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Phylogeny and Evolution of Butterflies of the Genus *Parnassius*: Inferences from Mitochondrial 16S and ND1 Sequences

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ABSTRACT—Phylogenetic relationships among species of the genus *Parnassius* and its related taxa were analyzed by comparing nucleotide sequences of mitochondrial 16S ribosomal RNA (504 sites) and NADH-dehydrogenase subunit 1 (469 sites). In the phylogenetic trees, *Parnassius* was found to be most closely related to *Hypermnestra helios*, whereas *Archon apollinus*, which has been classified in the tribe Parnassiini together with *Parnassius* and *Hypermnestra*, was more closely related to members of the tribe Zerynthiini. Within the *Parnassius* clade, six major clades corresponding to species groups were well supported, although the phylogenetic relationships among them were not clear. Although the results of the present study were in agreement with those of a previous phylogenetic study based on mitochondrial NADH-dehydrogenase subunit 5 sequences, our study strongly supported a close relationship between *Parnassius* and *Hypermnestra*, which was not well supported in the previous study.

Key words: Parnassian butterflies, mitochondrial DNA, phylogenetic tree

INTRODUCTION

The butterflies of the genus *Parnassius* are among the most popular swallowtail butterfly groups of the family Papilionidae. The appearance of these butterflies is quite unique, as the adults have nearly transparent wings without a “tail” on the hind-wing (Fig. 1a, b). They have attracted a large number of biologists due to their geographical variation with respect to wing pattern, and also because of the rarity of certain species that occur in the remote mountainous areas of the Himalayas, Central Asia, Tibet, and other parts of northern Eurasia.

According to traditional studies, the genus *Parnassius* is classified together with two other genera, *Archon* (Fig. 1c) and *Hypermnestra* (Fig. 1d), into the tribe Parnassiini of the subfamily Parnassiinae (Munroe, 1961; Hancock, 1983). The subfamily consists of two tribes: one is Parnassiini, described above, and the other is Zerynthiini, which includes the genera *Zerynthia* (Fig. 1e), *Allancastria* (Fig. 1f), *Sericinus*, *Bhutanitis*, and *Luehdorfia*. However, previous studies have called into the question the monophyly of Parnassiinae (Häuser, 1983; Yagi *et al*., 1999; Caterino *et al*., 2001). In addition, Omoto *et al*. (2004) have recently presented phylogenetic trees in which *Parnassius* and *Hypermnestra* comprise a monophyletic group, whereas *Archon* is clustered with members of the tribe Zerynthiini rather than with those of the tribe Parnassiini, thus raising taxonomic controversy. To date, the phylogenetic relationship between *Parnassius* and its related genera remains controversial.

Phylogenetic relationships among species or species groups within *Parnassius* have also remained uncertain. The genus consists of about fifty species, and up to ten species groups or subgenera have been proposed based on previous morphological and behavioral studies (Bryk, 1935; Eisner, 1958, 1968; Munroe, 1961; Ackery, 1975; Hancock, 1983; Weiss, 1992–1998). The morphological characteristics primarily used in the classification of species and species groups include wing pattern, venation, male genitalia, fore-tibial epiphysis, and sphragis, i.e., the attachment to the end of the female abdomen made by the male secretion during copulation (see Hancock, 1983). However, despite these previous efforts, a number of conflicting placements have occurred in the classification of species and/or species

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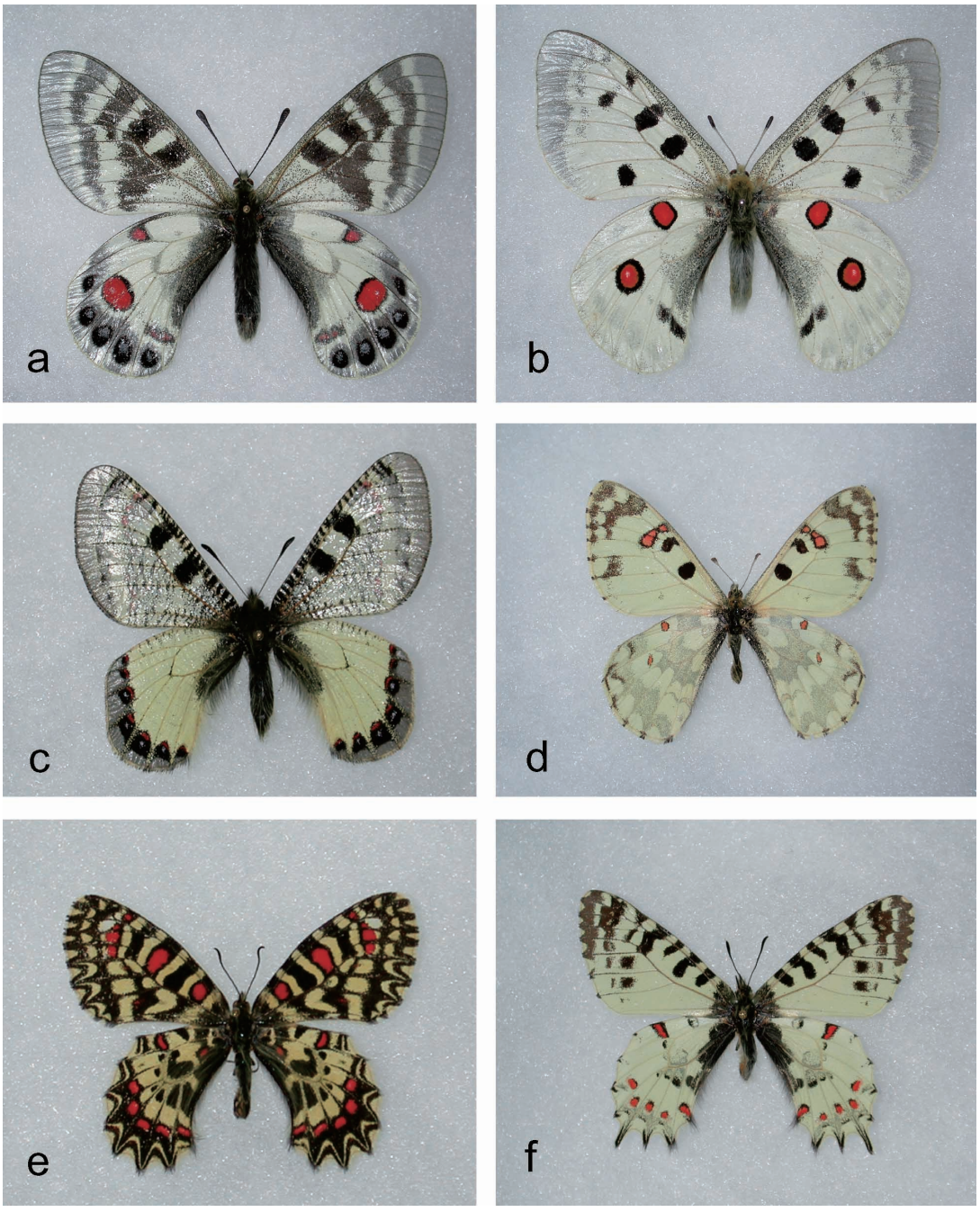

Fig. 1. Butterflies of the subfamily Parnassiinae. (a) *Parnassius charltonius*. (b) *Parnassius apollo*. (c) *Archon apollinus*. (d) *Hypermnestra helios*. (e) *Zerynthia rumina*. (f) *Allancastria cerisyi*.

groups in the genus *Parnassius*. In order to solve this problem, Omoto *et al.* (2004) recently analyzed the phylogenetic relationships among nearly all species of *Parnassius* using molecular data. Their study revealed that several clusters recognized in the *Parnassius* lineage corresponded well to those of the species groups recognized based on traditional studies. However, the monophyly and relationships of certain species or species groups remain unclear, and therefore more data will be required for further analyses.

In this study, we analyzed the nucleotide sequences of 16S ribosomal RNA (rRNA) and NADH-dehydrogenase subunit 1 (ND1) in mitochondrial DNA (mtDNA) from representative species of *Parnassius* and its related genera. Based on the phylogenetic trees constructed, we discuss here the phylogeny and evolution of *Parnassius* and its related taxa.

MATERIALS AND METHODS

Samples

A total of 34 adult butterfly specimens, including 27 species of *Parnassius*, were included in the present study (Table 1). Most of these specimens were the same ones used by Omoto *et al.* (2004).

Table1. Butterfly samples used in this study

Subfamily	Tribe	Genus	Species	Subspecies	Locality	Accession number	
						16S	ND1
Parnassinae	Parnassiini	*Parnassius*	*acco*	gemmifer	Karo-la, S. Tibet, China	AB186145	AB186179
			acdestis	*lampidius*	Karo-la, S. Tibet, China	AB186156	AB186190
			actius	*actius*	Tianshan, Xinjiang, China	AB186143	AB186177
			ariadne		Altai, Russia	AB186160	AB186194
			autocrator		Tajikistan	AB186158	AB186192
			bremeri	*bremeri*	Middle Amur, F. E. Russia	AB186140	AB186174
			cephalus	*elwesi*	Qamdo, E. Tibet, China	AB186148	AB186182
			charltonius	*charltonius*	Tibet, China	AB186154	AB186188
			charltonius	*romanovi*	Kyrgyzstan	AB186155	AB186189
			delphius	*juldussica*	Tianshan, Xinjiang, China	AB186151	AB186185
			epaphus	*tsaidamensis*	Qilianshan, Gansu, China	AB186142	AB186176
			eversmanni	*eversmanni*	Magadan, Russia	AB186163	AB186197
			glacialis	*glacialis*	Hokkaido,Japan	AB186165	AB186199
			glacialis	*tsingtaua*	Sichuan, China	AB186164	AB186198
			hardwickii	*hardwickii*	E. Nepal	AB186144	AB186178
			hide	*aksobhya*	Wenquan, Qinghai, China	AB186153	AB186187
			huberi		Tanggulashan, C. Tibet, China	AB186146	AB186180
			hunningtoni		Karo-la, S.Tibet, China	AB186147	AB186181
			imperator	*musagetus*	Qinghai, China	AB186157	AB186191
			maharaja	*labeyriei*	Qinghai, China	AB186150	AB186184
			mnemosyne	*giganteus*	Kyrgyzstan	AB186159	AB186193
			nomion	*mandschuricus*	Primorye, F. E. Russia	AB186141	AB186175
			nordmanni		Dombay, N. Caucasus, Russia	AB186161	AB186195
			orleans	*groumi*	Qinghai, China	AB186162	AB186196
			patricius	*xinjiangensis*	Tianshan, Xinjiang, China	AB186152	AB186186
			phoebus	*ochotskensis*	Magadan, F. E. Russia	AB186139	AB186173
			schultei	*schultei*	Karo-la, Tibet, China	AB186149	AB186183
		Archon	*apollinus*	*apollinus*	Izmir, Turkey	AB186168	AB186202
		Hypermnestra	*helios*	*helios*	Uzbekistan	AB186166	AB186200
	Zerynthiini	*Allancastria*	*cerisyi*	*cerisyi*	Izmir, Turkey	AB186169	AB186203
		Luehdorfia	*japonica*		Kyoto, Japan	AB186171	AB186205
			puziloi	*yessoensis*	Hokkaido, Japan	AB186170	AB186204
		Zerynthia	*rumina*	*rumina*	Aix-en-Provence, France	AB186167	AB186201
Papilioninae		*Papilio*	*machaon*		Tibet, China	AB186172	AB186206

DNA was extracted according to a method described by Omoto *et al*. (2004). All of the DNA samples examined in this study are preserved in the laboratory of T. Y. at the Research Institute for Advanced Science and Technology, Osaka Prefecture University, Osaka, Japan.

PCR amplification and DNA sequencing

Partial sequences of the mitochondrial 16S and ND1 genes were amplified by the polymerase chain reaction (PCR). The nucleotide sequences of the primers were those cited in Aubert *et al*. (1999). PCR reactions were carried out in 100-ul reaction volumes, containing 10 mM Tris-HCl pH8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 uM of each dNTP, 0.5 or 1 uM of each primer, approximately 1 ug template DNA, and 2.5 units of Taq DNA polymerase (Life Technologies or Takara). PCR amplification was performed for 35 cycles using a PCR Thermal Cycler (Takara) under the following conditions: 94°C for 1 min, 52°C or 54°C for 1 min, and 72°C for 2 min. The amplification products were purified using an UltraClean GelSpin DNA Purification Kit (MO BIO).

The nucleotide sequences of all samples were directly determined in both directions. Cycle sequencing was carried out using a Perkin Elmer/ABI Dye Terminator Cycle Sequencing Kit. The primers used for cycle sequencing were the same as those used for the PCR amplification. The cycle sequencing products were cleaned by phenol/chloroform extraction and ethanol precipitation. The cleaned products were sequenced with an ABI 377-18 automated sequencer.

Phylogenetic analysis

The nucleotide sequences were aligned by multiple alignment using Clustal X 1.83 (Thompson *et al*., 1997) with the default settings, gap opening=10 and gap extension=0.20, followed by adjustment by eye. Phylogenetic analyses were performed using minimum-evolution (ME), maximum-parsimony (MP), and maximum-likelihood (ML) methods. Optimal ME trees were obtained from close-neighbor-interchange (CNI) searches (Nei and Kumar, 2000) starting with a topology given by the neighbor-joining (NJ) method (Saitou and Nei, 1987), using MEGA 2.1 (Kumar *et al*., 2001). Optimal MP trees were obtained through 100 heuristic search replicates, with starting trees generated by random stepwise addition, followed by TBR branch swapping, using PAUP* 4.0b10 (Swofford, 2002). Optimal ML trees were obtained by with a local rearrangement search starting with a topology given by the NJ method, using MOLPHY 2.3b3 (Adachi and Hasegawa 1996); the HKY model (Hasegawa *et al*., 1985) of nucleotide substitution was employed. Bootstrap values for ME and MP trees were obtained from 1000 replicates using Felsenstein's (1985) method, whereas those for the ML trees were the local bootstrap values (bootstrap values given to a node by fixing the relationships in other parts of the tree) obtained from 1000 replicates using the RELL method (Adachi and Hasegawa, 1996).

In order to verify possible incongruence between the 16S and ND1 regions, we performed an incongruence length difference (ILD) test (Farris *et al*., 1994), which is referred to as a partition homogeneity test in PAUP*. The test was implemented under parsimony with 1000 heuristic search replicates for each of 10 starting trees generated by random stepwise addition in order to generate the null distribution. It should be noted, however, that the utility of the ILD test has been strongly argued (see Hipp *et al*., 2004). Therefore, to determine whether the two regions had different phylogenetic signals, we also analyzed combined and partitioned data separately.

RESULTS AND DISCUSSION

Data characteristics

In the 16S region, alignment gaps were observed at several nucleotide sites. Therefore, these sites were excluded and the remaining 504 sites were used for further analyses. In contrast, no alignment gaps were observed in the ND1 region, such that the consecutive 469 sites could be used for further analyses. The G+C contents of the 16S and ND1 regions throughout the taxa were 22.4±0.5% and 20.8±0.5%, respectively. The χ^2 test of base frequencies across taxa revealed no heterogeneity among the samples (16S: χ^2=8.6, df=99, P=1.0; ND1: χ^2=11.5, df=99, P=1.0). The ILD test yielded no significant difference between the 16S and ND1 regions (sum of tree length, 906; P=0.833).

Phylogenetic trees

For the combined 16S and ND1 data, the ME tree (Fig. 2) was obtained using Tamura's (1992) distance, which is generally used for the phylogenetic analysis of mtDNA in insects. *Papilio machaon* was used as an outgroup according to Omoto *et al*. (2004). While ME searches were also conducted with more parameter-rich models such as GTR+I+Γ using PAUP*, the topology remained essentially unchanged. In the ME tree, 27 samples belonging to the genus *Parnassius* formed a monophyletic group (80% bootstrap) that emerged as a sister group to *Hypermnestra helios* (98% bootstrap). Although *Archon apollinus* has been classified in the tribe Parnassiini together with *Parnassius* and *Hypermnestra*, this species emerged as a sister clade to that of the genus *Luehdorfia*, which is a member of the tribe Zerynthiini. Within the *Parnassius* clade, we found six major clades, which corresponded entirely with those described in Omoto *et al*. (2004), as follows: I, the *apollo* group; II, the *hardwickii* group; III, the *acco* group, V, the *delphius* group; VI, the *charltonius*/*imperator* group; VIII, the *mnemosyne* group. As noted in Omoto *et al*. (2004), these clades corresponded with those species groups or subgenera described based on their morphological and behavioral characteristics (Bryk, 1935; Munroe, 1961; Ackey, 1975; Hancock, 1983). Among the six major clades, clades V and VI were found to be the closest sister taxa with respect to each other (95% bootstrap); however, the remaining relationships were not well supported due to their low bootstrap values. When the 16S and ND1 sequences were analyzed separately, the ME trees consistently showed the monophyly of *Parnassius* as well as its sister relationship to *Hypermnestra*, although the branching patterns among the six major clades of *Parnassius* were unstable (Fig. 3).

Two equally optimal MP trees were found (232 parsimony informative sites, 911 steps in length, CI=0.47, RI=0.59, RC=0.27). The strict consensus tree of these trees is shown in Fig. 4. In the consensus MP tree, *Parnassius* formed a monophyletic group including *Hypermnestra* (99% bootstrap), whereas *Archon* emerged as a sister taxon to *Luehdorfia* (95% bootstrap). In addition, within the *Parnassius* clade, the six clades (I, II, III, V, VI, and VIII) were again recognized with high bootstrap values (at least 90%). Thus, the phylogenetic tree obtained by the present MP analysis was largely compatible with that obtained by the ME analysis. However, some clades in the MP tree were not congruent with those in the ME tree, although most of these clades were not well supported by either the ME and/or MP analyses. Among these clades, the phylogenetic position of *Hypermnestra* as a sister taxon to *P. hardwickii* was noteworthy in the MP tree. However, in the MP tree, this relationship was weakly supported by a low bootstrap value (<50%). Furthermore, the position of *Hypermnestra* in the MP tree was also found to be unstable when the 16S and ND1 sequences were analyzed separately (Fig. 3).

The ML tree obtained using the combined 16S and ND1 data is shown in Fig. 5. In general, the phylogenetic tree constructed by the ML analysis was quite compatible with those obtained from the ME and MP analyses. In the ML tree, *Parnassius* was shown to be monophyletic (95% bootstrap), and it emerged as a sister taxon to *Hypermnestra* (99% bootstrap), although *Hypermnestra* was included in the *Parnassius* clade when the 16S data was considered separately (Fig. 3). As was consistently shown in the ME and MP trees, *Archon* emerged as a sister taxon to *Luehdorfia*, with a high bootstrap value (99%). The six major clades of *Parnassius* observed in the ME and MP trees were also well supported in the ML tree (at least 97% bootstrap),

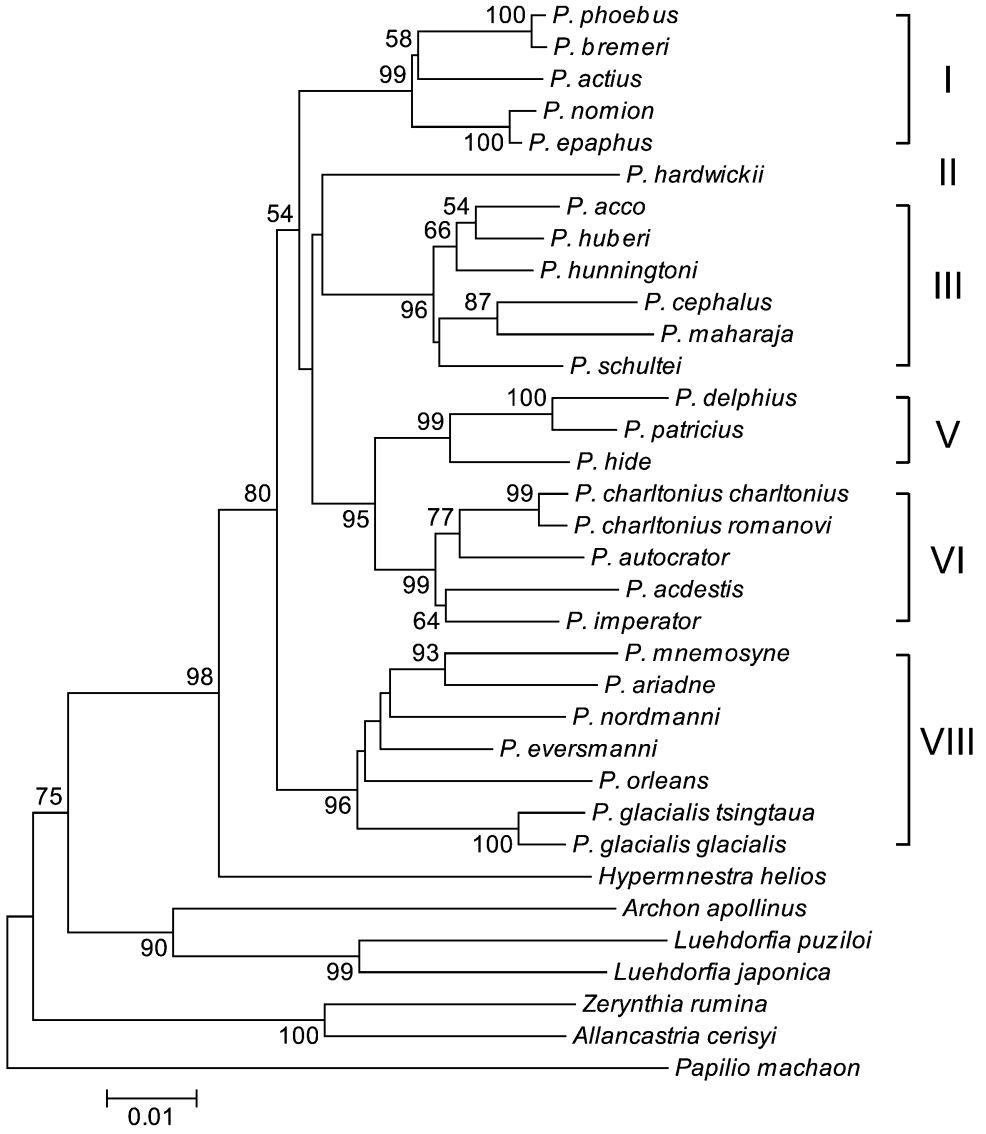

Fig. 2. Minimum evolution (ME) tree with Tamura's (1992) three-parameter distances based on the combined 16S and ND1 data. Bootstrap values larger than 50%, based on 1000 replicates, are shown. The clades denoted as roman letters (I, II, III, V, VI, and VIII) correspond to those presented by Omoto *et al*. (2004).

although clade VI was paraphyletic to clade V when the 16S data were considered separately (Fig. 3).

In summary, our phylogenetic trees consistently revealed the following results: (1) *Parnassius* has a close relationship with *Hypermnestra*; (2) *Archon* has a closer relationship with the members of Zerynthiini than with those of *Parnassius* and *Hypermnestra*; (3) the six major clades observed within *Parnassius* corresponded quite well with the previously described species groups and subgenera. However, the phylogenetic relationships among these *Parnassius* clades remain uncertain, with the exception of the close relationship demonstrated between clades V (the *delphius* group) and VI (the *charltonius*/*imperator* group).

Origin and evolution of *Parnassius*

According to the traditional view, *Parnassius* has been classified together with *Archon* and *Hypermnestra*, into the tribe Parnassiini of the subfamily Parnassiinae (Munroe, 1961; Hancock, 1983). However, our phylogenetic study revealed that Parnassiini is not necessarily a monophyletic group. In the phylogenetic trees obtained in the present study, *Parnassius* and *Hypermnestra* were shown to share close genetic affinity with each other, whereas *Archon* was more closely related to the members of Zerynthiini than to those of Parnassiini. It is of note that, although the same results were obtained by previous phylogenetic analyses using mitochondrial ND5 data (Omoto *et al*., 2004), closeness between *Parnassius* and *Hypermnestra* was not so clear in the previous study. Thus, the present study enabled us to clarify a close relationship between them. This finding may be surprising, as the larva of *Hypermnestra* are known to be quite distinct in form from those of *Parnassius* (Iga-

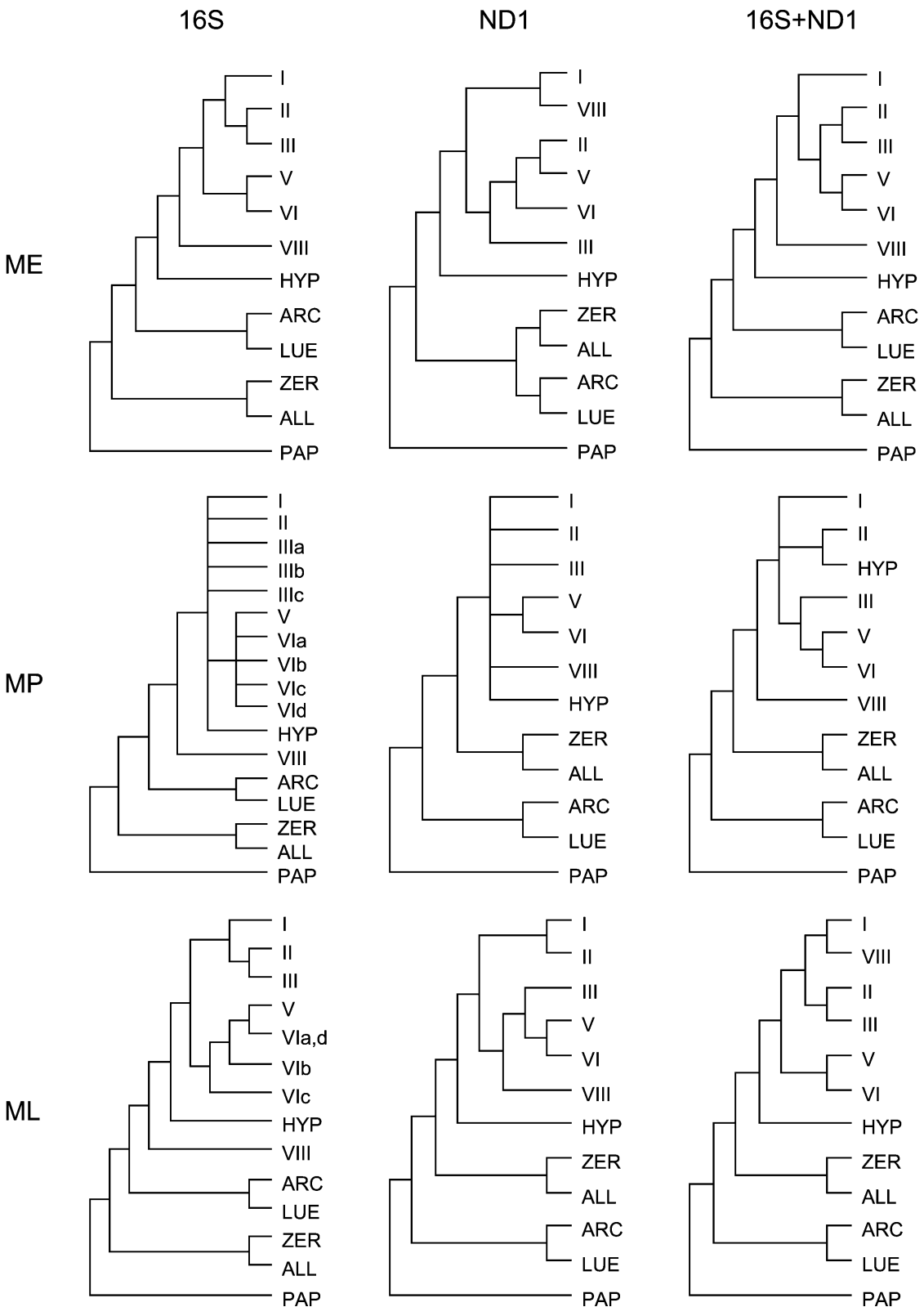

Fig. 3. Summarized tree topologies inferred from the ME, MP and ML analyses for the partitioned and combined 16S and ND1 data sets. I=*apollo* group; II=*hardwickii* group; III=*acco* group (a=*P. acco*, *P. huberi*, and *P. hunningtoni*; b=*P. cephalus* and *P. maharaja*; c=*P. schultei*); V=*delphius* group; VI=*charltonius*/*imperator* group (a=*P. charltonius charltonius* and *P. charltonius romanovi*; b = *P. acdestis*; c=*P. imperator*; d=*P. autocrator*); VIII=*mnemosyne* group; HYP=*Hypermnestra helios*; ARC=*Archon apollinus*; LUE=*Luehdorfia japonica* and *Luehdorfia puziloi*; ZER=*Zerynthia rumina*; ALL=*Allancastria cerisyi*; PAP=*Papilio machaon*. The roman letters correspond to those labeled in Fig. 2.

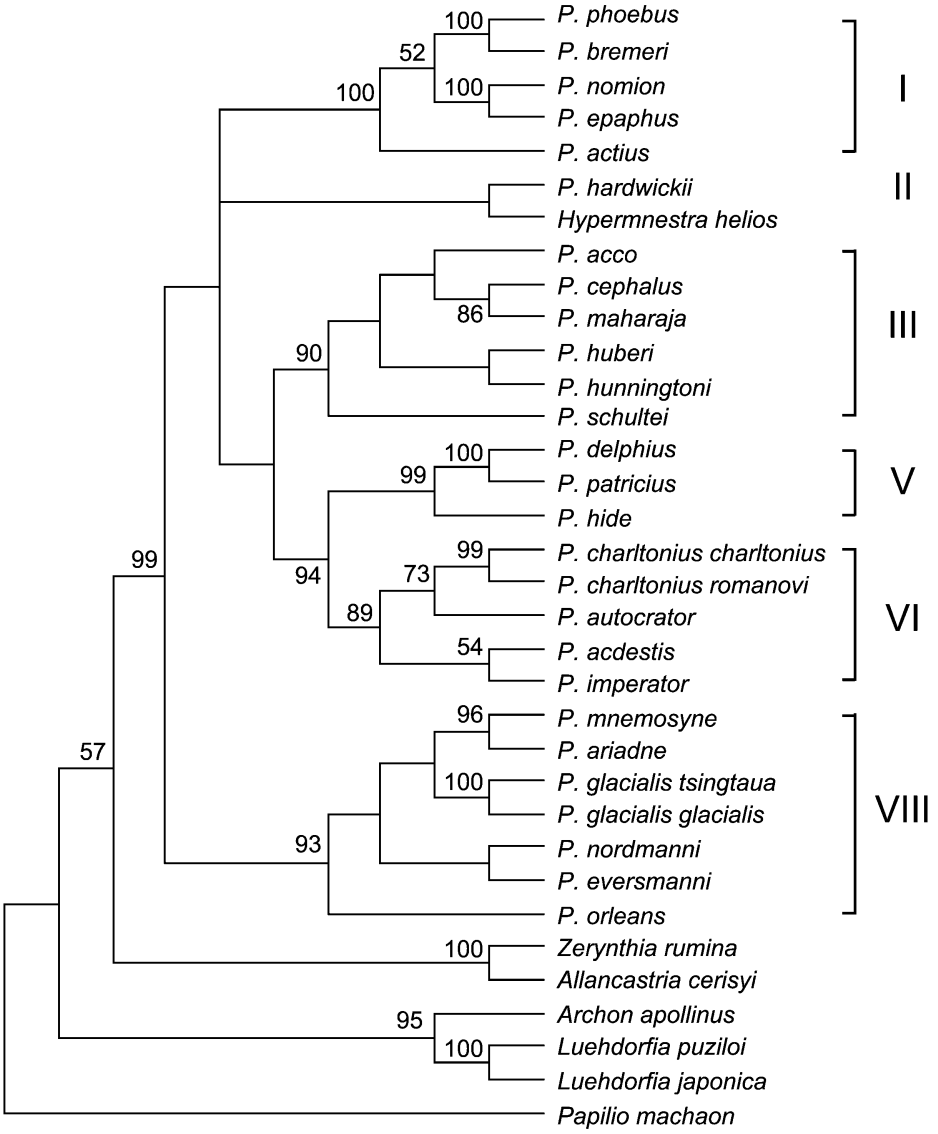

Fig. 4. Strict consensus tree of two equally parsimonious trees based on the combined 16S and ND1 data. Bootstrap values >50% are shown, based on 1000 replicates. The roman letters correspond to those labeled in Fig. 2.

rashi, 1979); moreover, *Hypermnestra* feeds on the plants of the family Zygophyllaceae, whereas the plant food sources of most Parnassian butterflies are Papaveraceae and Crassulaceae. However, a recent phylogenetic study has suggested that many of the features of swallowtail butterflies are of polyphyletic origin, with multiple gains and losses of particular morphological or ecological traits, including larval morphology and sources of food (Zakharov *et al*., 2004). Thus, our findings also demonstrate the complex nature of the evolution of characteristics among butterfly species.

Within the *Parnassius* clade, six major clades with high bootstrap support were recognized in our phylogenetic trees. These clades corresponded to those observed in Omoto *et al*. (2004), and they also corresponded with the species groups or subgenera based on morphology and ecology (Bryk, 1935; Munroe, 1961; Ackey, 1975; Hancock, 1983). However, except for the close relationship demonstrated between clades V (the *delphius* group) and VI (the *charltonius*/*imperator* group), no particular relationship was observed among the *Parnassius* clades even in the present study. Notably, according to both the ME and ML trees, these clades appeared to be connected to each other by relatively short internal branches. Generally, this branching pattern is expected when clades have radiated within a relatively short time during a specific period. This premise appears to be compatible with Hancock's (1983) study, in which it was noted that *Parnassius* underwent considerable speciation since the early Tertiary period, although this conclusion is still tentative, and therefore more data will be required for further analyses.

It should be noted that our findings are derived solely from the analysis of mitochondrial sequence data. Furthermore, in the present study, we were unable to analyze the species of *Bhutanitis*, which may be a key taxon for gaining

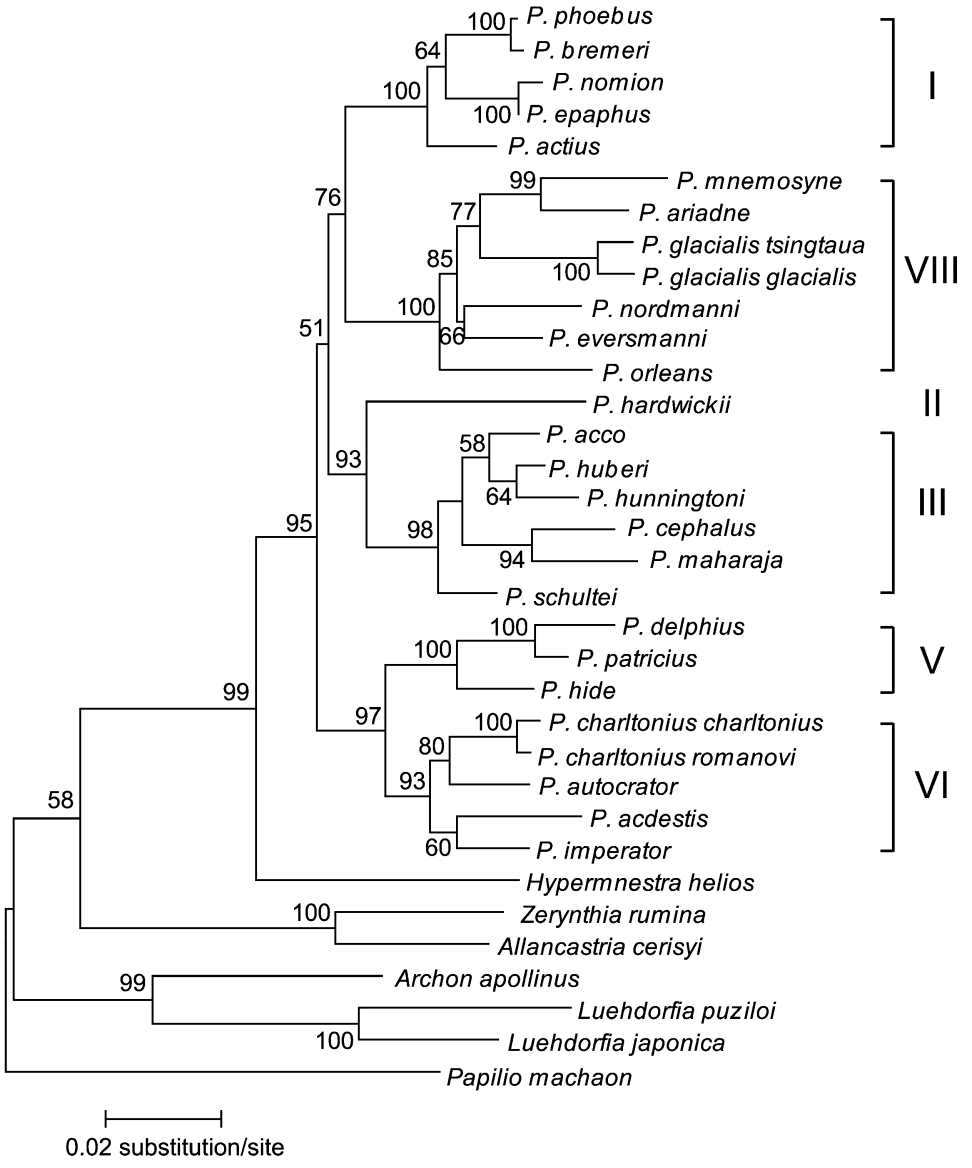

Fig. 5. Maximum likelihood (ML) tree with the HKY model (Hasegawa et al. 1985) based on the combined 16S and ND1 data. Local bootstrap values >50% are shown, based on 1000 replications using the RELL method (Adachi and Hasegawa, 1996). The roman letters correspond to those labeled in Fig. 2.

a detailed understanding of the relationship between *Archon* and Zerynthiini. Therefore, further studies investigating a larger number of species as well as data from other genes will be required for a more comprehensive understanding of the origin and evolution of Parnassian butterflies.

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