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Molecular systematics and evolution of the recently discovered "Parnassian" butterfly (*Parnassius davydovi* Churkin, 2006) and its allied species (Lepidoptera, Papilionidae)

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ABSTRACT

The nucleotide sequence of 807 bp of the mtDNA-*ND5* locus of *Parnassius davydovi* (Churkin, S. 2006. A new species of *Parnassius* Latreille, 1804, from Kyrgyzstan (Lepidoptera, Papilionidae). Helios (Moskow) 7,142-158), was determined. This butterfly was unexpectedly discovered recently in Kyrgyzstan, and we wished to shed light on its molecular phylogenetic relationship to other Parnassian butterflies, as well as to the related taxa in the subfamily Parnassiinae of the family Papilionidae. Using the ML method with the GTR+I+Γ model, we inferred the phylogenetic tree for 60 *Parnassius* individuals together with materials of the related genera in the subfamily Parnassiinae (*Hypermnestra*, *Archon*, *Luehdorfia*, *Bhutanitis*, *Allancastria*, *Zerynthia* and *Sericinus*) with *Papilio machaon* as an out-group. It was found that *P. davydovi* is a distinct species most closely related to *P. loxias* in clade VI among the eight clades, or species groups of *Parnassius*. The morphological diversity in the form of sphragis, the attachment to the female abdomen formed by the male during copulation, is characteristic to this clade, and we inferred the order of emergence of the different sphragis forms during evolution. Attempts to estimate the divergence times between related taxa were also made. It was inferred that the relatively rapid radiation of Parnassian butterflies started at about 24 MYA BP, while *P. davydovi* diverged from *P. loxias* at about 10 MYA BP.

1. Introduction

In July 2005, a new species of the genus *Parnassius* (Lepidoptera, Papilionidae) was discovered unexpectedly in the Mold-Too Mts. in Kyrgyzstan, Central Asia, and the news spread rapidly with excitement among the butterfly collectors of the world (Churkin, 2006).

The butterflies of this genus, often called "Apollos," are so popular among collectors and taxonomists that the term Parnassiology has been in use since the 1920s. About fifty species are known for this butterfly group, occurring mostly in high altitude areas of Central Asia, the Himalayas, and Western China. Because of enthusiastic searching by "Apollo" hunters for over 100 years in remote mountain areas of Eurasia, it was considered that a "new" species would not be found any more.

The first molecular systematic study of this genus was carried out by Professor Omoto and his colleagues who attempted to shed light on the evolutionary history of the subfamily Parnassiinae of the family Papilionidae (Omoto et al., 2004). They examined mitochondrial DNA of essentially all the fifty species of the genus *Parnassius* and most species of the related genera (*Hypermnestra* and *Archon*) of the tribe Parnassiini of the subfamily Parnassiinae, together with the tribe Zerynthiini (*Zerynthia*, *Allancastria*, *Sericinus* and *Luehdorfia*).

In the study, using the sequence data of about 800 bp of the *ND5* locus, the NJ and MP molecular phylogenetic trees were inferred. It was shown that the genus *Parnassius* was a monophyletic group comprised of eight clades, or species groups, which were mostly found to correspond to the subgenera hitherto proposed by a number of "Parnassiologists", with some exceptions. Judging from the branching pattern in the molecular phylogenetic tree, the genus *Parnassius* was assumed to have experienced a relatively rapid radiation at a certain geological time, probably during the late Tertiary period (20–30 MYA BP). The sister genus to *Parnassius* was found to be *Hypermnestra*, rather than *Archon*, the butterfly which superficially looks exceedingly similar to those of *Parnassius*. Contrary to the previously supported classification based on morphological characteristics (Hancock, 1983; Häuser, 1993), the genus *Archon* was found to belong to the tribe *Zerynthiini,* with very different looking butterflies such as of the genus *Luehdorfia*, rather than *Parnassiini*, at the DNA level (Omoto et al., 2004).

Further study adding data of the *16S* and *ND1* sequences, as well as more detailed statistical analyses, confirmed the result mentioned above (Katoh et al., 2005). Nazari and his colleagues reported detailed molecular systematic studies using a large set of data of five mtDNA

Abbreviations: NJ, neighbor joining; ML, maximum likelihood; BP, bootstrap probability; MYA BP, million years before present.

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loci (*COI*, *COII*, *ND5*, *ND1*, *16S*) together with two nuclear DNA loci (*EF1*α and *wg*). They confirmed the sister-group relationships between *Parnassius* and *Hypermnestra*, as well as between *Archon* and *Luehdorfia* (Nazari et al., 2007). More recently, Michel et al. (2008) reported molecular phylogenetic analyses of Parnassiinae using the sequence data of four mtDNA loci (*16S*, *ND1*, *ND5*, *COI*). Their results essentially confirm the previously mentioned results with more detailed discussion about the relationships among species of the genus *Parnassius*.

The present study aims at determining the molecular systematic position of *Parnassius davydovi*, and by examining the molecular versus morphological diversities, shedding more light on the evolutionary history of some butterflies of the genus *Parnassius*. Moreover, based on the data given by Nazari et al. (2007), but using a different statistical method, we report some new estimates for divergence times among the taxa in the Parnassiinae.

2. Materials and methods

2.1. Material, DNA extraction and sequencing

One male *Parnassius davydovi* specimen, collected in July, 2006, by Sergei Churkin at altitude 2,500–2,600 m in the Moldo-Too Mts., Tien Shan Mountain Range, Kyrgyzstan, was obtained in a dried and mounted condition (Fig. 1). Two legs were taken from the specimen and sent to the laboratory of one of us (T. S.) for mtDNA sequencing. Extraction of the whole genomic DNA from the leg samples was carried out by means of the method commonly used for *Parnassius* and other Papilionid groups (Yagi et al., 1999, 2001, 2006).

Part of the *ND5* locus of mitochondrial DNA was amplified by PCR and the nucleotide sequence of 807 bp was determined using a 377-18 DNA sequencer with a Big-Dye Terminator Cycle Sequence Kit (Applied Biosystems). The detailed procedures of PCR and sequencing followed that of Omoto et al. (2004).

2.2. Phylogenetic analyses

The nucleotide sequence data used in this study are summarized in Table 1. These data were aligned automatically by Clustal W (Thompson et al., 1994) and carefully checked by eye. All ambiguous sites were excluded from the analyses. The phylogenetic trees were inferred with the ML method (Felsenstein, 1981), the Bayesian method (Yang and Rannala, 1997), and the NJ method (Saitou and Nei, 1987). In all the trees, *Papilio machaon hippocrates* (Papilioninae) was used as an out-group. The ML analysis was carried out using RAxML ver. 7.0.4 (Stamatakis et al., 2008) with the GTR+I+Γ model (Rodriguez et al., 1990, Yang, 1996). The Bayesian analysis was carried out using Mr. Bayes ver. 3.1.2 (Ronquist and Huelsenbeck, 2003) with the GTR+I+Γ model under two sets of four simultaneous chains of 1,000,000 generations (Burnin was set to be 100,000 generations. Sample frequency was set to be per 100 generations.). The convergence of the log-likelihood was confirmed by checking that the PSRF (the potential scale reduction factor) values of all parameters were close to 1.0. NJ analysis was carried out by MEGA ver. 4.0 (Tamura et al., 2007) with the MCL (*M*aximum *C*omposite *L*ikelihood)+Γ model (Tamura et al., 2004, Yang, 1996). The shape parameter (α) of gamma distribution, which was estimated by RAxML, was applied to this analysis. The bootstrap analyses were carried out with 100 replications for the ML method and 1,000 replications for the NJ method to assess the confidence of internal nodes.

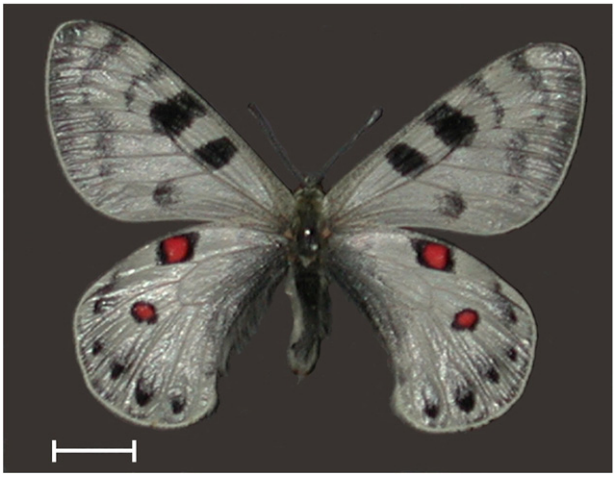

Fig. 1. *Parnassius davydovi* Churkin, 2006. Male, collected in the Moldo-Too Mts, Tien Shan Mountain Range, Kyrgyzstan in July, 2006. Scale bar indicates 10 mm.

2.3. Estimation of divergence time

We estimated divergence times following two different methods.

For the higher-taxa relationships (genus or tribe level), the divergence times were estimated using the method of Thorne et al. (1998), which does not assume the constant rate of the molecular clock. For this analysis, we used TTT (*T*hornian *T*ime *T*raveler; http://abacus.gene.ucl.ac.uk/). This software requires two stages of analyses. The first stage is the estimation of the branch lengths with the ML method and the second stage is the estimation of the divergence time with the Bayesian method.

The nucleotide sequence data of *ND1*, *ND5*, *COI*, *COII*, *16S*, *Leu-tRNA*, and *EF1*α (accession numbers are summarized in supplemental Table S1) were used in this analysis. The gaps and missing sites were completely excluded from the analyses.

To take account of the different tempo and mode among different genes and among different nucleotide sites of codon, we separated gene data into several partitions. The branch lengths of each partition and their variance-covariance matrix were independently estimated using the ESTBNEW program of TTT with the HKY+Γ model (Hasegawa et al., 1985, Yang, 1996). The parameters of this model were estimated using the BASEML program of PAML ver. 4.0 (Yang, 2007) in advance. Then the divergence times were estimated using the MULTIDIVTIME program of TTT under the chains of 1,000,000 generations (Burnin was set to be 100,000 generations. Sample frequency was set to be per 100 generations.)

The constraints of the divergence times were given as follows. The split of Papilionini (*Papilio*) and Troidini (*Troides*) was between 82.5 and 89.1 MYA BP (Gaunt and Miles, 2002). The initial split of genus *Papilio* (*P. machaon* and *P. thoas*) was between 35 and 65 MYA BP (Zakharov et al., 2004). The split of *Allancastria cretica* and *A. cerisyi* was between 3 and 11 MYA BP (Nazari et al., 2007).

For the lower-taxa relationship (species level), we assumed a molecular clock. In this analysis, we used the BASEML program of PAML ver. 4.0 with GTR+Γ model. The parameters (and rate matrix) were estimated for each nucleotide codon site.

3. Results and discussions

3.1. The molecular systematic position of *P. davydovi*

In the ML tree in Fig. 2, it is shown that *P. davydovi* is closest to *P. loxias* belonging to clade VI as mentioned below. The NJ and Bayesian trees showed essentially the same branching pattern (data not shown). Concerning the Bayesian tree, the PSRF value of the all parameters ranged from 1.00 to 1.03. In the original description of *P. davydovi*, S. Churkin already mentioned the morphological similarity between these two species, and comparing the wing patterns and male genitalia he concluded that the two species are “good” species, rather than subspecies (Churkin, 2006).