Morphology, molecules and fritillaries: approaching a stable phylogeny for Argynnini (Lepidoptera: Nymphalidae)

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We examine the phylogenetic relationships among 29 species of Argynnini based on 141 previously published morphological characters and new data from the mitochondrial gene COI and the two nuclear genes EF-1a and *wingless*. We investigate the stability and robustness of the resulting phylogenetic hypotheses through various combinations of the 4 functionally separate datasets. Increasing the number of datasets in combined analyses led to increased support for clades, sometimes substantially. We find that the tribe Argynnini is a well-supported, robust, monophyletic clade with the following internal subtribal structure: (Euptoietina (Yrameina (Boloriina Argynnina))). Our analyses support the classification of argynnine species into six robust and stable genera: *Euptoieta, Yramea, Boloria, Issoria, Brenthis* and *Argynnis*. We suggest that for moderate amounts of data, a total evidence approach is always best.

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Introduction

Researchers working on the evolutionary history of a group of species are interested in generating robust phylogenetic hypotheses for their taxon. A robust phylogenetic hypothesis can be seen as one that does not change when new characters are added to the data matrix. When inferring phylogenetic hypotheses, the combined analysis of data from various sources is commonly considered to lead to the most robust hypothesis (Kluge 1989). Since the molecular revolution in systematics, large amounts of new data have been generated by finding and sequencing new gene regions, although positive effects of combining morphological data with molecular data have been reported (Miller *et al.* 1997, Baker & Gatesy 2002, Wahlberg & Nylin 2003, Wahlberg *et al*. 2005). Combining different kinds of data into a single analysis is still debated, but it is now understood that separate analysis of biologically delimited data sets is a way of investigating the strength of the phylogenetic signal apparent in the total evidence analysis (DeSalle & Brower 1997; Gatesy *et al.* 1999).

Here we investigate the relative contributions of morphological characters and DNA sequences from three gene regions to the pattern of phylogenetic relationships among butterflies in the tribe Argynnini of the subfamily Heliconiinae. We are specifically interested in the effects of adding new molecular data to an already published morphological dataset (Simonsen 2006a). Do we really need to add data from 20 gene regions, as suggested by Rokas *et al*. (2003), to arrive at a robust phylogenetic hypothesis for this tribe of butterflies?

Phylogenetic assessments of relationships among various clades in the nymphalid subfamily Heliconiinae have been increasing over the last few years. The work of Penz & Peggie (2003) established a clear hypothesis for the higher level diversification and relationships of the major lineages in the subfamily, although problems still exist. Based on a large morphological data set, Penz & Peggie (2003) divided Heliconiinae into four tribes: Acraeini, Heliconiini, Vagrantini and Argynnini. Although these clades did not receive strong bootstrap support, they do conform to the intuitive groupings used by many authors prior to that study.

Most recent studies of relationships of taxa in Heliconiinae have concentrated on the tribe Heliconiini (Brower 1994; Brower 1997; Brower & Egan 1997; Penz 1999), but lately some attention has been given to the mainly Holarctic tribe Argynnini. Since the precladistic works of Warren (1944, 1955), Dos Passos & Grey (1945) and Warren *et al*. (1946) and the early cladistic or systematic works of Shirôzu & Saigusa (1973, 1975) and Higgins (1975), only Aubert *et al.* (1996) have dealt with the group in a modern cladistic way, using an outgroup and computer analyses. However, two morphology based phylogenetic studies of the tribe Argynnini and its subtribes have recently been concluded (Simonsen 2005, 2006a), and both morphology and DNA-based phylogenetic studies of the Nearctic subgenus *Speyeria* are in preparation (J. Dunford, *pers. comm.*). The only molecule based phylogenetic study of the group so far (Aubert *et al.* 1996) was limited to western Palaearctic taxa, and the results should be considered preliminary (H. Descimon, *pers. comm.*). No attempt to combine morphological and molecular data has hitherto been published for the tribe Argynnini.

The Argynnini comprise 100+ species, historically placed in up to 19 genera, although currently placed in 6 genera (Simonsen 2006a). Almost all species are found in temperate, arctic and/or alpine areas, mainly in the Palaearctic and Nearctic biomes. A few species are found in the high Andes of South America, or the mountains of East Africa, and a single species (*Argynnis hyperbius*) occurs widely from Japan to Australia to Eastern Africa. There are two previously published studies with taxon sampling relevant to our study, both based on morphological data. Penz & Peggie (2003) sampled 18 species of Argynnini which they classified in 12 genera, and found that the enigmatic genus *Euptoieta* was nested within the tribe Argynnini. Simonsen (2006a) found *Euptoieta* to be the sister-group to the rest of the Argynnini, and that there were additionally two distinct clades, which were termed the subtribes Yramiina and Argynnina.

In this contribution we present a phylogenetic hypothesis of the Argynnini at the species group level, based on the adult genitalia and wing morphology from Simonsen (2006a) as well as the mitochondrial gene *cytochrome oxidase subunit I* (COI) and the nuclear genes *elongation factor-1α* (EF-1α) and *wingless*.

Material and methods

Taxon sampling

29 ingroup taxa representing all major groups within the tribe (17 "genera") and 7 heliconiine outgroup taxa used by Simonsen (2006a) are included in the analysis of the morphological dataset. A complete list of the species including specimen data is given in Table 1. The morphological character matrix and character list can be found in Simonsen (2006a).

Preparation and terminology

Morphological characters. – Preparation techniques for morphological characters are found in Simonsen (2006a).

Taxonomy. – We initially adopt the argynnine classification proposed by Simonsen (2006a) but propose some changes based upon the current results.

Molecular characters. – We extracted DNA either from one or two legs or from the thorax musculature of freshly frozen, dried or alcohol conserved butterflies using QIAgen's DNEasy extraction kit.

For each of the 36 species we sequenced COI, EF-1α and *wingless*. Primers for COI were taken from Wahlberg & Zimmermann (2000), for EF-1α from Peña *et al*. (2006), and for *wingless* from Brower and DeSalle (1998). We performed all PCRs in a 20-ml reaction volume. The cycling profile for both COI and *wingless* was 95°C for 5 min, 35 cycles of 94°C for 30 s, 47°C for 30 s, 72°C for 1 min 30 s, and a final extension period of 72°C for 10 min. For EF-1α the cycling profile was 95°C for 7 min, 35 cycles 95°C for 1 min,

55°C for 1 min, 72°C for 2 min and a final extension period of 72°C for 10 min. For all three gene-regions the PCR primers were also used for sequencing. Sequencing was done with a Beckman-Coulter CEQ8000 capillary sequencer (Stockholm University) or an ABI Prism 377 DNA sequencer (University of Leiden). The resulting chromatograms were verified using the program BioEdit (Hall 1999) and the sequences were aligned by eye. The sequences are available on GenBank (Accession numbers in Table 1).

Phylogenetic analyses

Phylogenetic analyses were carried out in TNT 1.0 (Goloboff *et al*. 2003) using maximum parsimony and a heuristic search algorithm. Molecular data were equally weighted and unordered; morphological data were coded as in Simonsen (2006a). Heuristic searches were run with 1000 random-addition replicates using TBR branch swapping. The four datasets were analysed separately and in combination. The effects of adding new data to a published phylogenetic dataset (Simonsen 2006a) was analysed by sequentially adding the molecular datasets to the morphological dataset in all combinations, including a total evidence analysis with all four datasets combined in a single analysis. The molecular data were also combined and analysed without the morphological data.

Robustness of the clades in the resulting cladograms was evaluated with Bremer support values (BS) (Bremer 1988; Bremer 1994). The scripting feature of TNT was used to calculate BS values (see Peña *et al*. 2006). The contribution of each data partition to the BS values of the combined analyses was assessed using partitioned Bremer support (Baker & DeSalle 1997; Baker *et al.* 1998; Gatesy *et al.* 1999) using another script in TNT (scripts available from N. Wahlberg). Basic sequence statistics and genetic distances were calculated in MEGA2 (Kumar *et al.*, 2001). The distribution of homoplasy within and among partitions was examined with the ILD statistic (Mickevich & Farris 1981). The Genbank accession codes for the gene sequences from the different species are shown in Table 1.

Results

General

Of the 141 morphological characters from Simonsen (2006a), 125 were parsimony informative with the current taxon sampling.

1450 bp from COI, 1240 bp from EF-1α and 400 bp from *wingless* were sequenced for all species. The nucleotide base frequencies for each gene are given in Table 2. The AT bias for COI is in accordance with the general AT bias for insect mt genes (DeSalle *et al.*, 1987; Liu & Beckenbach 1992, Simon *et al.* 1994). The tree statistics for each individual data partition and the combined sets (including tree lengths, number of trees and total PBS contribution) are given in Table 3. Almost all homoplasy is intrinsic to individual data partitions, rather than due to incongruence among partitions when they are combined. Between 48% and 67% of tree lengths for the analyses of individual partitions are due to homoplastic character state transformations, while the combination of the three gene regions adds 1%, and the addition of the morphological data to the combined DNA data adds another 1%.

Phylogenetic patterns in Argynnini

The analyses of each data partition both separately and combined in various ways revealed stable patterns of relationships (Figs 1-4). All analyses recover the following clades: *Euptoieta, Yramea, Boloria, Brenthis* and the clade comprising all Argynnini except *Euptoieta*. The only analysis in which the monophyly of the tribe Argynnini is not resolved is when COI is analysed on its own (Fig. 1B). The monophyly of the subtribe Argynnina is not resolved when morphology and the *wingless* are analysed separately (Figs 1A, D), nor when these two are combined (Fig. 2C), but the subtribe is found to be monophyletic in all other analyses. Monophyly of *Issoria* is likewise unresolved in the separate analyses of morphology and *wingless* (Figs 1A, D), but is found to be monophyletic in the combined analysis of these two partitions (Fig. 2C), as well as in all other analyses. The genus *Argynnis* (sensu Simonsen, 2006a) is not found to be monophyletic in the analysis of the EF-1α partition (Fig. 1C), and its monophyly is unresolved in the analysis of *wingless* (Fig. 1 D), and in the combined analyses of morphology+EF-1α and morphology+EF-1α+*wingless* (Figs 2B, F).

The combined molecular dataset (1 tree, 3318 steps) recovers the Argynnini as monophyletic (Fig. 3) and only differs from the total evidence analysis in the internal phylogeny of a subordinate

Table 1. A taxonomic list of the species and origin of the specimens used in the present analysis. Ingroup taxa listed to subgenera following Simonsen (2006a) with generic names in bold. BMNH: Natural History Museum, London, Great Britain. RMNH: National Museum of Natural History, Leiden, The Netherlands. ZMUC: The Natural History Museum of Denmark (Zoology), Copenhagen, Denmark.

Species	Locality DNA specimen	Locality Morphology specimen	Museum collection	Genbank Accession numbers COI	EF-1α	wingless
Outgroup						
Cupha erymanthis (Drury, 1773)		Java	♂+♀-ZMUC			
Cupha prosope (Fabricius, 1775)	Australia			DQ922839	DQ922871	DQ922808
Phalanta phalanta (Drury, 1773)	Tanzania	Nyasaland, Nigeria	♂+♀-ZMUC	DQ922840	DQ922872	DQ922809
Cethosia cydippe (Linnaeus, 1767)		Java	♂+♀-ZMUC			
Cethosia cyane (Drury, 1773)	Bangladesh			DQ922870	DQ922902	DQ922838
Vindula arsinoe (Cramer, 1777)	Australia	Philippines, Malaysia	♂+♀-ZMUC	AY090204	AY090170	AY090137
Agraulis vanillae (Linnaeus, 1758)	USA	Cuba, Brazil	♂+♀-ZMUC	DQ922841	DQ922873	AF169921
Dryas iulia (Fabricius, 1775)	Costa Rica	Brazil	♂+♀-ZMUC	DQ922842	DQ922874	DQ922810
Heliconius charitonia (Linnaeus, 1767)		Panama, Venezuela	♂+♀-ZMUC			
Heliconius hecale (Fabricius, 1775)	Costa Rica			AY090202	AY090168	AY090135
Ingroup						
Subtribe Argynnina						
Argynnis s.l						
Argynnis paphia (Linnaeus, 1758)	Sweden	Denmark	♂+♀-ZMUC	AY090200	AY090166	AY090133
Argyreus hyperbius (Linnaeus, 1763)	Japan	China, India	♂+♀-ZMUC	DQ922843	DQ922875	DQ922811
Argyronome laodice (Pallas, 1771)	Russia	Estonia	♂+♀-ZMUC	DQ922844	DQ922876	DQ922812
Argyronome ruslana, Motschulsky, 1866	Japan	Japan	♂+♀-ZMUC	DQ922845	DQ922877	DQ922813
Childrena childreni (Gray, 1831)	China	Sikkim, Assam	♂+♀-ZMUC	DQ922849	DQ922881	DQ922817
Damora sagana (Doubleday, 1847)	Japan	Russia, Japan	♂+♀-ZMUC	DQ922850	DQ922882	DQ922818
Fabriciana adippe (Denis & Schiff., 1775)	Finland	Denamrk	♂+♀-ZMUC	DQ922852	DQ922884	DQ922820
Fabriciana niobe (Linnaeus, 1758)	Denmark	Denmark	♂+♀-ZMUC	DQ922851	DQ922883	DQ922819
Fabriciana kamala (Moore, 1875)	India	Kashmir	♂+♀-BMNH	DQ922853	DQ922885	DQ922821
Mesoacidalia aglaja (Linnaeus, 1758)	Sweden	Denmark	♂+♀-ZMUC	DQ922860	DQ922892	DQ922828
Nephargynnis anadyomene (Feld. & Feld., 1862)	Japan	Japan	♂+♀-ZMUC	DQ922861	DQ922893	DQ922829
Pandoriana pandora (Denis & Schiff., 1775)	Russia	France, Spain	♂+♀-ZMUC	DQ922862	DQ922894	DQ922830
Speyeria cybele (Fabricius, 1775)	USA	USA	♂+♀-ZMUC	DQ922863	DQ922895	DQ922831
Brenthis						
Brenthis daphne (Denis & Schiff., 1775)	Spain	France, Romania	♂+♀-ZMUC	DQ922848	DQ922880	DQ922816
Brenthis ino (Rottemburg, 1775)	Finland	Denmark	♂+♀-ZMUC	DQ922847	DQ922879	DQ922815
Brenthis hecate (Denis & Schiff., 1775)	Kirgisia	Hungary	♂+♀-ZMUC	DQ922846	DQ922878	DQ922814
Issoria						
Issoria lathonia (Linnaeus, 1758)	France	Denmark	♂+♀-ZMUC	DQ922854	DQ922886	DQ922822
Issoria eugenia Eversmann, 1847	Russia	Nepal	♂+♀-RMNH	DQ922857	DQ922889	DQ922825
Issoria smaragdifera (Butler, 1895)	Tanzania	Nysaland, Nigeria	♂+♀-BMNH	DQ922855	DQ922887	DQ922823
Issoria hanningtoni Elwes, 1889	Tanzania	British East Africa	♂+♀-BMNH	DQ922856	DQ922888	DQ922824

Subtribe Boloriina						
Boloria s.l.						
Boloria aquilonaris (Stichel, 1908)	Denmark	Denmark	♂+♀-ZMUC	DQ922867	DQ922899	DQ922835
Boloria pales (Denis & Schiff., 1775)	Italy	Switzerland	♂+♀-ZMUC	DQ922866	DQ922898	DQ922834
Clossiana euphrosyne (Linnaeus, 1758)	Spain	Denmark	♂+♀-ZMUC	DQ922868	DQ922900	DQ922836
Clossiana selene (Denis & Schiff., 1775)	Sweden	Denmark	♂+♀-ZMUC	AY090201	AY090167	AY090134
Proclossiana eunomia (Esper, 1799)	Spain	Norway, Switzerland	♂+♀-ZMUC	DQ922869	DQ922901	DQ922837
Subtribe Yrameiina						
Yramea						
Yramea cytheris (Drury, 1773)	Chile	Argentina	♂+♀-ZMUC	DQ922858	DQ922890	DQ922826
Yramea inca (Staudinger, 1894)	Peru	Peru	♂+♀-ZMUC	DQ922859	DQ922891	DQ922827
Subtribe Euptoietina						
Euptoieta						
Euptoieta claudia (Cramer, 1775)	USA	USA	♂+♀-ZMUC	DQ922864	DQ922896	DQ922832
Euptoieta hegesia (Cramer, 1779)	Brazil	Costa Rica, Cuba	♂+♀-ZMUC	DQ922865	DQ922897	DQ922833

Table 2. Empirical base frequencies (%) for the three molecular data sets in 36 species of Heliconiinae included in the analysis.

	Empirical base frequencies (%)			
Gene	A	G	C	T
COI	31.2	13.6	14.5	40.6
EF-1α	26.8	23.5	26.1	23.6
wingless	25.1	28.0	25.2	21.7

group within *Argynnis*, where *A. pandora* is found to be the sister taxon of a group comprising *A. paphia, A. sagana, A. anadyomene, A. laodice, A. ruslana, A. hyperbius* and *A. childrena*, in which (*A. paphia A. sagana*) in turn are found to comprise the sister group of (*A. anadyomene* (*A. laodice A. ruslana*)).

The total evidence analysis yielded one most parsimonious tree of 3724 steps (Fig. 4). Compared to the molecules only analysis, the support for almost all nodes in the total evidence tree is increased, sometimes substantially (Fig. 5). Adding morphological data decreases the BS at only four nodes: BS for the two nodes within the *Issoria* clade is decreased by 3, support for the sister relationship of *A. hyberbius* and *A. childreni* is decreased by 1, and the support for the sister relationship of Argynnina and Bolorina is decreased by 1. There is a clear trend of increasing BS values for the major clades (genera and subtribes) as additional characters are added to the combined analyses (Fig. 5).

In the total evidence analysis (Fig. 4), the Argynnini are monophyletic with high Bremer support (BS 15). The first split within the Argynnini is between *Euptoieta* and the remaining in-group genera. The latter clade is well supported (BS 20). *Euptoieta* is very strongly supported with a very high BS (50). *Yramea* is also very strongly supported with a very high BS (87). The sister group of *Yramea*, a clade comprising *Boloria* and Argynnina, is fairly well supported with a BS of 7. *Boloria* appears very strongly supported with a BS of 18. Within *Boloria* s.l., the position of *B. eunomia* is unstable to method of analysis, being sister to *Boloria* s. str. (BS 5). *Boloria* s. str. is strongly supported by a BS value of 37.

The Argynnina is monophyletic and well supported with a high BS of 10. Within Argynnina, *Issoria* s.l. is the sister group of a clade comprising *Brenthis* and *Argynnis*. *Issoria* s.l. is well sup-

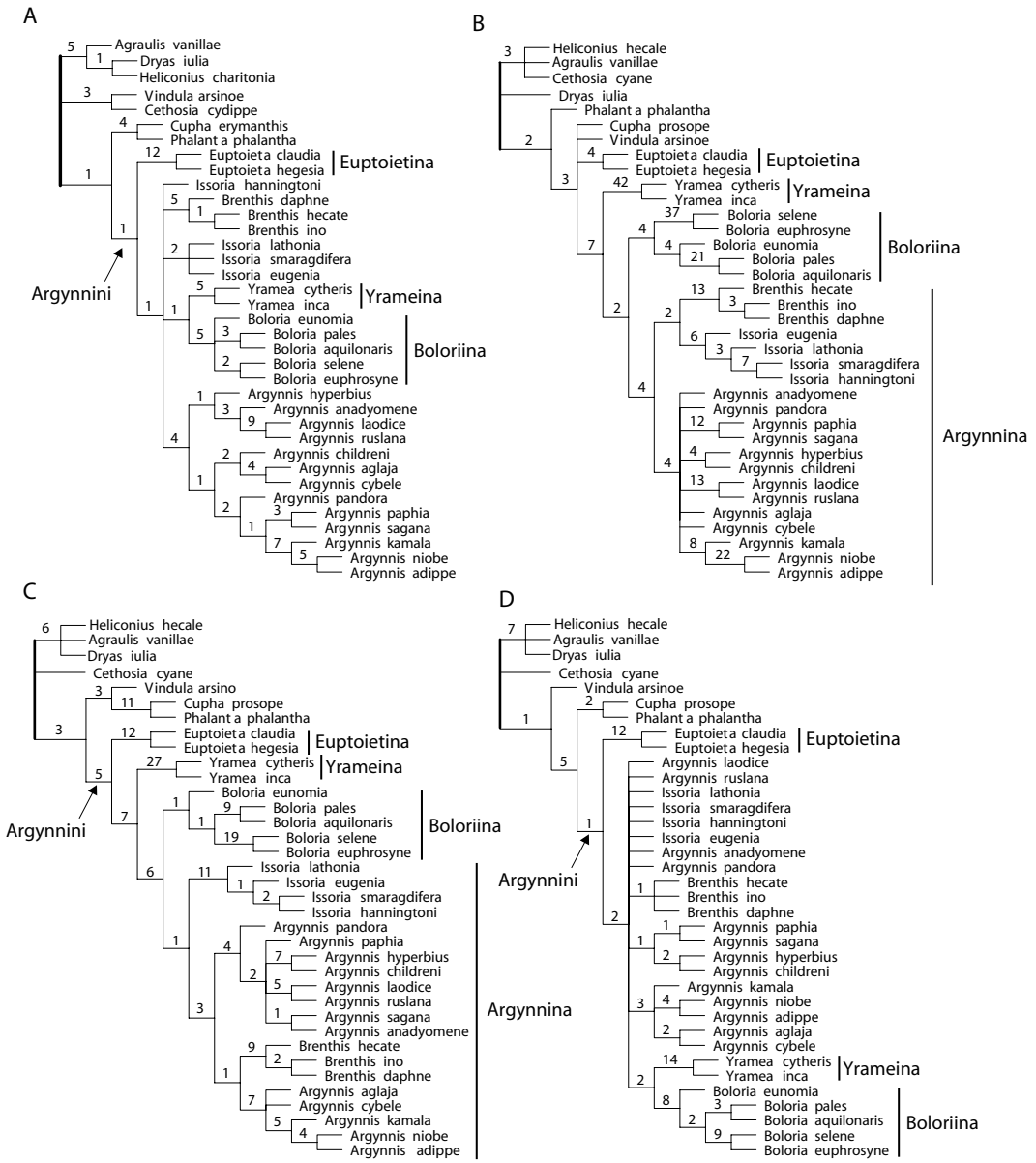

Fig. 1. Strict consensus trees from the separate analyses of the four datasets. **A** Morphology (8 trees, each 376 steps with CI = 0.41 and RI = 0.69), **B** COI (26 trees, each 2127 steps, CI = 0.36, RI =0.43), **C** EF-1α (4 trees, each 772 steps, CI = 0.52, RI = 0.68), **D** *wingless* (6 trees, each 388 steps, CI = 0.51, RI = 0.63). The numbers above the nodes are Bremer support values.

ported by a high BS of 26. Within *Issoria*, *I. eugenia* is the sister group of the remainder of the genus, which is modestly supported with a BS of 4. *I. lathonia* is the sister group of the two African species *I. smaragdifera* and *I. hanningtoni*, a clade modestly supported (BS 5). The clade comprising *Brenthis* and *Argynnis* has a fairly high BS of 8. *Brenthis* is well supported (BS 26).

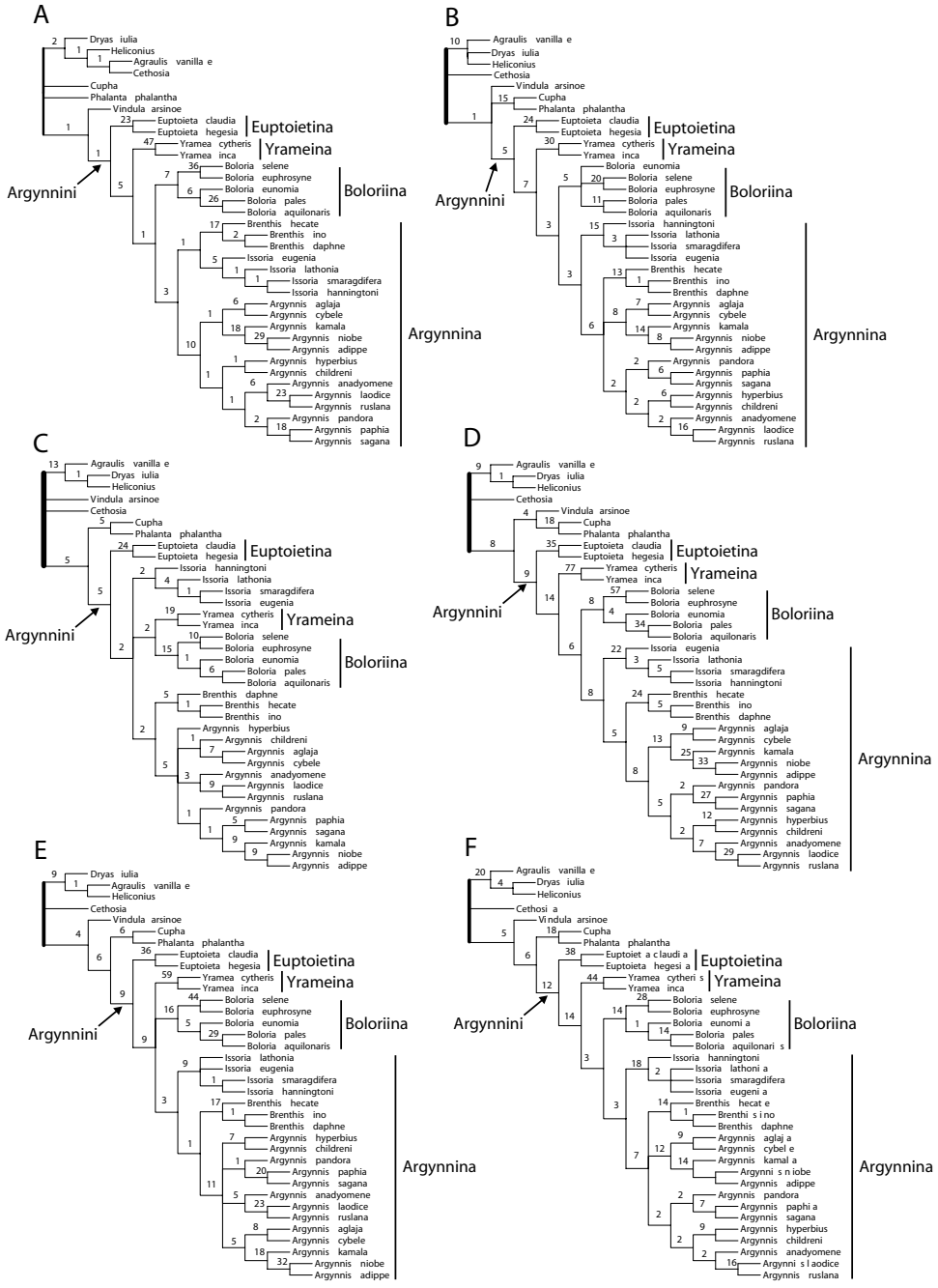

Fig. 2. Strict consensus trees from various combinations of the four datasets. **A** Morphology and COI (2 trees, each 2544 steps, CI = 0.36 and RI = 0.48), **B** Morphology and EF-1α (12 trees, each 1179 steps, CI = 0.47 and RI = 0.67), **C** Morphology and *wingless* (8 trees, each 778 steps, CI = 0.46 and RI = 0.66), **D** Morphology, COI and EF-1α (1 tree, 3327 steps, CI = 0.40 and RI = 0.53), **E** Morphology, COI and *wingless* (6 trees, each 2940 steps, CI = 0.38 and RI = 0.50), **F** Morphology, EF-1α and *wingless* (3 trees, each 1573 steps, CI = 0.48 and RI = 0.66). The numbers above the nodes are Bremer support values.

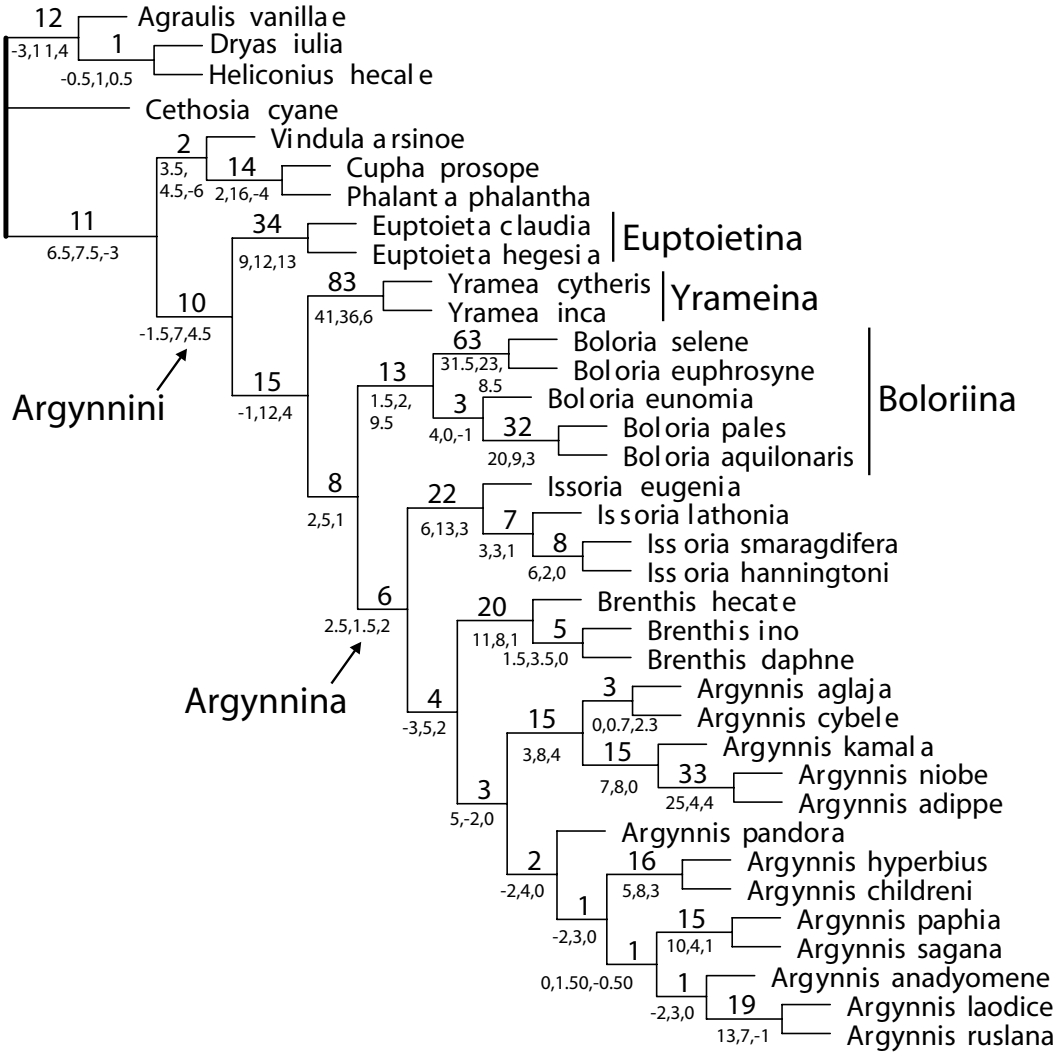

Fig. 3. The combined analysis of the three molecular datasets. The single most parsimonious tree (3318 steps, CI = 0.41, RI = 0.52). The numbers above the nodes are Bremer support values, whereas the numbers below the nodes are partitioned Bremer support values yielded by COI, EF-1α and *wingless* respectively.

Argynnis s. l. as defined by Simonsen (2006a) is reasonably well supported and has a fairly high BS (8). The first split within *Argynnis* is between a clade comprising the two subgenera *Fabriciana* (represented by *A. kamala, A. niobe* and *A. adippe*) and *Speyeria* (represented by *A. aglaja* and *A. cybele*) and a clade comprising the remaining *Argynnis*. The *Fabriciana*+*Speyeria* clade is well supported with a very high BS (17). The clade comprising the two representatives of subgenus *Speyeria* is well supported and the BS (11) is high. The subgenus *Fabriciana* is well supported and the BS (25) is very high. The clade comprising the remaining *Argynnis* is only moderately supported (BS 5). The basal split within this clade is between a clade comprising *A. pandora, A. sagana* and *A. paphia* and a clade comprising *A. hyperbius, A. childreni, A. anadyomene, A. laodice* and *A. ruslana*. The latter is poorly supported with a low BS (2). Within this clade, *A. hyperbius* and *A. chil-*

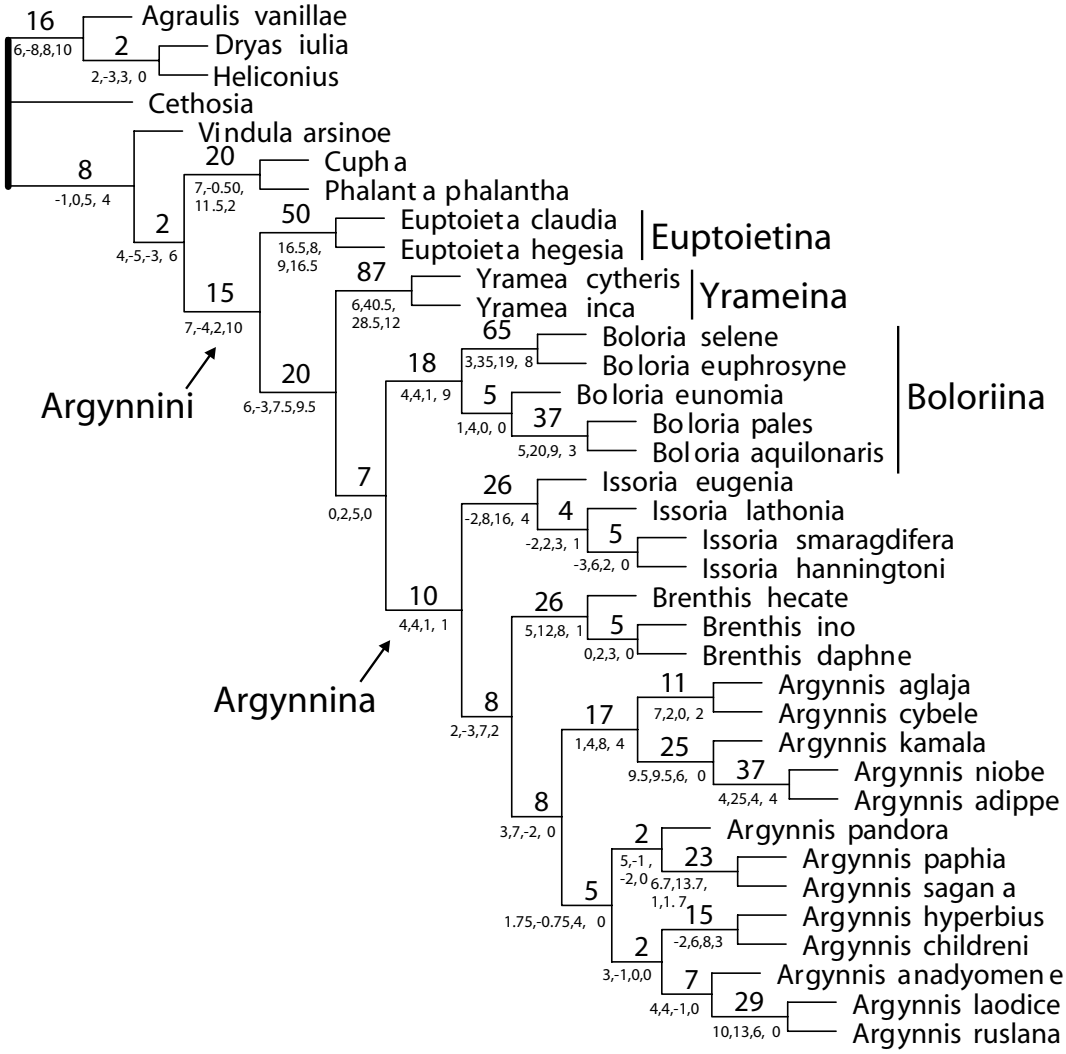

Fig. 4. The combined analysis of all four datasets. The single most parsimonious tree (3724 steps, CI = 0.41, RI = 0.54). The numbers above the nodes are Bremer support values, whereas the numbers below the nodes are partitioned Bremer support values yielded by morphology, COI, EF-1α and *wingless* respectively.

dreni comprise the sister group of *A. anadyomene, A. laodice* and *A. ruslana*. *A. hyperbius* and *A. childreni* form a well supported clade with a high BS (15). The clade comprising *A. anadyomene, A. laodice* and *A. ruslana* is well supported and the BS (7) is fairly high. *A. laodice* and *A. ruslana* (subgenus *Argyronome*) form a strongly supported clade (BS 29). The clade comprising *A. pandora, A. sagana* and *A. paphia* is weakly supported, with a BS of 2, while the clade comprising *A. sagana* and *A. paphia* is well supported with a BS of 24.

The positive partitioned total support values show that all datasets contributed significantly to the final result (Table 3). However, the datasets were conflicting or ambiguous at some nodes (Fig. 4). Of 28 nodes, 12 were unanimously supported by all datasets. In 4 cases the three molecular datasets unanimously supported the node, whereas the morphology was in conflict (3 of these nodes are in the *Issoria* clade). There was no node for

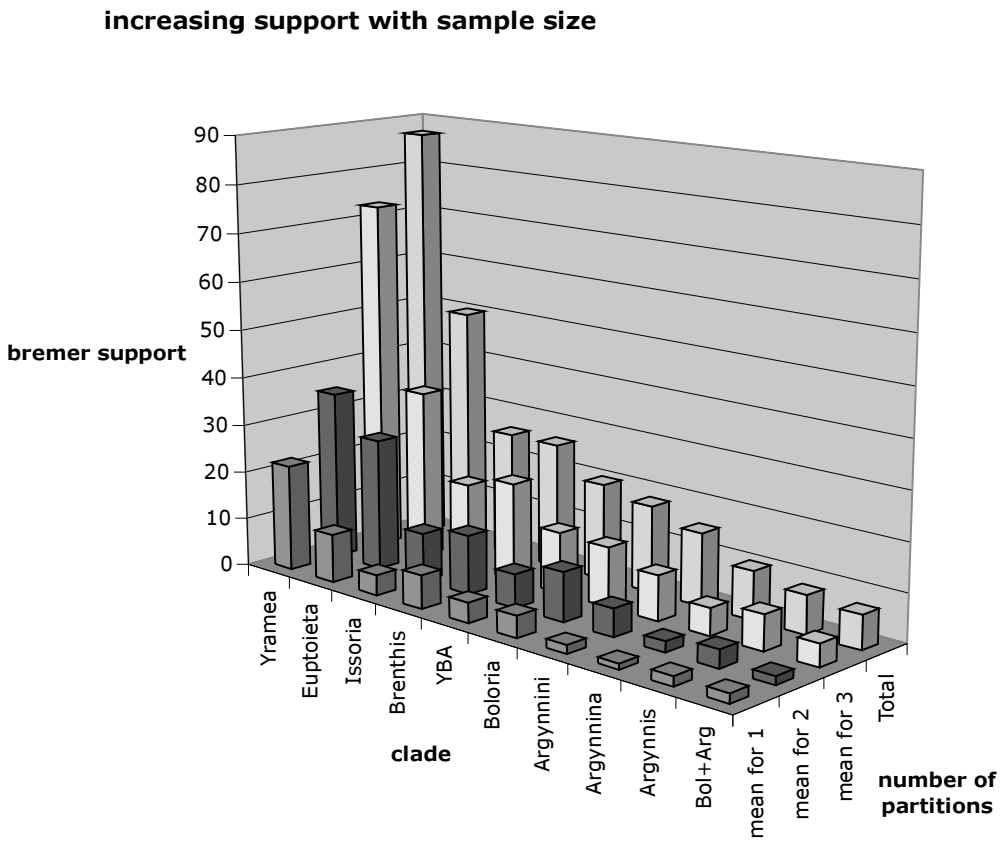

Fig. 5. The development of Bremer support values at major nodes as the number of partitions increase. Bol+Arg = Boloriina+Argynnina, YBA = Yrameina+Boloriina+Argynnina.

which the three molecular datasets unanimously yielded negative PBS. In one case (the position of *A. pandora* as sister to *A. paphia* and *A. sagana*) only morphology supported a node (the molecular datasets were either conflicting or ambiguous). The extremely small number of extra steps entailed by combining the molecular data partitions, and subsequently the molecular and morphological partitions (Table 3), also gives a clear indication of the high degree of congruence among these different sources of evidence.

Discussion

Combining data to increase support

The question of how much data is needed to arrive at a robust phylogenetic hypothesis is still debated (Rokas et al. 2003, Gatesy & Baker 2005). In our study we have shown that increasing the number of functionally separate datasets also increases the stability and robustness of the resulting phylogenetic hypothesis. All four datasets have considerable positive impact on the results of the combined analysis. By sequentially adding the new molecular datasets to the already published morphological dataset, we have been able to discover which clades are very stable (i.e. unlikely to change no matter how much new data we add), which clades tend to stabilise as new data are added (Bremer supports increase substantially in combined analyses) and which clades are still relatively uncertain.

When analysed separately, each of the datasets show incongruence, both among one another and with the combined result. However, this is likely to be caused by the intrinsic properties of each finite dataset, i.e. the number of variable characters is

Table 3. Parameters for the individual and combined data partitions. D homoplasy is the additional steps due to incongruence among data partitions when they are combined. The total support is the sum of PBS values across all branches of the combined tree for a given partition.

Data partition	No. Char.	Variable Char.	Informative Chars	No. of Trees	Total Tree length	Intrinsic homo-plasy	D homo-plasy	Total support in TE tree
Morphology	141	140	125	8	376	222 (59%)		123.4
COI	1450	550	421	26	2127	1361 (64%)		202.4
Ef-1a	1240	305	225	4	772	371 (48%)		177.5
Wingless	400	136	112	6	388	190 (49%)		113.7
Comb. DNA	3090	991	233	1	3318		31 (1%)	493.6
Tot. evidence	3231	1131	248	1	3724		30 (1%)	617

limited and homoplasy is high (as in all of our datasets). Combining the various datasets allows the underlying phylogenetic signal to come out (Gatesy *et al.* 1999, Baker & Gatesy 2002). In our analyses, this phenomenon is striking in the total evidence analysis (Fig. 5). Recent studies have suggested that incongruence between molecular datasets may be due to real differences in evolutionary histories of genes (e.g. Holland *et al.* 2004, Jeffroy *et al.* 2006). Such studies have taken advantage of very large datasets that are not yet tractable for many groups of organisms. Our study is typical of most studies today (about 3000 characters) and we suggest that in such cases a total evidence approach is not only appropriate but also necessary. It is likely that instances of real incongruence will only be reliably inferred with larger numbers of independent datasets.

In our case, by combining morphological characters and sequences from three genes, a more resolved, well-supported phylogenetic hypothesis is inferred than when any of the data partitions are analyzed alone. As shown in Fig. 5, the support for all higher clades increased as character sampling increased. Our study emphasizes the desirability of analyzing large datasets to arrive at robust phylogenetic hypotheses. Single gene studies, particularly using short mitochondrial gene regions, should be viewed with circumspection (Brower 2006).

Phylogeny

Although the present results are largely in agreement with those of Simonsen (2006a), some very important differences are obvious and deserve attention. There is agreement that Argynnini are monophyletic and that the subtribe Euptoietina comprises the sister group of the remaining taxa. The placement of *Yramea* as the sister group of *Boloria* + Argynnina, however, conflicts with the earlier results. In Simonsen (2006a), *Yramea* was sister of *Boloria,* and these two genera were placed in a subtribe named Yrameina. However, according to our current results this name should be reserved only for the genus *Yramea*. The internal phylogeny of *Boloria*, where *B. eunomia* is the sister group of (*B. pales B. aquilonaris*) is in conflict with Simonsen (2006a) where *B. eunomia* was placed as the sister group of (*B. selene B. euphrosyne*), but is congruent with the morphology based analysis focused on *Boloria* by Simonsen (2005).

This analysis corroborates the hypothesis that the subtribe Argynnina is monophyletic. However, in contrast to Simonsen (2006a), we found that the genus *Issoria* s.l. is monophyletic. In part due to the absence of the 'rectal plate' in two African species (*I. baumanni* and *I. hanningtoni*), Simonsen (2006a) found that these two species formed the sister group to the rest of Argynnina and they were thus placed in the genus *Prokuekenthaliella*. Our results here suggest that the two African species are nested well within *Issoria*. The rectal plate is a very complex structure associated with the tegumen and uncus in Palaearctic *Issoria* and the African species *I. smaragdifera* (Simonsen 2006b), but absent in other Heliconiinae. Given the simple structure of the uncus and tegumen in *I. hanningtoni* and *I. baumanni* the present results seem to indicate that these structures (and hence the rectal plate) have been secondarily reduced in these two species compared to other *Issoria*.

There is agreement that *Brenthis* form the sister group of a monophyletic *Argynnis* s.l.. The genus *Brenthis* is well supported here though the internal

relationships of the genus differ from Simonsen (2006a).

Monophyly of *Argynnis* s.l. is moderately well supported, but the internal phylogeny of the genus contradicts the results of Simonsen (2006a), where a clade comprising *(A. hyperbius (A. anadyomne (A. laodice A. ruslana)))* is the sister group of the remaining species in the genus. In our total evidence analysis the first split in *Argynnis* is between a clade comprising the two subgenera *Fabriciana* and *Speyeria* (including *Mesoacidalia*) and a clade comprising the remaining subgenera. Though contradicting Simonsen (2006a), a close relationship between *Fabriciana* and *Speyeria* was suggested by Penz & Peggie (2003) and here the clade is well supported by all datasets. Although not found by Simonsen (2006a), the clade comprising the remaining *Argynnis* bears some similarities to the previous results, but also conflicts with these. The clade comprising *A. pandora, A. sagana* and *A. paphia* is supported in both analyses and the sister group relationship between the latter two suggested by Simonsen (2006a) is strongly supported here. The sister clade that here comprises *A. hyperbius, A. childreni, A. anadyomene, A. laodice* and *A. ruslana* contradicts Simonsen (2006a) where *A. childreni* (and its sister species *A. zenobia*) is placed with the subgenus *Speyeria*. However, Penz & Peggie (2003) placed *A. childreni* with *A. hyperbius*. We agree with Simonsen (2006a) that *A. ruslana* and *A. laodice* form a strongly supported clade and that their sister group is *A. anadyomene.*

Classification

The present results necessitate three changes in the classification proposed by Simonsen (2006a). Since the clade comprising *Boloria* and *Yramea* is not supported here we suggest that the name Yrameina should be reserved for a subtribe comprising only *Yramea*. Given its phylogenetic position, *Boloria* should be placed in its own subtribe. The name Boloriina (Warren 1944, Warren et al. 1946) is available for this genus and should be assigned to it. The inclusion of the re-established genus *Prokuekenthaliella* (Simonsen 2006a) in *Issoria* removes the need for retaining *Prokuekenthaliella* as a separate genus.

The fairly well supported clade comprising the *Argynnis* s.l. species supports the unification of all the "larger fritillaries" in one genus. As argued by Simonsen (2006a) there is little justification for the large number of generic names traditionally applied to various members of that group, and one large, unified genus *Argynnis* seems to be the only stable and "natural" solution for this problem. Not only is *Argynnis* as defined here well delimited and easily recognized based on the highly specialized male alar androconials (Barth 1944), it is also supported in the morphological analysis, the combined molecular analysis and the total evidence analysis. Additionally, no alternative division of *Argynnis* into two or three genera seems ideal. The combined analyses do split *Argynnis* into two reciprocally monophyletic groups. However, these two groups are contradicted by morphology and not easily and immediately recognizable as a unit.

In summary, the tribe Argynnini is a well supported monophyletic group comprising of the subtribes Euptoietina, Yrameina, Boloriina and Argynnina. Six monophyletic, well-supported, robust and morphologically well-defined clades are termed genera in this study. These are *Euptoieta, Yramea, Boloria, Issoria, Brenthis* and *Argynnis*. We feel that this classification of Argynnini will be stable to the addition of new data based on our analyses in this paper.

Correction added in proof

Just as the paper was being typeset, the authors became aware that the *Boloria euphrosyne* specimen from which the DNA sequences were obtained was in fact a misidentified, slightly aberrant, *Boloria selene* (identified by T. J. Simonsen). This does not affect the results and conclusions, since the internal relationships of *Boloria* were outside the scope of the study.

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