptocephalinae, Lamprosomatinae, Eumolpinae and Cassidinae; d, outgroups. [Colour figure can be viewed at wileyonlinelibrary.com].

of spurious long-branch repulsion of otherwise closely related lineages. However, the lack of the unique tRNA inversion in Cassidinae (Figs 1,2) is strong evidence against the sister relationship with Cryptocephalinae, and thus supports the mitogenome tree over the nuclear rRNA data in one of the few discrepancies with Gómez-Zurita *et al*.'s (2007, 2008) studies.

We also assessed levels of heterogeneity using the AliGROOVE method, which identifies terminals for which overall nucleotide variation deviates from most others in pairwise comparisons, but this analysis revealed only minor deviations from uniform variation and the removal of a small number of the most heavily affected sequences had no effect on tree topology. However, when different algorithms and models of evolution were used, substantial differences at the base of the tree were obvious, whereas relationships within the major clades were largely consistent across methodologies, particularly in the various RAxML and MrBayes trees.

The justification for the selection of any of the methods required extensive data exploration. We approached the problem of tree selection by firstly assessing the strength of the conflict. Keeping a single model, implemented in IQ-tree (which is identical to that used in RAxML), the tree topologies obtained with PhyloBayes (and less clearly the topology of MrBayes) were rejected with high significance, indicating strongly different likelihoods of the models employed by these methods. Choosing between these competing models and topologies requires additional criteria. Only the PhyloBayes analysis recovered the three major clades, including the sagrine clade, which broke up in the other searches, either by placing Bruchinae as sister to all others (RAxML) or by placing Sagrinae within the eumolpine clade (MrBayes). On the grounds of topology, we therefore preferred the PhyloBayes tree. The heterogeneous mixture model employed by PhyloBayes presumably adds greater complexity as the model employs multiple GTR processes rather than the single process that is applied across all data in the other two algorithms. We therefore also assessed whether greater model complexity in the other methods could improve the tree in a similar way. Firstly, we used increasing numbers of partitions (by genes and codons) in the RAxML analysis, but this had virtually no effect on the topology (Appendix S14). Secondly, we applied different models in MrBayes, comparing the HKY (two rate parameters) and GTR (six rate parameters) models. The latter moved the topology slightly towards that of the PhyloBayes tree (resulting in monophyly of the sagrine and eumolpine clades), but other differences among the trees remained. Finally, we tested the effect of excluding the presumably most biased third codon positions. However, no great effect on tree topology was observed (*13PCGs* vs. *13PCGs-codon12* in Table 1).

By contrast, coding the sequence data as amino acids in PhyloBayes analyses produced a different resolution of the three clades, supporting (chrysomelines (eumolpines + sagrines)) versus (eumolpines (chrysomelines + sagrines)) obtained with the same software using nucleotide datasets. The former relationships were also supported by FcLM analyses using both amino acid and nucleotide data. The method applies the same GTR model as the RAxML search, and the result is consistent with the RAxML tree searches, which also generally favoured the early split of chrysomelines (with both datasets based on amino acids and nucleotides, respectively). It is unclear why nucleotide- and amino acid-based coding produce different tree topologies, but the fact that they do shows that support for either of the two competing hypotheses is weak; this is further complicated by the unclear positions of *Timarcha* and Synetinae, which represent two further deep lineages branching near the base of the tree and were occasionally grouped together as two unlikely sister taxa. Thus, despite our detailed analysis of the basal relationships, the resolution of basal nodes remains unsatisfactory, particularly in the light of studies on nuclear genes that support the third possible topology of an early split of sagrines first from a grouping of eumolpines and chrysomelines. This topology has been

obtained in various previous papers mainly based on nuclear rRNA genes (Farrell, 1998; Duckett *et al*., 2004; Gómez-Zurita *et al*., 2007, 2008), but also by a set of nearly 100 nuclear protein coding markers (although based on the inclusion of very few taxa) (Zhang *et al*., 2018). None of the mitochondrial analyses supported this arrangement, which perhaps indicates some hitherto undefined bias in mitogenome sequences of Chrysomelidae.

Implications for the phylogeny and classification of Chrysomelidae

Mitogenomes broadly support the subfamily classification of Chrysomelidae and virtually all 13 subfamilies in the taxonomy of Löbl & Smetana (2010) were monophyletic (although four subfamilies were represented by a single exemplar only). The results broadly agree with Gómez-Zurita *et al*.'s (2007, 2008) extensive study of chrysomelid relationships based on nuclear 18S and 28S rRNA genes, which included a short fragment of the mitochondrial *rrnL* gene and slightly greater taxon sampling in particular in the Eumolpinae and Chrysomelinae, but which lacked two subfamilies, Sagrinae and Lamprosomatinae. Mitogenomes corroborate the three major clades of Chrysomelidae proposed by Gómez-Zurita *et al*. (2007). The major subdivision of Chrysomelidae detected with molecular data also matches the classification of Chen (1964), who proposed separate families for each of the three basal clades, including a narrowly defined Chrysomelidae sensu stricto represented by Chrysomelinae, Galerucinae and Halticinae, a family Crioceridae represented by Sagrinae, Donaciinae and Criocerinae (in addition to several groups that are now considered to be part of the wider superfamily Chrysomeloidea), and the family Eumolpidae represented by the same subfamilies as those found to constitute the eumolpine clade in the current study, with the exception of Cassidinae, which was placed in a fourth, distant family. The mitogenome study was partly confounded by the fairly weak and sometimes contradictory support for the placement of Sagrinae and Bruchinae (of the sagrine clade), and two small groups, *Timarcha* and Synetinae, which have been difficult to place in previous work, were found to lie outside these three major clades.

Specifically, *Timarcha* has been classified as a member of Chrysomelinae in the recent literature (Reid, 1995, 2000), but was proposed as a separate subfamily by Jolivet & Verma (2008), who also cited earlier research in support of this elevated status, including papers by Sharp & Muir (1912), Powell (1941) and Verma (1998), and reiterated this position more recently (Jolivet *et al*., 2013). The subfamily status was also supported by molecular studies of Haddad & McKenna (2016) and Gómez-Zurita *et al*. (2007, 2008), although this was never formalized. *Timarcha* is morphologically isolated from all other chrysomelids and differs from other Chrysomelinae in the primitive structure of the male genitalia and the presence of a ring-like tegmen (Jolivet, 2008).

A curious finding was the affinity of *Timarcha* with Synetinae seen in several analyses as these formed a deep-branching lineage as sister of the entire sagrine + chrysomeline clades. The affinity with Synetinae may indicate long-branch attraction between two isolated lineages branching near the base of the Chrysomelidae. The two groups are very different except in terms of their long life cycles and the fact that their distributions are confined to the Palearctic and Nearctic. In the preferred PhyloBayes tree (Fig. 2), *Timarcha* was placed as sister to the chrysomeline clade, a position corroborated by FcLM which placed *Timarcha* with chrysomelines rather than the two other major clades (Appendix S6). The position of Synetinae remains very unclear but, in the preferred tree, Synetinae is sister to the eumolpine + sagrine clade. It was placed tentatively with the sagrines by the rRNA study (Gómez-Zurita *et al*., 2007), and grouped with Eumolpinae by Reid (1995) and Farrell (1998). The placement of Synetinae should ultimately be resolved in the context of the relationships among the three major clades, the positions of which relative to one another remain unclear.

In terms of the internal relationships in each major clade, in the chrysomelines the subfamily relationships of (Chrysomelinae (Galerucinae + Alticinae)) are stable. The result agrees with other molecular (Gómez-Zurita *et al*., 2007, 2008; Hunt *et al*., 2007; Ge *et al*., 2011, 2012; Bocak *et al*., 2014; Nie *et al*., 2018) and morphological (Chen, 1964; Reid, 1995) studies, and several morphological traits, including the shape of the anterior coxae, venation type and archaic male genitalia, strongly support the monophyly of these three subfamilies. Given its distant position and unclear placement, our study adds to arguments for the status of *Timarcha* to be defined as that of a distinct subfamily separate from Chrysomelinae, as proposed by Jolivet *et al*. (2013).

In the 'sagrine' clade, we consistently recovered the two main monocot-feeding subfamilies Donaciinae and Criocerinae as sister groups, which is supported by previous studies (Duckett *et al*., 2004; Hunt *et al*., 2007; Bocak *et al*., 2014; Song *et al*., 2017), but inconsistently resolved in Gómez-Zurita *et al*. (2007, 2008) and not supported in Farrell (1998). The two remaining subfamilies, Bruchinae and Sagrinae, were placed together with the other sagrines only in the PhyloBayes analysis, and only in the *13PCGs-AA* tree are they sister groups to one another. Both subfamilies have more than one male accessory gland and a reduced number of larval stemmata (Reid, 1995). Most larvae of Sagrinae feed on the stems of Leguminosae and are gallicolous within the stems of host plants, which indicates feeding habits similar to those of the bruchid genera *Rhaebus* and *Eubaptus* (Jolivet, 1988).

Relationships in the 'eumolpine' clade strongly support Cryptocephalinae s.l. as sister to Lamprosomatinae; both combined are sister to Eumolpinae. This clade had been proposed by Chen (1964) based on shared larval habits of Cryptocephalinae s.l. and Lamprosomatinae, which are case-bearers (Chen, 1964; Jolivet, 1988; Chamorro, 2014), and adult morphological characters, such as the concave frontoclypeus and the hypognathous orientation of the mouth parts. Here, this was confirmed by the translocation of tRNA-Arg and tRNA-Ala narrowly confined to this clade. All molecular phylogenetic trees split the Eumolpinae from the other two subfamilies; Lamprosomatinae could be considered to be in an intermediate position because its larval habits (as a case-bearer) resemble those of the Cryptocephalinae rather than the Eumolpinae, the larvae of which are free-living

and feed below ground, whereas some adult morphological characters (such as the non-narrowing middle of the second, third and fourth abdominal segments and the more or less oval body shape) are closer to those of the Eumolpinae (Chen, 1964). The Cassidinae should now be considered to be outside this lineage, in contrast with the findings of Gómez-Zurita *et al*. (2007), which recovered Cassidinae in a more terminal position as a sister group of (Cryptocephalinae s.l. + Lamprosomatinae) within a paraphyletic Eumolpinae. The subfamily Cassidinae is unique among Chrysomelidae because it is the only group with an eutetrameran tarsus, whereas all other species exhibit a pseudotetrameran tarsus. Chen (1940) inferred the nearest relatives of Cassidinae to be Eumolpinae because both groups show similarities in head type (opisthognathus), wing venation (the development of Cu1) and male genitalia (the tegmen is directly attached to the ventral edge of the median foramen). The analysis also provides a stable position for the small family Spilopyrinae as a sister to all other members of the eumolpine clade. The latter are confirmed by a derived type of genitalia; among those taxa with the 'incomplete' type of lateral lobes, the eumolpine clade (excluding Spilopyrinae) exhibits a tegmen attached to the ventral edge of the median foramen, by contrast with all other subfamilies (including Spilopyrinae), in which the tegmen shows the median lobe to be attached more distantly from the ventral edge or posterior edge of the median foramen. In addition, the three subfamilies Sagrinae, Donaciinae and Timarchini exhibit the 'complete' type of lateral lobe, which may be plesiomorphic.

Conclusions

Mitogenomes have proven to be powerful markers in defining the major chrysomelid lineages at subfamily level and below, but struggle to resolve the deepest nodes that define the relationships of five major lineages [i.e. the three major clades of Gómez-Zurita *et al*. (2007) plus *Timarcha* and Synetinae]. Irrespective of the final resolution of these issues, the monocot-feeding lineages (Bruchinae, Criocerinae, Donaciinae and Cassidinae including Hispinae) separate into at least two distant groups, supporting the hypothesis of multiple monocot colonization and thus a less stringent co-evolutionary scenario, in agreement with Gómez-Zurita *et al*. (2007). In a next step these findings should be integrated into the geological time scale, which is still not entirely established for the evolution of angiosperms (Zeng *et al*., 2014; Li *et al*., 2019), and the recent discovery of ancient fossils (RN and XKY, unpublished data, 2019) may also change existing conclusions about the time tree of Chrysomelidae.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Primers used for PCR.
Appendix S2. Samples and register of gene information.
Appendix S3. The likelihood value of the RAxML tree under different partition schemes.
Appendix S4. *t*-test comparing substitution rates of subfamily Cryptocephalinae with those in other subfamilies.
Appendix S5. *t*-test comparing GC contents of subfamily Cryptocephalinae with those in other subfamilies.
Appendix S6 Four-cluster likelihood mapping (FcLM) results based on the different group partition schemes. Partition i: a, chrysomeline clade (Chrysomelinae, Galerucinae and Alticinae); b, sagrine clade: Sagrinae, Criocerinae, Donaciinae and Synetinae, Burchinae; c, eumolpine clade: Cryptocephalinae, Lamprosomatinae Eumolpinae and Cassidinae; d, Timarchini, Synetinae. Partition ii: a, chrysomeline clade (Chrysomelinae with Timarchini, Galerucinae and Alticinae); b, sagrine clade: Sagrinae, Criocerinae, Donaciinae and Synetinae; c, eumolpine clade: Cryptocephalinae, Lamprosomatinae Eumolpinae and Cassidinae; d, Bruchinae.
Appendix S7. PhyloBayes tree based on combined data of *15genes*.
Appendix S8. PhyloBayes tree based on combined data of *13PCGs*.
Appendix S9. PhyloBayes tree based on combined data of *13PCGs-codon12*.
Appendix S10. Mr Bayes tree based on combined data of *15genes* partitioned by genes and by codons (36 parts) and HKY model (nst = 2). Numbers above each node are posterior probabilities.
Appendix S11. Mr Bayes tree based on combined data of *15genes* by genes (36 parts). Nst = 6.
Appendix S12. Mr Bayes tree based on combined data of *13PCGs* by genes (39 parts). Nst = 6.
Appendix S13. Mr Bayes tree based on combined data of *13PCGs-codon12*. Nst = 6.
Appendix S14. The consensus RAxML tree based on *15genes* from the RaxML trees of 0, 14, 15, 36, 41 partition schemes of *15genes*.
Appendix S15. RAxML tree based on combined data of *13PCGs* by gene by codon.
Appendix S16. RAxML tree based on combined data of *13PCGs-codon12*.
Appendix S17. RAxML tree based on combined data of *13PCGs-AA*.

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The authors declare no conflicts of interest.

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