Mitochondrial DNA divergence and phylogeography in western Palaearctic Parnassiinae (Lepidoptera: Papilionidae): How many species are there?

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We inferred the phylogeny and historical biogeography of Parnassiinae species from the western Palaearctic using 825 bp DNA sequence from the mitochondrial protein-coding gene *cytochrome oxidase* I. Investigation of genetic variation revealed several cases of overlap in extent of divergence between traditionally applied taxonomic ranks. In particular, we found deep divergences between populations of *Archon apollinus* (Herbst) from Turkey and Israel, *Zerynthia rumina* (Linnaeus) from Spain and North Africa, *Zerynthia polyxena* (Denis & Schiffermüller) from Italy and other parts of its range, and *Hypermnestra helios* (Nickerl) from Iran and Central Asia. Due to incomplete sampling and weak morphological support, we only report the possibility of more than one species within each of these four taxa. The origin of ancestral *Archon* and *Allancastria/Zerynthia* is found to lie in the Iranian region. Diversification within genera is postulated to be the result of complex tectonic interactions between Eurasia and Africa during the past 20 million years, involving multiple dispersal and vicariance events.

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Introduction

The rich geological history of western Eurasia and its tectonic interactions with Africa and Arabia have had substantial effects on the evolution of many organisms inhabiting Europe and western Asia (Steininger and Rögl, 1996). Studies that correlate phylogenies with paleotectonic events may provide clues to geological events that have gone unnoticed (eg. Sanmartin, 2003; Guo *et al.*, 2005; Cosson *et al.* 2005). Among other organisms, butterflies have been the subject of broad-scale research on biodiversity and contemporary geography in the region (e.g. Dennis *et al.*, 2000; Grill and Cleary, 2003; Schmitt *et al.* 2005).

The readily identifiable phenotypic diversity that butterflies offer through their wing patterns has put them among the few groups that have been studied meticulously throughout modern history by a vast array of enthusiasts, from amateur collectors to professional biologists and taxonomists. Nonetheless, at a systematic level, lack of a clear definition for what constitutes a lower taxonomic category (species and subspecies) has had a considerable effect on butterfly taxonomy. Over the past century a multitude of "varieties" and "forms" of European Parnassiinae have been studied and named based on the slightest morphological differences; extreme instances are *Zerynthia polyxena* (Schiffermüller) (see Nardelli and Hirschfeld, 2002) and *Parnassius apollo* (Linnaeus) (see e.g.

Eisner, 1974). These infra-specific names (i.e. trinomials) may be described on the basis of limited specimens and only characters from their wing pattern, and are often subsequently sunk as junior synonyms of older available names (e.g. see Hesselbarth *et al.,* 1995). On the other hand, some subspecies gain species-level recognition upon further examination of life history and internal morphology (e.g. *Archon apollinaris* (Staudinger), De Freina, 1985). The phylogenetic status of many such infra-specific names, however, remains unclear.

Geographically, swallowtail butterflies of the subfamily Parnassiinae are divided into an eastern Palaearctic group of genera (*Bhutanitis* [4 spp], *Luehdorfia* [4 spp], *Sericinus* [1 sp]) and a western Palaearctic group of genera (*Archon* [3 spp], *Allancastria* [5 spp], *Zerynthia* [2 spp], and *Hypermnestra* [1 sp]), although the genus *Parnassius* - which is not the focus of this study - is widespread in the Holarctic region with about 50 species. This east-west disjunction has been associated with the collision of the Indian plate into Eurasia about 65 million years ago (Nazari *et al.*, 2007), which created the Himalayas and split the range of the last common ancestor of the subfamily. The number of infra-specific names described for the western group of genera is larger than that for the eastern Palaearctic group (for example, see Bryk, 1934, 1935). A previous study on Parnassiinae based on morphology and sequence data from seven genes found that all genera within the subfamily are monophyletic (Nazari *et al.*, 2007).

The genus ***Archon*** has three species: *A. apollinus* (Herbst) (type locality: Izmir, W. Turkey) distributed from Bulgaria to Greece and western Turkey, Syria, Palestine and Israel, *A. apollinaris* (Staudinger) (type locality: NE. Turkey) from eastern Turkey, west and northwest Iran, and northern Iraq, and *Archon bostanchii* (De Freina & Naderi) (type locality: Lorestan, W. Iran). All three species depend on *Aristolochia* as larval host, and are morphologically similar. *Archon apollinaris* was separated as a distinct species through a comprehensive study of genitalic characters (De Freina, 1985), an idea that was further supported by comparison of immature stages (Carbonell, 1991). Although Nazari *et al.* (2007) recognized only two species, *Archon bostanchii* has recently been given a specific rank based on distinctive morphological and ecological characters as well as DNA sequence data (Carbonell and Michel, 2006). At least nine sub-specific names within *A. apollinus*, mostly described from Anatolia (e.g. Koçak, 1982) have been synonymized with the nominal subspecies by Hesselbarth *et al.* (1995), who list only three subspecies for Turkey.

Five species are recognized within the genus ***Allancastria*** (after Häuser *et al.*, 2005): *A. cerisy* (Godart), *A. deyrollei* Oberthür, *A. cretica* (Rebel), *A. caucasica* (Lederer), and *A. louristana* (Le Cerf). All species feed on various *Aristolochia* at the larval stage. For a long time, the genus was considered to have only a single species, *A. cerisy* (type locality: W. Turkey), with many local "forms" and subspecies described under this name. Le Cerf (1913) was the first to re-evaluate whether these entities should be given specific status, prompted by his discovery of *A. louristana* a few years earlier (type locality: W. Iran), and its striking similarity to *Hypermnestra*. He conducted a comparative analysis of morphological characters of the adult and life stages of *A. louristana, A. apollinus, H. helios* (Nickerl), and *Parnassius tenedius* (Eversmann), trying to determine the phylogenetic relationship between them. A comprehensive review of *Allancastria* was later carried out by Bernardi (1970), who maintained the monotypy of the genus, listed every variety and locality known to that date, and described *A. c. eisneri*. He also pointed out the co-habitation of two subspecies (*A. c. eisneri* Bernardi, and *A. c. speciosa* Stichel) in Jerusalem, hesitating to give them species rank. This situation was immediately noted by Larsen (1973) who assigned specific status to *A. deyrollei* (type locality: NE. Turkey) based on its co-existence with *A. cerisy* in Lebanon. He also suggested that *A. louristana* should be a subspecies of *A. deyrollei*, and that *A. caucasica* (type locality: Georgia, Caucasus Mts.) should be given specific status as well. However, some publications in later years overlooked Larsen's suggestions. Eisner (1974) and Ackery (1975) both maintained previous viewpoints that the genus was monotypic with several subspecies. Koçak (1975) described *A. c. abanti* for a population of *A. cerisy* in northeastern Turkey, but Larsen (1976) suggested that this population is closer to *A. caucasica* than *A. cerisy.* Kuhna (1977), based on genitalia and wing morphology for the first time elevated *A. caucasica* to species level, suggested species status for *A. louristana*, and described two new subspecies for *A. cerisy.* De Freina (1979) further reinforced the specific status and subspecies assignments of *A. caucasica, A.*

deyrollei and *A. louristana*, and presented a detailed discussion of the biology of these species. The taxon *A. cretica* (type locality: Crete [Kriti], Greece) remained a subspecies of *A. cerisy* until Koçak (1981) gave it species rank. He also sank *Allancastria* as a subgenus of *Zerynthia*, which was accepted by some (e.g. Hesselbarth *et al.,* 1995) and ignored by others (e.g. Hancock, 1983; Miller, 1987). More recent studies by Carbonell (1996a, b), Hürter (2001) and others have provided a comparative analysis of many morphological and biological characters of the species of *Allancastria*, including life stages and male/female genitalia, as well as artificial hybrids, e.g. between *A. cretica* and *A. cerisy* (Hürter, 2001).

The genus ***Zerynthia*** has two species: *Z. polyxena* (Denis & Schiffermüller) and *Z. rumina* (Linnaeus). *Z. polyxena* (type locality: Austria) has a wide distribution from southern, central and eastern Europe to southwestern Russia and Kazakhstan. There are 31 available subspecific names for *Z. polyxena*, with the highest diversity in Italy (Nardelli and Hirschfeld, 2002). The range of *Z. rumina* (type locality: S. Europe) extends from southern France to Spain, Portugal, Morocco, Algeria and Tunisia. The larvae of both species feed on *Aristolochia.* Previous work has demonstrated morphological differentiation between populations of *Z. rumina* within Africa and those in Europe (Tarrier *et al.,* 1994). There are at least 11 available subspecific names for the species, the majority of which refer to Spanish populations (Sabariego and Martinez, 1991), though two have their type localities in Africa (Binagot and Lartigue, 1998).

The genus ***Hypermnestra*** is monotypic, and its species, *Hypermnestra helios* (Nickerl), inhabits a narrow range of dry desert foothill habitats in Iran, Afghanistan, Pakistan, Turkmenistan, Tajikstan, Kazakhstan, Kirghizistan and Uzbekistan (type locality: Kazakhstan). The centre of origin of *Hypermnestra* has been suggested by Korb (1997) to lie "in the Turan arid zone which was located at E Tetis coast"; *H. helios* "strictly followed the distribution pathway [of its host plant] when spreading from the center of its origin". Korb (1997) also suggested that, subsequent to climatic changes in Central Asia in the Miocene, *H. helios* switched its food-plant as well as its flight pattern, while remaining on the plains. It is the only butterfly known to feed on *Zygophyllum* (Zygophyllaceae) at the larval stage.

We investigated the pattern of divergence within and between western Palaearctic species of Parnassiinae based on an 825 base-pair fragment of mitochondrial DNA (mtDNA), in order to find further evidence of their divergence and evaluate the rank of some of their currently used sub-specific names. In interpreting divergences, we particularly focused on geological events (within the past 10 MYA) that could have caused disjunctions and limited the opportunities for gene exchange between populations that are now on their way to complete divergence as separate species.

Materials and methods

Specimens

Dead, dried specimens of Parnassiinae from the western Palaearctic were procured by purchase, trade, or gift from as many localities as possible considering their availability, mostly as unrelaxed specimens (Table 1). In addition to the 27 specimens of Parnassiinae reported by Nazari *et al.* (2006), the present study used 43 new specimens. Most of the new material (36) was from the western genera *Hypermnestra, Archon, Zerynthia* and *Allancastria,* but 7 new specimens for the eastern genera (*Luehdorfia, Sericinus* and *Bhutanitis*) were also used to provide a better basis for comparison. In the case of the genus *Parnassius*, only representatives from major species groups (after Omoto *et al.* 2004) were selected, with the exception of *Parnassius hardwickii* (Gray) for which no specimens were available. Despite an exhaustive search, some critical populations of *H. helios, Z. polyxena,* and *A. apollinus* could not be sampled. DNA degradation also was a problem with some of the procured specimens.

The outgroup, *Baronia brevicornis* (Salvin), was selected as the closest sister taxon to Parnassiinae after Nazari *et al.* (2007). All voucher specimens and extracted DNA samples are deposited in the E. H. Strickland Entomological Museum, University of Alberta. Remains of available specimens of *A. apollinus, Z. rumina, Z. polyxena* and *H. helios* were re-examined for morphological character variability. Voucher information can be viewed at http://www.biology.ualberta.ca/old_site/uasm//Vouchers/index.html. Male genitalia of all available specimens were prepared and examined using previously described methods (Winter, 2000; Du *et al.* 2005). Wing pattern elements were also examined in all specimens, and previously

Table 1. Specimens examined, with their collection data and associated Genbank numbers.

	Species	subspecies	Locality	Specimen ID	GenBank
1	*Baronia brevicornis*		Mexico: Teacalco, btw Guerrero-Morelos, 07.1988	FS-a-167	AF170865
2	*Archon apollinus*	*apollinus*	Turkey: Bursa, Yaliciftilik, 11.03.2003	FS-b-2059	DQ383990
3	*Archon apollinus*	*apollinus*	Turkey: Mügla., Ölüdinez, 9.04.1999	FS-b-1868	DQ351031
4	*Archon apollinus*	*apollinus*	Greece: Samos, 2006	FS-b-2124	DQ875936
5	*Archon apollinus*	*bellargus*	Israel: Emaus, 2 km NE Latrun, 10.03.2000	FS-b-2024	DQ383989
6	*Archon apollinaris*	*apollinaris*	Iran: Kermanshah, Rijab, 9.04.1998	FS-b-2025	DQ351032
7	*Archon apollinaris*	*apollinaris*	Turkey: 33 km Mardin-Diyarbakir, 15.04.1987	FS-b-2060	DQ383991
8	*Archon bostanchii*		Iran: Lorestan, Poledokhtar, 10.04.2003	FS-b-2063	DQ351033
9	*Luehdorfia japonica*	*japonica*	Japan: Shimizu Pass, Muikamichi, Niigata, 2.05.2002	FS-b-2033	DQ383987
10	*Luehdorfia japonica*	*japonica*	Japan: Kanazawa, Ishikawa, 20.02.1991	FS-a-335	AF170867
11	*Luehdorfia puziloi*	*puziloi*	Russia: Primoriye, Vladivostok	EZ-2-11	DQ351035
12	*Luehdorfia puziloi*	*lingjiangensis*	China: Liaoning, Nanzamu, 26.04.2005	FS-b-2118	DQ383988
13	*Luehdorfia taibai*	*taibai*	China: Shaanxi, Qinling, 06.2002	FS-b-2102	DQ351034
14	*Hypermnestra helios*	*helios*	SE Kazakhstan: Ili Rive, Bakanas village, 1-15.05.1998	FS-b-1597	DQ351025
15	*Hypermnestra helios*	*helios*	SE Kazakhstan: Ili Rive, Bakanas village, 1-3.05.2002	FS-b-2071	DQ383986
16	*Hypermnestra helios*	*maxima*	Tajikistan: Kurgan-Tube, 20 km S Dzhilikul, 10.05.2000	FS-b-2070	DQ383985
17	*Hypermnestra helios*	*maxima*	Uzbekstan: Fergana Valley, Komsomolabad, 20.05.2002	FS-b-2069	DQ383984
18	*Hypermnestra helios*	*hyrcana*	Iran: Tehran, Karaj, Jaroo Mtn., 11.05.2002	FS-b-2067	DQ383982
19	*Hypermnestra helios*	*bushirica*	Iran: Hormozgan, 40 km N Bandarabbas, 16.03.1998	FS-b-2068	DQ383983
20	*Parnassius phoebus*		Canada: Alberta, Plateau Mtn., 08.1986	FS-a-8	AF170872
21	*Parnassius schultei*		China: Tibet, Trans-himalaya, Karola Pass, 22-28.06.1994	FS-b-1978	DQ351026
22	*Parnassius tenedius*		Kirghizstan: Altai Mts., Aktash village, 16.05.1997	FS-b-1784	DQ351027
23	*Parnassius delphius*		Kirghizstan: Tian-Shan, Naryntoo Mts., 1-10.07.1996	FS-b-1775	DQ351028
24	*Parnassius autocrator*		Tajikistan: E. Pamir, Muzkoi Mts., W Morgav village, 08.2000	FS-b-1983	DQ351029
25	*Parnassius simonius*		Kirghizstan: Transalai Mts., 1-20.07.1998	FS-b-1777	DQ351030
26	*Parnassius clodius*		USA: Washington, Okanagen Co., Chinook Pass, 7.03.1986	FS-a-375	AF170871
27	*Bhutanitis mansfieldi*	*mansfieldi*	China: Sichuan, E'mei Mtn., 07.2000	FS-b-1589	DQ351036
28	*Bhutanitis mansfieldi*	*mansfieldi*	China: Sichuan, E'mei Mtn., 07.2001	FS-b-2041	DQ383994
29	*Bhutanitis thaidiana*	*thaidiana*	China: Sichuan, Daba Mtn., 07.2000	FS-b-1591	DQ351037
30	*Bhutanitis thaidiana*	*thaidiana*	China: Sichuan, Daba Mtn., 06.2002	FS-b-2043	DQ383995
31	*Bhutanitis lidderdali*	*lidderdali*	China: Yunnan, Dongchuan County, 10.2002	FS-b-2044	DQ351038
32	*Sericinus montela*	ssp.	China; emg. 04.2006	FS-b-2123	DQ875937
33	*Sericinus montela*	*montela*	Japan: Tanashi, near Tokyo, emg. 4.04.1991	FS-a-399	AF170867
34	*Sericinus montela*	*koreanus*	Korea: Near Seoul	FS-b-2028	DQ383992
35	*Sericinus montela*	*amurensis*	Russia: Primoriye, 5-9.08.1998	FS-b-2090	DQ383993

	Species	subspecies	Locality	Specimen ID	GenBank
36	*Zerynthia polyxena*	*latevittata*	Italy: Sicily, Mt. Etna, nr. Raglana, 25.04.2006	FS-b-2122	DQ875940
37	*Zerynthia polyxena*	*cassandra*	Italy: E. Imola, Voltana, near Lugo di Romagna, emg. 04.2006	FS-b-2121	DQ875939
38	*Zerynthia polyxena*	*albanica*	Kosovo: Prizren, 8-9.05.2003	FS-b-2072	DQ384005
39	*Zerynthia polyxena*	*bosniensis*	Serbia: Petlovo Brdo, 27.05.2003	FS-b-2073	DQ384006
40	*Zerynthia polyxena*	*macedonica*	Greece: Florina, 9-10.05.2003	FS-b-2066	DQ384004
41	*Zerynthia polyxena*	*bryki*	Montenegro: Lovten, Crna Gora, 29.05.2003	FS-b-2045	DQ384003
42	*Zerynthia polyxena*	*petri*	Ukraine: Zaporozhje env., 25.05.2002	FS-b-2075	DQ384008
43	*Zerynthia polyxena*	*petri*	Russia: District of Stravropol, 12.04.2002	FS-b-2074	DQ384007
44	*Zerynthia polyxena*	*petri*	Russia: District of Voronezh, 1-5.05.1998	FS-b-1596	DQ351039
45	*Zerynthia rumina*	*africana*	Morocco: Atlas Mts., near Casablanca, 06.2002	FS-b-2040	DQ383996
46	*Zerynthia rumina*	*tarrieri*	Morocco: Anti Atlas, E. Askaou, Djebel Sirouna, emg. 04.2006	FS-b-2120	DQ875938
47	*Zerynthia rumina*	*castiliana*	Spain: Logroño, Islallana, La Rioja, 10.05.2003	FS-b-2053	DQ383997
48	*Zerynthia rumina*	*castiliana*	Spain: N. Burgos, Nidaguila, 20.05.2003	FS-b-2054	DQ383998
49	*Zerynthia rumina*	*castiliana*	Spain: N. Burgos, Temiño, 29.04.2003	FS-b-2055	DQ383999
50	*Zerynthia rumina*	*castiliana*	Spain: S. Burgos, Hortigüela, 6.04.1995	FS-b-2056	DQ384000
51	*Zerynthia rumina*	*cantabricae*	Spain: Cantabria, Aldea de Ebro, Pozazal, 15.05.2003	FS-b-2052	DQ384002
52	*Zerynthia rumina*	*rumina*	Spain: Malaga (1), emg. 5.11.1989	FS-a-88	AF170870
53	*Zerynthia rumina*	*rumina*	Spain: Malaga (2), Ronda Prov., Gaucin, 22.03.2002	FS-b-2057	DQ384001
54	*Allancastria louristana*	*louristana*	Iran: Lorestan, Malavi, 04.04.1999	FS-b-2037	DQ351040
55	*Allancastria cretica*	*cretica*	Greece: Kriti Island, Lassithi, 4.05.2003	FS-b-2038	DQ351041
56	*Allancastria caucasica*	*caucasica*	Turkey: Bolu, Bolu Daglari, 21.04.2001	FS-b-2046	DQ351042
57	*Allancastria cerisy*	*cerisy*	Turkey: Bursa, Davultlar, 8.04.2002	FS-b-2077	DQ384016
58	*Allancastria cerisy*	*speciosa*	Israel: Karen Hacarmel, Karmel Mt., 7.04.2000	FS-b-2034	DQ384014
59	*Allancastria cerisy*	*cypria*	Greece: Cyprus, Paphos Prov., Polis, 3-9.05.1999	FS-b-2076	DQ384015
60	*Allancastria cerisy*	*huberi*	Greece: Florina, 9-10.05.2003	FS-b-2078	DQ384017
61	*Allancastria cerisy*	*huberi*	Macedonia: Bitola, 12.05.2003	FS-b-2079	DQ384018
62	*Allancastria cerisy*	*ferdinandi*	Macedonia: Katlanovo, 5.05.2003	FS-b-2080	DQ384019
63	*Allancastria cerisy*	*ferdinandi*	Kosovo: Kachanik, 7.05.2003	FS-b-2081	DQ384020
64	*Allancastria cerisy*	*ferdinandi*	Macedonia/Bulgaria/Greece border: Belasica Mt., 15.05.2003	FS-b-2082	DQ384021
65	*Allancastria cerisy*	ssp.	Greece: Thessaloniki, 1990	FS-a-342	AF170869
66	*Allancastria deyrollei*	*deyrollei*	Turkey: Yozgat, vic. Yerkoy, 2003	FS-b-2088	DQ384012
67	*Allancastria deyrollei*	*deyrollei*	Turkey: Van, W Gevas, Kushunkiran Pass, 2003	FS-b-2087	DQ384011
68	*Allancastria deyrollei*	*eisneri*	Iran: Kermanshah, Rijab, 5.04.1999	FS-b-2036	DQ384009
69	*Allancastria deyrollei*	*eisneri*	Iran: W Azarbaijan, Takab, 23.05.2003	FS-b-2068	DQ351043
70	*Allancastria deyrollei*	*eisneri*	Iran: W Azarbaijan, E Marand Rd. Boukan, 2003	FS-b-2089	DQ384013
71	*Allancastria deyrollei*	*eisneri*	Israel: Canada Park, Latrun, 15.06.2003	FS-b-2083	DQ384010

published photographs (e.g. Bang-Haas, 1938; Wyatt, 1961; Hesselbarth *et al.*, 1995; Tschikolovets 1998, 2000, 2003) were checked in order to evaluate further variation.

Molecular techniques

Amplifications of 825 bp from the 3' end of the mitochondrial cytochrome oxidase subunit I (*COI*) were obtained for all taxa that had not been sequenced before, with the exception of one specimen (*A. cerisy huberi* from Greece, FS-b-2078) for which only the first 402 nucleotides could be amplified. *COI* was selected based on its demonstrated phylogenetic utility in previous studies on swallowtail butterflies (e.g. Caterino & Sperling, 1999; Caterino *et al.,* 2001, Zakharov *et al.,* 2004b; Matsumura *et al.,* 2005; Braby *et al.,* 2005; Silva-Brandao *et al.,* 2005). All new sequences have been deposited on GenBank (Accession numbers DQ383982-DQ384021 and DQ875936-DQ 875940).

We extracted total genomic DNA using the QIAGEN QIAamp DNA mini kit, and in all cases we used tissue from legs or the thorax of the specimens. Polymerase chain reactions (PCRs) were conducted on a T-personal PCR thermocycler (Biometra GmbH, Germany), using primers described previously (Table 2). For the most part we added *Taq* polymerase at the end of the initial 2-5 min denaturation at 95°C, which was then followed by 35 cycles of 94°C for 1 min, 45°C for 1 min, 72°C for 1 min, and a final extension period of 72°C for 10 min. PCR products were then evaluated on an agar gel and purified only when a single strong band was observed, using a QIAGEN QIAquick PCR purification kit. Sequencing reactions were then conducted using an Applied Biosystems Big Dye terminator cycle sequencing kit (ABI, Foster City, CA). All fragments were sequenced in both directions. We filtered the sequencing products through Sephadex-packed columns and dried them using a speed-vacuum. Final products were re-suspended and fractionated on an ABI Prism® 377 automated sequencer. The resulting chromatograms were evaluated in Sequencher® 4.1; sequences were aligned in ClustalX 1.81 (Thompson *et al.* 1997), and converted to nexus format in Se-Al 2.0 (Rambault, 2002). Alignments were then evaluated by eye.

Phylogenetic analyses

Phylogenetic analyses of the DNA data was conducted for the most part in PAUP* 4.0b10 (Swofford, 2002) under neighbor joining (NJ), maximum parsimony (MP) and maximum likelihood (ML) criteria. Heuristic searches were conducted for MP analysis with all characters equally weighted and under the TBR swapping algorithm with 100 random addition sequences. Bootstrapping of 100 replicates was conducted under the parsimony criterion with the default setting starting with a random seed and TBR branch swapping algorithm. The initial ML tree was generated using the parameters of the best-fit model (GTR+Γ+I) selected under Modeltest 3.0 (Posada and Crandell, 1998).

A second ML analysis was performed with topological constraints enforced to represent the

Table 2. Primers used in this study.

Location*	Name	Source	F/R	Sequence (5'-3')
1751	RonIII	Caterino & Sperling 1999	F	GGA GCA CCT GAC ATA GCT TTC CC
2183	Jerry	Simon *et al.*1994	F	CAA CAT TTA TTT TGA TTT TTT GG
2329	K525	Simon *et al.*1994	R	ACT GTA AAT ATA TGA TGA GCT CA
2329	K525.2	Caterino *et al.* 2001	R	ACA GTA AAT ATA TGA TGA GCT CA
2329	K525.4	Caterino *et al.* 2001	R	ACT GTG AAT ATG TGA TGG GCT CA
2495	BrianXXI	Caterino *et al.* 2001	F	CCT CAA TTT TAT GAA GAT TAG G
2658	Mila7	Caterino & Sperling 1999	R	GAA AGT CCA GTA AAT AAA GG
2837	George	Bogdanowicz *et al.* 1993	F	ATA CCT CGA CGT TAT TCA GA
3014	Pat	Simon *et al.*1994	R	TCC AAT GCA CTA ATC TGC CAT ATT A
3014	PatII	Sperling *et al.* 1996	R	TCC ATT ACA TAT AAT CTG CCA TAT TAG

* Positions relative to *Drosophila yakuba* mtDNA (Clary and Wolstenholme, 1985).

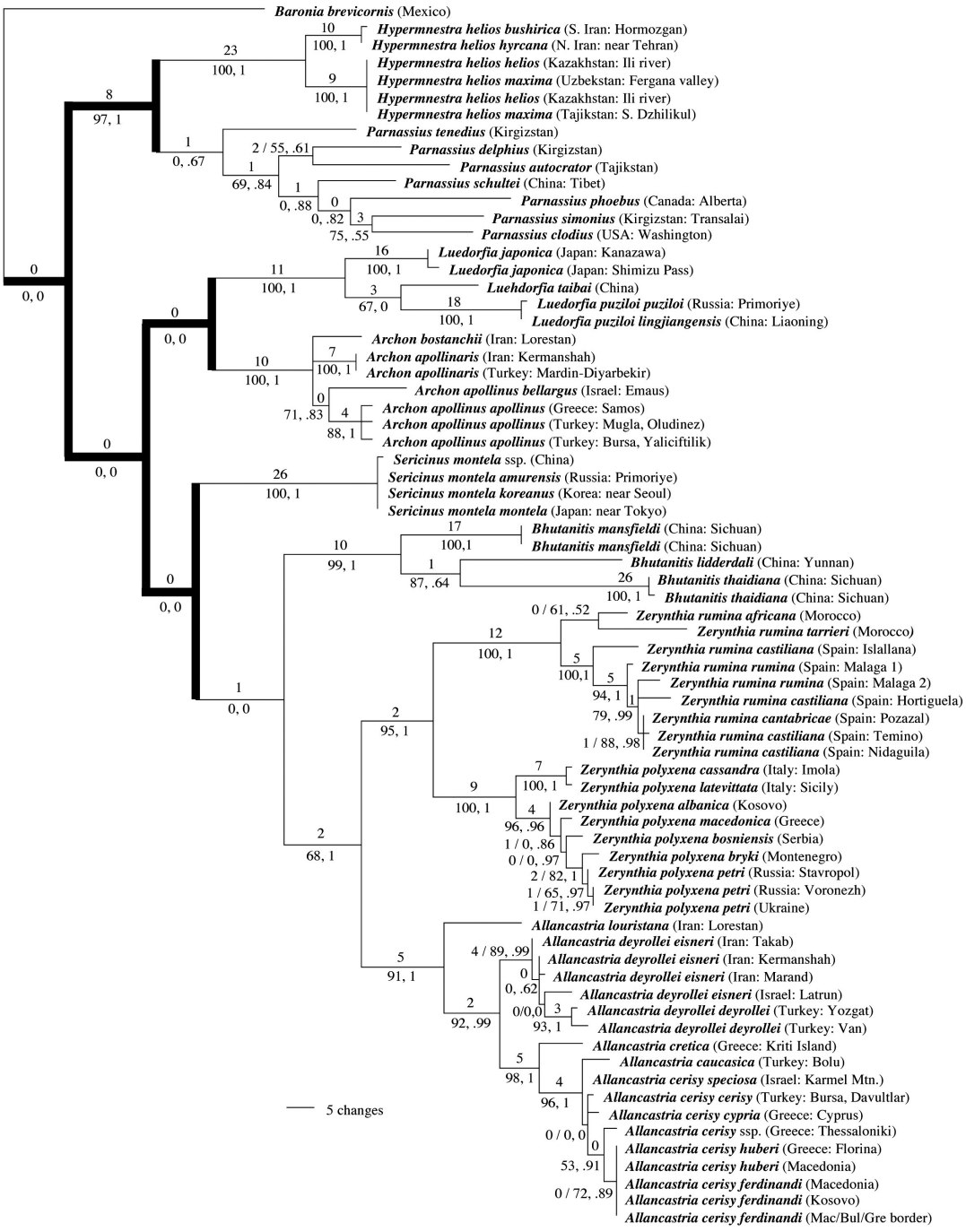

Fig.1. Maximum likelihood tree obtained with internal nodes constrained (shown as thick branches) to reflect the higher-level phylogeny determined by Nazari *et al.*, 2006 (TL= 932, CI= 0.428, RI= 0.838). Numbers above the branches indicate Bremer support and those below the branches represent Bootstrap values and Bayesian posterior probabilities.

tribal-level relationships proposed by Nazari *et al.* (2007), using a tree file created in MacClade 4.0 (Maddison and Maddison, 2000). In order to test whether there was a significant difference between the two topologies, we used the Shimodaira-Hasegawa test (Shimodaira and Hasegawa, 1999) in PAUP* with 1000 replicates and full optimization.

Bayesian analysis was conducted in MrBayes 3.04 (Huelsenbeck and Ronquist, 2001) under the GTR+Γ+I model and 4 (one cold and three heated) simultaneous Markov chains for 1,500,000 generations, starting with random initial trees and sampling every 100 generations. Substitution rates were freely estimated as part of the analysis from default priors. The burnin value was estimated prior to the initiation of the MCMC chain and the first 2000 trees were discarded. The majority rule consensus tree was generated using the remaining trees. Alignments as well as the resulting ML phylogeny were subsequently deposited on Treebase (www.treebase.org).

The pairwise distances were corrected in PAUP* with parameters from the best-fit model (GTR+Γ+I). Decay values for all trees were calculated using the program TreeRot (Sorenson, 1999). We also examined genetic divergences at various taxonomic levels by plotting the uncorrected *p* distances in a cumulative graph using SYSTAT 11.0.

Results

Despite numerous attempts, some specimens - including *H. helios* from Kopet Dagh mountains (Turkmenistan), *Z. rumina* from southern France, Portugal, and some Spanish populations, as well as *A. caucasica* from the Caucasus mountains - did not yield any usable DNA. Only those from which sequence was obtained are listed on Table 1.

The 825 base-pair fragment of mtDNA sequenced here was homologous to positions 2183 to 3014 at the 3' end of the *COI* gene (positions relative to *Drosophila yakuba* Burla mitochondrial DNA; Clary & Wolstenholme, 1985). No insertions or deletions were present in our dataset and, as expected for mitochondrial DNA (Harrison, 1989; Liu & Beckenbach, 1992; Simon *et al.,* 1994), the nucleotide base frequencies in our dataset were found to be significantly different (A=0.32, C=0.14, G=0.12, T=0.42; χ^2=51.7, P=1.000). Of the total of 825 base pairs, 534 were constant, 46 were parsimony uninformative, and 245 (29.7%) were parsimony informative, of which 10 were on first, 50 on second, and 185 on third codon positions.

The trees resulting from NJ, MP, ML and Bayesian analyses had somewhat different topologies, mainly in the deeper nodes. None of the trees reflected all the three tribes recognized for the subfamily proposed by Nazari *et al.* (2007). ML analysis produced a single tree, while MP analysis resulted in 1957 equally parsimonious trees of 925 steps. Only Parnassiini (*Parnassius* + *Hypermnestra*) was consistently recovered in all analyses, and only the NJ analysis reflected Zerynthiini (*Sericinus* + *Bhutanitis* + *Zerynthia* + *Allancastria*) as a monophyletic group. Luehdorfiini (sensu Nazari *et al.,* 2007) was never recovered as a clade, with *Luehdorfia* and *Archon* inconsistently appearing as sister to other Zerynthiini or Parnassiini genera. The position of *Sericinus* was also unstable through MP, ML and Bayesian analyses, often appearing as basal to other genera.

Since our study used only 825 base pairs in one gene, compared to the 5775 base pairs in seven genes used in the higher-level study of Nazari *et al.* (2006), we chose to constrain the tribal-level clades. We repeated the ML analysis with a constrained tree where three tribes (Parnassiini, Zerynthiini and Luehdorfiini) were fixed according to Nazari *et al.* (2006) but all the shallower nodes were allowed to vary. The Shimodaira-Hasegawa test showed that the constrained tree was not significantly longer ($P > 0.05$) than the unconstrained ML phylogeny (Δ –ln L = 9.5343, P=0.346). As the higher phylogeny was not of concern in the present study, and the lower nodes were not significantly different across these analyses, only the constrained maximum likelihood tree is shown (Fig. 1).

Large divergences (>2%) were noted between the mtDNA of populations of *Hypermnestra helios* from Iran and Central Asia, *A. apollinus* from Israel and Turkey, *Z. rumina* from North Africa and Spain, and *Z. polyxena* from Italy and other parts of its range, mostly supported by high decay values and Bayesian posterior probabilities. Average uncorrected pairwise (*p*) distances within and between species of Parnassiinae (Table 3), similarly showed a high degree of divergence (2.6%, 2.7%, 2.1-4.5% and 2.4%, respectively) for the above cases, larger than the distance between many established species of Parnassiinae, e.g. *Archon apollinus* and *A. apollinaris* (2.0%), or *A. cerisy* and *A. caucasica* (1.0%). To evaluate

Table 3. Average uncorrected pairwise distances between species in Parnassiinae based on 825 bp of *COI*. Bold values in boxes are cases of relatively low genetic diversity between recognized pairs of species, and those highlighted are the ones noted through this study as being high for variation within species (*H. helios, A. apollinus, Z. rumina*). N indicates number of specimens examined in this study, and numbers on the diagonal represent mean genetic variation among speci-

		N	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
Parnassiini	1 *P. phoebus*	1	-																														
	2 *P. schultei*	1	5.8	-																													
	3 *P. tenedius*	1	7.8	6.3	-																												
	4 *P. delphius*	1	5.8	4.7	5.7	-																											
	5 *P. autocrator*	1	6.8	5.1	7.0	5.0	-																										
	6 *P. simonius*	1	6.1	4.6	6.5	5.5	5.8	-																									
	7 *P. clodius*	1	5.9	4.6	7.3	5.8	6.7	4.6	-																								
	8 *H. helios* C Asia	4	9.6	8.6	8.6	9.0	9.1	9.5	8.7	0.0																							
	9 *H. helios* Iran	2	8.6	8.9	8.8	8.9	9.2	9.8	8.7	2.6	0.1																						
Luehdorfiini	10 *L. japonica*	2	9.9	8.6	10.2	9.7	9.8	9.9	8.9	11.8	11.5	0.2																					
	11 *L. puziloi*	2	9.8	8.6	9.5	9.2	10.1	10.5	8.9	11.2	11.6	5.5	0.1																				
	12 *L. taibai*	1	9.2	7.4	9.1	8.6	8.7	8.8	7.6	10.8	11.2	4.9	4.4	-																			
	13 *A. apollinus* Israel	2	10.4	9.7	10.7	9.7	11.2	10.8	10.8	9.9	10.4	8.4	9.3	9.0	-																		
	14 *A. apollinus* [others]	1	10.2	9.5	10.6	9.9	10.5	10.8	10.5	10.5	10.6	7.8	8.8	8.3	2.7	0.5																	
	15 *A. ap. apollinaris*	2	10.1	9.5	10.1	9.3	10.8	10.7	10.4	10.3	10.3	7.8	8.2	8.1	3.0	**2.0**	0.0																
	16 *A. bostanchii*	1	10.4	9.2	9.8	9.7	10.6	10.3	10.1	10.4	10.4	7.9	8.9	8.5	3.2	2.3	2.1	-															
Zerynthiini	17 *S. montela*	3	9.4	9.1	9.9	9.7	10.7	10.1	9.4	10.5	11.0	9.7	9.9	9.5	9.1	9.2	8.0	9.1	0.1														
	18 *B. mansfieldi*	2	12.1	10.8	11.0	11.5	11.9	11.8	10.8	11.9	12.2	10.7	11.1	10.8	9.3	8.9	8.7	8.2	10.5	0.0													
	19 *B. thaidiana*	2	11.6	11.3	12.4	11.5	12.8	12.1	11.3	11.9	12.4	11.5	11.2	11.0	10.4	9.4	9.2	10.0	10.0	7.5	0.1												
	20 *B. lidderdalii*	1	12.7	11.8	11.5	11.0	12.1	12.1	12.0	12.7	12.7	12.0	12.3	11.4	10.2	10.0	9.3	9.2	10.6	7.3	7.7	-											
	21 *Z. rumina africana*	1	11.7	11.0	10.9	11.5	12.0	11.7	11.0	12.4	12.7	11.1	10.9	10.0	10.4	9.8	9.8	9.7	10.5	10.2	10.7	10.9	-										
	22 *Z. rumina tarrieri*	1	12.1	11.2	11.9	11.9	12.4	11.8	11.9	13.0	13.4	11.5	11.2	10.7	11.2	10.7	10.3	10.4	10.9	10.8	11.3	11.5	2.5	-									
	23 *Z. rumina* Islallana	1	12.6	11.4	11.2	12.0	11.9	11.9	11.6	12.4	12.7	11.8	12.2	11.0	10.6	9.9	9.9	9.6	10.7	10.6	11.2	10.6	3.2	4.5	-								
	24 *Z. rumina* [others]	6	12.8	11.7	11.8	12.4	12.3	12.3	11.6	12.5	12.8	11.9	12.0	11.2	11.1	10.5	10.4	10.2	10.9	10.7	11.1	11.1	3.3	4.3	2.1	0.6							
	25 *Z. polyxena* Italy	2	10.8	10.1	10.9	10.7	11.3	11.2	10.6	12.4	13.0	10.7	11.0	9.5	9.8	8.9	8.8	8.1	9.4	9.1	10.5	9.6	6.3	6.6	6.9	6.8	0.2						
	26 *Z. polyxena* [others]	7	10.9	10.6	11.4	11.2	11.3	11.9	11.4	12.3	12.9	10.7	10.9	9.9	10.0	9.1	8.8	8.4	9.1	9.4	10.7	10.3	6.1	6.4	6.8	6.4	2.4	0.7					
	27 *A. louristana*	1	10.5	10.2	11.3	11.0	11.6	11.2	10.5	10.8	11.5	10.4	10.7	9.9	8.8	8.2	8.0	8.4	8.8	9.9	10.7	10.4	7.9	8.5	8.1	8.8	6.7	7.0	-				
	28 *A. devrollei*	6	10.8	10.4	10.9	11.0	11.1	11.3	10.7	11.6	11.7	10.6	10.7	9.9	9.2	8.4	8.3	8.7	8.7	9.6	11.1	10.3	8.3	9.1	8.9	8.8	7.6	7.7	3.9	0.8			
	29 *A. cretica*	1	10.9	10.4	11.2	10.9	11.6	11.4	11.0	11.6	11.8	10.7	11.0	10.2	9.2	9.0	8.4	9.0	8.8	9.5	10.5	10.1	8.6	8.8	9.2	9.6	7.8	7.8	4.5	3.0	-		
	30 *A. caucasica*	1	10.9	10.9	10.9	11.6	12.1	11.9	11.5	11.4	11.8	11.3	11.0	10.4	9.6	9.4	8.7	9.3	8.8	10.3	11.2	10.6	9.2	9.3	9.7	9.8	8.0	8.2	4.6	3.4	2.3	-	
	31 *A. cerisy*	9	11.1	10.7	11.1	11.5	11.9	11.4	10.9	11.3	11.6	11.0	10.9	10.3	9.4	9.4	8.8	9.2	9.1	10.1	10.9	10.0	9.2	9.3	9.3	9.5	7.7	8.0	4.3	3.3	2.3	**1.0**	0.5

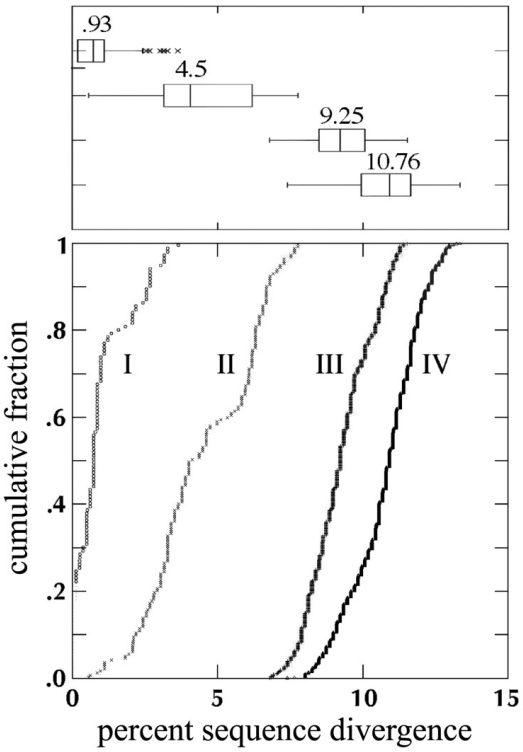

Fig. 2. Cumulative pairwise uncorrected *COI* divergences within species (I), between species within genera (II), between genera within tribes (III) and between tribes (IV) of Parnassiinae examined in this study. Box plots above the graph summarize the variation. The central vertical line in each box 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 manual for details). The outliers in the first category are those discussed in this paper as being potentially distinct species, since they fall within the range of genetic differentiation commonly found between established species.

whether species-level recognition was justified, morphological characters were investigated in these four cases.

To examine species-level genetic variation within the subfamily, we plotted uncorrected *p* distances within and between established species, between genera, and between tribes against the cumulative fraction of the values in a quadratic graph (Fig. 2). Overlap in values between categories was observed in every case. The few outliers in the "within species" distance category, corresponding to the species listed above (*H. helios, A. apollinus, Z. rumina*, and *Z. polyxena*), mostly overlapped with the central 50% of the "between species" distance category.

Further examination of morphological traits in specimens from the unusually divergent populations of the above four species showed some differences in wing-pattern as well as in internal structures, but this variation was not comparable to the amount of divergence normally observed between different species in the subfamily. Most of the variable traits noted were those utilized by original authors in their descriptions of the subspecies in question. Most prominently, we observed that the tip of the aedeagus of *H. helios* had fewer teeth (1 or 2 per side) in Iranian populations compared to those from Central Asia (a series of 3 or 4 per side) (Fig. 3).

Discussion

Species definitions

The concept of what constitutes a species continues to be debated among systematists (Bock, 2004; Coyne and Orr, 2004; Hebert *et al.,* 2004; Queiroz, 2005). Many species definitions incorporate an implicitly genetic component (Cracraft, 1989; Mallet, 1995; Sperling, 2003). A 2% sequence divergence in mtDNA is sometimes used as the benchmark for delimiting species, with the argument that intra-specific divergences are rarely greater than 2% (Avise, 1994; Hebert *et al.,* 2003). However, due to heterogeneity in divergence rates, the potential for introgression and retained polymorphism between recently diverged species, the strict use of percent sequence divergence in drawing boundaries between species is widely recommended against (Sperling, 2003; Funk and Omland, 2003; Meyer and Paulay, 2005; Rubinoff and Holland, 2005; Cognato, 2006). It has been shown that swallowtail species that are distinct by most conventional species definitions may show no significant divergence in mtDNA sequences or other molecular characters (Hagen and Scriber, 1991; Sperling 1993, 2003). mtDNA divergence between some *Colias* species is also less than 1% (DeChaine and Martin, 2005). On the other hand, a 3.8% differentiation in *COI+COII* between the Australian subspecies of *Papilio demoleus* (Linnaeus) and others from Southeast Asia has been reported, without making any taxonomic decisions (Zakharov *et al.,* 2004b).

Such evidence against the utility of strict

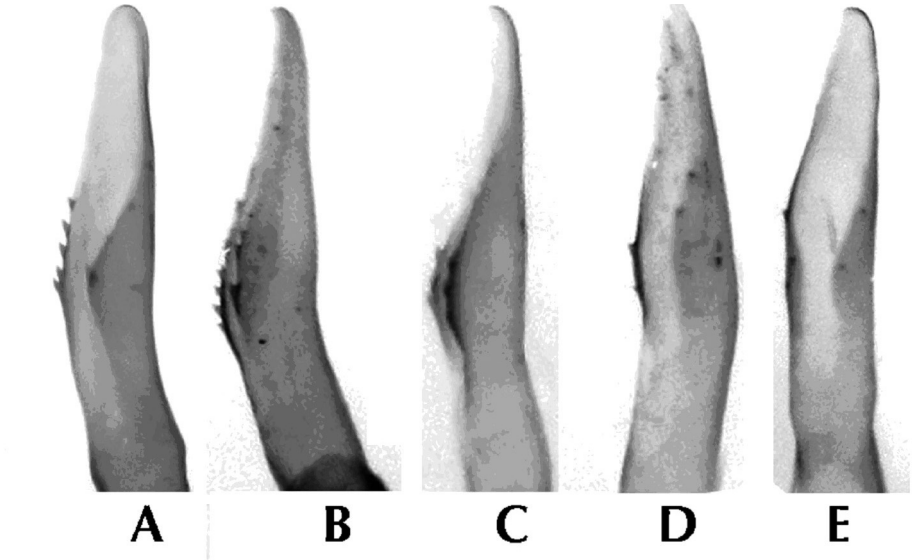

Fig. 3. Tip of aedeagus in specimens from various populations of *Hypermnestra helios.* A) Uzbekistan, B) Tajikistan, C) Turkmenistan, D) N. Iran, E) S. Iran.

mtDNA cutoffs in species delimitations does not support the consistent application of a subspecies concept, which in addition to the criterion of geographic separation implies genetic divergence (Wilson & Brown, 1953). Subspecies have often been considered arbitrary (Ehrlich, 1961) and are unpopular in many groups of insects (e.g. see Colless, 1976; Carpenter, 1987; Crosskey & Howard, 2006). However, subspecies have a rich history in Lepidoptera, and the concept has been put on a somewhat consistent footing since Rothschild and Jordan (1906). In well known groups like butterflies, subspecies provide a useful preliminary sorting convention that allows the recognition of geographic variants that may later be recognized as species (Sperling, 1987, 2003).

On a molecular level, unusually diverged mitochondrial sequences between subspecies can provide clues to potential speciation events that should be investigated further through other molecular characters, morphology and life history. Such divergences should not be used as the sole criterion in delimiting species boundaries or, for that matter, any taxonomic category. Other recent studies provide evidence of substantial overlap in DNA sequence divergence limits between higher taxonomic categories (Zakharov *et al.*, 2004a, Nazari *et al.*, 2007).

From a phylogenetic perspective, monophyly is now generally considered the single most important criterion for constituting any taxonomic category (Hennig, 1966; Mayr *et al.,* 1953; Mayr, 1999; but see Davis & Nixon, 1992; Brower, 1999). If taxa are found to be paraphyletic, they are often split into smaller categories (Mayr, 1999). In our study, all traditionally recognized genera and species were monophyletic based on sequence from 825 bp of the *COI* gene, although our results do not recover the three tribes of Parnassiinae as proposed by Nazari *et al.* (2007). The establishment of these tribes was based on a much larger dataset both in terms of genes and outgroup selection, and therefore it is not surprising that our inferred phylogeny based on a relatively short mitochondrial fragment does not reflect the higher classification of the subfamily. Furthermore, mitochondrial protein coding genes have faster divergence rates than nuclear genes that are generally used in higher phylogenetic analyses and also have more pronounced sequence saturation at higher taxonomic levels (Simon *et al.,* 1994; Caterino *et al.,* 2000; Nazari *et al.,* 2007). This is supported by the fact that the *COI* uncorrected pairwise distances between the three tribes were observed to be very close to, and largely overlapping with, those between genera (Fig. 2).

Archon

Although extensive sampling across all subspecies of *A. apollinus* was not possible, there were several obvious cases of high divergences in mtDNA among the subspecies that were included. First, we observed a large gap (2.7%) between the Israeli (ssp. *bellargus* Staudinger) and the Turkish (ssp. *apollinus* Herbst) populations of *Archon apollinus*, which exceeded the average divergence between *A. apollinus* and *A. apollinaris* (2.0%). The taxon *bellargus* (type: Turkey, Hatay), together with many other names, has previously been synonymized with the nominal *apollinus* based on similarity of genitalia (De Freina, 1985). In light of present molecular evidence, we suggest that further work is needed to support this synonymy. Examination of specimens across other subspecies of the *Archon apollinus* complex may reveal further instances of high divergence within this species.

Branch lengths for mtDNA within the *Archon* clade (Fig. 1) provide further evidence that the taxon *Archon bostanchii* (De Freina & Naderi, 2003) from Iran, originally described as a subspecies of *Archon apollinus*, is as diverged as *A. apollinus* or *A. apollinaris,* and is likely a separate species as suggested by Carbonell and Michel (2007). Although our MP and ML reconstructions fail to resolve the position of this subspecies relative to *A. apollinus* and *A. apollinaris*, *A. bostanchii* appears as sister to all other *Archon* in our NJ and Bayesian reconstructions, albeit with weak support. This indicates a possible Iranian origin for *Archon,* with further dispersal to the west. Divergence of *Archon* from the most recent common ancestor of Luehdorfiini has previously been estimated to have taken place about 30 MYA (Nazari *et al.*, 2007).

Allancastria

The topology of the *Allancastria* clade in our mtDNA phylogeny is congruent with the one inferred by Nazari *et al.* (2007), with *A. louristana* as basal species, and *A. deyrollei, A. cretica, A. caucasica* and *A. cerisy* branching off consecutively. We observed limited divergence within two of the species for which we had multiple samples (*A. deyrollei* = 0.8%, *A. cerisy* = 0.5%). For *A. deyrollei,* the Turkish populations (ssp. *deyrollei*) form a well-supported clade, and specimens from western Iran and Israel (ssp. *eisneri*) also group together but with weak support. Based on the ML phylogeny (Fig. 1), the most parsimonious hypothesis for dispersal of *A. deyrollei* is that the ancestral stock dispersed from Iran to Turkey and Israel.

The short branch length and consistently close alliance of *A. caucasica* with *A. cerisy* in all of our phylogenies are evidence against separate specific status for *A. caucasica,* since mtDNA of this species seems to be part of the larger variation within *A. cerisy.* A recent molecular study (Nazari *et al.*, 2006) using multiple nuclear and mitochondrial genes (including *COI*) also found strong support for alliance between the two species. However, previous work on morphology and genitalia (Kuhna, 1977), as well as the biology of *A. caucasica* (Carbonell, 1996b) have demonstrated differentiation of *A. caucasica* from *A. cerisy. A. caucasica* flies together with *A. cerisy* in many localities in Turkey (Hesselbarth *et al.,* 1995). The limited divergence observed between *A. caucasica* and *A. cerisy* could be an artifact of the population sampling of our study, and so we suggest that further work is needed to evaluate the separate specific status of *A. caucasica.* The ancestral distribution of the *A. cerisy* clade cannot be unambiguously determined based on current data, as it is equally likely that the ancestral stock of *A. cerisy* either dispersed northwards from Israel to Turkey and Europe, or from Europe to the south. Our mtDNA data also show no divergence between the Greek and Macedonian populations of *A. cerisy* assigned to ssp. *huberi* (Sala & Bollino), compared to those assigned to ssp. *ferdinandi* (Stichel), and therefore do not support a separate status for these two taxa.

In order to provide a biogeographic hypothesis for the distribution of *Allancastria*, we compared the results of a previous divergence/vicariance and molecular clock analysis (Nazari *et al.*, 2007) with the geological and tectonic history of the Mediterranean basin (after Steininger and Rögl, 1996). The most parsimonious hypothesis for dispersal and vicariance for this genus would be that the ancestral *Allancastria* probably originated in the Iran-Anatolian plate in the early Miocene (21-19 MYA), and dispersed into the Afro-Arabian region upon extension of the Fars Formation across the Mesopotamian Trough (~ 17 MYA). The separation of the Greece-Turkey-Yugoslavia landmass from Eurasia, and removal of the Fars Formation (16-15 MYA) would have subsequently isolated the three populations and gradually given rise to three ancestral species: *A. louristana* (Iran), *A.*

deyrollei (Anatolia), and *A. cerisy* (Afro-Arabia). During the middle Miocene (15-14 MYA), and upon formation of land bridges between the Middle East and Eurasia, ancestral *A. cerisy* would have dispersed into Turkey and Greece. The Island of Crete [Kriti] later disconnected from the mainland around 11 MYA, giving rise to *A. cretica.* The populations of *A. caucasica* were isolated only in the Pliocene (3.5-3 MYA) upon flooding of the Mediterranean Sea, which created water connections between the Mediterranean, Black and Caspian seas, leaving the Caucasus Mountains in the middle as islands.

Zerynthia

Based on a previous molecular clock analysis (Nazari *et al.*, 2006), the two species of *Zerynthia* would have originated from a common ancestor around 14.5-18.5 MYA after a vicariance event (formation of the Mediterranean Basin) widely separated the ancestral range. The northern populations would have evolved into *Z. polyxena,* and the southern ones given rise to *Z. rumina.*

For *Z. polyxena,* we were able to sample only a limited number of populations from Italy, Greece and Eastern Europe as well as Russia and the Ukraine. We observed a well-supported gap between the Italian populations compared to those from the other regions (2.4%), much higher than the divergence between all other populations of this species (0.7%). The phylogeny indicates that the Italian and all other east European populations shared a common ancestor, but the eastern populations split relatively early from the Italian populations. The eastern populations may then have dispersed further to the north and east, since the specimens from Ukraine and SW Russia form a clade in a crown node.

Our phylogenetic reconstructions provide no meaningful distinction among the Spanish subspecies of *Z. rumina*, and the mtDNA of specimens assigned to various populations of *Z. r. castiliana* (Rühl) seems to be paraphyletic with respect to other subspecies. The mitochondrial haplotype of the specimen from Islallana seems to be sister to all other Spanish populations and shows a notable divergence (2.1%) compared to the rest (0.6%). The status of other European subspecies, including *lusitanica* (Bryk) (Portugal) and *australis* (Esper) (southern France) remains unknown.

The two African lineages of *Zerynthia rumina* studied here (ssp. *africana* Stichel and ssp. *tarrieri* Binagot and Lartigue) were sister to all Spanish populations with a considerable degree of divergence (3.2-4.5%). They were also highly diverged from one another (2.5%). These values are comparable to the divergence between most closely related species within the Parnassiinae (Table 3 and Fig. 2). The large gap and the basal position of the two African populations of *Z. rumina* suggest early dispersal of the ancestral stock of this species between Africa and Europe. A similar pattern of dispersal has also been noted in nymphalid butterflies of the genus *Pararge* (Weingarther *et al.,* 2006). Paleogeographical reconstructions show that the last known contact between Iberia and Africa occurred at the end of Miocene, with the formation of the Gibraltar arc which completely disconnected the Mediterranean sea from the Atlantic Ocean, causing extensive evaporation of the Mediterranean during the Messinian age (7-5.3 MYA) (also known as "the Messinian salinity crisis", Sanmartin, 2003). This event temporarily closed the water corridors between Africa and Iberian Peninsula, and permitted biotic exchange between the two continents (Krijgsman, 2002). The barrier was restored when Gibraltar re-opened by the beginning of the Pliocene (5 MYA). This short period of connection between the two continents probably accounts for vicariance between the North African and the Iberian lineages of *Z. rumina*. This event has been suggested as a plausible explanation for vicariance between African/ Iberian lineages of fishes in the Cyprinidae (Doadrio *et al.,* 1998) and beetles in the Pachydeminae (Sanmartin, 2003).

Binagot and Lartigue (1998) present a scenario for how the two African subspecies of *Z. rumina* differentiated from the same ancestral stock after dispersal and subsequent isolation of the two populations on the two sides of the Moroccan Atlas Mountains. They estimate that the event occurred between 25 to 22 thousand years ago. This scenario can be tested using molecular clock estimates derived from Parnassiinae and other insects (Nazari *et al.,* 2007). The pairwise sequence divergence of mtDNA in *Heliconius* butterflies and other insects has previously been estimated to be about 2.3% per million years (Brower, 1994). Based on an independently derived divergence date of 14.5-18.5 MYA estimated for the last common ancestor of *Z. rumina* and *Z. polyxena* (Nazari *et al.,* 2007), and the average sequence

divergence of 6 to 6.5% between the two species observed in the present study, we calculated the rate of mtDNA sequence divergence in *Zerynthia* to be between 2.3-3.1% per million years. This estimate is remarkably consistent with the general rate suggested by Brower (1994). Considering the observed 2.5% divergence between the African subspecies of *Z. rumina*, we conclude that the two subspecies have diverged from a common ancestor around 1.2 to 0.9 MYA, considerably earlier than the dates suggested by Binagot and Lartigue (1998).

Nonetheless, morphology does not support a species-level distinction between the two African populations, or for that matter, between African and Spanish populations. Slight differences in wing markings noted previously in describing subspecies, and almost uniform genitalia across the entire range of *Z. rumina*, are not strong arguments for supporting species-level recognition. Further studies on the biology and immature stages, as well as molecular work using different gene regions, might ultimately provide satisfactory evidence for the elevation of these populations of *Z. rumina* as separate species.

Hypermnestra

The range of *H. helios* can be roughly divided into two regions: a) the Iranian plateau, an area delimited by the Zagros mountains in the west, Kopet-Dagh and the Lesser Caucasus mountains in the north, and the Pamir mountains in the east; and b) Central Asia, also known as the Turanian or Transcaspian region, extending from Turkmenistan to Kazakhstan, which in this case includes the remaining range of *H. helios*. The Central Asian populations of this species studied here (*H. h. helios* Nickerl and *H. h. maxima* Grum-Grshimailo) demonstrate no mtDNA difference among specimens (0.0%), which supports their synonymy as suggested previously (Tschikolovets, 1998). The Iranian populations, however, are clearly distinct from Central Asian ones (2.6%) and show some variation as well (0.1%).

Comparison of morphological characters between the two populations by the first author has shown that wing pattern elements are somewhat variable and not reliable for taxonomic work. The reduced number of teeth on the tip of the aedeagus in Iranian populations compared to those from Central Asia is suggestive of more substantive differences. However, this may still turn out to be a variable trait considering the limited number of specimens examined here (Table 1). The Pakistani populations of *H. helios* hold the oldest available name for the Iranian Plateau group (*H. h. balucha* Moore) which should be used as the species name if this population is found to be part of the Iranian group. However, if these - and other populations from Afghanistan - are discovered to be part of the Central Asian group, the valid name to use for the Iranian group would be *H. h. bushirica* Bang-Haas, with *H. h. hyrcana* Sheljuzhko as the subspecies from Northern Iran.

The separation of an ancestral *Hypermnestra* lineage from the last common ancestor of Parnassiini has been estimated to have taken place around the same time that India collided into Eurasia (65-42 MYA) (Nazari *et al.*, 2007), which resulted in confinement of the ancestral *Hypermnestra* in the lowlands of Asia. Given the average divergence of the *COI* gene between *Parnassius* species studied here and *Hypermnestra* (9%), and given the average age of about 50 MY estimated for that event (sensu Nazari *et al.*, 2007), the divergence of the two lineages within *Hypermnestra* (2.6%) can be roughly estimated to have taken place at least 12 MY ago. Formation of the Iranian plateau, which today stands as the main barrier between the two lineages, is known to have begun around 10 MYA after the collision of the Arabian plate into Eurasia, resulting in the uplift of the Zagros mountains in the Miocene, and subsequently the Lesser Caucasus and Kopet-Dagh mountains in the early Pliocene (5 MYA) (Sanmartin, 2003). Our results suggest that the two lineages of *H. helios* separated during or right before the formation of the Iranian plateau. This event has previously been suggested as the best explanation for evolutionary divergences observed between Iranian and Central Asian populations of agamid lizards (Macey *et al.* 1998) and beetles in the Pachydeminae (Sanmartin, 2003).

Conclusion

Our results show large gaps in mitochondrial *COI* DNA sequence divergence among populations within *Archon apollinus* (2.7%), *H. helios* (2.6%), *Z. rumina* (2.1-4.5 %), and *Z. polyxena* (2.4%), but more limited divergences between some previously established species, i.e. *Archon apollinus* and *A. apollinaris* (1.9%), and *Allancastria cerisy* and *A. caucasica* (1.0%). Our attempt to find additional

morphological evidence to independently confirm the large divergences within species was not successful. Although we believe that these high divergences present good indications of potential speciation events, we refrain from making any taxonomic conclusions before more comprehensive morphological investigations are conducted, as well as examination of further molecular characters in more populations. We also observed several cases of substantial overlap in the range of uncorrected pairwise distances in the mitochondrial *COI* gene between higher taxonomic categories in Parnassiinae butterflies.

We suggest that a revision of the genus *Archon,* based on further biological and molecular research, is needed to evaluate the synonymies proposed for infra-specific names within *Archon apollinus*, since the present molecular data support recognition of a significant distinction between the Israeli and Turkish subspecies while these have been previously proposed as synonyms (De Freina, 1985). No decision can be made on the taxonomic status of the diverged populations within *H. helios*, *Z. rumina* and *Z. polyxena* without a thorough examination of specimens from a broader range, including populations of *H. helios* from Afghanistan and Pakistan, and *Z. rumina* from other localities in northern Africa as well as Europe.

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