Phylogenetically informative rearrangements in mitochondrial genomes of Coleoptera, and monophyly of aquatic elateriform beetles (Dryopoidea)

Martijn J.T.N. Timmermans a,b,* , Alfried P. Vogler a,b

*Department of Entomology, Natural History Museum, Cromwell Road, London SW7 5BD, UK
aDivision of Biology, Imperial College London, Silwood Park Campus, Ascot SL5 7PY, UK

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Mitochondrial gene order in Coleoptera has been thought to be conservative but a survey of 60 complete or nearly complete genomes revealed a total of seven different gene rearrangements (deletions, gene order reversals), mainly affecting tRNA genes. All of these were found to be limited to a single taxon or a subclade of Coleoptera. The phylogenetic distribution of a translocation of tRNAPro in three species of elateriform beetles was investigated further by sequencing three nearly complete mitochondrial genomes (Dascillusidae, Byrrhidae, Limnichidae) and ten additional individuals for a ~1370 bp diagnostic fragment spanning the relevant region. Phylogenetic analysis consistently recovered the monophyly of families previously grouped in the contentious superfamily Dryopoidea, a group of approximately 10 beetle families with mainly aquatic lifestyles. The Byrrhidae (moss beetles) were not part of this lineage, although they may be its sister group, to recover the widely accepted Byrrhoidea. The tRNAPro translocation was present in all members of Dryopoidea, but not in any other Elateriformia, providing independent support for this lineage and for a single origin of aquatic habits.

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1. Introduction

Gene order rearrangements in the mitochondrial genome have been extremely useful phylogenetic markers (Boore and Brown, 1998). However, their utility seems to be greater in some lineages than others because of differences in the rate at which these rearrangements occur (Dowton et al., 2009). In Coleoptera (beetles) the available sequences suggested a uniform gene order corresponding to the ancestral holometabolan arrangement (Friedrich and Muqim, 2003; Sheffield et al., 2008). This situation differs from other major groups of insects such as the Hymenoptera or Psocoptera that show extensive levels of rearrangements (Dowton et al., 2009; Shao et al., 2003). Yet, the denser taxonomic sampling of mitochondrial genome sequences increases the chances of finding deviations in gene order. Already, inversions of tRNA gene order and a deletion of a tRNA gene are known from recent studies in weevils (Curculionidae) (Song et al., 2010) and the tenebrionoid family Mordellidae (Cameron et al., 2009). Although these conditions each represent a derived character state that is phylogenetically uninformative, they could represent synapomorphies of particular subclades.

Further rearrangements may remain undetected in the mitochondrial genome sequences of Coleoptera, in particular where the relative position of tRNA genes is affected, because phylogenetic analysis of mitogenomes has usually been based on the protein coding regions only that were aligned individually without considering the gene order (Pons et al., 2010; Sheffield et al., 2009; Song et al., 2010; Timmermans et al., 2010). Here, we searched all mitogenome sequences currently available for the Coleoptera for deviations from the presumed panarthropodan (Braband et al., 2010) gene order. This resulted in the discovery of several additional cases of translocations and deletions of tRNA genes.

The phylogenetic distribution of one of these rearrangements, in the superfamily Elateriformia, was investigated in more detail for its taxonomic extent. The Elateriformia is one of the five Series (infraorders) of Polyphaga that includes well-known families such as click beetles (Elateridae), soldier beetles (Cantharidae), jewel beetles (Buprestidae) and glow worms (Rhagophthalmidae, Lampyridae). Basal relationships in the Elateriformia remain uncertain, despite recent progress in particular in the superfamily Elateroidea (Bocakova et al., 2007; Kundrata and Bocak, 2011). Several smaller families have been grouped variously in the superfamilies Byrrhoidea and Dryopoidea whose circumscription conflicts which each other. The Dryopoidea, as defined by Crowson (1981), includes several small families of beetles that are affiliated with aquatic and riparian habitats either in the adult, larval or both stages. However, their
monophyly has been rejected in all recent cladistic analyses (Beutel, 1995; Costa et al., 1995; Lawrence, 1988; Lawrence et al., 2011), notably through the inclusion of the morphologically divergent family Byrrhidae (moss beetles). Consequently the superfAMILY Byrrhoidea of Lawrence and Newton (1995) refers to a Dryopoidea expanded by the Byrrhidae, and it was considered closely allied with the Buprestidae (=Buprestoidea). The Byrrhoidea sensu Lawrence and Newton (1995) usually are found to be split into two major groups, sometimes referred to as “Psphenoida” and “Byrrhoida” (Costa et al., 1995; Lawrence, 1988). The cladistic analyses performed to date in all cases therefore resulted in the polyphyly of the aquatic lineages. Whether or not the aquatic lineages are monophyletic is important for an understanding of the major transitions in life style of Coleoptera and how these shifts would have contributed to the great species richness of this group.

2. Material and methods

2.1. PCR amplification and sequencing

Nearly complete mitochondrial genomes were newly obtained for species of Limnichidae (Limnichidae gen. sp), Byrrhidae (Byrrhus sp.) and Dascillidae (Dascillus cervinus), using long-range PCR and 454/Roche sequencing as described previously (Timmermans et al., 2010). Seven additional species (Cytilus sericus, Byrrhidae; Limnius volkmari, Elmidae; Oulimninus rivularis, Elmidae; Potamodytes sp., Elmidae; Chelonarion sp., Chelonarionidae; Callirhipis sp., Callirhipidae; Pitloactyta serricornis, Pitloactytaidae), two species of Buprestidae (Trachys minuta, Julodinae sp.) and one specimen of Dascillidae (D. cervinus) were selected from the NHM frozen DNA collection to target a smaller genomic fragment of approximately 1370 bp (tRNA^Thr, tRNA^Pro, tRNA^Nds, and cob) to test the gene order in this particular region. PCR was performed with primer CB4 (located in cob; Barraclough et al., 1999) and either primer tRNA-Thr1 5′-ATT ATT GGT CTT GTA AAY C-3′ or tRNA-Thr2 5′-AAT ATT GGT CTT GTA AAY C-3′ (located in tRNA^Thr) under the following conditions: 3 min at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 50 °C and 90 s at 72 °C; a final extension of 10 min at 72 °C. Amplicons were purified (Millipore) and bi-directionally sequenced by an in-house sequencing facility using ABI technology. Reads from Sanger sequencing were edited in Sequencher (GeneCodes Corp.). All sequences were submitted to GenBank (Accession Numbers JQ034407-JQ034419).

2.2. Gene order

Mitochondrial genome sequences included a set of 25 complete or nearly complete sequences from the Genome database; 28 partially annotated mitochondrial fragments from 454/Roche sequencing (Timmermans et al., 2010); seven sequences generated by Song et al. (2010) newly available on GenBank only since the compilation of Timmermans et al. (2010); and three species newly sequenced in this study. The combined set includes 63 species that represent most major lineages of Coleoptera, including all four suborders and the five Series of Polyphaga, and multiple representatives of some larger families such as Chrysomelidae and Curculionidae (see Timmermans et al., 2010). tRNA sequence prediction was performed with the COVE software package (http://selab.janelia.org/software.html), using Coleoptera specific covariance models generated based on the tRNA annotations of the genomes in GenBank. These models were applied to annotate newly acquired and incompletely annotated sequences, and gene orders were then compared together with the fully annotated mitochondrial genomes available at GenBank using custom-designed BioPerl (http://bioperl.org) scripts.

2.3. Phylogenetic analysis

Sequences of Elateroidea, Buprestoidea and Byrrhoidea were selected from the data matrix of 12 protein coding sequences of Timmermans et al. (2010) and combined with the newly generated sequence data (Table 1). Phylogenetic analyses were performed on

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**Table 1**

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BMNH, British Museum (Natural History); PCG, all protein coding mitochondrial genes except 5′ cox1 and nad2. *cox1 unavailable. ** nad5, nad6, nad4L and nad6 unavailable.
the cob and nad6 fragments, which avoids missing data, and on the full matrix of 12 protein-coding regions.

Models of sequence evolution best suited to the data were determined with MrAIC (Nylander, 2004) for each of the three codon positions; for first and second codon position combined; and for the full dataset. The selected model for each codon position, if analyzed separately or combined was GTR + I + G for the data set composed of 12 protein coding genes. Likewise, GTR + I + G was selected for the cob–nad6 fragment, but 1st and 3rd codon positions analyzed separately required the HKY + I + G model, and the 2nd codon position required the GTR + I + G model. Tree searches using unpartitioned data were performed in Maximum Likelihood (ML) analyses using Phyml 3.0 (Guindon et al., 2005), with 100 bootstrap replicates to establish nodal support. For Bayesian inferences, for each of the codon positions an independent model was selected with unlinked parameters for the partitions based on the Bayesian Information Criterion (Posada and Buckley, 2004). Searches were conducted in MrBayes version 3.1.2 (Huelsenbeck and Ronquist, 2001), either using all codon positions or under RY-recoding (Hassanin, 2006) of first codon positions and removal of third positions (Pons et al., 2010). In each case, two independent searches were conducted using a single chain (no heating) and the default priors starting with random trees running for 20 million generations sampled at intervals of 1000 generations. Following the MrBayes Manual, heating is frequently not needed for analyses involving less than 50 taxa, which we can confirm from conducting the analysis several times and always receiving the same tree and very similar values for likelihood and posterior probabilities. The searches from the constrained and unconstrained tree searches on the dataset with RY coded first codon positions and removed third codon positions.

3. Results

The survey of published mitogenome sequences revealed several deviations from the ancestral arthropod gene order, particularly in a cluster of six tRNA genes bracketed by the nad3 and nad5 genes (Fig. 1). These changes include: (1) the reversal of order of the tRNAAla and tRNAAsn genes in Pephoptera acromnalis (Chrysomelidae) and Naupactus xanthographus (Curculionidae); (2) an order reversal of tRNAAsn and tRNAArg in Mordella atrata (Mordellidae); (3) an order reversal of tRNAAsn and tRNAArg in tRNAser in Ischialia sp. (Anthicidae); (4) a deletion of tRNAle in N. xanthographus and Sphenophorus sp. (Curculionidae); (5) an insertion of nearly 150 bp of extraneous sequence into the (highly divergent) tRNAlav gene in Merolycus dentipes (Lycidae), (6) an order reversal of tRNAPro and nad6 in Eulichas sp., Heterocerus fenestratus and Elmidae sp. (Byrrhoidea).

Only two of these rearrangements are potential synapomorphies for more than one of the sequenced taxa, in Curculionidae and Byrrhoidea. Given that representatives of three families of Byrrhoidea showed the tRNApro – nad6 translocation, we investigated if this rearrangement is a synapomorphy for certain subclades within Byrrhoidea. Three nearly complete mitochondrial genomes were obtained and 10 additional representatives of Byrrhoidea covering most established families were sequenced for a ~1370 bp region covering the cob to tRNAAla fragment that covers the relevant region. We found the tRNApro – nad6 translocation to be present in all representatives of Elmidae, Pitlodziactyliidae, Eulichaadidae, Chelonariidae, Callirhipiidae, Heteroceridae and Limnichidae, but to be absent from the two representatives of Byrrhoidea and all other taxa.

Phylogenetic analysis was conducted under ML and Bayesian methods on the cob + nad6 sequences (no missing data) or on the full matrix of 12 protein-coding regions (with missing data for taxa sequenced for cob + nad6 only). Each of the analyses was performed either on all nucleotide positions; on 1st (RY coded) and 2nd positions only; and under partitioning by nucleotide position (Bayesian analysis only) (Table 2). All analyses recovered three superfamilies of Elateriformia as monophyletic, including the Dascilloidea, Elateroidea (including Cantharoida) and Buprestoidea, although the elateroid Melasis buprestoides (Eucinetidae) was grouped with Buprestoidea in some cases (Table 2). In contrast, the Byrrhoidea sensu Lawrence and Newton (1995) was consistently paraphyletic because of the separation of Byrrhoidea from the remaining families, while the aquatic families (Dryopoidea of Lawrence and Newton, 1995) were monophyletic. The relationships of these five major lineages differed, in particular the relative

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**Fig. 1.** Gene order in mitochondrial genomes of Coleoptera. The panel at the top shows the map of protein coding, tRNA and tRNA genes representing the ancestral gene order. Arrows pointing to the right (and black bars for tRNA genes) represent genes on the plus strand, arrows pointing to the left (and gray bars for tRNA genes) represent genes on the minus strand. Taxa that deviate in their gene orders are given in the bottom panel. tRNA genes are labeled with a single-letter code. Dashes indicate missing data. ▲ denotes a missing tRNA gene. 1, sequenced in the current study.
position of Dryopoidea, Byrrhidae and Buprestidae, which were either placed in a clade (Elateroidea (Dascilloidea (Byrrhidae (Dryopoidea, Buprestidae)))) when analyzing the cob–nad6 fragment only, or included the Dascilloidea for (Elateroidea, Buprestidae (Dryopoidea (Byrrhidae, Dascilloidea)))) when using all data (Fig. 2). However, if Dryopoidea + Byrrhidae were constrained for monophyly, i.e. enforcing a monophyletic Byrrhoidea, the resulting trees were only marginally worse (log_{10}BF = 1.03–1.13) and the Byrrhidae remained the sister to all other families in this lineage (Fig. 3). In all analyses the Dryopoidea of Lawrence and Newton (1995) was highly supported (pp: 1.0), which confirmed the cob–nad6 translocation as a synapomorphy of the Dryopoidea.

4. Discussion

The mitochondrial genomes of Coleoptera initially have been thought to be largely unchanged from the ancestral gene order (Friedrich and Muqim, 2003; Sheffield et al., 2008), but denser taxon sampling is now revealing several rearrangements that may be phylogenetically informative. In 63 mitogenome sequences available publicly, we found a total of seven types of gene orders based on six different translocations or deletions (Fig. 1). These changes affected almost exclusively the tRNA genes, which is expected due to their higher apparent mobility relative to protein coding genes (Moritz et al., 1987). In addition, several mitogenome
The tRNAIle deletion in on molecular clock analysis of the Coleoptera (Hunt et al., 2007). Analysis pointed to a deep origin of this character, in the ancestral limits using PCR amplification of the relevant region. The of existing mitogenomes revealed multiple taxa with the tRNAPro markers was clearly evident in the Dryopoidea. The initial screen showing major taxa and the phylogenetic placement of the tRNAPro translocation.

Fig. 3. Bayesian tree from search constrained for a monophyletic Byrrhoidea (•), showing major taxa and the phylogenetic placement of the tRNAα2 translation.

sequences were incomplete for the control region and the regions around the ribosomal RNA genes and nad2 genes (Fig. 1), and hence may contain additional deviations from the ancestral gene order not detected here. The changes also included a deletion of a tRNA gene while in another case a disruption of tRNAα1 presumably renders the gene product non-functional. These deletions and insertions are curious, as they suggest gene loss without the presence of functional copies elsewhere in the genome, although they might involve translocations to unsequenced portions (Song et al., 2010). A further rearrangement (an order reversal of tRNAPhe and tRNAGlu) involving translocations to unsequenced portions (Song et al., 2010), whereas a complicated picture was obtained in a study of Elateriformia (which are now known to include the soft-bodied Cantharoidea) is well supported by morphological (Lawrence and Newton, 1982) and molecular (Bocakova et al., 2007) data. The Dascilloidea also are well established as a basal branch of the Elateriformia but their precise affinities remain unclear (Lawrence and Newton, 1982) and in the current analysis they show strong affinities to Dryopoidea (Fig. 2). In Lawrence & Newton’s (1995) widely used classification, the superfamily Byrrhoidea is proposed to include the aquatic families plus Byrrhididae, but it is considered separate from the closely allied Buprestidae (=Buprestoidea). Lawrence et al.’s (2011) recent morphology-based phylogeny of Coleoptera inserts Buprestidae into the dryopoid clade, rendering Dryopoidea polyphyletic. The Dryopoidea were split into two separate clades, separating the Psephenoidae (Psephenidae, Ptinidae) from the remaining Dryopoidea (Elmidae, Heteroceridae, Linnichidae), the latter being sister to Buprestidae and Byrrhididae (Lawrence et al., 2011). Molecular studies to date have been equivocal; whereas a complicated picture was obtained in a study of Elateriformia (mainly focused on other questions in this group) (Friedrich and Muqim, 2003), but after our reanalysis of the sequence this conclusion appears to be based on an incorrect annotation of the tRNAα2 gene.

The utility of mitochondrial gene rearrangements as clade markers was clearly evident in the Dryopoidea. The initial screen of existing mitogenomes revealed multiple taxa with the tRNAα2 – nadβ translocation, which was further explored for the precise clade limits using PCR amplification of the relevant region. The analysis pointed to a deep origin of this character, in the ancestor of a lineage that has diversified as early as the Jurassic, based on molecular clock analysis of the Coleoptera (Hunt et al., 2007). The tRNAα2 deletion in Naupactus and Sphenophorus is a further potential synapomorphy for a subgroup of weevils (Curculionidae). This kind of character may help eventually to establish the extent of sub-familial groupings of the Curculionidae that have been notoriously difficult to separate from each other (McKenna et al., 2009). In addition, the tRNAα2 – tRNAα3 change in gene order is shared between Naupactus and Pseudoptera, a member of Chrysomelidae (leaf beetles), potentially joining the Phytophaga. However, this represents a case of homoplasies, as each of these species have close relatives (including Sphenophorus in the case of Naupactus) that exhibit the ancestral gene order. All other rearrangements affect just a single taxon represented in the study, but this does not preclude a wider distribution of these markers, considering that the current taxon sampling is limited to isolated lineages. For example, two different rearrangements were found in Mordella and Ischalia in the superfamilies Notonectoidea and Dryopidae. They are taxonomically distant from each other and rearrangements in either species may still define larger clades. Similarly, the tRNAα2 – tRNAα3 gene order change in Pseudoptera and Naupactus, despite being homoplastic, may well define larger groups, e.g. at the subfamily level in both Chrysomelidae and Curculionidae that remains sparsely sampled.

While not entirely free of homoplasies, at least in the case of Dryopoidea the genomic rearrangement provided an important clade marker. Traditional taxonomists grouped all (semi)aquatic families of Elateriformia as “Dryopoidea” (e.g. Crowson, 1978), but their monophyly subsequently was not confirmed (Beutel, 1995; Lawrence, 1988; Lawrence et al., 2011). The relationships of the dryopoids to the other elateriform superfamilies, Buprestoidea, Dascilloidea, Elateroidea and Cantharoidea have also been difficult to resolve. Affinities of Buprestoidea with dryopoids were first recognized by Crowson, 1982, superseding earlier views that linked them to other hard-bodied groups of the Elateroidea including Elateridae (click beetles) and Eucnemidae in the “Sternoxia”. The phylogenetic separation of dryopoids from the Elateroidea (which are now known to include the soft-bodied Cantharoidea) is well supported by morphological (Lawrence and Newton, 1982) and molecular (Bocakova et al., 2007) data. The Dascilloidea also are well established as a basal branch of the Elateriformia but their precise affinities remain unclear (Lawrence and Newton, 1982) and in the current analysis they show strong affinities to Dryopoidea (Fig. 2). In Lawrence & Newton’s (1995) widely used classification, the superfamily Byrrhoidea is proposed to include the aquatic families plus Byrrhididae, but it is considered separate from the closely allied Buprestidae (=Buprestoidea). Lawrence et al.’s (2011) recent morphology-based phylogeny of Coleoptera inserts Buprestidae into the dryopoid clade, rendering Dryopoidea polyphyletic. The Dryopoidea were split into two separate clades, separating the Psephenoidae (Psephenidae, Ptinidae) from the remaining Dryopoidea (Elmidae, Heteroceridae, Linnichidae), the latter being sister to Buprestidae and Byrrhididae (Lawrence et al., 2011). Molecular studies to date have been equivocal; whereas a complicated picture was obtained in a study of Elateriformia (mainly focused on other questions in this group) (Bocakova et al., 2007), Hunt et al.’s (2007) tree of Coleoptera finds a monophyletic Dryopoidea, as sister to Buprestoidea and a distantly related Byrrhididae.

The genomic rearrangement now resolves these various possibilities in favor of a monophyletic Dryopoidea, whose sister group are either Byrrhididae or possibly Dascilloidea or Buprestidae (Table 2, Fig. 2). The monophyly of the aquatic families is also supported by the presence of a unique type of wing folding (“dryopoid” type of Forbes, 1926), the loss of functional spiracles on the 8th abdominal segment in adults (with a reversal to functional spiracles in Dryopoidea, Heteroceridae and Linnichidae) and the unilobate tarsus in the larva. Despite a high degree of ecological diversification, all families in this group are associated with aquatic habitats, ranging from fully aquatic in all stages (e.g. Elmidae), fully aquatic larva and terrestrial adults (e.g. Psephenidae, Ptinidae), fully aquatic adults and terrestrial larvae (some Dryopoidea), or both adults and larvae riparian (some Linnichidae, Heteroceridae) (Crowson, 1981; Jäch, 1998). There are also several reversals to a terrestrial lifestyle within fully aquatic groups, usually affecting isolated taxa only. The phylogenetic analysis and the finding of
a clade marker defining the aquatic clade supports an evolutionary scenario in which the aquatic lifestyle originated only once in Elateriformia.

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