

## [REVIEW]

# Molecular Phylogeny of Polyneoptera Based on the Mitochondrial Genome and Focused on Phasmatodea\*

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## 1. Molecular phylogeny based on the mitochondrial genome

Traditionally, insects have been classified based on comparisons of morphological characters and estimations of evolutionary trends. However, the use of morphological traits may not be appropriate for the analysis of taxa with ancient radiation patterns which makes it difficult to identify their synapomorphies. Convergence can also be problematic and may contribute to erroneous phylogenetic relationships. Alternatively, the use of molecular phylogenetics can be advantageous because the use of molecular data easily produces a large number of unambiguous traits for comparison.

Early molecular phylogenetic studies used a few genes, such as ribosomal DNAs (rDNAs), to estimate phylogeny (Carmean *et al.*, 1992; Kjer, 2004); conserved sequences and multiple copies within the genome make it easy to clone rDNAs. However, longer sequence data from increasing taxa has been analyzed in more recent phylogenetic studies. Mitochondrial DNA (mtDNA) has been used in various insect phylogenetic studies (Nardi *et al.*, 2003; Cook *et al.*, 2005; Kômoto *et al.*, 2011, 2012; Tomita *et al.*, 2011). Nucleotide sequences of mtDNA longer than 10 kb can be obtained after PCR-based cloning or random pyrosequencing relatively easily because there are many mitochondria in the cytoplasm and primers can be designed in conserved regions (Rasmussen and Noor, 2009; Nabholz *et al.*, 2010). The absence of recombination, introns, and paralogs helps to precisely identify orthologous genes (Gissi *et al.*, 2008).

Recently, phylogenomic approaches have been applied to various arthropods (Meusemann *et al.*, 2010; Rota-Stabelli *et al.*, 2011; Letsch *et al.*, 2012). Sequencing technology is improving rapidly, and extremely large data sets, such as those in the genome or transcriptome, are becoming available in various species. Ongoing genome projects, such as i5K and 1KITE, will usher in a new era of insect phylogenetic studies, but mitochondrial genome phylogenetics will still play an

important role, especially in detailed analyses of taxa not covered by the genome projects.

## 2. Monophyly of Polyneoptera

Polyneoptera is a group of hemimetabolous insects that includes 11 orders: Blattodea (cockroaches), Dermaptera (earwigs), Embioptera (webspinners), Grylloblattodea (ice crawlers), Isoptera (termites), Mantodea (praying mantises), Mantophasmatodea (heelwalkers), Orthoptera (grasshoppers, crickets, etc), Phasmatodea (stick and leaf insects), Plecoptera (stoneflies), and Zoraptera (angel insects). The phylogenetic relationships of these orders and even the monophyly of Polyneoptera have long been debated. Although the enlarged anal fan of the hindwing is proposed as one of the autapomorphies of Polyneoptera, Embioptera and Zoraptera do not have it, and Grylloblattodea and Mantophasmatodea are apterous (Grimaldi and Engel, 2005). Ross (1955) hypothesized that Zoraptera and Paraneoptera form a monophyletic clade while Plecoptera is sister to the clade Paraneoptera + Holometabola. Kristensen (1975) reviewed the preceding hypotheses and concluded that the monophyly of Polyneoptera was unclear. The unstable placement of the polyneopteran orders in insect evolution is caused by deep branching, which resulted in few synapomorphies and is assumed to date back to the late Paleozoic (Grimaldi and Engel, 2005). Recently, Yoshizawa (2011) showed that Polyneoptera is monophyletic, based on a comparison of wing base structures.

For phylogenetic analyses of taxa that share few morphological traits because of deep branching or extreme convergence, molecular phylogenies can help by providing neutral traits. For example, the monophyly of Polyneoptera has also been examined molecularly by many studies. Among the various genes applied to insect molecular phylogenetics, ribosomal DNAs (rDNAs) were preferred in early studies because conserved sequences among various species allow easy cloning and sequence alignment. Many studies using

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rDNAs to analyze insect phylogeny determined that Polyneoptera was paraphyletic, or at least failed to support monophyly. For example, Kjer (2004) compared all polyneopteran orders, except Zoraptera and Mantophasmatodea, using 18S rDNA, and the resulting tree neither supported nor rejected the monophyly of Polyneoptera, while Yoshizawa and Johnson (2005) included Zoraptera and determined that Polyneoptera is paraphyletic. The phylogenetic tree made up of 18S rDNA, however, showed that Zoraptera had an extraordinarily long branch. Further, a study comparing all 11 polyneopteran orders based on the 28S rDNA sequences also showed Polyneoptera to be paraphyletic (Wang *et al.* 2013). In this tree, however, Polyneoptera and Holometabola formed a clade, which is inconsistent with the general idea that Holometabola is closer to Paraneoptera than to Polyneoptera.

Molecular phylogenies of insects have also been determined using the mitochondrial genome. Carapelli *et al.* (2007) used all 13 protein-coding genes of the mitochondrial genome to reconstruct phylogenetic trees of Pancrustacea. In these trees, Polyneoptera—including Blattodea, Grylloblattodea, Mantodea, Mantophasmatodea, Orthoptera, Phasmatodea, and Plecoptera—was polyphyletic or paraphyletic. However, it is possible that this does not reflect true evolutionary history because Hexapoda and Crustacea are reciprocally paraphyletic, perhaps affected by the high evolutionary rate of the mitochondrial genome.

Recently, protein-coding nuclear genes have been used in insect molecular phylogenetics. Ishiwata *et al.* (2011) used three new nuclear genes (DPD1, RPB1, and RPB2), all of which are relatively large, contain many variable sites, and do not have paralogs. The authors reconstructed the phylogenetic trees of the three genes from all insect orders, including the 11 polyneopteran orders, and found Polyneoptera to be monophyletic. Sasaki *et al.* (2013) included Entognatha and similarly concluded that Polyneoptera is monophyletic. Sequencing methods are progressing rapidly and transcriptome and/or genome data have become available for phylogenomic analysis. Simon *et al.* (2012) analyzed transcriptomic data of insects, including Blattodea, Isoptera, Orthoptera, Dermaptera, Plecoptera, and Zoraptera, and found that these polyneopteran taxa form a monophyletic clade. To improve the robustness of molecular phylogenies, studies must increase the number of taxa and/or the size of data sets, while avoiding rogue taxa, such as those with long branches and paralogous genes.

### 3. Interordinal relationships of Polyneoptera

In addition to the debated monophyly of Polyneoptera, other phylogenetic relationships of polyneopteran orders have been controversial because of a lack of appropriate morphological characters, stemming from deep branching. To overcome the constraint of morphological classification, molecular phylogenetic approaches have been applied to the interordinal phylogenies of Polyneoptera using nuclear genes (Whiting *et al.*, 2003; Yoshizawa and Johnson, 2005; Ishiwata *et al.*,

2011). The mitochondrial genome was also used to reconstruct the phylogenetic relationship of polyneopteran orders, except Zoraptera and Dermaptera (Tomita *et al.*, 2011; Kômoto *et al.*, 2012). We recently added a partial sequence of the mitochondrial genome from these two orders to compare the phylogenetic relationships of all 11 polyneopteran orders (Fig. 1 depicts preliminary data). Another way of performing phylogenetic analysis, combining morphological characters and molecular data, is termed 'total evidence analysis' (Wheeler *et al.*, 2001; Terry and Whiting, 2005; Kjer *et al.*, 2006). However, this method should be interpreted carefully because the addition of morphological characters can bias the resulting trees, spoiling the significance of molecular data as an alternative and neutral way.

Dictyoptera, consisting of the orders Isoptera, Blattodea, and Mantodea, is one of the most robust monophyletic clades within Polyneoptera, which is supported by various studies (Kristensen, 1981; Engel and Grimaldi, 2005). Molecular phylogenies of mitochondrial genome also support the monophyly of Dictyoptera (Tomita *et al.*, 2011; Kômoto *et al.*, 2012; Fig. 1), and show that Isoptera was included in Blattodea, which became a paraphyletic group. The monophyly of Dictyoptera has also been supported by 18S rDNA or three nuclear genes (Yoshizawa and Johnson, 2005; Ishiwata *et al.*, 2011). The paraphyly of Blattodea has also been determined by a comprehensive analysis of Dictyoptera based on two mitochondrial (12S rDNA and COII) and three nuclear genes (18S, rDNA, 28S rDNA and histone H3) (Inward *et al.*, 2007).

A mitochondrial genome phylogeny shows a close relationship between Phasmatodea and Embioptera, which form a monophyletic clade; Eukinolabia was named by Terry and Whiting (2005) (Kômoto *et al.*, 2012; Fig. 1). Note that the paraphyletic status of Phasmatodea will be discussed in the next section. Many morphological studies do not support this relationship. For example, Crampton (1926) grouped Phasmatodea with Dermaptera and Orthoptera, and Embioptera with Plecoptera, based on the structure of the head and its appendages. A close relationship between Embioptera and Zoraptera was also proposed (Engel and Grimaldi, 2000; Yoshizawa, 2011). Recently, Bradler (2009) performed a phylogenetic analysis using morphological traits and proposed a sister group relationship between Phasmatodea and Embioptera. Jintsu *et al.* (2010) came to the same conclusion based on egg structure. Recent molecular analyses of nuclear genes and those of transcriptome data support the monophyly of Eukinolabia (Whiting *et al.*, 2003; Ishiwata *et al.*, 2011; Letsch *et al.*, 2012). Morphological comparisons have often grouped Phasmatodea with Orthoptera (Crampton, 1926; Engel and Grimaldi, 2000; Yoshizawa, 2011), but according to a mitochondrial genome phylogeny, these two orders diverged early in polyneopteran evolution (Kômoto *et al.*, 2012; Fig. 1). The monophyly of Orthoptera has been consistently supported by various molecular phylogenetic analyses (Fenn *et al.*, 2008; Ishiwata *et al.*, 2011; Kômoto *et al.*, 2012)

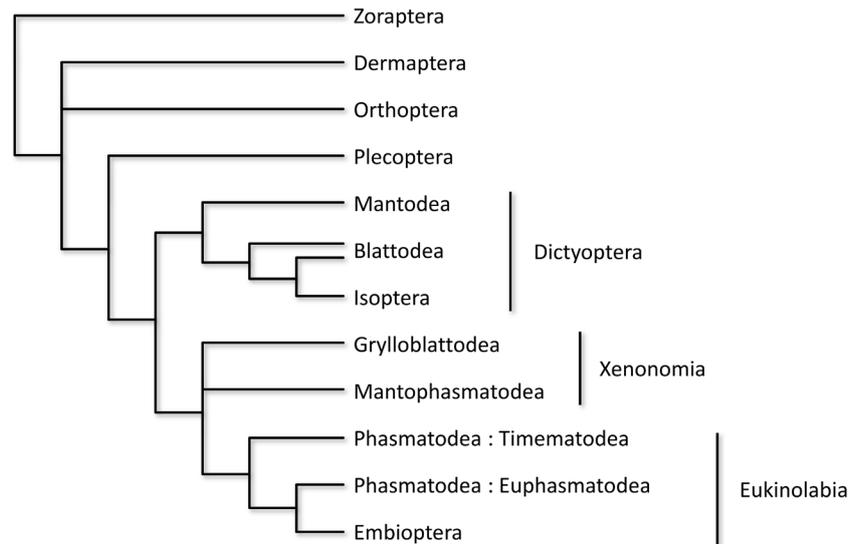


Fig. 1 A schematic diagram of a polyneopteran phylogeny based on partial mitochondrial genomic data.

When Mantophasmatodea was first described as a new insect order, morphological analysis suggested that it was related to Grylloblattodea and Phasmatodea (Klass *et al.* 2002). A mitochondrial genome phylogeny also shows that Mantophasmatodea, Grylloblattodea and Eukinolabia (Phasmatodea + Embioptera) form a sister clade to Dictyoptera (Kômoto *et al.*, 2012; Fig. 1). The branching order of Mantophasmatodea and Grylloblattodea, however, is not stable in molecular phylogenetics. Other mitochondrial genome phylogenies also preferred this branching order (Cameron *et al.*, 2006; Plazzi *et al.*, 2011; Tomita *et al.*, 2011), but the application of three protein-coding nuclear genes led to a monophyletic clade: Xenonomia, consisting of Mantophasmatodea and Grylloblattodea (Ishiwata *et al.*, 2011). The monophyly of Xenonomia has not necessarily been supported by molecular analyses: 18S rDNA comparisons under the alignment with CLUSTAL did not support the monophyly of Xenonomia, while 28S rDNA data did (Terry and Whiting, 2005). To confirm the relationship between Mantophasmatodea and Grylloblattodea, it will be necessary to increase the number of taxa whose mitochondrial genome is fully sequenced.

The positions of the orders Plecoptera, Dermaptera, and Zoraptera are difficult to resolve in mitochondrial genome phylogenies because they generally branch at the base of Polyneoptera (Kômoto *et al.*, 2012; Fig. 1). Without Dermaptera and Zoraptera, mitochondrial genome phylogenies placed Plecoptera in the position of sister to Orthoptera (Tomita *et al.*, 2011; Kômoto *et al.*, 2012). The addition of a partial sequence of the mitochondrial genome of Zoraptera and Dermaptera changed the position of Plecoptera and defined it as sister to Dictyoptera + Xenonomia + Eukinolabia, the branch opposite of Orthoptera (Fig. 1). Wan *et al.* (2012) sequenced the full mitochondrial genome of a dermapteran species and constructed phylogenetic trees showing the sister relationship between Dermaptera and

Plecoptera branching from other polyneopteran orders, except Zoraptera which is absent in their trees. Our preliminary data placed Zoraptera sister to the other polyneopteran orders, and showed trifurcate branching of Dermaptera, Orthoptera and the others (Fig. 1). Ishiwata *et al.* (2011) was not able to define the branching orders of Plecoptera, Dermaptera, and Zoraptera in Polyneoptera, but many morphological studies have placed Zoraptera close to Embioptera (Engel and Grimaldi, 2000; Yoshizawa, 2011; Mashimo *et al.*, 2014). To elaborate on these molecular phylogenies using the mitochondrial genome, it is important to increase the number of taxa, especially within Zoraptera and Plecoptera.

#### 4. Phasmatodea phylogeny

Phasmatodea is a polyneopteran order whose synapomorphies are a short mesonotum, the first abdominal segment that is fused with the metanotum, five tarsomeres, two prothoracic exocrine glands, *etc.* (Zompro, 2004). Its prominent adaptation for crypsis, *e.g.* resembling twigs or leaves, can easily result in morphological convergence, which makes it difficult to resolve intraordinal relationships.

The phylogenetic position of *Timema* is problematic in Phasmatodea systematics. *Timema* has been thought of as a 'basal' taxon of Phasmatodea because the first abdominal segment is not fused with metanotum, the tarsomeres are trimeric, and so on (Zompro, 2004). The egg structure of *Timema* species indicates its close relationship with Embioptera, supporting the idea that Embioptera and Phasmatodea form a monophyletic clade, Eukinolabia (Zompro, 2004; Jintsu *et al.*, 2010). Molecular phylogenetic analyses on nuclear genes have shown that *Timema* is sister to the remaining phasmatodean taxa (Whiting *et al.*, 2003). On the other hand, our mitochondrial data showed that Euphasmatodea and Embioptera form a sister clade to *Timema*, suggesting the paraphyly of Phasmatodea (Kômoto

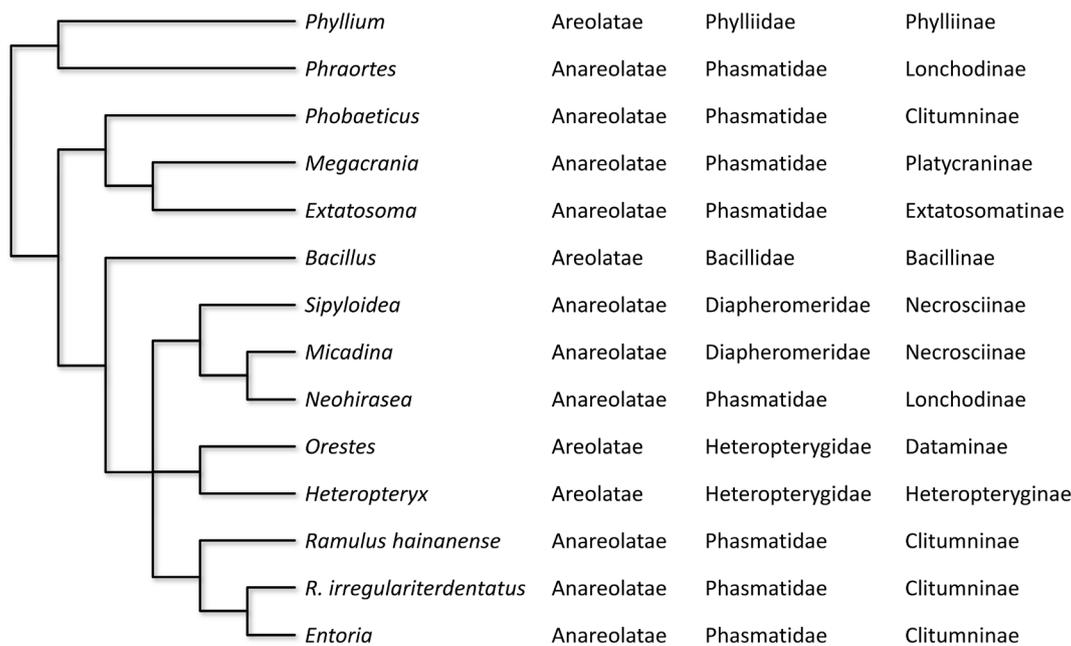


Fig. 2 A schematic diagram of a phasmatodean phylogeny based on the full sequence of the mitochondrial genome. Modified from Tomita *et al.* (2011).

*et al.*, 2012; Fig. 1). However, the tree topology should be interpreted carefully because of the long branch of Embioptera (Kômoto *et al.*, 2012); long branches of some taxa can cause distortion of the phylogenetic trees (Philippe *et al.*, 2011). To overcome long branch attraction (LBA), we attempted different evolutionary models or increasing the number of taxa, all resulting in a failure to avoid the long branch (data not shown). Phylogenomic approaches may help in the understanding of the branching order of these taxa. We tried a preliminary analysis of Eukinolabia based on recently available EST data of *Timema*, *Phyllium*, and Embioptera (Comeault *et al.*, 2012; Letsch *et al.*, 2012), and results showed that *Timema* and *Phyllium* form a clade sister to Embioptera, consistent with the monophyly of Phasmatodea (data not shown).

Another issue to be re-evaluated is the intraordinal classification of Phasmatodea. Phasmatodea has been divided into two groups, Areolatae and Anareolatae, defined by the presence and absence, respectively, of an impression on the tip of the tibiae called the area apicalis (Brunner von Wattenwyl and Redtenbacher, 1906–1908). Although this basic classification has been accepted for many years (Bradley and Galil, 1977; Günther, 1953), the dichotomic classification has been questioned recently. Cladistic analyses based on morphological traits have suggested that both Areolatae and Anareolatae are polyphyletic (Tilgner, 2002; Bradler, 2009), supported by our molecular phylogenetic studies based on the mitochondrial genome (Kômoto *et al.*, 2011; Tomita *et al.*, 2011; Fig. 2). Phylogenetic analyses based on the nuclear genes, 18S rDNA, 28S rDNA and histone H3, have also concluded that the two groups are polyphyletic (Whiting *et al.*, 2003). Another hypothesis is that area apicalis is a

pleisiomorphy that has been lost independently multiple times throughout phasmatodean evolution, supported by the presence of a similar impressed area in Embioptera and the egg morphology of Phasmatodea (Zompro, 2004). The area apicalis, however, is not suitable for phasmatodean classification.

Families and lower taxa in phasmatodean taxonomy have also been challenged. Anareolatae is known to consist of two families: Diapheromeridae and Phasmatidae, but the subfamily Lonchodinae was proposed to be moved from Diapheromeridae to Phasmatidae based on male genital structures (Hennemann and Conle, 2008). Four of six families and 9 of 19 subfamilies, including Lonchodinae, described by Bradley and Galil (1977), were shown to be polyphyletic based on comprehensive cladistic analyses of morphological and behavioral traits (Bradler, 2009). The tree topology of mitochondrial phylogenies also differs from conventional systematics (Kômoto *et al.*, 2011, 2012; Tomita *et al.*, 2011; Fig. 2). For example, Diapheromeridae and Phasmatidae are paraphyletic and polyphyletic, respectively. Two genera within Lonchodinae, *Phraortes* and *Neohirasea*, are separated in distantly related branches. *Neohirasea* is sister to *Micadina*, which belongs to the paraphyletic subfamily Necrosciinae. *Ramulus* is also a paraphyletic group, which forms a monophyletic clade with *Entoria*. Molecular phylogenetics based on nuclear genes also contradicts conventional taxonomy, showing similar results to mitochondrial analyses (Whiting *et al.*, 2003). For example, both Diapheromeridae and Phasmatidae are polyphyletic, and *Neohirasea* is sister to Necrosciinae species. In the meantime, it is better to be cautious when considering the surprising hypothesis that wings were recovered independently several times from

apterous phasmatodean lineages (Whiting *et al.*, 2003). It should be noted that wing loss and recovery cannot be weighed equally in parsimonious analysis (Stone and French, 2003; Zompro, 2004). The direct optimization method, in which sequence alignment and tree searches are conducted simultaneously, may also cause artificial alignment (Simmons, 2004; Ogden and Rosenberg, 2007; Yoshizawa, 2010).

## 5. Conclusion

This review has shown that the mitochondrial genome is a useful tool for determining polyneopteran phylogeny, especially in intraordinal relationships. It should be applied carefully, however, to ancient divergence such as the interordinal phylogeny of Polyneoptera. In particular, extremely different substitution rates or base compositions can lead to misinterpretation of evolutionary history. The position of Embioptera is expected to be verified based on other nuclear genes and phylogenomics with increased number of taxa. The intraordinal systematics of Phasmatodea need to be reexamined by molecular phylogenies. We will use the mitochondrial genome of more phasmatodean species to determine further important information which, united with morphological classification, may resolve phasmatodean evolution.

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