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Phylogeny, historical biogeography, and taxonomic ranking of Parnassiinae (Lepidoptera, Papilionidae) based on morphology and seven genes

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Abstract

We tested the taxonomic utility of morphology and seven mitochondrial or nuclear genes in a phylogenetic reconstruction of swallowtail butterflies in the subfamily Parnassiinae. Our data included 236 morphological characters and DNA sequences for seven genes that are commonly used to infer lepidopteran relationships (*COI + COII, ND5, ND1*, 16*S, EF-1α*, and *wg*; total 5775 bp). Nuclear genes performed best for inferring phylogenies, particularly at higher taxonomic levels, while there was substantial variation in performance among mitochondrial genes. Multiple analyses of molecular data (MP, ML and Bayesian) consistently produced a tree topology different from that obtained by morphology alone. Based on molecular evidence, sister-group relationships were confirmed between the genera *Hypermnestra* and *Parnassius*, as well as between *Archon* and *Luehdorfia*, while the monophyly of the subfamily was weakly supported. We recognize three tribes within Parnassiinae, with *Archon* and *Luehdorfia* forming the tribe Luehdorfiini Tutt, 1896 [stat. rev.]. Three fossil taxa were incorporated into a molecular clock analysis with biogeographic time constraints. Based on dispersal-vicariance (DIVA) analysis, the most recent common ancestor of Parnassiinae occurred in the Iranian Plateau and Central Asia to China. Early diversification of Parnassiinae took place at the same time that India collided into Eurasia, 65–42 million years ago.
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1. Introduction

Phylogenetic studies of insects have used DNA sequences from a multitude of gene regions, whether mitochondrial, nuclear, protein-coding or ribosomal, with the aim of finding regions that provide informative data useful for resolving phylogenies at various levels. These studies generally only assess the utility of individual gene regions or compare them to one or two others in a phylogenetic context (Simon et al., 1994; Brower and De Salle, 1994, 1998; Vila and Bjorklund, 2004; Danforth et al., 2004, 2005; Silva-Brandao et al., 2005; Wahlberg et al., 2005a; Whin-

nett et al., 2005; Wilkerson et al., 2005; but see Mallarino et al., 2005; Giribet and Edgecome, 2005). The popularity of some genes, particularly those encoded by mitochondrial DNA, may primarily be due to lab tradition or the ease of their amplification, and not necessarily the phylogenetic information contained in them (Caterino et al., 2000; Sperling, 2003). However, the choice of sub-optimally informative genes for a particular taxonomic level, together with incomplete sampling and missing data, can contribute to a poorly resolved phylogeny.

Here we examine divergence patterns of five mitochondrial and two nuclear genes in a phylogenetic analysis that includes all genera and subgenera of the swallowtail butterfly subfamily Parnassiinae. These butterflies comprise eight extant genera and about 70 species with a primarily Palaearctic distribution. The Parnassiinae have been considered the sister group of all remaining swallowtails

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(Papilionidae) except for the monotypic genus *Baronia* (Hancock, 1983). *Allancastria*, *Zerynthia*, *Archon*, and *Hypermnestra* range from Europe to central Asia, while *Sericinus*, *Luehdorfia*, and *Bhutanitis* are distributed from Bhutan to eastern Russia and Japan (Fig. 1). *Parnassius* has a Holarctic distribution with the highest diversity in the Himalayas (Bryk, 1935; Weiss, 1991, 1992, 1999, 2005). The larvae of most Parnassiinae genera feed on *Aristolochia* and other Aristolochiaceae, although larvae of *Hypermnestra* feed on Zygophyllaceae and those of *Parnassius* feed on Papaveraceae and Crassulaceae (Igarashi, 1984).

Although not generally considered to belong to Parnassiinae, the phylogenetic relationship of the genus *Baronia* to the subfamily remains intriguing. The only member of the subfamily Baroniinae, *Baronia brevicornis* is found in Mexico, and is often considered to be a "living fossil" (Collins and Morris, 1985; Tyler et al., 1994; Scriber et al., 1995; Eisner, 2003). The presence of many plesiomorphic traits in *Baronia* supports its position as the most primitive living swallowtail (Munroe, 1961; Hancock, 1983). The only DNA sequence study that included *Baronia*, based on *COI–COII* and *EF-1α* sequences, failed to resolve its position relative to the Parnassiinae (Caterino et al., 2001). Here we examine whether additional DNA sequence evidence would help to resolve the phylogenetic placement of *Baronia*.

The subfamily Parnassiinae is generally considered to comprise two tribes (Fig. 2): Parnasiini (including *Archon*, *Hypermnestra*, and *Parnassius*), and Zerynthiini (including *Allancastria*, *Sericinus*, *Zerynthia*, *Luehdorfia*, and *Bhutanitis*) (e.g. Ehrlich, 1958; Munroe, 1961; Ackery, 1975; Igarashi, 1984), although there has been some disagreement over whether these tribes should be regarded as separate subfamilies (Bryk, 1934; Talbot, 1939; Ford, 1944b; Chu-

nsheng, 2001) or even families (Clench, 1955; Hemming, 1960; Eisner, 1974).

Lack of a well resolved phylogeny for the Parnassiinae (Fig. 3), together with taxonomic uncertainties within the group, has contributed to confusion over the classification of the subfamily. Despite numerous attempts to infer the phylogeny of the group using characters from morphology (adult anatomy, wing venation and wing pattern, genitalia, immature stages, and ecological or biochemical characters) as well as DNA (Fig. 4), the phylogenetic relationships within Parnassiinae remain largely unresolved. Earlier morphological studies have produced contradictory results (e.g. Hiura, 1980 and Hancock, 1983), and all molecular studies published so far lacked representatives of some genera (Caterino et al., 2001; Omoto et al., 2004; Katoh et al., 2005). Moreover, the monophyly of the subfamily itself has frequently been questioned (Häuser, 1993; Hesselbarth et al., 1995; Yagi et al., 1999; Caterino et al., 2001; Stekolnikov and Kuznetsov, 2003).

At the tribal level, correct positions of the genera *Archon*, *Hypermnestra* and *Luehdorfia* have been particularly controversial (Fig. 2). *Archon* has sometimes been included in Zerynthiini (Eisner, 1974; Higgins, 1975) or in a separate sub-tribe within Parnassiini (Koçak, 1989). Häuser (1993) suggested a separate subfamily for *Hypermnestra* based on a number of morphological and ecological autapomorphies, a view previously expressed by Dujardin (1965), Hiura (1980), and Korshunov (1990) (as reported by Korb, 1997). A recent study of genitalic characters (Stekolnikov and Kuznetsov, 2003) recognized the tribe "Hypermnestriini Hiura, 1980" and gave subfamily status (Luehdorfiinae Tutt, 1896) to *Luehdorfia* based on putatively primitive genitalic characters (also see Ford, 1944b). Recent findings, including the discovery of
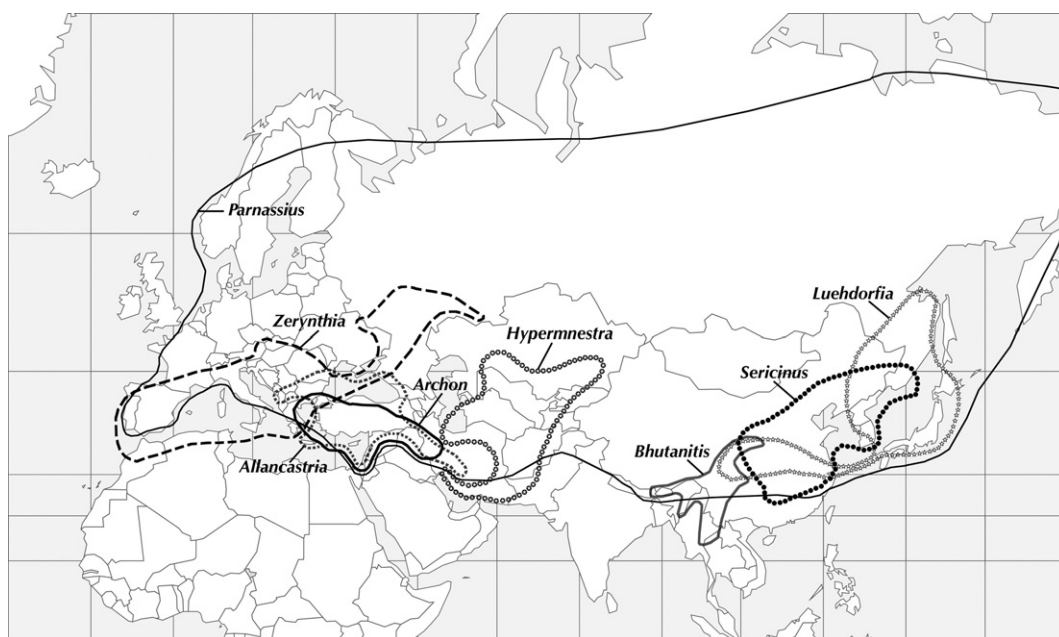

Fig. 1. Geographical distribution of Parnassiinae genera in the Palaearctic (summarized from Kudrna, 2002; Nazari, 2003; Weiss, 1992, 1999, 2005; Tshikolovets, 1998, 2000, 2003; Hesselbarth et al., 1995; Igarashi, 2003). The range of *Parnassius* also extends into western North America (Opler and Warren, 2003).
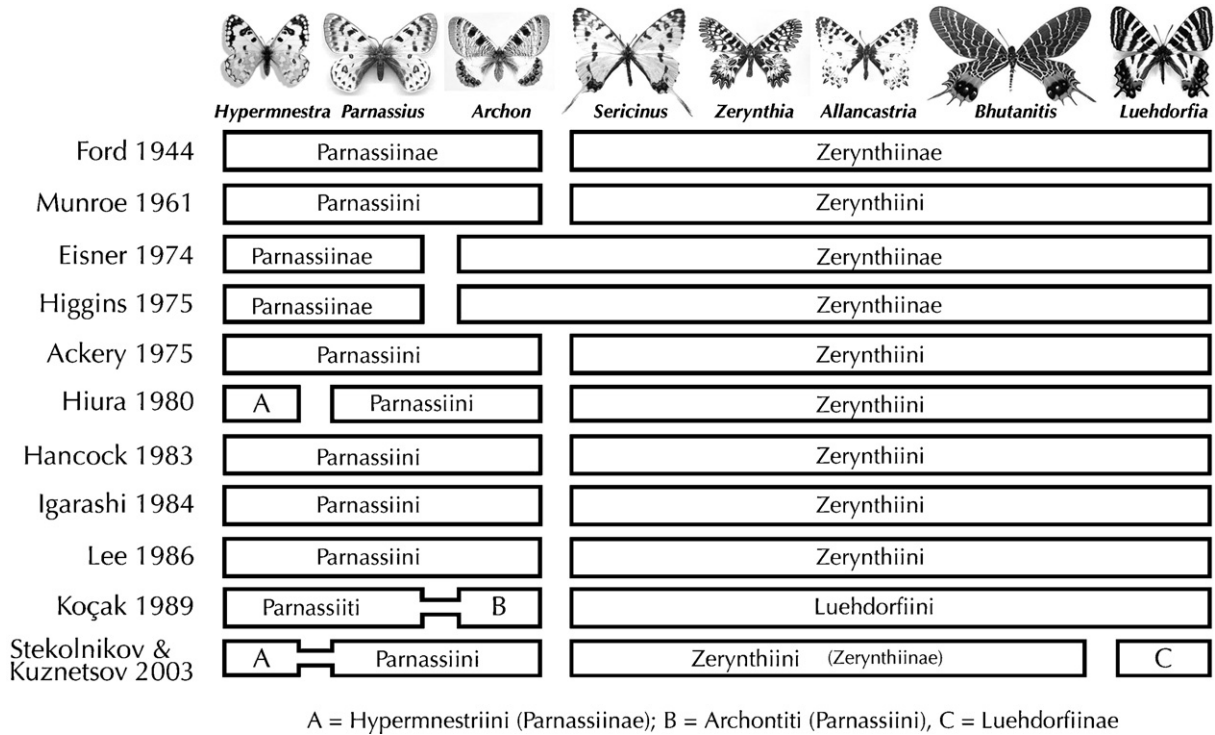
Fig. 2. Previous classifications of Parnassiinae. Connected boxes indicate subdivisions within tribe Parnassiini (Koçak, 1989) and subfamily Parnassiinae (Stekolnikov and Kuznetsov, 2003).
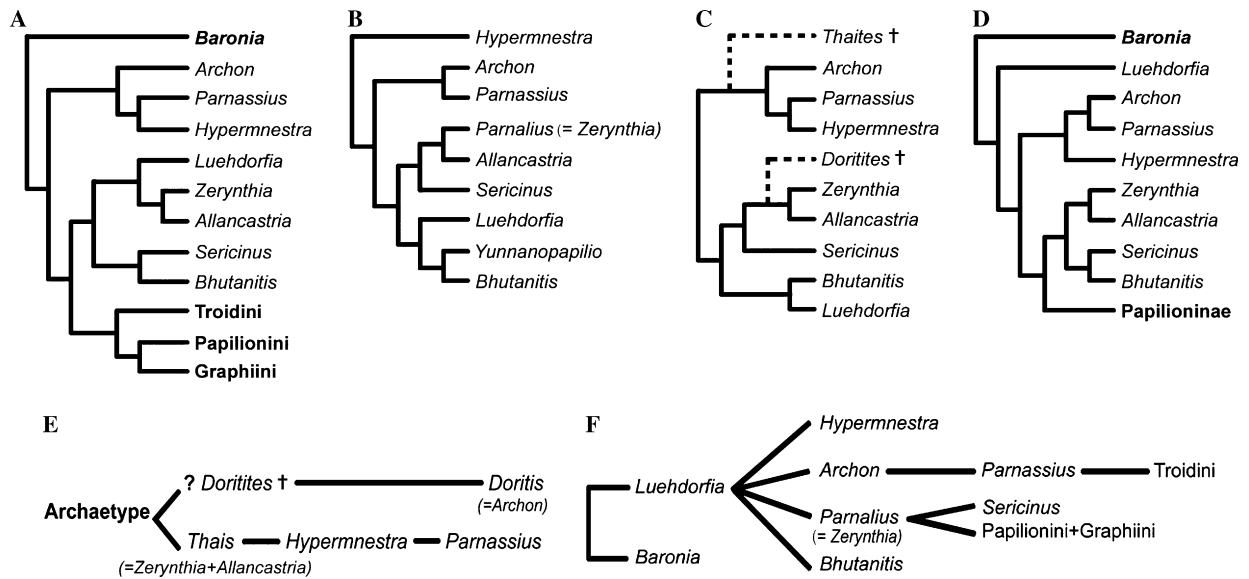

Fig. 3. Previous phylogenetic hypotheses for Parnassiinae based on morphological characters; (A) Ford, 1944a,b (simplified), (B) Hiura, 1980, primarily based on wing pattern, (C) Hancock, 1983, (D) Stekolnikov and Kuznetsov, 2003, primarily based on genitalia, (E) Le Cerf, 1913; (F) Igarashi, 1984 (simplified), primarily based on immature stages.

mitochondrial DNA sequence affinity between *Archon* and *Luehdorfia* (Omoto et al., 2004; Katoh et al., 2005) complicate the picture further.

Moreover, the phylogenetic significance of two well-documented fossil species within Parnassiinae (*Thaites ruminiana* Scudder, 1875 from the lower Oligocene, Aix-en-Provence, southern France, and *Doritites bosniaskii* Rebel, 1898 from Miocene, Tuscany, Italy) has generally been overlooked. Despite suggestions by their original

describers and Hancock (1983), the position of these fossils in the Parnassiinae remains uncertain.

Taxonomic disagreements also exist over the composition and ranking of most genera in the Parnassiinae. Although the genus *Parnassius* sensu lato is generally considered monophyletic (cf. Omoto et al., 2004; Katoh et al., 2005), the clade is often divided into smaller genera or species-groups based on minor morphological differences (Bryk, 1935; Korb, 1997) that are of questionable validity
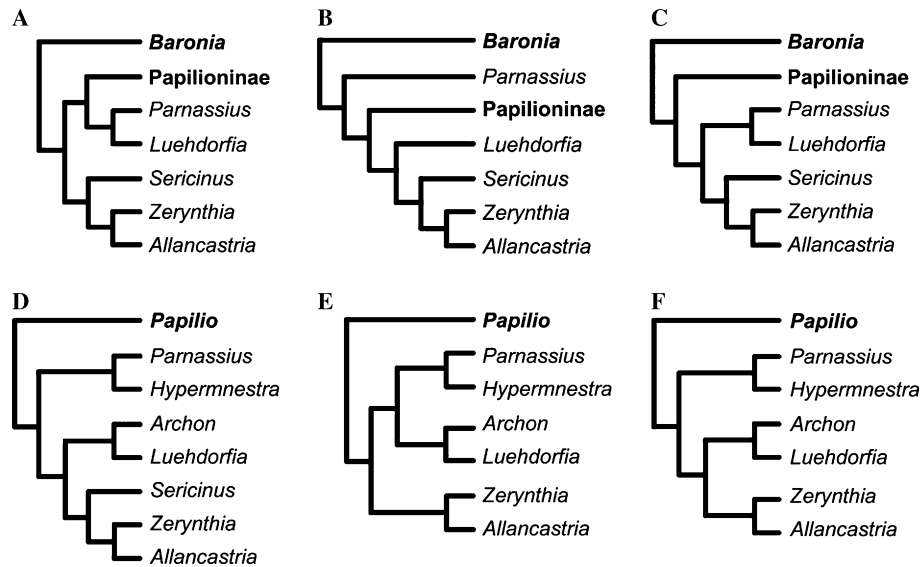
Fig. 4. Previous phylogenetic hypotheses for Parnassiinae based on DNA evidence. (A–C) Caterino et al., 2001, maximum parsimony trees based on *COI–COII* (A), *EF-1α* (B), and combined data (C); (D) Omoto et al., 2004, neighbor joining tree based on ND5; (E and F): Katoh et al., 2005, trees based on ND1 and 16S sequences, using minimum evolution (*E*), maximum parsimony and maximum likelihood (*F*) methods.

(Hesselbarth et al., 1995). Over a century ago, the mono-typic genus *Hypermnestra* was placed in the genus *Parnassius* (e.g. Doubleday and Westwood, 1847–1848; Gray, 1853; Moore, 1895) or *Doritis* (= *Archon*) (e.g. Herrich-Schäffer, 1856). *Allancastria* has been treated as a synonym, or a subgenus, of *Zerynthia* (Hesselbarth et al., 1995), or the two have been collectively called *Parnalius* (Ackery, 1975). Species in the genus *Bhutanitis* have received several generic names (*Yunnanopapilio*, *Sinonitis*, and *Bhutanitis*; Lee, 1986).

Our study is the most comprehensive attempt to date, in terms of characters as well as sampling of genera and species, to infer the phylogeny of Parnassiinae. We re-examine morphological characters used by previous workers and combine all informative morphological characters with 5775 bp of mitochondrial and nuclear DNA sequences in order to resolve several long-standing controversial issues concerning the classification of the subfamily. The purpose of this study is to: (a) establish the phylogeny and classification of Parnassiinae; (b) compare the efficacy of several widely used genes for phylogenetic reconstruction, and (c) reconstruct the biogeography and evolutionary path of selected ecological characters of the Parnassiinae.

2. Materials and methods

2.1. Taxon sampling

Sampling was comprehensive for all genus or subgenus level taxa within the traditional Parnassiinae (Table 1). Except for *Parnassius*, we attempted to sample every known species within the subfamily. For *Parnassius*, single species from each of the 8 major species-groups (Omoto et al., 2004) were selected. No specimens of *P. hardwickii* could be obtained; only the sequences available on Gen-

Bank were used. Outgroups were chosen to represent major butterfly families (Hesperiidae, Lycaenidae, Nymphalidae and Pieridae). Members from all subfamilies within Papilionidae, including *Baronia brevicornis* (Baroniinae) and three representatives from each of the three main tribes within the Papilioninae, were selected as additional ingroup taxa for all analyses. Selection was to some extent based on the availability of previously published sequences (Caterino et al., 2001; Wahlberg et al., 2005a). Three fossil taxa were included: *Praepapilio colorado* Durden and Rose, 1978 (Colorado, Middle Eocene), *Thaites ruminiana* Scudder, 1875 (Aix-en-Provence, Lower Oligocene), and *Doritites bosniaskii* Rebel, 1898 (Tuscany, Miocene); morphological and other data on each of these taxa were obtained using original descriptions and figures.

Dried, un-relaxed specimens were received as donations or purchased from international suppliers. Permits were obtained for all species listed under the Convention on International Trade of Endangered Species (CITES). *Bhutanitis lidderdalei* specimens were solicited by the first author from confiscated material deposited at the Canadian National Collection in Ottawa. No specimens of *Bhutanitis ludlowi* were available, and despite several attempts, some specimens (including *L. chinensis*) did not yield any usable DNA. Voucher specimens and extracted DNA samples are deposited in the E.H. Strickland Entomological Museum, University of Alberta.

2.2. Morphological characters

Morphological characters used by previous workers (Ford, 1944a,b; Ehrlich, 1958; Munroe, 1961; Hiura, 1980; Saigusa and Lee, 1982; Hancock, 1983; Igarashi, 1984; Miller, 1987; De Jong et al., 1996; Kato, 1998; Ackery et al., 1999) were re-examined wherever possible,

Table 1
Taxa, collecting data, specimen identification, and Genbank accession numbers

	Species	Locality (for specimens sequenced in this study)	Specimen ID	*COI–COII*	ND5	ND1	16S	EF-1α	Wingless
1	*Pyrgus communis* (Hesperiidae)	USA: CA: Solano County, NE Vallejo, 21.08.1997	FS-b-901	AF170857	DQ351044	U25880	DQ351078	AF173396	AY569043
2	*Hylephila phyleus* (Hesperiidae)	USA: CA: Berkeley, 27.08.1998	FS-b-989	AF170859	DQ351045	DQ351059	DQ351079	AF173398	DQ351124
3	*Coenonympha tullia* (Nymphalidae)	USA: CA: Oakland Hills	FS-b-984	AF170860	DQ351047	AF229952[1]	DQ351081	AF173399[a]	DQ351126
4	*Plebejus acmon* (Lycaenidae)	USA: CA: San Diego	FS-b-969	AF170864	DQ351046	DQ351060	DQ351080	AF173404	DQ351125
5	*Colias eurytheme* (Pieridae)	Canada: ON: Ottawa	FS-b-543	AF044024	DQ351048	U32456	AB194748[2]	AF173400[a]	AY569040
6	*Pieris napi* (Pieridae)	USA: CA: Redwood Canyon, Alameda Co., 28.06.1996	FS-b-943	AF170861	AB044594[3]	DQ351061	DQ351082	AF173401	AY569041
7	*Eurytides marcellus*	USA: FL: Ocala State Forest, 23.03.1988	FS-a-7	AF044022	AB088651[4]	—	DQ351087	AF044815[a]	DQ351128
8	*Graphium agamemnon*	SE Asia (Country Unknown); 12.1996	FS-b-900	AF170874	AB059508	DQ351062	DQ351086	AF173414	AY569046
9	*Iphiclides podalirius*	France: 1988	FS-a-6	AF170873	AB059546	AJ224087	DQ351088	AF173413[a]	DQ351129
10	*Battus philenor*	USA: VA: Bedford County	FS-a-3	AF170875	AB027573	AJ224086	DQ351083	AF173415[a]	DQ351130
11	*Parides photinus*	Costa Rica: Villa Colon, 03.02.[1988]	FS-a-149	AF170877	AB027578[5]	DQ351064	DQ351085	AF173417	DQ351127
12	*Troides helena*	Malaysia: 10.09.1997	FS-b-974	AF170878	AB084430	DQ351063	DQ351084	AF173418[a]	AY569047
13	*Papilio demoleus*	Malaysia: Penang Island, 16.05.1989	FS-a-68	AF044000	AB013159	AJ224099	DQ351090	AF044825	AY569114
14	*Papilio machaon*	France: Coudoux, 18.02.1988	FS-a-27	AF044006	AB013150	AB186206	AB186172	AF044828	AY569124
15	*Papilio thoas*	French Guiana: Pointe Macouria, 30.05.1990	FS-a-302	AY457601	DQ351049	DQ351065	DQ351089	AY457632	AY569126
16	*Baronia brevicornis*	Mexico: Teacalco, btw Guerrero-Morelos, 07.1988	FS-a-167	AF170865	DQ351050	—	DQ351091	AF173405[a]	AY569044
17	*Hypermnestra helios*	SE Kazakhstan: Ili River, Bakanas village, 1–15.05.1998	FS-b-1597	DQ351025	AB095659	AB186200	AB186166	DQ351106	DQ351131
18	*Parnassius phoebus* (I)	Canada: AB: Plateau Mt., 08.1986	FS-a-8	AF170872	AB063354	AB186173	AB186139	AF173412[a]	AY569045
19	*Parnassius hardwickii* (II)	E. Nepal	—	—	AB094969	AB186178	AB186144	—	—
20	*Parnassius schultei* (III)	China: Tibet, Trans-himalaya, Karola Pass, 22–28.06.1994	FS-b-1978	DQ351026	AB095619	AB186183	AB186149	—	—
21	*Parnassius tenedius* (IV)	Kirghizstan: Altai Mts., Aktash village, 16.05.1997	FS-b-1784	DQ351027	AB095658	DQ351066	DQ351092	DQ351107	DQ351132
22	*Parnassius delphius* (V)	Kirghizstan: Tian-Shan, Naryntoo Mts., 1–10.07.1996	FS-b-1775	DQ351028	AB095655	AB186185	AB186151	DQ351108	—
23	*Parnassius autocrator* (VI)	Tadjikstan: E. Pamir, Muzkoi Mts., W Morgav village, 08.2000	FS-b-1983	DQ351029	AB095634	AB186192	AB186158	DQ351109	DQ351133
24	*Parnassius simonius* (VII)	Kirghizstan: Transalai Mts., 1–20.07.1998	FS-b-1777	DQ351030	AB095649	DQ351067	DQ351093	DQ351110	—
25	*Parnassius clodius* (VIII)	USA: WA: Okanagen Co., Chinook Pass, 7.03.1986	FS-a-375	AF170871	AB095624	DQ351068	DQ351094	AF173411[a]	DQ351134
26	*Archon apollinaris apollinaris*	Iran: Kermanshah, Rijab, 9.04.1998	FS-b-2025	DQ351032	DQ351051	DQ351069	DQ351095	DQ351112	DQ351136
27	*Archon apollinaris bostanchii*	Iran: Lorestan, Ploedokhtar, 10.04.2003	FS-b-2063	DQ351033	DQ351052	DQ351070	DQ351096	DQ351113	DQ351137
28	*Archon apollinus*	Turkey: Oludinez, 9.04.1999	FS-b-1868	DQ351031	AB095661	AB186202	AB186168	DQ351111	DQ351135
29	*Luehdorfia chinensis*	China: Zhejiang, Lishui, 06.2002	—	AB179872	AB016826	AB071942	—	—	—
30	*Luehdorfia japonica*	Japan: Kanazawa, Ishikawa; emg. 20.02.1991	FS-a-335	AF170867	AB013142	AB186205	AB186171	AF173407[a]	DQ351138
31	*Luehdorfia puziloi*	Russia: Primorye, Vladivostok, 05.1999	EZ-2-11	DQ351035	AB013143	AB186204	AB186170	DQ351115	DQ351139
32	*Luehdorfia taibai*	China: Shaanxi, Qinling, 06.2002	FS-b-2102	DQ351034	AB016828	AB071944	—	DQ351114	—
33	*Sericinus montela*	Japan: Tanashi, near Tokyo, 4.04.1991	FS-a-399	AF170868	AB095665	DQ351071	DQ351100	AF173408[a]	DQ351143

Table 1 (*continued*)

	Species	Locality (for specimens sequenced in this study)	Specimen ID	COI–COII	ND5	ND1	16S	EF-1α	Wingless
34	*Bhutanitis lidderdali*	China: Yunnan, Dongchuan, 10.2002	FS-b-2044	DQ351038	DQ351053	DQ351072	DQ351099	DQ351118	DQ351142
35	*Bhutanitis mansfieldi*	China: Sichuan, East of Mei Mtn, 07.2000	FS-b-1589	DQ351036	AB026727	AB071945	DQ351097	DQ351116	DQ351140
36	*Bhutanitis thaidiana*	China: Sichuan, Daba Mtn, 07.2000	FS-b-1591	DQ351037	AB026728	AB071946	DQ351098	DQ351117	DQ351141
37	*Zerynthia polyxena*	Russia: District of Voronezh, 1-5.05.1998	FS-b-1596	DQ351039	DQ351054	DQ351073	DQ351101	DQ351119	DQ351145
38	*Zerynthia rumina*	Spain: Malaga; emg. 5.11.1989	FS-a-88	AF170870	AB095660	AB186201	AB186167	AF173410[a]	DQ351144
39	*Allancastria caucasica*	Turkey: Bolu Pro., Bolu Daglari, 21.04.2001	FS-b-2046	DQ351042	DQ351057	DQ351074	DQ351104	DQ351122	DQ351149
40	*Allancastria cerisyi*	Greece: Thessaloniki, 1990	FS-a-342	AF170869	AB095662	AB186203	AB186169	AF173409[a]	DQ351146
41	*Allancastria cretica*	Greece: Crete Island, Lassithi, 4.5.2003	FS-b-2038	DQ351041	DQ351056	DQ351076	DQ351103	DQ351121	DQ351148
42	*Allancastria louristana*	Iran: Lorestan, Malavi, 1000m, 4.04.1999	FS-b-2037	DQ351040	DQ351055	DQ351075	DQ351102	DQ351120	DQ351147
43	*Allancastria deyrollei*	Iran: West Azerbaijan, Takab, 2000m, 23.05.2003	FS-b-2086	DQ351043	DQ351058	DQ351077	DQ351105	DQ351123	DQ351150

[1–5]Replacement sequences for fragments that could not be amplified: 1 = *Coenonympha dorus*, 2 = *Eurema hecabe*, 3 = *Pieris rapae*, 4 = *Eurytides asius*, 5 = *Parides montezuma*.

[a] EF-1α sequences extended by 245 basepairs at 5′ end.

but in some cases taken from the literature (Appendix 1). Some new characters were proposed, whereas several traditionally used characters were excluded if they were considered too difficult to score (Appendix 2) or invariant among the ingroup. Geographical distribution, larval food plant, larval gregariousness and habitat type were excluded from the primary phylogenetic analysis and instead reconstructed on the best-supported phylogeny. In most cases, vouchers for the specimens used in DNA extractions were the same ones used for coding and checking morphological characters (Appendix 3). Gross morphological characters were examined under a Wild-Heerbrugg dissecting microscope, and wing scales were prepared and examined with a Leitz *Laborlux S* compound microscope. Morphological character coding is presented in Appendix 4.

To facilitate reliable and consistent coding of wing pattern elements, several wing pattern models previously proposed for Parnassiinae and Papilionidae were evaluated (Eimer, 1889–1895; Verity, 1911; Hiura, 1980, 1981; Smith and Vane-Wright, 2001). Wherever possible, homologies were incorporated in a basic model that included all 3 subfamilies of Papilionidae, but these codings were not extended to outgroups due to difficulties in unambiguously assigning character states. Although there were some coding errors in Hiura's (1980) analysis of Parnassiinae wing pattern (due to simplified template patterns used in his analysis that did not take into account individual variation), this system for naming bands and other wing markings was found to be the most practical. The model used in our study is therefore a modified version of Hiura's (1980) model. All characters used by Hiura and other workers were re-analyzed, but some were discarded due to unreliability or inapplicability.

Genitalia were prepared and photographed for both males and females. In some cases where male or female specimens were not available for examination or particular structures were used up in DNA extraction (e.g. legs and thorax), character states were coded as missing data. Life history, ecology, and fossil characters were coded using the available literature. All specimens used for evaluation of morphological characters are deposited in the E.H. Strickland Entomological Museum, University of Alberta. Specimen data is also available at: http://www.biology.ualberta.ca/old_site/uasm//Vouchers/index.html.

2.3. DNA sequences

Amplifications were obtained for any taxa and genes that had not previously been sequenced and available on GenBank, including mitochondrial cytochrome oxidase subunit I (*COI*) + *t*RNA-Leucine + cytochrome oxidase subunit II (*COII*) (2310 bp), NADH-dehydrogenase subunit 5 (*ND5*) (816 bp), NADH-dehydrogenase subunit 1 (*ND1*) (472 bp), and 16*S* ribosomal RNA (533 bp), as well as the nuclear protein-coding genes elongation factor 1 α (*EF-1α*) (1240 bp) and wingless (*wg*) (404 bp) (total 5775 bp). These genes were selected based on their wide phylogenetic utility in published studies on swallowtail butterflies (e.g. Aubert et al., 1999; Yagi et al., 1999; Caterino et al., 2001; Zakharov et al., 2004b, Katoh et al., 2005). In a few cases where amplifications could not be obtained, the respective fragment was coded as missing data. For outgroups, sequences from closely related taxa were used in the data matrix if the desired sequence was not available (e.g. *Eurytides asius* instead of *E. marcellus*). Overall, more than 56% of the sequence used was new. Some of the previously available *EF-1α* sequences were extended for 245 base pairs at the 5′ end (Table 1).

2.4. Laboratory techniques

Total genomic DNA was extracted using the QIAGEN QIAamp DNA mini kit, and in all cases legs or thorax tissue were used. Polymerase chain reactions (PCRs) were conducted on either a T-gradient or a T-personal PCR thermocycler (Biometra GmbH, Germany), using a variety of primers, most of which have been described previously (Appendix 5). Most primers used in this study were taken from Bogdanowicz et al. (1993), Carroll et al. (1994), Caterino and Sperling (1999), Cho et al. (1995), Reed and Sperling (1999), Sperling et al. (1994, 1995, 1996), with primer positions for mtDNA following Clary and Wolstenholme (1985). *Taq* Polymerase was added at the end of an initial 2–5 min denaturation at 95 °C, which was followed by 35 cycles of 94 °C denaturing for 1 min, 45–52 °C (depending on primer combinations) annealing for 1 min, 72 °C extension for 1 min, and a final extension period of 72 °C for 7–10 min. PCR products were tested by electrophoresis on an agar gel, and if a single band was observed, were purified using a QIAGEN QIAquick PCR purification kit. If more than one band was present, the appropriately sized PCR product was cut from the gel and extracted using a QIAGEN QIAEX II gel extraction kit. Sequencing reactions were then conducted using Big Dye terminator cycle sequencing (Applied Biosystems, Foster City, CA) under manufacturer's recommendations. Sequencing products were filtered through Sephadex-packed columns and dried using a vacuum centrifuge. Final products were re-suspended in formamide and fractioned on an ABI prism® 377 automated sequencer.

All fragments were sequenced in both directions. Resulting chromatograms were evaluated for miscalls and ambiguities and assembled into contigs in Sequencher® 4.1 (GeneCodes Corp., Ann Arbor, MI). Consensus files were aligned using Clustal X 1.81 (Thompson et al., 1997) and the alignment files were converted to nexus format with the aid of Se-Al 2.0 (Rambault, 2002). Initial multiple sequence alignments obtained from Clustal X with the default settings (gap opening = 10, gap extention = 0.20) were examined by eye and manually adjusted in some regions that contained gaps. *EF-1α*, *wg* and *ND1* had no alignment gaps, but several small indels were present in *COI + tRNA-leu + COII*, *ND5* and *16S*. The alignment of *16S* sequences was particularly problematic because of the presence of multiple indels. Other than known introns and previously reported indels (Zakharov et al., 2004a; Katoh et al., 2005), no other insertions or deletions were found in the alignments. In all analyses, all data was incorporated with no sites deleted. Individual datasets were assembled into a combined nexus file and analyzed in PAUP* 4.0b8-b10 (Swofford, 2002). Alignments as well as the morphology partition of the data matrix were subsequently deposited on TreeBase (www.treebase.org). MacClade 4.0 (Maddison and Maddison, 2000) was used, in addition to PAUP*, to trace morphological character changes.

2.5. Phylogenetic analysis

Neighbor joining (NJ), maximum parsimony (MP), maximum likelihood (ML), and bootstrap analyses were all conducted in PAUP* 4.0b10 (Swofford, 2002). NJ was used for a preliminary rapid evaluation of sequences for each gene before assembling the combined data matrix, particularly to detect any possible sequence chimerism due to PCR contamination or sequence misalignment. Any questionable sequences were re-amplified and re-sequenced several times; this included *Baronia brevicornis*. Congruence in gene partitions on the final data matrix was tested using the partition homogeneity test of PAUP*, also known as incongruence length difference (ILD) test (Farris et al., 1994). The test was conducted under parsimony with 100 random addition sequences of taxa and 100 replicates. Considering the unreliability of the ILD test (Graham et al., 1998; Darlu and Lecointre, 2002), each data partition was also analyzed separately to determine alternative phylogenetic hypotheses. In all analyses, 6 taxa (*P. communis*, *H. phyleus*, *C. eurytheme*, *P. napi*, *P. acmon* and *C. tullia*) were predefined as outgroups.

2.5.1. MP analysis

Parsimony analyses used heuristic searches, starting trees determined by 100 random taxon addition, tree bisection-reconnection (TBR) branch swapping algorithm, gaps treated as missing data, multiple character states in the same taxon treated as uncertainty, and all characters equally weighted. Parsimony hypotheses were evaluated by bootstrap analysis (Felsenstein, 1985) with 100 repetitions under the same parameters as for initial parsimony searches. Bootstrap searches were conducted for each data partition, combined mitochondrial and combined nuclear partitions, and the total data set. Decay values were calculated using the program TreeRot (Sorenson, 1999) in conjunction with PAUP*, and partitioned Bremer support (PBS) was also calculated for each combined set of partitions. We traced morphological character evolution for internal nodes on the combined phylogenetic reconstruction in PAUP* under the accelerated transformation (ACCTRAN) character-state optimization criterion.

2.5.2. ML analysis

A hierarchical likelihood ratio test was conducted using MODELTEST 3.0 (Posada and Crandell, 1998) to examine the fit of 56 different evolution models to the partitioned and combined data, ranging from simple Jukes–Cantor to general time reversible (GTR) models. In each case, parameters from the best model were used in ML phylogenetic reconstruction, with the GTR plus gamma distribution plus invariable site (GTR + Γ + I) model found to be most appropriate in all cases. Independent ML analyses for each gene partition and combined data were then performed in PAUP* 4.0b10 for UNIX (Swofford, 2002). Support values for ML trees were estimated with 100 bootstrap replicates

and, under the best fit model, with the ML tree selected as the starting seed.

2.5.3. Bayesian analyses

Bayesian posterior probabilities were calculated for partitioned and combined data sets using MrBayes 3.04 (Huelsenbeck and Ronquist, 2001) under the GTR + Γ + I model and 4 (one cold and three heated) simultaneous Markov chains for 1,000,000 generations, starting with random initial trees and sampling every 100 generation. Substitution rates were estimated as part of the analysis from default priors, and model parameters were allowed to vary for analysis of the combined data. Trees corresponding to the burnin values estimated prior to initiation of each MCMC chain (varying from 800 to 5000, depending on gene partition) were discarded, and the majority rule consensus tree was generated using the remaining trees with posterior probabilities plotted on each node. The Bayesian approach in phylogenetic analysis of combined datasets is under continuous scrutiny (e.g. Yang and Yoder, 2003; Pickett and Randle, 2005; Mossel and Vigoda, 2005; Ronquist et al., 2006). The decay values for partitioned and combined analyses were also calculated and are presented alongside the Bayesian posterior probabilities in Table 4.

2.5.4. Usefulness of genes

We investigated the relative utility of the genes we used for phylogenetic inference at various taxonomic levels from two perspectives. First, using PAUP∗, we calculated the tree length (TL), log likelihood ($-\ln L$), consistency index (CI) and retention index (RI) values for all of the phylogenies inferred in our analyses, with outgroups included. Second, we obtained uncorrected p distances with PAUP∗ for each gene partition and then plotted them against *COI*, with a saturation curve fitted to the data range in SYSTAT using a simple saturation model ($y = bx/(a + x)$, where a equals half-saturation and b equals the rise in maximum value). *COI* was selected for purposes of comparison, based on its extensive use in phylogenetic studies as well as in DNA barcoding (see www.barcodinglife.org). Average uncorrected p distances for *COI* between species, genera and tribes of Papilionidae subfamilies used in this study were also calculated and plotted against a cumulative graph of all genes. Furthermore, for every gene we calculated the genetic distance between tribes in Papilioninae and Parnassiinae as well as their distance from Baroniinae.

2.5.5. DIVA analysis

The center of origin of the subfamily Parnassiinae has been previously suggested to lie "in the Turan arid zone which was located at E Tetis coast and included territories of the present day Aral Sea and Lower Syrdar'ya and Amudar'ya Rivers" (Korb, 1997). This hypothesis was tested through a dispersal and vicariance analysis with the aid of the computer program DIVA (Ronquist, 1997). This method has frequently been used in reconstruction of ancestral distributions (given a phylogeny), including swallowtail and nymphalid butterflies (e.g. Zakharov et al., 2004b; Wahlberg et al., 2005b). Outgroups and non-Parnassiinae species were excluded from DIVA analysis due to limited sampling; fossils were also removed due to uncertainty of their phylogenetic status, although their positions – as inferred in this study based on combined data – were plotted later on the DIVA reconstruction.

Areas selected in this study were based on the geographic distribution of the species but were also largely congruent with areas of endemism previously proposed for the western Palaearctic (Sanmartín, 2003). Areas included: (A) North America, (B) southern Europe, extending from Portugal and Spain to France, Italy, and Greece, (C) northern Africa, (D) the island of Crete [Kriti] in Greece, (E) Anatolia, Lebanon and Israel, (F) Zagros Mountains, extending from Iran to northern Iraq and SE Turkey, (G) Caucasus Mountains, (H) the Iranian Plateau, including eastern Iran, Afghanistan, and the central Asian plains, (I) the Himalayas as one unit, including Pamir, Tianshan, Tibet, Altai, etc., (J) northern India, Bhutan, Bangladesh, Burma and northern Thailand, (K) mainland China, Korea, and eastern Russia, and (L) Japan.

Distribution data for each species was compiled in a nexus file in MacClade 4.0 as presence/absence for each region, with the ML phylogeny for all DNA sequences used for the analysis. Analysis was conducted with and without restriction of maximum number of areas for ancestral nodes. DIVA assigns a cost of zero to vicariance (allopatric speciation) and duplication (sympatric speciation) events and a cost of 1 per unit area to any dispersal and extinction events; thus the best reconstructions are those that minimize the number of dispersals and extinctions under a parsimony criterion. Under the default setting, DIVA accumulates distribution areas towards the root, and the initial analysis produced an ancestral distribution of ABCDEFGHIJKL for the last common ancestor of Parnassiinae. Constraints of 2, 3, 4 and 5 unit areas were then imposed as the ancestral distribution, and the best construction (with the least number of dispersals = 26) was obtained when the maximum number of unit areas in ancestral distributions was set to three.

2.5.6. Molecular clock analysis

A likelihood ratio test (Felsenstein, 1988) was conducted in PAUP with the ML tree topology, and with and without enforcing a molecular clock to test the data for clocklike behavior among taxa. Divergence times were estimated using the r8S program (Sanderson, 2002) with semiparametric rate smoothing and a penalized likelihood approach applied to the phylogeny inferred from combined molecular data. The utility of the penalized likelihood approach in estimation of divergence times has been well demonstrated (e.g. Zakharov et al., 2004a; Dumont et al., 2005). Initial results were obtained under default settings with cross-validation enforced. The rate smoothing with the lowest cross-validation scores was selected and the dating procedure was repeated. Standard deviations were obtained by bootstrapping

data 1000 times using the seqboot module from PHYLIP 3.6 (Felsenstein, 1989) and with the aid of the r8s bootstrap kit (Eriksson, 2002). The constrained initial topology was used to re-estimate branch lengths for each node. This procedure was repeated for each tree, and the statistics were summarized using the "profile" command in r8S.

The molecular clock was calibrated using previously hypothesized divergence dates for Papilionidae taxa as well as fossils and major geological events relevant to this study. Dates used here are: 82.5–89.1 MYA (fixage) for the last common ancestor of Papilionini and Troidini (Gaunt and Miles, 2002); 35–65 MYA (constraint *a*) for the initial split in the genus *Papilio* (Zakharov et al., 2004a); 39.8–45.1 MYA (constraint *b*) for the split between ancestral *P. machaon* and *P. demoleus* (Zakharov et al., 2004a); and 9.6 ± 1.2 MYA (constraint *c*) for last common ancestor of *Luehdorfia* (Makita et al., 2000).

The relict distribution of *Allancastria cretica* was also used as a calibration point; separation of Crete from mainland Greece and Turkey has been estimated at 11 million years (Dennis et al., 2000), although the island has existed in the form of several smaller islands until 3 million years ago due to tectonic uplifts of the southern Aegean (Papazachos and Kiratzi, 1996; Stöckhert, 1999). The speciation of *A. cretica* has also previously been hypothesized to have taken place before the Pleistocene (Olivier, 1993). We thus constrained the *A. cretica* node to 11–3 MYA (constraint *d*).

The correct phylogenetic positions of fossil taxa used in this study are unclear; our morphological and combined analyses also fail to provide strong support for the position of these taxa due to the large amount of missing data. We therefore only used the upper limit of the Miocene (5.3 MYA) as the minimum age to constrain the split in ancestral *Archon* (constraint *e*). Constraints were applied separately and in several possible combinations. Divergence dates proposed by Braby et al. (2005) for Troidini were tested separately due to age conflict with some of the other nodes; these are 90 MYA for the initial split in Troidini (Braby et al., 2005) and 64.9 ± 6.88 for the split between *Troides* and *Parides* (Braby et al., 2005).

3. Results

Maximum parsimony analysis of 236 characters in the morphological dataset yielded 41 most parsimonious trees (TL: 826), the consensus of which is shown on Fig. 5. The resulting topology is mostly congruent with previous morphological hypotheses, in that *Luehdorfia* groups with other Zerynthiini, and *Hypermnestra* with *Parnassius*. The positions of *Archon*, as well as two of the fossil taxa, remain uncertain as they fall into a polytomy. *Praepapilio*, however, is the sister group to a monophyletic Papilionini, with weak support.

During initial analyses on molecular data, a previously published *ND1* sequence for *Parnassius clodius* (GenBank number **U32464**; Weller et al., 1996) was found to belong to

another species (*P. smintheus*). We deposited a new *ND1* sequence for *P. clodius* on GenBank (Table 1).

Base frequencies among ingroup taxa were found to be significantly different (*P* = 1.00) in all genes except *wg* (*P* = 0.87), with mitochondrial genes having much higher A/T frequencies and *EF-1α* having slightly higher A/C frequencies. The ILD test demonstrated no heterogeneity among gene partitions (sum of lengths for original partition = 11438, *P* = 0.01). Exclusion of partitions one at a time also revealed no heterogeneity among remaining partitions (*P* = 0.01) in every case. Regardless, all analyses were performed both on partitioned and combined data to assess any undetected incongruence. Inferred phylogenies from the combined molecular data consistently demonstrated much better resolution and branch support values.

The total number of parsimony-informative characters in the combined dataset with outgroups excluded was 1875 (31.2%), with *wg* having the highest (41.0%) and 16*S* and *t*RNA-Leu the lowest (24.6% and 11.3%) proportion of informative sites in DNA sequence (Table 2). The morphology partition consisted mostly of parsimony informative characters (67.4%) within the ingroup, due to our selective approach. In the combined mitochondrial (with outgroups, *t*RNA-Leu and 16*S* excluded) and nuclear DNA datasets, respectively, 63.6% and 87.2% of informative characters were third codon positions.

The estimated model parameters were comparable to previous estimates for swallowtails (Zakharov et al., 2004a,b), with the GTR + *Γ* + *I* model found to be the best fit in every case (Table 3). Both Modeltest and PAUP* predicted extremely high substitution rates for *COII* under GTR + *Γ* + *I* (best fit) and GTR + *I* models but not for other models. Examination of the character change ratio in the *COII* dataset under an NJ tree revealed relatively few G–T substitutions (only 1.15%). As demonstrated previously, a low G–T reference rate can lead to an overestimation of the five other relative rates under a GTR model (Zwickl and Holder, 2004). We further contrasted substitution rates obtained through Modeltest and PAUP* with those estimated freely in Mr. Bayes under Bayesian criteria, and these did not seem to show any anomalies.

Simplified tree topologies for partitioned and combined molecular data are shown in Fig. 6, and the support values for important nodes are listed in Table 4. Monophyly of Parnassiinae was supported mostly by Bayesian analyses, particularly by *ND5* and *wg* gene partitions. In maximum parsimony searches, Parnassiinae appeared as a monophyletic group only for *wg*. Heuristic searches conducted under the maximum likelihood criterion produced trees with monophyletic Parnassiinae for *ND5* and *wg*, as well as combined mitochondrial and combined nuclear data, although these were not supported (Bremer support = 0) and subsequently collapsed in bootstrap consensus trees.

Combined morphological and molecular data yielded a tree with the same topology as the tree obtained from only the combined molecular data under the parsimony criterion (Fig. 7). No DNA sequences were available for fossils and
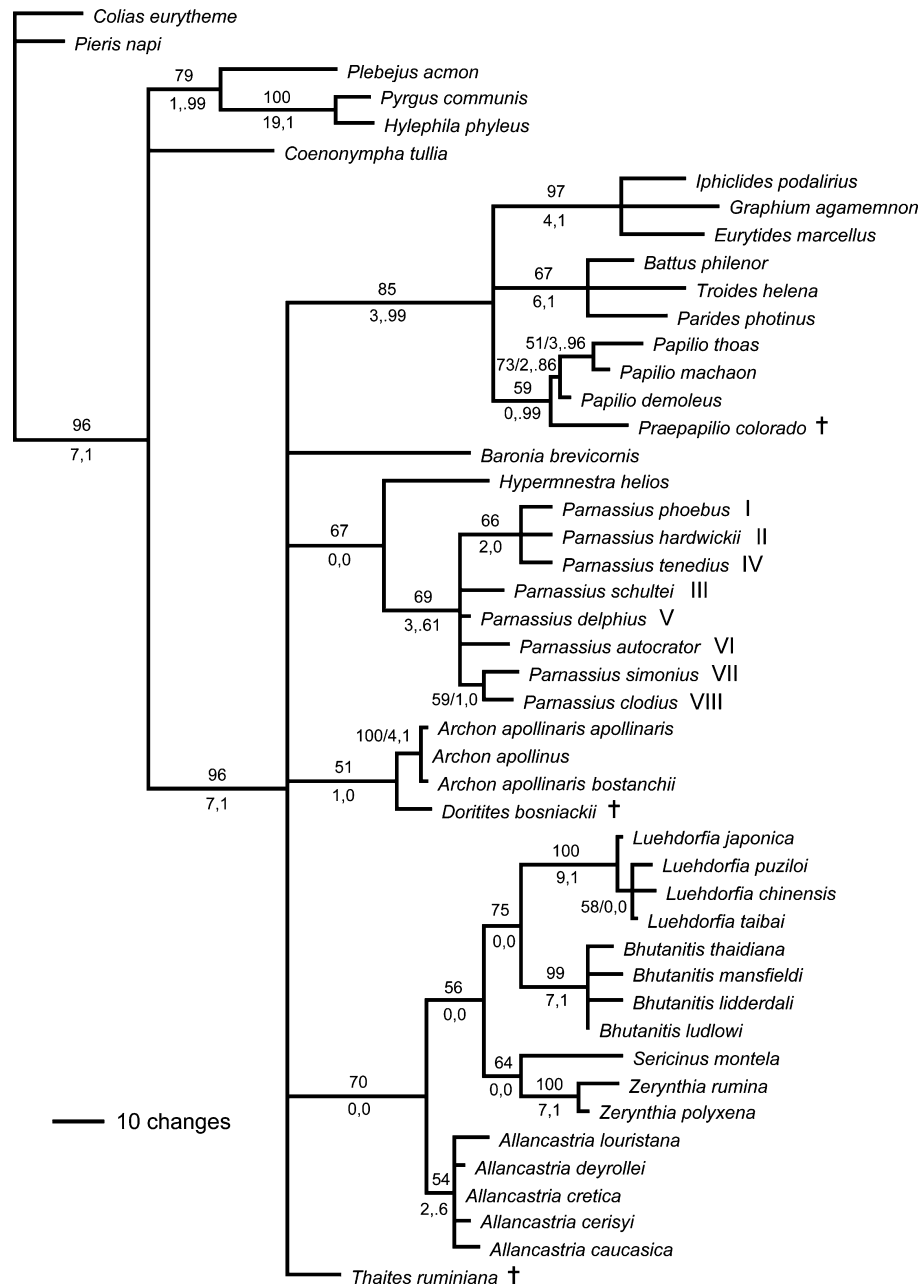
Fig. 5. Maximum parsimony phylogeny based on 236 morphological characters, shown as the strict consensus of 41 most parsimonious trees (TL: 826, CI: 0.384, RI: 0.683). Bootstrap support values are plotted above and Bremer support/Bayesian posterior probabilities are below the branches. Neither MP nor Bayesian analyses on morphological data support monophyly for Parnassiinae.

Bhutanitis ludlowi, hence these do not appear in subsequent figures. The sum of partitioned Bremer support for the MP tree from combined molecular and morphological data did not match the total Bremer support obtained from the same tree, and the supports estimated for fossil nodes were incorrect as TreeRot predicted support from molecular partitions for these taxa when there were no such data. The same problem was encountered in calculating Bremer support for the Bayesian reconstruction of all data. It has been shown that shared missing data can serve as shared character states and consequently affect the support computation (Wilkinson, 1995; Makovicky, 2000).

Terminal branch lengths were relatively longer than internal branches in the ML tree based on combined mitochondrial data compared to those inferred from combined nuclear data (Fig. 8). The deeper node topology for Parnassiinae was identical in both trees. In the combined molecular dataset, partitioned Bremer support values from different genes were highly variable (Fig. 9). In many of our inferred phylogenies (Figs. 6–8A), Papilioninae formed a well-supported group, as expected based on previous work (Caterino et al., 2001; Zakharov et al., 2004a). The position of *Baronia brevicornis*, however, was highly unstable, with all gene partitions, combinations and analyses reconstructing distinctly different phylogenetic positions for this taxon.

Table 2
Summary of character partitions for protein coding genes (also by codon position), RNA genes, and morphology, with outgroups excluded

Gene partition:	COI				COII				COI + COII				ND5				ND1			
Codon position:	All	1st	2nd	3rd	All	1st	2nd	3rd	All	1st	2nd	3rd	All	1st	2nd	3rd	All	1st	2nd	3rd
Total characters	1547	516	516	515	683	228	287	228	2230	744	743	743	816	272	272	272	472	157	157	158
Constant	966	408	392	166	392	153	193	46	1359	561	585	212	427	158	200	46	249	88	129	30
Uninformative	130	30	40	60	86	19	20	47	216	49	60	107	115	40	38	52	53	17	9	27
Informative	451	78	84	289	205	56	14	135	656	134	98	424	274	74	34	174	170	52	19	101
% Informative	29.2				30.0				29.4				33.6				36.0			

Gene partition:	16S	tRNALeu	EF-1α				wg				Morphology	All data
Codon position:			All	1st	2nd	3rd	All	1st	2nd	3rd		
Total characters	533	80	1240	413	413	414	404	135	134	135	236	6011
Constant	357	60	838	377	391	67	194	80	108	3	50	3533
Uninformative	45	11	88	16	14	56	46	24	15	6	27	603
Informative	131	9	314	20	8	291	164	31	11	126	159	1875
% Informative	24.6	11.3	25.3				41.0				67.4	31.2

Table 3
Substitution model parameters[a] from partitioned and combined data sets estimated under ML and Bayesian approach and General Time Reversible model

Partition	Estimation	Base frequencies				Substitution rates						Γ	I
		A	C	G	T	A–C	A–G	A–T	C–G	C–T	G–T		
COI	ML	0.339	0.069	0.127	0.465	114.515	40.658	68.714	51.836	1732.026	1.000	0.468	0.473
	Bayesian	0.361	0.082	0.084	0.474	0.650	1.940	2.021	1.342	47.871	1.000	0.183	
COII	ML	0.401	0.060	0.079	0.460	6.965×10^6	2.648×10^6	7.628×10^5	2.412×10^6	3.543×10^7	1.000	0.396	0.381
	Bayesian	0.392	0.073	0.035	0.501	4.576	15.488	0.824	8.100	44.245	1.000	0.144	
COI + COII	ML	0.363	0.065	0.111	0.460	296.937	93.848	92.143	105.917	2859.617	1.000	0.441	0.451
	Bayesian	0.374	0.777	0.789	0.470	4.464	3.197	2.251	1.852	48.122	1.000	0.199	
ND5	ML	0.418	0.036	0.061	0.485	0.076	6.811	0.400	3.379	1.233	1.000	0.609	0.336
	Bayesian	0.415	0.014	0.056	0.515	1.181	15.899	0.308	34.691	13.839	1.000	0.215	
ND1	ML	0.395	0.078	0.110	0.417	0.149	7.770	1.939	1.878	1.362	1.000	0.492	0.171
	Bayesian	0.379	0.017	0.056	0.548	1.685	21.112	0.124	24.553	26.414	1.000	0.160	
16S	ML	0.462	0.081	0.055	0.402	0.052	11.354	1.988	0.581	0.521	1.000	0.523	0.500
	Bayesian	0.501	0.075	0.036	0.389	0.002	45.138	0.028	0.070	0.022	1.000	0.130	
All mtDNA	ML	0.411	0.046	0.062	0.482	3.110	10.176	1.686	7.864	30.261	1.000	0.506	0.445
	Bayesian	0.406	0.047	0.079	0.467	4.522	9.317	2.707	7.465	45.787	1.000	0.227	
EF1-α	ML	0.259	0.269	0.233	0.239	0.719	4.909	2.091	0.555	7.051	1.000	1.433	0.614
	Bayesian	0.252	0.268	0.233	0.246	0.921	6.302	2.483	0.711	7.906	1.000	0.187	
Wg	ML	0.186	0.309	0.316	0.189	1.136	3.841	1.425	0.364	4.673	1.000	1.036	0.388
	Bayesian	0.165	0.317	0.333	0.185	1.979	9.241	3.267	0.500	7.837	1.000	0.163	
All ncDNA	ML	0.242	0.283	0.249	0.226	0.791	4.124	1.663	0.574	5.763	1.000	1.292	0.556
	Bayesian	0.238	0.291	0.245	0.228	0.812	4.661	1.748	0.628	5.581	1.000	0.218	
All DNA	ML	0.324	0.139	0.144	0.393	1.231	8.732	5.790	3.408	11.290	1.000	0.872	0.473
	Bayesian	0.315	0.147	0.150	0.390	1.242	8.882	6.223	3.141	10.771	1.000	0.268	

[a] $\Gamma = \alpha$, estimated shape parameter; I = proportion of invariable sites.

Due to this labile behavior, most gene fragments for *Baronia* were re-sequenced to confirm the authenticity of the obtained sequences. In the combined ML analysis of all molecular data (Fig. 9), *Baronia* is sister only to Parnassiinae with relatively strong support (bootstrap 93, Bremer 20, Bayesian 1.0).

The results of our molecular and combined data provide only weak support for the monophyly of Parnassiinae (Table 4). However, Parnassiinae appears as a monophyletic group in our *wg* and combined molecular heuristic searches using MP, ML or Bayesian reconstructions (except for mtDNA MP analysis), despite a lack of Bremer support (Figs. 6 and 9), even though the group collapses

after bootstrapping. Nonetheless, three distinct, highly supported clades are observed within Parnassiinae: (1) *Hypermnestra + Parnassius*, (2) *Archon + Luehdorfia*, and (3) *Sericinus + Bhutanitis + Zerynthia + Allancastria* (Figs. 8 and 9).

3.1. Usefulness of genes

Bar graphs of consistency and retention indices (Fig. 10) demonstrate notable variation among different genes, although there is little variation between different analytical approaches. Nuclear genes, particularly *wingless*, show the highest CI and RI and the lowest log likelihood values.

Table 4
Support values for important nodes obtained from decay analysis (Bremer support, BR), bootstrap on maximum parsimony tree (BSMP), bootstrap on maximum likelihood tree (BSML), and Bayesian posterior probability (BPP)

Node	COI BR	BSMP	BSML	BPP	COII BR	BSMP	BSML	BPP	COI + tRNALeu + COII BR	BSMP	BSML	BPP	ND5 BR	BSMP	BSML	BPP	ND1 BR	BSMP	BSML	BPP	16S BR	BSMP	BSML	BPP	All mtDNA BR	BSMP	BSML	BPP
Papilionidae	5	55	77	1	0	<50	<50ª	0.67	4	68	85	1	1	<50	<50*	0.94	3	<50	<50	0.52	4	53	56	0.92	20	99	100	0.99
Papilioninae	0	<50	<50	0	0	<50	<50	0	0	<50	<50	0	0	<50	<50*	0.85	0	<50	<50	0	0	<50	<50	0	8	70	90	0.99
Graphiini	5	<50	66	1	0	<50	<50	0	0	53	75	1	0	<50	<50*	0	2	<50	<50	0.84	0	<50	<50	0	7	86	100	0.99
Troidini	0	<50	<50	0	0	<50	54	0	2	<50	72	1	2	56	61	0.98	0	54	<50	0.69	1	<50	58	0.98	5	86	95	1
Papilionini	2	65	100	1	0	<50	56	0.58	4	68	100	1	0	<50	<50*	0	3	70	80	0.99	2	55	<50*	0.58	24	98	100	1
Troi + Papil	2	51	<50*	0.84	0	<50	<50	0	4	59	<50	0.99	0	<50	<50*	0	0	<50	<50	0.99	0	<50	74	1	11	97	100	1
Parnassiinae	0	<50	<50	0	0	<50	<50	0	0	<50	<50	0	0	<50	<50*	0.94	0	<50	<50	0.62	0	<50	<50	0	0	<50	54	0.65
Hyp + Parn	17	100	97	1	3	81	73	0.99	16	100	100	1	2	<50	<50*	0.83	5	74	84	0	4	91	66	0.96	17	100	100	1
Arch + Lueh	2	<50	69	0.95	0	51	73	1	4	71	91	1	5	70	88	1	3	66	<50	0	0	<50	<50*	0	15	100	100	1
S + B + A + Z	1	56	83	1	0	<50	<50*	0	5	74	89	1	3	<50	59	0.99	0	<50	<50	0.99	0	<50	59	0.93	13	93	100	1
Allan + Zeryn	11	100	100	1	3	79	<50	0	14	100	100	1	8	89	94	1	0	53	57	0.99	4	93	<50*	0	26	100	100	1

Node	Ef1a BR	BSMP	BSML	BPP	wg BR	BSMP	BSML	BPP	All ncDNA BR	BSMP	BSML	BPP	All DNA BR	BSMP	BSML	BPP	Morphology BR	BSMP	BSML	BPP	All data BR	BSMP	BSML	BPP
Papilionidae	8	74	86	1	4	75	100	1	17	98	99	1	43	100	100	1	7	96	—	1	5	96	—	1
Papilioninae	0	<50	<50	0	0	<50	<50	0	0	<50	<50	0	0	<50	100	0	3	85	—	0.99	0	63	—	0.94
Graphiini	12	94	99	1	6	87	98	0.99	18	100	99	0	35	100	100	1	4	97	—	1	4	100	—	1
Troidini	3	52	71	1	0	<50	<50ª	0.84	2	<50	81	1	16	95	100	1	6	67	—	1	5	96	—	1
Papilionini	8	91	84	1	12	100	99	1	16	100	100	1	38	100	100	1	2	73	—	0.86	1	95	—	0.86
Troi + Papil	0	<50	<50	0	0	<50	<50	0	0	<50	<50	0	16	95	100	1	0	<50	—	0	0	73	—	0.91
Parnassiinae	0	<50	<50	0	8	99	68	0.97	8	78	<50ª	0.95	0	<50	86	1	0	<50	—	0	0	<50	—	0.93
Hyp + Parn	9	97	98	1	10	99	97	1	19	100	100	1	35	100	100	1	0	67	—	0	6	97	—	1
Arch + Lueh	10	100	93	1	6	95	84	1	8	100	98	1	47	100	100	1	0	<50	—	0	0	<50	—	0.70
S + B + A + Z	0	<50	92	0.92	4	84	86	1	8	81	94	1	27	100	100	1	0	<50	—	0	3	87	—	0.99
Allan + Zeryn	8	97	98	1	4	99	91	1	15	100	99	1	39	100	100	1	0	<50	—	0	13	99	—	1

Values with asterisks are nodes that were present in heuristic searches, but collapsed in bootstrap consensus. S + B + A + Z represents *Sericinus*, *Bhutanitis*, *Allancastria* and *Zerynthia*. Bootstrap values are for 100 replicates.
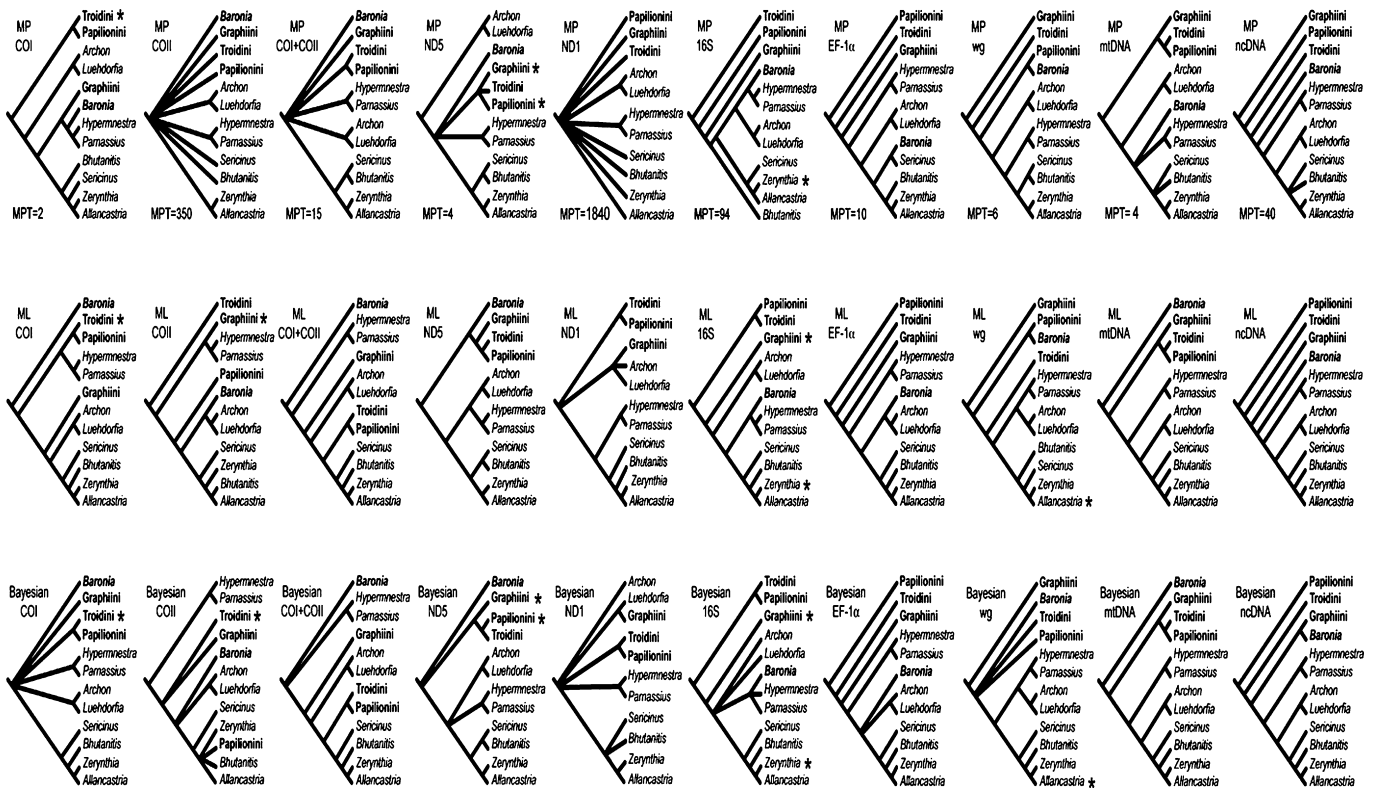
Fig. 6. Simplified phylogenies resulting from MP, ML and Bayesian analyses on partitioned and combined data, with outgroups removed after analysis. For maximum parsimony analyses, consensus of the most parsimonious trees (MPT) is shown. Parnassiinae genera and Papilioninae tribes were monophyletic in all cases except where indicated by an asterisk (∗); these taxa were paraphyletic with respect to their sister group.

Among mitochondrial genes, the lowest and highest CI values belong to *COI* and *ND1*, respectively. *ND5* and *ND1* show lower RI values compared to other mitochondrial genes. Tree length and log likelihood, which are essentially the same though on different scales, were highest in *COI + COII* and lowest in 16*S*.

We examined patterns of relative divergence and saturation for each gene by plotting uncorrected *p*distances against *COI* (Figs. 11 and 12). Three main categories of genes were evident. The first group (*COII*) demonstrated a divergence pattern virtually identical with *COI* for up to 15% divergence between taxa. The second group (*ND1*, *ND5*) initially showed similar rates of divergence to *COI* up to 7–9%, but continued to diverge to over 20% after *COI* saturated. The third group (*16S*, *EF-1α* and *wg*) was initially much slower to diverge than *COI*, then gradually approached the divergence of *COI* and intersected it at about 9% (*wg*) to 12% (16*S* and *EF-1α*), and continued to diverge further after *COI* saturated (16*S* = 15%, *EF-1α* = 20%, and *wg* = 31% maximum divergence). Despite a much smaller sample size for Papilioninae, sequence divergences in our analysis were comparable to those previously calculated for *COI–COII* for that subfamily (Zakharov et al., 2004a).

Comparison of uncorrected pairwise distances between tribes in Papilioninae and Parnassiinae, and among these two subfamilies and the Baroniinae (Fig. 13) also demonstrated substantial variation among genes. Despite some overlap, all genes (except 16*S* and *EF-1α*) consistently showed lower mean divergence between tribes in Parnassiinae compared to Papilioninae. The *Archon + Luehdorfia* clade (Luehdorfiini) was approximately equidistant from the other two clades in Parnassiinae, although with generally lower divergences than between the Parnassiini and Zerynthiini. In several cases, the medians of genetic distances between tribes in Papilioninae or Parnassiinae were higher than the medians of distances between subfamilies.

3.2. Divergence time estimation

The likelihood ratio test (Felsenstein, 1988) rejected a null hypothesis of rate constancy among taxa ($\delta = 367$, df = 41, $P < 0.0001$). Semiparametric rate smoothing was therefore conducted using optimal smoothing with the lowest χ^2 value estimated through cross-validation analysis.

We found that three of our calibration points did not have any significant impact on age estimates (Table 5 and Fig. 14). Even when they were not enforced, the resulting ages fell within the range proposed for the constraint; these included a minimum age for the ancestor of *Archon* (5.3 MY), the separation of Crete (3–11 MY), and the initial split in the genus *Papilio* (35–65 MY). We found that enforcing the estimated ages by Braby et al. (2005) in a separate analysis alongside other constraints did not have a substantive impact on ages estimated for Parnassiinae (Table 5).
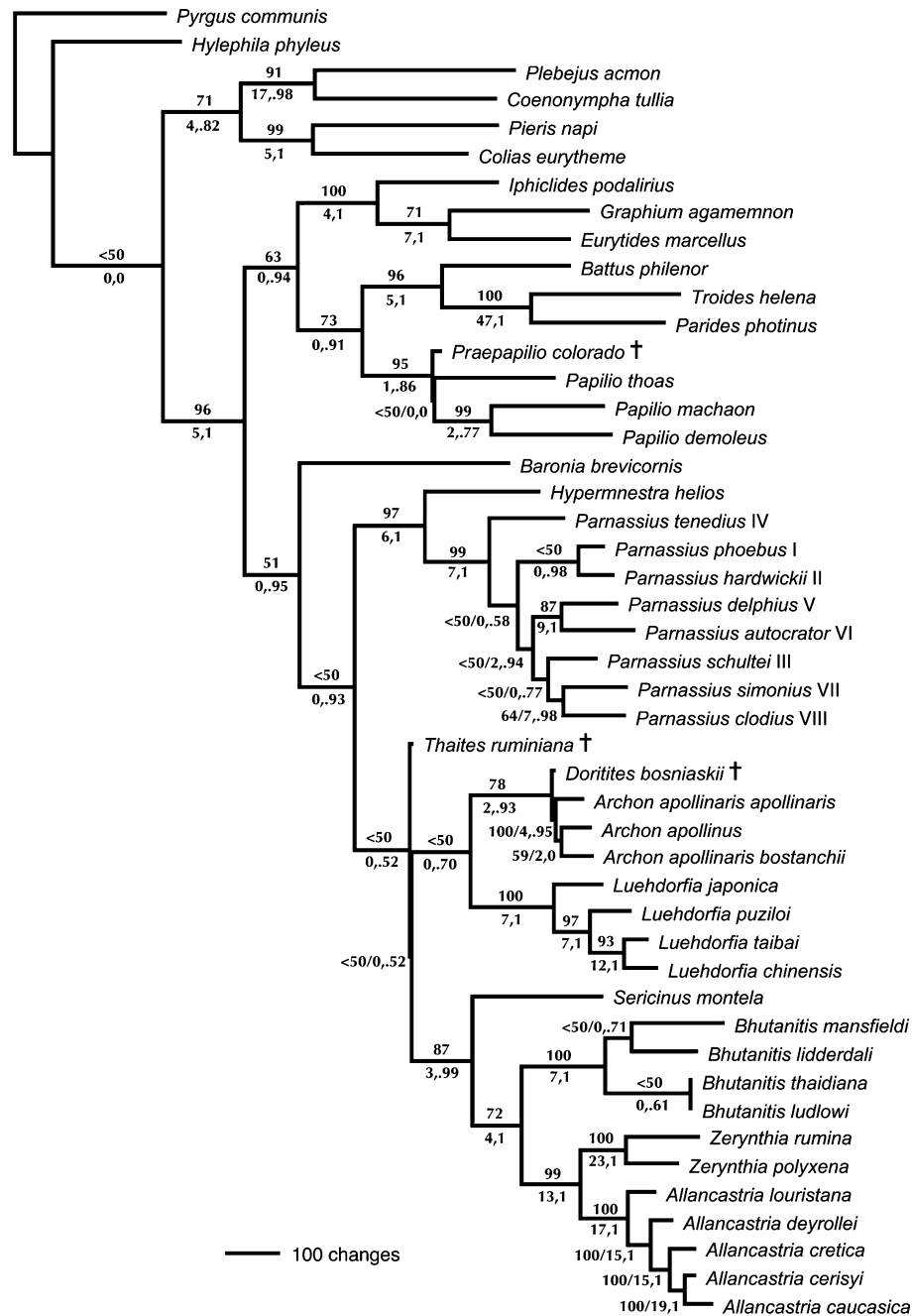
Fig. 7. Tree from Bayesian analysis of combined molecular and morphological data (TL = 11712, CI = 0.353, RI = 0.516). Numbers above branches are bootstrap values from parsimony analysis; numbers below branches are total Bremer support and Bayesian posterior probabilities.

4. Discussion

4.1. The challenge of ranking

The ambiguity of the criteria by which taxonomic ranks above the species level are determined is a serious problem in systematics (Hennig, 1966). A standard system for biological classification, which would allow objective comparison of such taxa among various phyla, has yet to be developed (Avise and Johns, 1999; Williams et al., 2001). One solution is to remove all indication of ranking from classifications, by naming and treating taxonomic names in accordance with a

system such as the Phylocode (Cantino and de Queiroz, 2004; Holmes, 2004). However, the Linnaean hierarchy remains the accepted framework for most current taxonomic work, and so there is incentive to make this ranking system more consistent, at least within family level taxa such as the Papilionidae.

A few fundamental elements have been considered in formally recognizing higher ranks: (1) monophyly, (2) geological age, (3) documentation of a decided gap, including genetic distance, separating the higher taxon from other taxa of the same rank, (4) taxonomic stability, and (5) inclusion of a consistent number of species (Hennig, 1966; Mayr and Ashlock, 1991; Mayr, 1999).
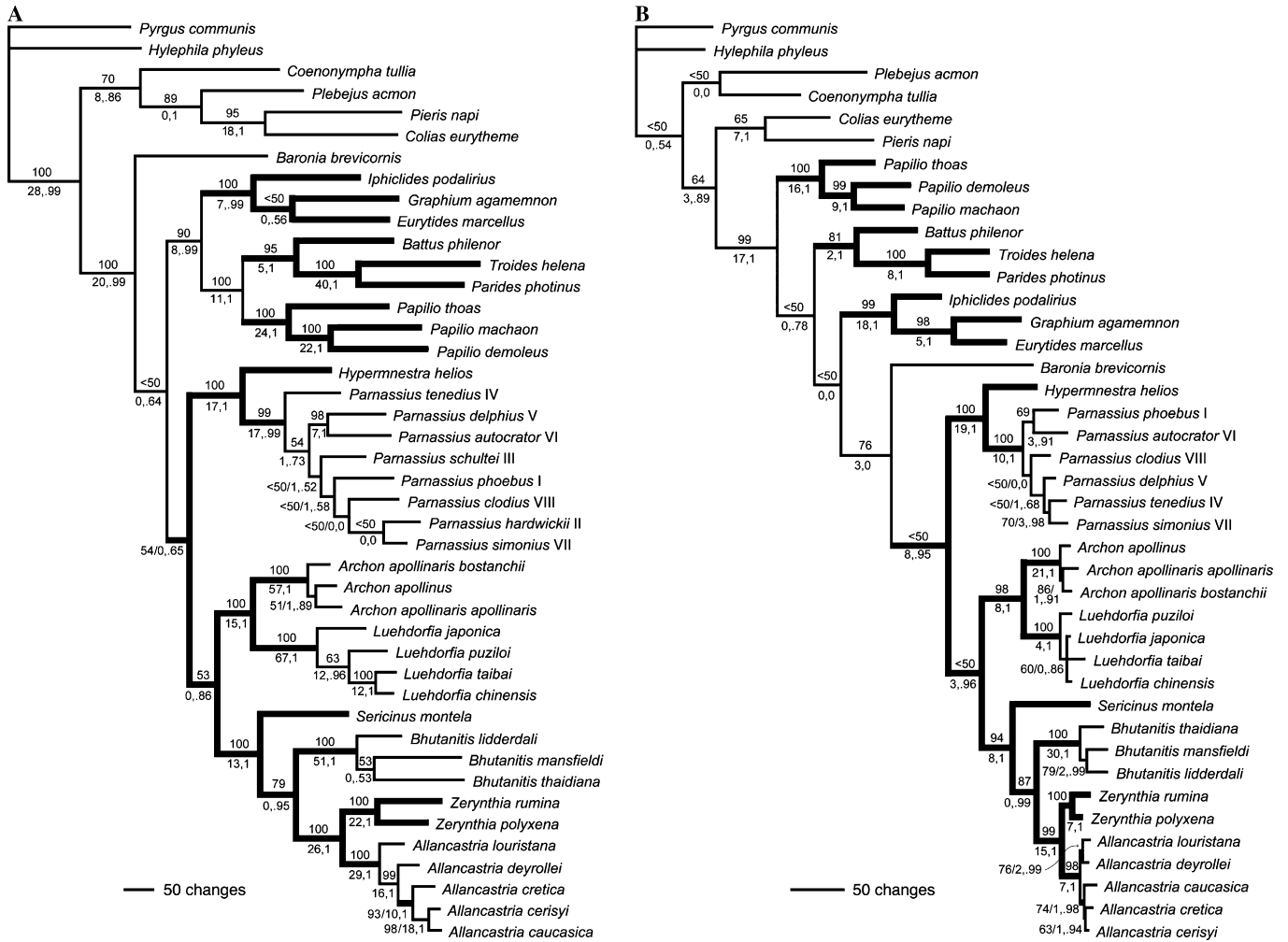
Fig. 8. Maximum likelihood trees obtained for combined mitochondrial (A) (TL = 8043, CI = 0.327, RI = 0.461) and combined nuclear data (B) (TL = 2675, CI = 0.382, RI = 0.587), with bootstrap values plotted above and Bremer support values/Bayesian posterior probabilities below the nodes. Ingroup nodes shown with thick lines are shared in both phylogenies.

Monophyly is currently considered the single most important criterion to be satisfied in recognizing a taxon at a higher rank, whether this taxon is to be called a genus, a tribe, or a family (Hennig, 1966). If a higher taxon is found to be paraphyletic, it is generally split into smaller monophyletic categories that are diagnosable via synapomorphies (Mayr et al., 1953; Peleggrino et al., 2001). Such splitting may be welcomed—and encouraged—in the case of "monster genera" that include hundreds or thousands of species (e.g. Cobos, 1986; Thayer and Newton, 2005). Thus the number of species in a genus may also provide a basis for splitting that is not always fully articulated. In fact, most systematic arrangements are originally determined by the "experience, good judgment, and common sense" of a traditional taxonomist (Mayr and Ashlock, 1991).

It has been suggested that molecular distances can provide a "yardstick" for measuring the geological age of divergence between taxonomic counterparts at any level when traditional systematics falls short, although such rates can be very different between various classes of animals (Avise, 1994). Early molecular studies using protein

and allozyme data generated much interest in this concept. More recently, percent DNA sequence divergence between species is often calculated but rarely used in refining generic or higher level boundaries (e.g. King and Wilson, 1975); instead, such ranks are generally determined based on tree topology and branch support (e.g. Williams et al., 2001).

Tradition also plays an important role in the designation of higher ranks. The International Code for Zoological Nomenclature (ICZN) has considered priority (and other principles) as subservient to stability in its preamble and in numerous articles (ICZN, 1999). Attempts to re-name or re-classify taxa that have been historically stable thus may be resisted by the scientific community unless there is convincing evidence that such a change is necessary.

We believe that our results demonstrate the need for a reclassification of Parnassiinae, even though this historically well-established group is generally considered to contain two tribes, Parnassiini and Zerynthiini. Instead, our data supports recognition of three tribes: the Parnassiini, Luehdorfiini, and Zerynthiini (Fig. 9).
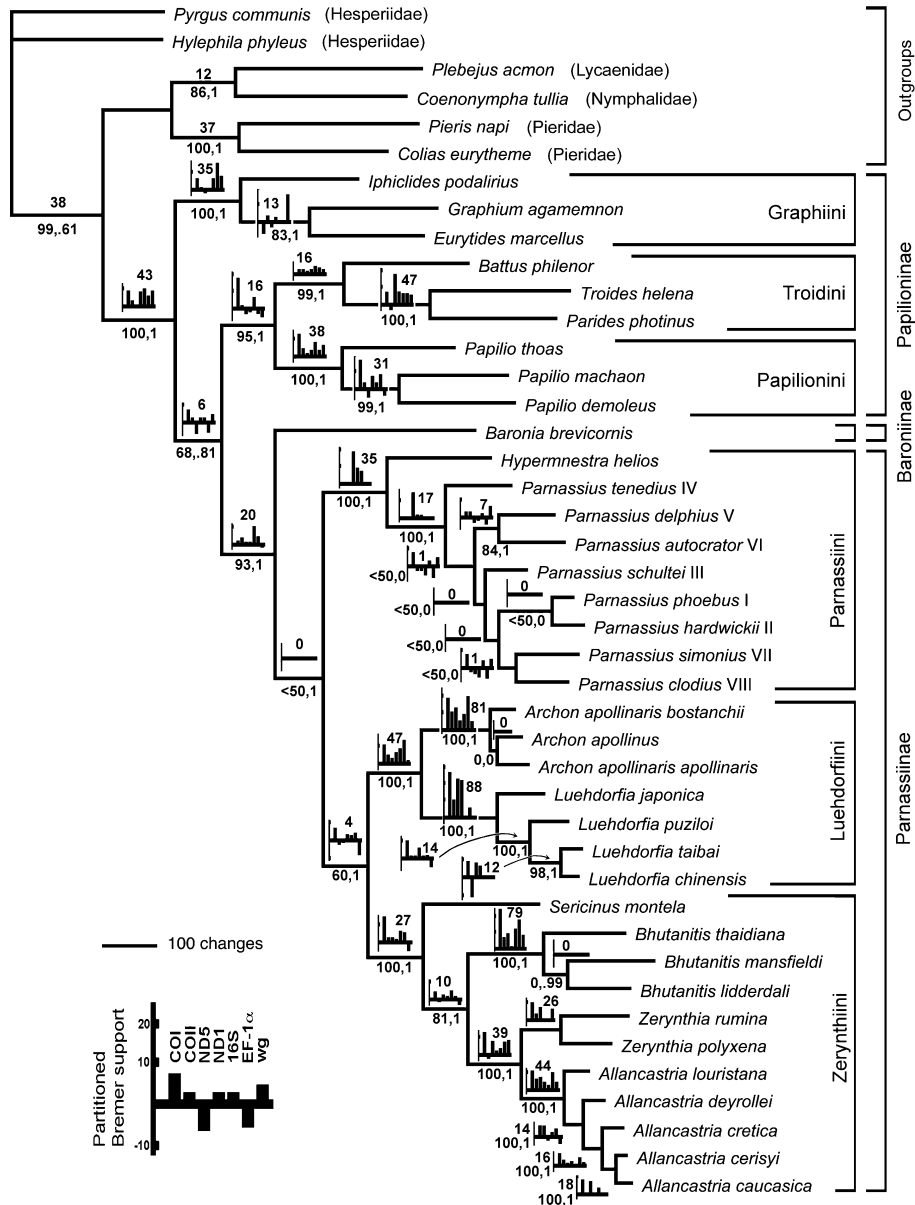
Fig. 9. Maximum likelihood reconstruction of Papilionidae phylogeny based on combined molecular data (TL = 10732, CI = 0.340, RI = 0.496). Graphs indicate partitioned Bremer support values for ingroup nodes, and the number above each graph represents the total Bremer support for each node. Numbers under each branch indicate MP bootstrap and Bayesian posterior probabilities from Bayesian analysis. The classification proposed in this study is shown to the right of tree.

In our best supported phylogenies, four genera (*Sericinus*, *Bhutanitis*, *Zerynthia* and *Allancastria*) always form a well supported clade, with *Sericinus* as the basal species and *Bhutanitis* as the sister taxon to *Zerynthia* + *Allancastria*. This is congruent with a previously established taxonomic classification (Fig. 2) but without *Luehdorfia*. We use the oldest available tribe name **Zerynthiini** Grote, 1899 for this clade.

An important result of our study is the strong support for the *Hypermnestra* + *Parnassius* clade, an alliance that has been questioned in the past (e.g. Hiura, 1980; Häuser, 1993). In our molecular phylogenies, *Hypermnestra* nearly always appears either basal to *Parnassius* or is located within the *Parnassius* clade. This also reflects a widely rec-

ognized group (Fig. 2), but without *Archon*. We use the oldest available tribe name **Parnassiini** Duponchel, [1835] for this clade.

Although phylogenetic relationships within the genus *Parnassius* were beyond the scope of this study, our data supports none of the previous hypotheses for the group (c.f. Hancock, 1983; Omoto et al., 2004; Katoh et al., 2005) except a few lower associations (*P. simonius* + *P. clodius*, *P. autocrator* + *P. delphius*). Further efforts should focus on more inclusive sampling of genes as well as more *Parnassius* species, since the currently available molecular data is unable to resolve the phylogeny of *Parnassius*.

The most intriguing outcome of our molecular analysis is the strongly supported association of *Archon* and
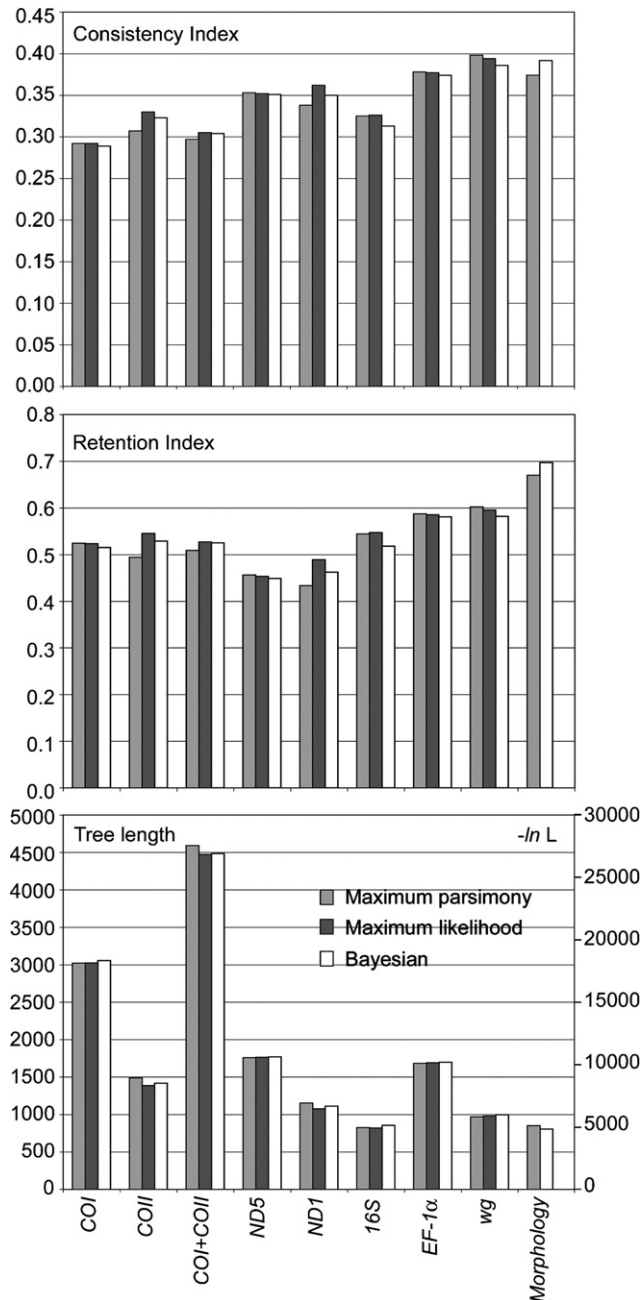
Fig. 10. Distribution of consistency index (CI), retention index (RI), tree length and log likelihood (−lnL) values for genes and morphology based on trees derived from each data partition with all taxa and outgroups included.

Luehdorfia (Fig. 9). A molecular relationship between these two genera has been demonstrated previously, although with limited taxon sampling (Omoto et al., 2004; Katoh et al., 2005). In all of our phylogenies, these two genera either group together or collapse in a polytomy. This is particularly interesting because *Archon*, commonly known as "the false apollo", has long been considered a primitive *Parnassius* (e.g. Higgins and Riley, 1970). In addition, *Luehdorfia* has often been associated with *Bhutanitis* based on similarities in wing pattern elements (e.g. Ford, 1944a; Hancock, 1983), although recent studies on

genitalia and early stages have challenged both of these assumptions (Saigusa, 1973; Igarashi, 1984; Stekolnikov and Kuznetsov, 2003). In contrast to the molecular characters, we have found no morphological synapomorphies supporting a relationship between *Archon* and *Luehdorfia*; even the lack of larval tubercles indicated by Häuser (1993) is shared by most other Papilionidae.

We rely on molecular data to recognize the tribe **Luehdorfiini** Tutt, 1899 **stat. rev.** as established previously (Stekolnikov and Kuznetsov, 2003), but now comprising *Luehdorfia* and *Archon*. Our strongest support for this recognition is the topology of the combined molecular phylogeny.

In order to quantify whether relative divergences provide support for erecting Luehdorfiini as a new tribe, we compared genetic divergences among tribes throughout the Papilionidae (Fig. 13). The uncorrected *p* distances from the *Archon* + *Luehdorfia* clade to both Zerynthiini and Parnassiini are similar, and yet somewhat lower than the distance between Zerynthiini and Parnassiini. These relative distances are incongruent with the consistent grouping of the *Archon* + *Luehdorfia* clade with the Zerynthiini, rather than Parnassiini, in phylogenetic analyses. This underscores the lack of homogeneity of divergence rates in the Parnassiinae. Furthermore, genetic distances among the three clades of Parnassiinae are not very comparable to those between tribes in Papilioninae for most genes. Limited taxon sampling for the Papilioninae is another potential factor affecting the pattern of *p* distances observed in our data. Thus the evidence for ranking the Luehdorfiini is ambiguous with respect to the magnitude of molecular divergences within Papilionidae. However, the monophyly of the *Archon* + *Luehdorfia* clade and the existence of equivalent gaps between tribal-level groups of Parnassiinae are distinctive features supporting recognition of Luehdorfiini as a new tribe.

The strongly supported position of *Baronia brevicornis* as the sister to only the Parnassiinae in our ML phylogeny of all molecular data contradicts several previous studies (e.g. Hancock, 1983; Tyler et al., 1994) but supports a much older statement that *Baronia* "belongs in the neighborhood of *Parnassius*" (Jordan, 1907–1908). The genetic distance of Baroniinae from the other two subfamilies of Papilionidae (Fig. 13) does not show greater divergence than between Parnassiinae and Papilioninae, and hence does not support a basal position for *Baronia*. The estimated age for the separation of the last common ancestor of Baroniinae and Parnassiinae on our phylogeny is between 75 and 82 MYA, corresponding to the late Cretaceous when the North American and Eurasian landmasses were still connected (Dietz and Holden, 1970). It is possible that *Baronia* is the sole extant member of an ancient sister lineage to Parnassiinae that survived the K/T mass extinction (Labandeira et al., 2002). Since the sparse sampling of Papilioninae in our study might have had an influence on the placement of *Baronia*, we refrain from further taxonomic conclusions on the position of the subfamily Baroniinae on the Papilionidae family tree.
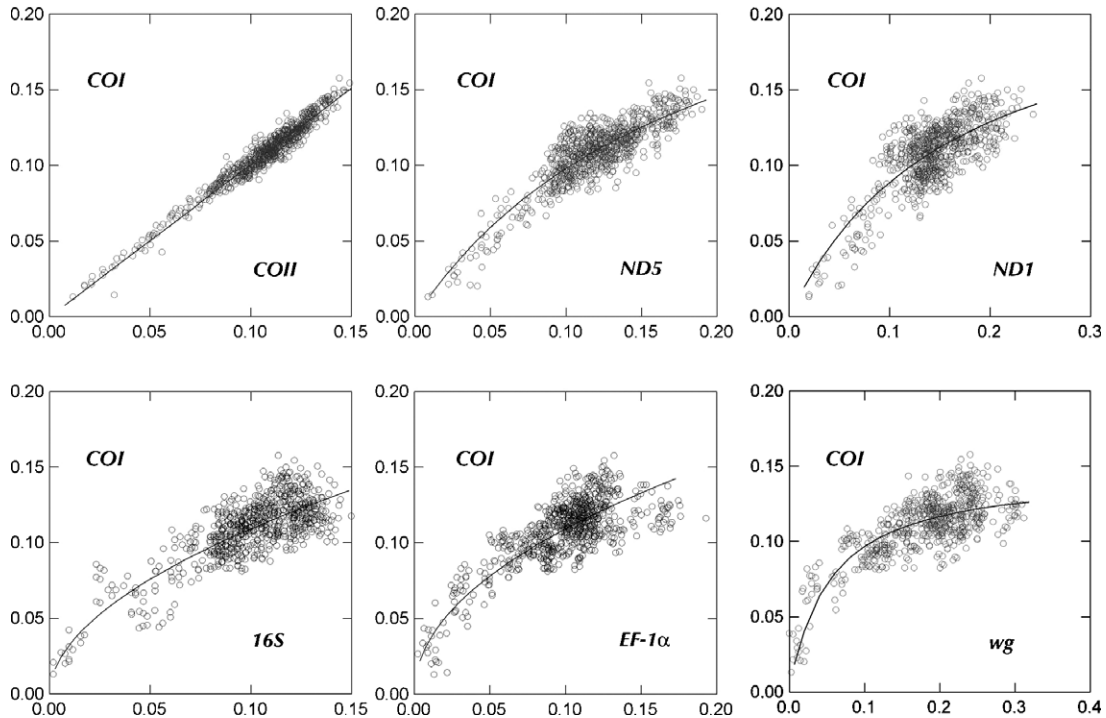
Fig. 11. Scatter plots of uncorrected *p* distances for each gene (*X* axis) plotted against *COI* (*Y* axis). Saturation curves are fitted to the data and limited to the data range. Note the differences in scale of the *X*-axes.
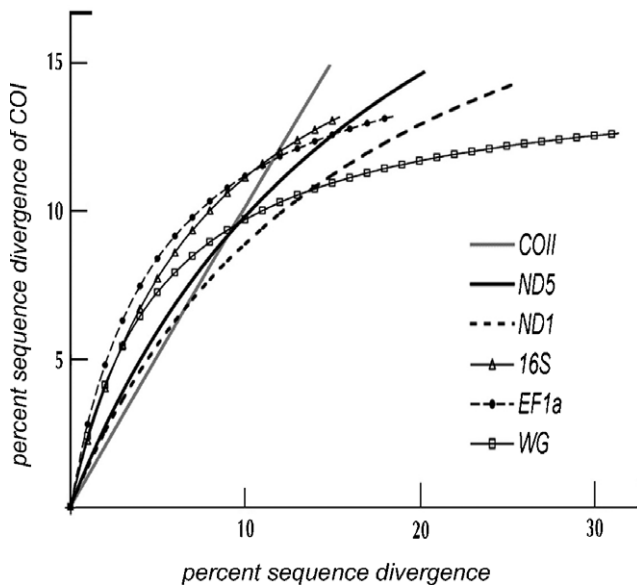

Fig. 12. Overlaid saturation curves (from Fig. 11) of uncorrected percentage sequence divergence of genes in relation to *COI*.

4.2. Usefulness of genes

Several methods have been previously proposed for estimating levels of homoplasy and systematic information content provided by genes in a combined nucleotide data set. The most commonly employed methods include estimation of partitioned Bremer support (Baker and DeSalle, 1997) and consistency and retention indices (Kluge and Farris, 1969; Farris, 1989). Other quantitative indices, such

as rescaled consistency (Farris, 1989) and data decisiveness (Goloboff, 1991) also give insights into phylogenetic signal provided by each gene (Creer et al., 2003; Danforth et al., 2005).

Consistency and retention indices for our phylogenies provide evidence that the two nuclear genes consistently perform slightly better than mitochondrial genes (Fig. 10). Among mitochondrial genes, *COI* data has the lowest agreement with its inferred phylogeny (lowest CI), but its ability to infer the proportion of potential synapomorphies retained on the phylogeny (RI) is better than *ND5* and *ND1*, both of which show higher CI values compared to *COI*.

The morphology partition in our dataset also shows very high CI and RI values from parsimony and Bayesian analyses, as might be expected for previously filtered data. It is interesting, however, that the CI of morphology is similar to that of nuclear genes. It should be noted that since outgroups were included in all trees used for estimation of CI and RI values, a saturation effect cannot be ruled out.

The partitioned Bremer support (PBS) values on our ML phylogeny of combined molecular data demonstrate substantial variation in the extent to which each gene partition contributes to the phylogeny (Fig. 9). Although PBS values indicate that none of the genes support or reject the monophyly of Parnassiinae, this node is moderately supported by *wg* as well as combined nuclear data in independent phylogenetic reconstructions and decay analysis (Table 4).

The utility of mitochondrial genes in phylogenetic analyses at lower taxonomic levels, and the strength of nuclear
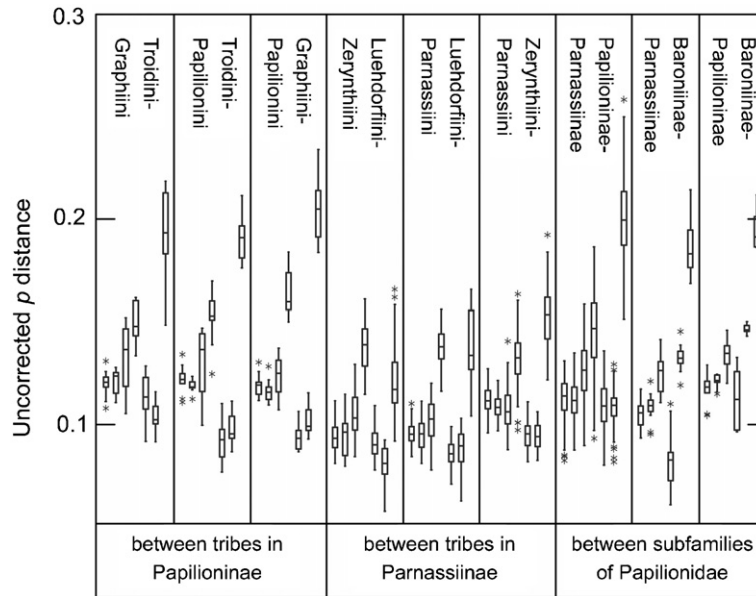
Fig. 13. Uncorrected *p* distances between tribes in Papilioninae and Parnassiinae, and between subfamilies of Papilionidae. Bars in each section represent (from left to right) distances based on *COI*, *COII*, *ND5*, *ND1*, *16S*, *EF-1α*, and *wg* sequence data, except for the last two panels where no *ND1* sequence for *Baronia* was available. The line in each box plot marks the median of the values; the length of the box shows the range within which the central 50% of the values fall; and whiskers show the range of values that fall within the inner fences (see SYSTAT, 2005 manual for details). Outside values are shown by asterisks.

and ribosomal gene regions in providing phylogenetic resolution for deeper nodes, have been reported previously (e.g. Simon et al., 1994; Brower and De Salle, 1998; Caterino et al., 2000). Comparison of uncorrected *p* distances in our data also shows that most protein coding mitochondrial genes (*COI*, *COII*, *ND1*, and *ND5*) show rapid increases in distance for recent divergences, but not for higher level relationships, although *ND1* and *ND5* continue to diverge at the genus level and above. On the other hand, *16S*, *EF-1α* and *wg* are more informative in genus- and tribal level analyses, and *EF-1α* and especially *wg* can still provide resolution in phylogenies at the taxonomic rank of subfamily (Fig. 12).

4.3. Biogeography, genetic divergence, and character evolution

Braby et al. (2005) critically review several previous divergence time estimates for Papilioninae and Troidini, and question the dates estimated by Gaunt and Miles (2002). Although their proposed age for the initial split in Troidini (*Battus* from other genera) at 90 MYA conflicts with the constraint for the last common ancestor of Papilionini and Troidini (82.5–89.1) (after Gaunt and Miles, 2002), we found enforcing this estimate alongside other constraints has no substantive impact on age estimates for Parnassiinae (Table 5).

Our dispersal/vicariance reconstruction and molecular clock analysis support a previous hypothesis (Korb, 1997) that the ancestral origin of Parnassiinae was in Central Asia. Optimizing the evolution of morphological characters at internal nodes using PAUP* under the MP criterion and

default ACCTRAN transformation indicates that the common ancestor of Parnassiinae flew at lower elevations, was distributed from the Iranian Plateau to central Asia and China, and had Aristolochiaceae feeding larvae (Fig. 14) (cf. Kreuzberg, 1994). However, a similar analysis using MacClade 4.0 suggests an equivocal state for the larval food plant of this ancestor, which could have been either Aristolochiaceae, Crassulaceae or Zygophyllaceae. Our phylogeny also supports previous assumptions that this ancestral species would have had a pale yellow ground color, no tails, asymmetrical pretarsi (Ehrlich, 1958), a narrow and heavily sclerotized aedeagus, a heavily sclerotized ostial region in females, an elongate third segment of labial palpus, and an incurved middle discocellular vein (mdc) on the forewing (Miller, 1987).

Based on maximum likelihood estimation of divergences (Fig. 14), the first split in ancestral Parnassiinae which gave rise to two lineages (Parnassiini and Zerynthiini + Luehdorfiini) would have occurred shortly after the initial collision of the Indian plate into Eurasia, which began around 65–55 MYA and continued until 54–42 MYA (Briggs, 2003). This event has also been used to explain the diversification in Eurasian Agamid lizards (Macey et al., 1999), *Latimeria* coelacanths (Springer, 1999; Inoue et al., 2005), and *Tetraponera* ants (Ward, 2001).

Based on our character optimizations, the ancestor of Parnassiini may have resembled *Hypermnestra*. It would also have had solitary larvae, cocooned pupae (Hancock, 1983), scaled antennae (Miller, 1987; after Hancock, 1983), sclerotized patagia, no mid-tibial spurs on hind tibiae, and a simple (= unforked) precostal vein on the hindwing (Hancock, 1983). The ancestral food plant of Parnassiini is

Table 5

Age estimates (with standard deviations) in millions of years for internal nodes using calibration points shown in Fig. 14 and model-corrected branch lengths

Node	Node 10 fixed at 89.1 MY		Node 10 fixed at 82.5 MY		Node 11 and 12 fixed[a]	Node 11 fixed[a]	None fixed
	No constraints	a+b+c+d+e	No constraints	a+b+c+d+e	5 constr. (n 10 free)	6 constr. (n 10 free)	6 constraints
Root	111.37 ± 0.57	111.37 ± 0.57	103.13 ± 0.53	103.14 ± 0.52	113.17 ± 3.1	112.48 ± 0.57	103.23 ± 1.52
2	107.27 ± 2.40	107.27 ± 2.46	99.30 ± 2.21	99.33 ± 2.26	108.73 ± 2.61	108.38 ± 2.45	100.02 ± 1.95
3	75.97 ± 18.75	76.27 ± 18.61	69.92 ± 12.16	70.45 ± 17.38	73.88 ± 18.28	77.23 ± 18.72	69.00 ± 16.52
4	51.58 ± 19.46	51.86 ± 19.32	47.66 ± 17.66	47.76 ± 17.89	47.79 ± 18.82	52.12 ± 19.39	42.31 ± 17.07
5	48.82 ± 19.90	48.72 ± 19.96	44.73 ± 18.08	45.08 ± 18.38	47.41 ± 18.05	49.25 ± 20.15	46.09 ± 16.89
6	104.04 ± 2.49	103.98 ± 2.53	96.23 ± 2.28	96.30 ± 2.32	104.832 ± 2.68	105.04 ± 2.58	96.62 ± 2.41
7	73.93 ± 17.45	73.49 ± 17.31	68.72 ± 16.33	68.20 ± 16.11	72.67 ± 16.90	74.47 ± 17.50	67.09 ± 16.72
8	47.49 ± 18.54	47.48 ± 18.80	44.25 ± 17.54	43.45 ± 17.11	46.35 ± 18.30	47.98 ± 18.92	45.54 ± 18.36
9	100.87 ± 2.39	100.90 ± 2.43	93.23 ± 2.13	93.40 ± 2.24	102.22 ± 2.65	101.90 ± 2.51	94.07 ± 2.34
10	**89.10 ± 0.00**	**89.10 ± 0.00**	**82.50 ± 0.00**	**82.50 ± 0.00**	98.50 ± 2.58	98.59 ± 2.71	***87.24 ± 1.29***
11	61.53 ± 15.72	61.58 ± 15.87	57.32 ± 14.88	57.09 ± 14.06	**90.00 ± 0.00**	**90.00 ± 0.00**	60.03 ± 16.02
12	39.06 ± 16.54	39.11 ± 16.17	36.41 ± 15.32	36.09 ± 15.14	**64.90 ± 0.00**	**66.77 ± 2.70**	38.96 ± 16.57
13	57.93 ± 14.86	***56.33 ± 4.26***	53.63 ± 13.66	***56.40 ± 4.26***	*57.77 ± 4.39*	*56.45 ± 4.31*	*57.45 ± 4.31*
14	36.21 ± 15.21	***43.10 ± 1.04***	33.19 ± 13.87	***43.10 ± 1.04***	*43.23 ± 1.13*	*43.10 ± 1.04*	*43.09 ± 1.13*
15	81.22 ± 11.71	82.34 ± 10.78	75.26 ± 10.85	76.56 ± 9.98	83.47 ± 10.37	83.46 ± 10.76	77.73 ± 8.94
16	64.32 ± 12.21	67.12 ± 11.29	59.34 ± 11.37	62.52 ± 10.30	68.26 ± 10.95	67.69 ± 11.53	63.33 ± 11.06
17	50.16 ± 13.22	52.07 ± 13.04	46.48 ± 12.06	48.51 ± 11.98	52.45 ± 11.16	52.70 ± 13.14	49.30 ± 11.83
18	37.59 ± 12.17	39.03 ± 12.15	34.83 ± 10.96	36.22 ± 11.29	39.14 ± 10.69	39.81 ± 12.02	36.90 ± 11.27
19	27.97 ± 10.04	28.89 ± 10.11	25.99 ± 9.21	26.84 ± 9.36	29.68 ± 10.28	29.58 ± 10.04	27.14 ± 9.13
20	16.89 ± 8.55	17.53 ± 8.74	15.55 ± 7.86	16.06 ± 8.11	18.53 ± 8.03	18.02 ± 9.06	17.60 ± 8.79
21	20.52 ± 8.85	20.83 ± 8.93	19.22 ± 8.17	19.59 ± 8.29	20.88 ± 8.95	21.50 ± 8.91	20.13 ± 8.68
22	14.37 ± 6.91	14.39 ± 6.77	13.48 ± 6.49	13.15 ± 6.26	14.42 ± 7.06	14.92 ± 6.80	13.44 ± 6.06
23	9.20 ± 5.60	9.15 ± 5.37	8.68 ± 5.32	8.64 ± 4.96	9.09 ± 5.25	9.53 ± 5.53	8.01 ± 4.40
24	9.27 ± 5.42	9.51 ± 5.63	8.77 ± 5.10	8.87 ± 5.21	9.60 ± 5.83	9.75 ± 5.57	8.70 ± 5.03
25	50.79 ± 12.23	54.64 ± 11.28	46.83 ± 11.07	50.87 ± 10.38	55.88 ± 10.77	55.20 ± 11.51	52.71 ± 10.67
26	37.86 ± 12.45	42.72 ± 11.41	34.87 ± 11.36	40.06 ± 10.44	43.53 ± 11.37	43.12 ± 11.57	41.85 ± 10.98
27	27.18 ± 11.40	***31.96 ± 10.69***	24.68 ± 10.43	***30.22 ± 9.83***	*31.55 ± 11.45*	*32.11 ± 10.82*	*29.57 ± 9.39*
28	16.89 ± 8.55	19.96 ± 8.95	15.52 ± 8.01	18.80 ± 8.24	20.51 ± 10.68	20.18 ± 9.12	18.40 ± 8.95
29	27.54 ± 10.90	***10.14 ± 0.36***	25.42 ± 10.10	***10.14 ± 0.36***	*10.14 ± 3.64*	*10.15 ± 36.09*	*10.20 ± 0.39*
30	18.96 ± 8.61	7.07 ± 1.79	17.47 ± 7.99	7.06 ± 1.83	6.94 ± 1.66	7.07 ± 1.77	7.10 ± 1.76
31	12.07 ± 6.71	4.56 ± 1.78	11.14 ± 6.24	4.50 ± 1.76	4.46 ± 1.81	4.60 ± 1.78	4.63 ± 1.84
32	40.26 ± 11.24	43.85 ± 10.67	37.12 ± 10.38	40.93 ± 9.86	44.69 ± 9.65	44.29 ± 10.79	41.81 ± 9.57
33	31.47 ± 9.86	34.71 ± 9.71	28.96 ± 8.96	32.56 ± 8.97	35.61 ± 9.02	35.15 ± 9.75	32.59 ± 9.13
34	20.27 ± 7.94	22.42 ± 8.08	18.78 ± 7.04	21.03 ± 7.44	24.72 ± 8.97	22.88 ± 8.13	22.69 ± 8.66
35	12.47 ± 6.30	13.65 ± 6.72	11.66 ± 5.82	12.90 ± 6.23	15.43 ± 7.90	13.78 ± 68.02	15.53 ± 8.03
36	24.16 ± 8.63	27.26 ± 8.62	22.07 ± 7.60	25.59 ± 7.94	28.08 ± 8.11	27.62 ± 8.63	24.93 ± 8.11
37	15.95 ± 8.11	18.12 ± 85.69	14.58 ± 6.74	16.97 ± 7.97	18.51 ± 7.38	18.09 ± 8.54	16.18 ± 8.37
38	17.78 ± 7.68	20.96 ± 7.68	16.19 ± 6.92	19.73 ± 7.09	21.58 ± 6.66	21.23 ± 7.70	19.16 ± 6.67
39	12.57 ± 6.54	15.64 ± 6.57	11.58 ± 5.95	14.85 ± 6.11	16.43 ± 5.49	15.89 ± 6.59	14.55 ± 5.89
40	8.88 ± 5.30	***8.29 ± 1.52***	8.22 ± 4.91	***8.18 ± 1.53***	*8.09 ± 12.57*	*8.25 ± 1.50*	*8.07 ± 1.40*
41	5.92 ± 3.82	5.49 ± 1.80	5.46 ± 3.56	5.44 ± 1.81	5.03 ± 1.69	5.46 ± 1.82	5.07 ± 1.78

Fixed nodes are shown in bold, constrained nodes are in bold italics. Constraints are: node 10 (82.5–89.1 MYA); a = node 13 (35–65 MYA); b = node 14 (39.8–45.1 MYA); c = node 29 (9.6 ± 1.2 MYA); d = node 40 (3–11 MYA); and e = node 27 (min age = 5.3 MYA).

[a] Fixed nodes after Braby et al., 2005 (node11 = 90 MYA, node12 = 64.9 ± 6.88 MYA), with all constraints imposed except for node 10 due to conflict of assumptions with that node.

also equivocal, although MP reconstruction in PAUP∗ suggests Crassulaceae as the ancestral larval host. The presence of many specialized characters in *Hypermnestra* is suggestive of a deep divergence with *Parnassius* and other Parnassiini, which is supported by our molecular clock analysis (Fig. 14 and Table 5).

Populations of the common ancestor of Parnassiini that remained in deserts could have adopted Zygophyllaceae as food plant and formed *Hypermnestra*, while those in higher altitudes began a rapid diversification under a new genus, *Parnassius*, with Crassulaceae feeding larvae. The ancestral *Parnassius* (node 18) in our analysis is estimated to be 34–39 MY old and originated in the Himalayas and China.

Our DIVA analysis shows that some species of *Parnassius* would have dispersed later into other parts of the world as far as Europe and North America, through a series of complex biogeographic and climatic events.

The uplift of the Tibetan plateau and formation of the Himalayas could have caused a complete geographic split in the range of the enigmatic *Aristolochia*-feeding ancestor of *Archon* and *Luehdorfia* about 42 MYA, leaving one lineage on each side of the high mountains. The fossil *Doritites bosniaskii*, which appears to be most closely related to *Archon*, demonstrates the expansion of an extinct ancestral lineage into southern Europe. Our DIVA analysis shows that the ancestor of *Archon* probably originated in the
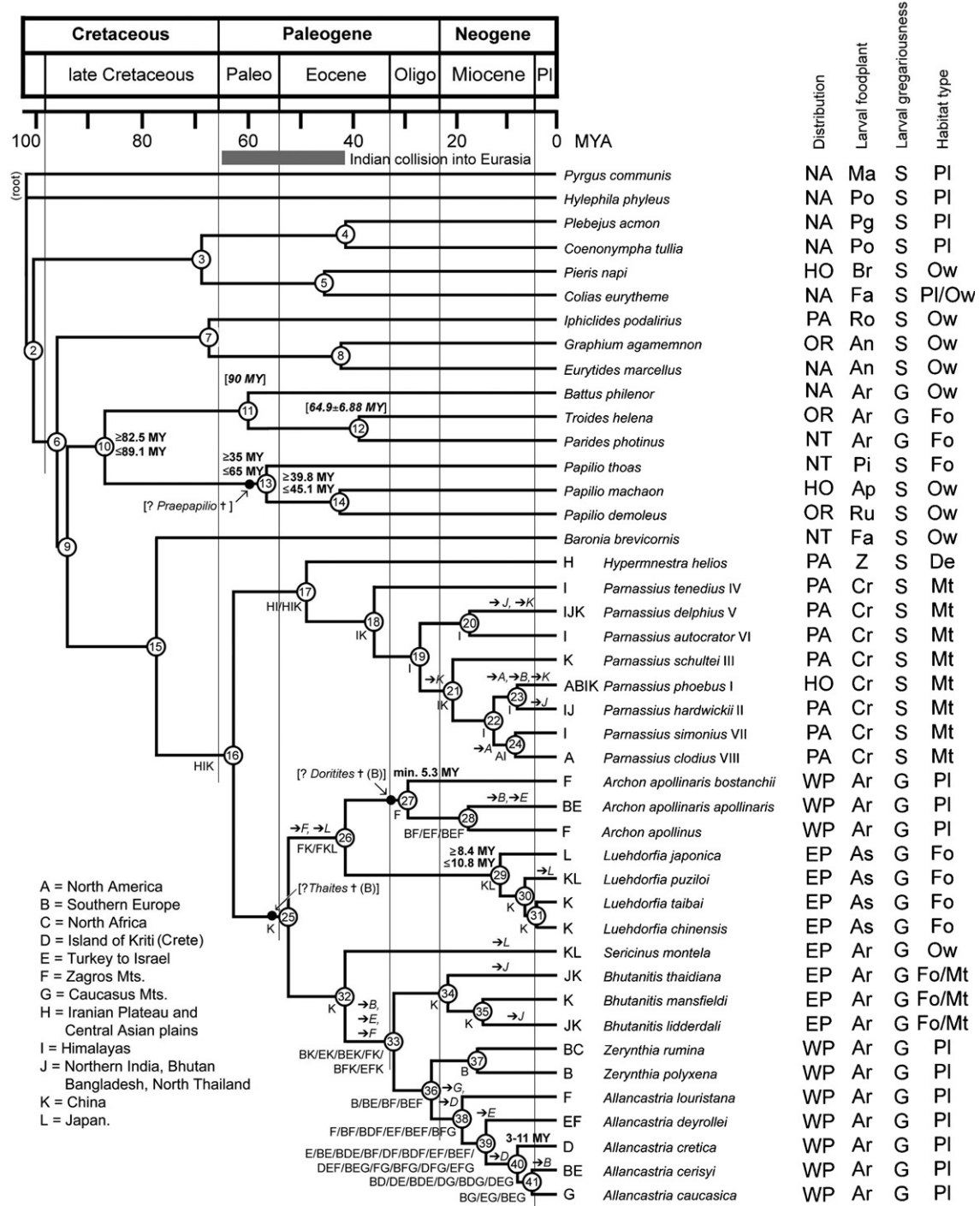
Fig. 14. Maximum likelihood chronogram for Papilionidae based on all molecular data with no fixed dates and six constraints imposed (last column in Table 5). Calibration points are printed in bold; dates in brackets are from Braby et al. (2005) and have been analyzed separately. Inferred positions for fossil taxa are shown with solid dots. For Parnassiinae, dispersal events into new areas are shown with arrows. The most parsimonious reconstruction of dispersal/vicarience events, as shown above, required 26 dispersal events. Ecological characters are indicated to the right: Distribution (NA = Nearctic, HO = Holarctic, NT = Neotropical, OR = Oriental, PA = Palaearctic, WP = Western Palaearctic, EP = Eastern Palaearctic); larval food plant (Ma = Malvaceae, Po = Poaceae, Pg = Polygonaceae, Br = Brassicaceae, Fa = Fabaceae, Ro = Rosaceae, An = Annonaceae, Pi = Piperaceae, Ap = Apiaceae, Ru = Rutaceae, Z = Zygophyllaceae, Cr = Crassulaceae and Papaveraceae, Ar = *Aristolochia* [Aristolochiaceae], As = *Asarum* [Aristolochiaceae]); larval gregariousness (G = gregarious, S = solitary); and primary habitat type (Ow = Open woodland, De = Deserts, Pl = Plains, Fo = Forests, Mt = Mountains). Characters were coded after Ford (1944a,b), Igarashi (1984) and Sillén-Tullberg (1988).

Zagros Mountains about 30 MYA and spread westwards into southern Europe and Israel. On the other hand, the lineage leading to *Luehdorfia* – which originated in China/ Japan – switched to feeding on *Asarum*. According to Makita et al. (2000), the common ancestor of extant *Luehdorfia* appeared relatively recently at about 9.6 ± 1.2 MYA,

although we found that alternative molecular clock analyses without enforcing this date as a constraint shows the split to have occurred as far back as 25–27 MYA (Table 5). Despite numerous phylogenetic hypotheses for the genus *Luehdorfia* (e.g. Takahashi, 1973; Saigusa, 1973; Hiura, 1978; Ishizuka, 1980, 1991; Shinkawa, 1991, 1999; Aoyama, 1994; Watanabe, 1996; Kato, 1998; Yashima et al., 1999; Matsumura et al., 2005), our results are only congruent with that of Makita et al. (2000).

It appears that larval gregariousness evolved in parallel with Aristolochiaceae feeding in the Parnassiinae (Fig. 14). Troidini (Papilionidae) also show this trait in addition to Zerynthiini and Luehdorfiini. The *Aristolochia*-feeding ancestor of Zerynthiini that remained in China may have been similar to *Sericinus*; the larva had fleshy segmental tubercles with setae, the pupa was slender; the adult had no scales on tibia and tarsi (Miller, 1987; after Hancock, 1983).

A further duplication event about 31 MYA produced ancestral *Bhutanitis* + *Zerynthia* + *Allancastria*. DIVA analysis suggests that this species had good dispersal capability as it expanded its range into Iran and further into Europe. The lineage that remained in China would have led to *Bhutanitis* by 20 MYA. The results of our combined analyses largely support a recent phylogenetic hypothesis for the evolution of the genus *Bhutanitis* based on *COI* alone (Zhu et al., 2005).

About 16 MYA, *Zerynthia* in Europe and *Allancastria* in the Zagros Mts would have diverged from a common ancestor. The ancestral *Allancastria*, which may have been similar to *A. louristana*, expanded its range through a series of dispersal events into southern Europe and the Middle East. The rich tectonic history of the Mediterranean (Steininger and Rögl, 1996) further assisted in speciation and produced today's complex distribution pattern of *Allancastria*, leaving *A. cretica* in Crete before its severance from the mainland about 8 MYA. A similar pattern of dispersal from Iran into Anatolia and Europe has been documented for Pachyderminae beetles (Sanmartín, 2003).

We also found that the estimated age of the node for the fossil *Thaites ruminiana* (~50 MYA) is somewhat older than the age of the fossil (Lower Oligocene, 30–38 MYA). Since the basal position inferred for the fossil is only weakly supported in this study, *Thaites* could belong to an extinct separate lineage for which apomorphies have not been preserved, or it may actually belong to either of the *Luehdorfia* + *Archon* or *Bhutanitis*/*Allancastria*/*Zerynthia* clades. Further studies on the fossil might provide new information and help clarify its phylogenetic position.

4.4. Conclusions

Our results demonstrate that the current higher classification of Parnassiinae does not reflect the phylogeny of the group. Our results provide strong support for monophyly of three groups within the Parnassiinae, and weak support for the monophyly of the subfamily. Divergence times estimated using several previously established calibration points show that the initial diversification of Parnassiinae genera occurred at about the same time that the Indian plate collided into Eurasia 65–42 MYA. These estimates correlate with other previously well-established dates of vicariance and tectonic events, while fossils may be unreliable sources for calibration due to uncertainties about their phylogenetic placement. Based primarily on molecular evidence, we propose the following classification of the subfamily:

Subfamily Parnassiinae Duponchel [1835]
Tribe Parnassiini Duponchel, [1835]
    Genus *Hypermnestra* Ménétriés, 1846
    Genus *Parnassius* Latreille, 1804
Tribe Luehdorfiini Tutt, 1896 (**stat. rev.**)
    Genus *Luehdorfia* Crüger, 1878
    Genus *Archon* Hübner, 1822
    † Genus *Doritites* Rebel, 1898
Tribe Zerynthiini Grote, 1899
    Genus *Sericinus* Westwood, 1851
    Genus *Bhutanitis* Atkinson, 1873
    Genus *Zerynthia* Ochsenheimer, 1816
    Genus *Allancastria* Bryk, 1934
[? † Genus *Thaites* Scudder 1875]

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/ j.ympev.2006.06.022.

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