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Exploring the molecular phylogeny of phasmids with whole mitochondrial genome sequences

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ABSTRACT

Phasmids are remarkable mimics of twigs, sticks, and leaves. This extreme adaptation for crypsis can easily lead to the convergent evolution of morphology, making it difficult to establish a taxonomic system of phasmids. Accordingly, there are multiple phylogenetic hypotheses that conflict with each other. Phylogenetic arrangements suggested by molecular data disagree with the morphology-based taxonomy in some instances. We collected 13 phasmatodean species, sequenced their mitochondrial genomes, and recovered their molecular phylogeny. Our analyses did not support the monophyly of Areolatae or Anareolatae, two major infraorders of Phasmatodea. The position of *Neohirasea* was also quite different from the conventional taxonomic systems, thus challenging the previously assumed monophyly of the subfamily Lonchodinae. The enigmatic taxon, *Timema*, was shown to be distantly related to other phasmatodeans.

1. Introduction

Phasmids are hemimetabolous insects and include more than 3200 species in about 570 genera (Zompro, 2004). Many inhabit tropical regions, and some are found in temperate areas (Brock, 1999). They are well known for crypsis by mimicking sticks, twigs, and leaves. Their eggs resemble plant seeds in size and shape. The frequent occurrence of apterous species in different lineages makes this group of insects a fascinating object for evolutionary studies of wing loss. However, the absence of firm systematics based on phylogenetic relationships interferes with the analysis of the molecular mechanisms underlying wing-loss events in an evolutionary developmental context. A previous molecular phylogenetic study of phasmids by Whiting et al. (2003) suggested that wings were regained secondarily in several different wingless lineages, although this hypothesis is controversial. For example, Stone and French (2003) argued that it is difficult to apply a parsimony-based explanation to wing evolution, even if the constructed tree reflects the correct phylogeny, because the difference in the probabilities of wing-loss and wing-gain is unknown.

The extreme adaptation for crypsis can easily lead to convergence of morphology, making it difficult to elucidate the taxonomy of phasmids. Parthenogenesis, which takes place in many phasmid taxa, also interferes with classification using male genitalia, which carries important traits for classifying many insect taxa. Phylogenetic work within Phasmatodea has long been lacking, and only recently have major revisions to the classification conducted by Bradley and Galil (1977), which largely followed Günther (1953), been advocated (Zompro, 2004; Hennemann and Conle, 2008).

Based on early works of taxonomy, phasmatodeans have been classified into two major groups, Areolatae and Anareolatae, which are characterized by the presence and absence, respectively, of an area apicalis, an impression on the tip of the tibia (Brunner von Wattenwyl, 1907; Redtenbacher, 1906, 1908). This dichotomic classification has been reexamined recently. Tilgner (2002) suggested that both Areolatae and Anareolatae are polyphyletic according to a cladistic study of morphological traits. Zompro (2004) pointed out the possibility that Anareolatae are included in Areolatae, assuming that the area apicalis has been lost several times independently. This hypothesis is supported by the similarity of the eggs of some Areolatae and Anareolatae species, e.g., *Prisopus* (Areolatae) and *Calvisia* (Anareolatae). Phylogenetic studies examining molecular data suggest that both Areolatae and Anareolatae are polyphyletic (Whiting et al., 2003).

The taxonomy of phasmatodeans is not stable even in lower levels. For example, a species formerly known as *Baculum irregulariterdentatum*, which appears in this study, has been moved to the genus *Ramulus* recently, based on the biogeographical consideration that *Baculum* and *Ramulus* are neotropical and oriental genera, respectively (Shiraki, 1935; Hennemann et al., 2008). The taxonomic positions of the two genera themselves have also been reconsidered recently. In the classification by Bradley and Galil

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(1977), *Baculum* and *Ramulus* belonged to different families, Heteronemiidae and Phasmatidae, respectively, although *Baculum* has been transferred to a neotropical subfamily, Cladomorphinae in Phasmatidae (Hennemann and Conle, 2008).

The taxonomic position of Timematidae is also controversial. It was assigned to Areolatae in Bradley and Galil's classification (1977), but Kristensen (1975) segregated it from other phasmid families, and today this group is generally treated as a separate suborder, Timematodea, versus all other phasmatodeans (Phasmatodea sensu Zompro, 2004). *Timema* has been considered a sister taxon to all the other phasmids because they share some characteristics with other phasmids such as the prothoracic exocrine gland and the area apicalis. This hypothesis is supported by the molecular phylogenetics of 18S rDNA, 28S rDNA, histone H3, and mitochondrial 16S rDNA, whereas another analysis of mitochondrial *cox1* suggests that Timematodea forms a clade with Plecoptera (Whiting et al., 2003; Damgaard et al., 2008). Comparison of egg morphology showed that *Timema* is more closely related to Embioptera than to Verophasmatodea (Areolatae + Anareolatae; Zompro, 2004).

Molecular phylogenetics will be a useful tool for examining the relationships among phasmids, for which morphology-based classification has proven difficult. Whiting et al. (2003) sampled extensive taxa and constructed phylogenetic trees using three nuclear genes, 18S rDNA, 28S rDNA, and histone H3. The trees recovered in their study are highly different from those of morphological studies. For example, *Agathemera* was placed in an Areolatae clade, whereas morphological studies indicate that it is a sister taxon to Verophasmatodea (Zompro, 2004). However, there are several nodes with relatively weak support in their molecular-based trees. Thus, it is necessary to reexamine the molecular phylogeny of phasmids using different data sets.

Previous studies have shown that the whole mitochondrial genome is useful for analyzing phylogenetic relationships within insect orders: for example, the intraordinal phylogeny of Hymenoptera (Castro and Dowton, 2007), Diptera (Cameron et al., 2007), Orthoptera (Fenn et al., 2008), and Coleoptera (Pons et al., 2010; Timmermans et al., 2010) has been resolved depending on whole mitochondrial genomes. There are various advantages to using the mitochondrial genome for inferring phylogeny, such as the absence of recombinations, introns, and paralogs, all of which often interfere with molecular phylogenetics. Castro and Dowton (2007) examined the efficacy of using the mitochondrial genome to recover the well-established phylogeny of hymenopteran taxa, and showed that Bayesian inference gave a more robust phylogeny than maximum parsimony analysis. They also found that analyses with amino acid sequences did not recover the expected phylogeny. On the other hand, the relatively high evolutionary rate of the mitochondrial genome can be a disadvantage when attempting to resolve deep relationships, such as Collembola and Insecta (Cameron et al., 2004) and the interordinal phylogeny of holometabolous insects (Castro and Dowton, 2007).

In the present study, we identified the intraordinal phylogeny of 13 phasmid species, most of which can be collected in Japan, using their mitochondrial genome sequences. Our results help elucidate the phylogenetic relationships of phasmids, whose convergent morphology may cause confusion for classification.

2. Materials and methods

2.1. Insects

Table 1 lists the insects used in this study. Insects collected in Japan were cultured in the laboratory prior to genomic DNA extraction. Those from Malaysia and Indonesia had been on display in an exhibition at Kitakyushu Museum of Natural History and Human History, Fukuoka, Japan; these were collected and frozen until use. All of the voucher specimens from which DNA was extracted are now maintained in our laboratory at Insect Genome Research Unit of National Institute of Agrobiological Sciences, Ibaraki, Japan.

2.2. DNA extraction, PCR, and sequencing

Genomic DNA was extracted from fresh or frozen specimens using common methods (Sambrook et al., 1989). Mitochondrial gene fragments shorter than 1.5 kb were initially amplified from the extracted genomic DNA with *Ex Taq*, PrimeSTAR GXL DNA polymerase (TAKARA BIO INC., Japan), or Phusion DNA polymerase (Finnzymes, Finland) according to the manufacturers' instructions using the primers listed in Table 2. PCR products were directly sequenced or cloned into plasmid vectors to be sequenced. After acquiring partial sequence data, species-specific primers were designed to amplify the fragments between the sequenced regions to cover the full genomic sequences with *LA Taq* (TAKARA BIO INC.) or PrimeSTAR GXL DNA polymerase according to the manufacturer's instructions. These long PCR products were sequenced by primer walking with specific primers for the identified regions, or cloned into plasmid vectors directly or after digestion with restriction enzymes. Long inserts in the plasmid vectors were sequenced by primer walking, subcloning, or random insertion of transposons using the GPS-1 Genome Priming System (New England Biolabs, USA). All of the sequences obtained in this study were deposited in DDBJ (see Table 1 for accession numbers).

2.3. Identification of genes

Nucleotide sequences of mitochondrial protein-coding, rRNA, and tRNA genes of the stick and leaf insects were identified through BLAST searches of the NCBI database. Gene arrangement was identified by comparing the sequences to those of *Timema californicum* (DQ241799) and *Bombyx mori* (AB070264, Lepidoptera, Yukuhiro et al., 2002). We identified 13 protein-coding, 2 rRNA, and 22 tRNA genes that were common among animal mitogenomes. The invertebrate mitochondrial genetic code was used to infer the amino acid sequences of the protein-coding genes (PCGs).

2.4. Sequence alignment

We used ClustalW (Thompson et al., 1994) in MEGA 4.1 (Tamura et al., 2007) to align nucleotide sequences of mitochondrial PCGs based on their translated amino acid sequences. Nucleotide sequences of the two mitochondrial rRNA genes were aligned using MUSCLE (Edgar, 2004) in EMBL-EBI. The alignments for *rrnS* and *rrnL* genes are provided in the Supplementary data as MEGA data files. The deduced amino acid sequences of individual genes were aligned using ClustalW in MEGA 4.1. All aligned sets of PCGs and rRNA genes were concatenated for use in subsequent phylogenetic analysis.

2.5. Sequence data sets

We constructed nucleotide data sets composed of 13,074 nucleotide sites from 13 concatenated mitochondrial PCGs and two rRNA genes. Because Cameron et al. (2007) suggested that a correct partitioning strategy is quite important for Bayesian analyses of mitogenomes data, we prepared two partitioning strategies for nucleotide data sets: one broken down by codon positions within each gene (CGP: first, second, and third codon positions for each of the 13 PCGs, *rrnS*, and *rrnL*) resulting in 41 partitions, and the other broken down by codon positions within each strand (CSP: first, second, and third codon positions for each of the concatenated PCGs from major and minor strands and two rRNA