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Phylogenetic relationships among the Nymphalidae (Lepidoptera) inferred from partial sequences of the *wingless* gene

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A cladistic analysis was performed on a 378 bp region of the *wingless* gene from 103 nymphalid species and three pierid outgroups in order to infer higher level patterns of relationship among nymphalid subfamilies and tribes. Although the data are highly homoplastic, in many instances the most parsimonious cladograms corroborate traditionally recognized groups. The results suggest that this short gene region provides a useful source of data for phylogenetic inference, provided that adequate effort is made to sample a diversity of taxa.

Keywords: cladistics; butterfly; molecular systematics; taxon sampling

1. INTRODUCTION

The morphologically and ecologically diverse nymphalid butterflies have been the subjects of prolonged and intense genetic, behavioural and ecological research for over a century. Since the time of Darwin and Bates, workers have repeatedly turned to this group for premier examples of natural selection, polymorphism and mimicry (Bates 1861; Poulton 1916; Goldschmidt 1945; Fisher 1958; Sheppard 1963; Turner 1977). The study of nymphalids continues to provide fundamental data for the diverse fields of ecological genetics, chemical ecology, community ecology, development, ethology and conservation biology (Gilbert 1991; Nijhout 1991; Carroll *et al.* 1994; Wahlberg *et al.* 1996; Monteiro *et al.* 1997; DeVries *et al.* 1999; see also the reviews in Vane-Wright & Ackery (1984)).

Yet even after more than a century of taxonomic study, the phylogenetic relationships among higher groups of nymphalids still confound systematic biologists. Unambiguous morphological synapomorphies, such as tricarinate antennae, diagnose the family Nymphalidae and further suites of characters permit the relatively uncontroversial recognition of numerous well-defined subfamilies and tribes (Ehrlich 1958; Ackery 1984; Scott 1985; De Jong *et al.* 1996; Ackery *et al.* 1999). However, the relative branching order among these intrafamilial clades remains uncertain and has been presented by most authors (e.g. Ackery 1984) as a largely unresolved node in the cladogram of butterfly relationships. It is not clear how existing data may be used to resolve these polytomies: despite morphological studies using a variety of data sets and analytical methods (Clark 1947; Ehrlich 1958; Kristensen 1976; Scott & Wright 1990; Harvey 1991; Ackery *et al.* 1999), the relationships among nymphalid tribes and subfamilies remain poorly understood. Both Harvey's (1991) classification and De Jong *et al.*'s (1996) cladogram (figure 1) suffer from a frustrating lack of basal resolution. Importantly, the lack of a stable phylogenetic hypothesis for nymphalids leaves in question the widely cited hypotheses on host use and coevolution (Brower & Brower 1964; Ehrlich & Raven 1965, Ackery 1988), which rely on the apparently concordant taxonomic distribution of these butterflies and their food plants.

Wing shapes, patterns and colours, which are frequently used for the recognition of nymphalid species, are notoriously labile in response to selection for recognition by predators (Brower 1984) and mates (Silberglied 1984) or even to differential weather conditions (e.g. Brakefield 1996). Nijhout (1991) lamented that 'The freedom with which butterfly species are able to shift, realign, hide, unmask and alter the color and shape of their pattern elements suggests that in principle there is no pattern to which that of another species could not converge' (p. 241). The outlook for additional, subtler morphological characters is hardly more sanguine. Indeed, the recent cladistic analysis of adult morphological characters by De Jong *et al.* (1996) offered less resolution among nymphalids than Ackery's (1984) tree. Ackery (1984) and DeVries *et al.* (1985) called for a detailed analysis of the larval morphology. Although Harvey's (1991) classification was partly based on examination of the larval filiform setae, no resolution of the relationships among nymphalid subfamilies was implied there either. Ackery *et al.*'s (1999) most recent summary concluded that 'most relationships within and between the various groups remain obscure . . . and continuing changes in higher classification must be anticipated, including alterations to limits of many of the subfamilies themselves' (p. 287).

Given the apparent intractability of the adult morphology and the current lack of larval specimens for many taxa, molecular data may provide a new, complementary approach to deciphering phylogenetic patterns in this large and complex group. Martin & Pashley (1992) and Weller *et al.* (1996) performed the only molecular systematic studies on higher-level relationships among the butterflies published to date. Martin & Pashley (1992) examined the relationships among eight of the 13 recognized nymphalid subfamilies by examining 212 sites in a region of the 28S ribosomal RNA gene from 15 species, and Weller *et al.* (1996) followed this up with a combined analysis of the 28S data, 320 bases of the mitochondrial ND-1 gene and a morphological data matrix of 50 characters compiled from Kristensen (1976), Scott (1985) and Robbins (1988, 1989). Although both papers were primarily focused on familial and superfamilial relationships, the

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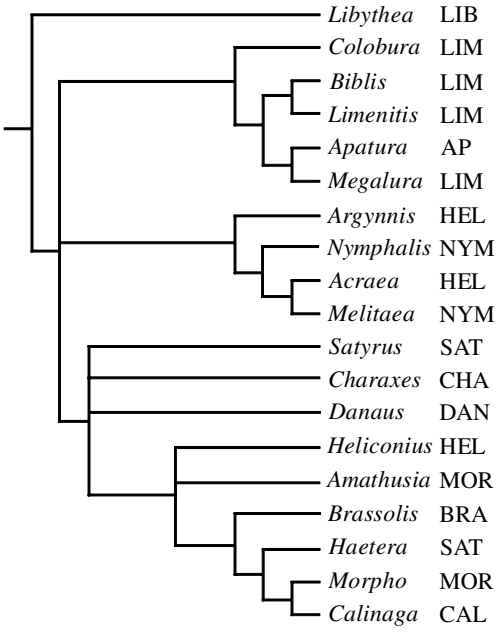

Figure 1. The nymphalid clade recovered as part of the strict consensus of the 1580 most parsimonious cladograms from De Jong *et al*. (1996, their fig. 4) based on 59 exemplar butterfly species and 103 morphological characters. Length = 473, CI = 0.321 and RI = 0.723. The subfamilies, as recognized by Harvey (1991) and Ackery *et al*. (1999), are indicated as follows: AP, Apaturinae; BRA, Brassolinae (included in the Morphinae by Ackery *et al*. (1999)); CAL, Calinaginae; CHA, Charaxinae; DAN, Danainae (including the Ithomiinae according to Ackery *et al*. (1999)); HEL, Heliconiinae; LIB, Libytheinae; LIM, Limenitidinae; MOR, Morphinae; NYM, Nymphalinae; SAT, Satyrinae.

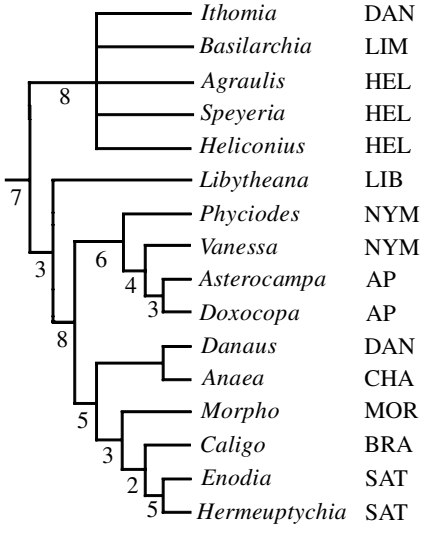

Figure 2. Strict consensus of the three trees for 16 nymphalid taxa pruned from the combined cladistic analysis of mtDNA ND-1, 28S rDNA and morphological characters for 29 Papilionoidea, redrawn from Weller *et al*. (1996, their fig. 3b). Length = 1058, CI = 0.44 and RI = 0.56. The numbers beneath the branches represent unambiguous synapomorphies. Subfamily abbreviations as in figure 1.

more recent paper included 16 species representing 11 nymphalid subfamilies, which were sampled for at least one gene (five species were missing one gene or the other). The strict consensus topology from combined cladistic analysis of all the data (figure 2) is almost fully resolved and for the most part agrees with traditional hypotheses of grouping, but the taxon sampling is so minimal that the monophyly of most subfamilies could not be tested. Of the four subfamilies represented by more than a single exemplar, Satyrinae and Apaturinae appear as monophyletic, while Heliconiinae and Nymphalinae are paraphyletic. The traditional association of the 'satyrid' subfamilies (Brassolinae plus Morphinae plus Satyrinae) is recovered, but Ithomiinae plus Danainae is not. Finally, it should be noted that none of the morphological characters employed by Weller *et al*. (1996) is relevant to the resolution of intrafamilial relationships: 'Nymphalidae' was a single terminal taxon in Kristenen's (1976), Scott's (1985) and Robbins' (1988, 1989) data matrices.

Here I address the problem of nymphalid phylogenetic relationships at the subfamilial and tribal levels using DNA sequence data from a coding region of the developmental patterning gene *wingless* (wg) (see the discussion in Brower & DeSalle (1998)). The family Nymphalidae currently comprises some 6000–6500 described species (Shields 1989; Ackery *et al*. 1999) in 350–450 recognized genera (Harvey 1991; Ackery *et al*. 1999), many of which are not demonstrably monophyletic and require revision. It is currently beyond the scope of any single study to incorporate all the species and it is even impractical to examine representatives of each putative genus. However, as noted by Wheeler (1992) and Judd (1998), appropriate sampling of taxa is fundamental to the inference of stable phylogenetic hypotheses. I have therefore sampled the diversity of the nymphalid butterflies rather intensively, obtaining taxa considered to be relevant to higher-level patterns of hierarchical grouping based on the most recent and comprehensive classification (Harvey 1991). Harvey's (1991) arrangement (table 1) serves as the null hypothesis of relationships for comparison with the results of my analyses.

2. MATERIAL AND METHODS

(a) *Specimens*

Individual butterflies were netted in the field and preserved in cryotubes in liquid nitrogen or in 2–7 ml vials of 100% ethyl alcohol (EtOH). I found the EtOH preservation technique to be equivalent to liquid nitrogen preservation in the quality and quantity of DNA obtained and superior from a logistical perspective. Specimens collected by colleagues in remote regions, preserved in EtOH and sent through the mail vastly expanded the taxonomic and biogeographical scope of the study. Locality data on voucher specimens are presented in table 1.

(b) *Outgroups*

Because the Nymphalidae and Lycaenidae have been considered paraphyletic with respect to one another in various ways (Ackery 1988; Robbins 1988; De Jong *et al*. 1996; Campbell *et al*. 2000), the Pieridae were selected as the nearest unambiguous outgroup clade. Three pierid genera were included as outgroups, although the cladograms were rooted with *Pieris rapae* alone. The problem of nymphalid–lycaenid relationships and the

Table 1. *Exemplar taxa included in this study, listed according to Harvey's (1991) higher-level classification of Nymphalidae*

(Genera within tribes are listed in Harvey's order. Genera of Satyrinae are placed in tribes based on Miller (1968). The '-ina' ending for subtribes replaces Harvey's '-iti' under ICZN article 29.2 (International Commission on Zoological Nomenclature 1999).)

subfamily	tribe	subtribe	genus species	sample code	GenBank accession	locality	voucher
Heliconiinae	Acraeini		*Acraea andromacha*	NSW4	AF246527	Australia: NSW, Evans Head	AMNH
			Actinote genitrix	B-5-1	AF246528	Brazil: Paraná, Quatro Barras	CUIC
			Actinote stratonice	C-17-2	AF014139	Colombia: Putumayo, 62 km North Mocoa	CUIC
			Cethosia hypsea	SA-2-1	AF246585	Malaysia: Sabah, Luasong	AMNH
	Heliconiini	Heliconiina	*Philaethrea dido*	RB283	AF014137	Brazil: Rondonia, Ariquemes	AMNH
			Podotricha telesiphe	C-17-4	AF169900	Colombia: Putumayo, 62 km North Mocoa	CUIC
			Dione juno	P-24-4	AF169909	Panama: Panamá, Lake Bayano	CUIC
			Agraulis vanillae	B-10-1	AF169921	Brazil: Amazonas, Manacapusu	AMNH
			Dryadula phaetusa	P-11-7	AF169902	Panama: Panamá, Gatún locks	CUIC
			Dryas iulia	E-4-3	AF169903	Ecuador: Pichincha, Tinalandia	CUIC
			Eueides vibilia	RB329	AF169918	Brazil: Rondonia, Ariquemes	AMNH
			Neruda metharme	RB275	AF169920	Brazil: Rondonia, Ariquemes	AMNH
			Laparus doris	E-4-5	AF014135	Ecuador: Pichincha, Tinalandia	CUIC
			Heliconius erato	JB1	AF169883	Colombia: Bolivar, Cartagena	CUIC
		(uncertain)	*Cirrochroa emalea*	SA-2-2	AF246582	Malaysia: Sabah, Luasong	AMNH
			Cupha erymanthis	JV3	AF014141	Indonesia: West Java, Bogor	AMNH
			Euptoieta claudia	RB248	AF014140	Brazil: Rondonia, Ariquemes	AMNH
		Argynnina	*Speyeria cybele*	VA2	AF169919	USA: Virginia, Blue Ridge	AMNH
		Boloriina	*Boloria bellona*	NY2	AF246530	USA: New York, Ulysses	AMNH
Nymphalinae	Nymphalini		*Vanessa atalanta*	NY6	AF246542	USA: New York, Ithaca, Coy Glen	AMNH
			Polygonia interrogationis	VA1	AF246569	USA: Washington, DC.	AMNH
			Hypanartia bella	PE-10-7	AF246590	Peru: Cuzco, Quebrada Chaupimayo	AMNH
			Symbrenthia hypatia	NP95-Y314	AF246609	Malaysia: (ex. Naomi Pierce)	MCZ
	Kallimini		*Siproeta steneles*	RB246	AF014142	Brazil: Rondonia, Ariquemes	AMNH
			Precis coenia	RB298	AF246570	Brazil: Rondonia, Ariquemes	AMNH
			Junonia iphita	SA-2-3	AF246593	Malaysia: Sabah, Luasong	AMNH
			Hypolimnas bolina	HBS7	AF246553	(reared in captivity)	AMNH
			Kallima limborgii	NP95-Y320	AF246594	Malaysia: (ex. Naomi Pierce)	MCZ
	Melitaeini	Euphydryina	*Euphydryas phaeton*	NY1	AF246529	USA: New York, Ulysses	AMNH
		Phyciodina	*Phyciodes tharos*	NY5	AF246540	USA: New York, Ithaca, Coy Glen	AMNH
			Eresia nauplius	G-36-5	AF246541	French Guiana: Kaw	AMNH
Limenitidinae	Coloburini		*Colobura dirce*	RB303	AF246533	Brazil: Rondonia, Ariquemes	AMNH
	Biblidini	Biblidina	*Biblis hyperia*	RB243	AF246534	Brazil: Rondonia, Ariquemes	AMNH
		Eurytelina	*Laringa castelnaui*	NP95-Y177	AF246595	Malaysia: (ex. Naomi Pierce)	MCZ
			Mestra dorcas	V25A	AF246598	Venezuela: Delta Amacuro, Barrancas	AMNH
			Vila semistalachtis	RB319	AF246538	Brazil: Rondonia, Ariquemes	AMNH

(*Cont.*)

Table 1. (*Cont.*)

subfamily	tribe	subtribe	genus species	sample code	GenBank accession	locality	voucher
		Epicaliina	*Myscelia cyaniris*	V22	AF246580	Venezuela: Delta Amacuro, Barrancas	AMNH
			Catonephele acontius	RB282	AF246536	Brazil: Rondonia, Ariquemes	AMNH
			Eunica sp.	RB244	AF246573	Brazil: Rondonia, Ariquemes	AMNH
		Ageroniina	*Hamadryas chloe*	RB262	AF246552	Brazil: Rondonia, Ariquemes	AMNH
			Panacea divalis	RB387	AF246537	Brazil: Rondonia, Ariquemes	AMNH
			Batesia hypochlora	RB225	AF246535	Brazil: Rondonia, Ariquemes	AMNH
		Epiphilina	*Temenis laothoe*	RB221	AF246572	Brazil: Rondonia, Ariquemes	AMNH
		Dynaminina	*Dynamine maeon*	RB348	AF246581	Brazil: Rondonia, Ariquemes	AMNH
		Catagrammina	*Diaethria clymena*	RB242	AF014143	Brazil: Rondonia, Ariquemes	AMNH
	Limenitidini	Limenitidina	*Limenitis arthemis*	NY3	AF246531	USA: New York, Caroline, Shindagin Hollow	AMNH
			Adelpha cytherea	G-36-4	AF246574	French Guiana: Kaw	AMNH
		Euthaliina	*Taenicia julii*	NP95-Y156	AF246610	Malaysia: (ex. Naomi Pierce)	MCZ
	Cyrestidini		*Marpesia orsilochus*	RB250	AF246532	Brazil: Rondonia, Ariquemes	AMNH
			Chersonesia rahria	NP95Y191	AF246586	Malaysia: (ex. Naomi Pierce)	MCZ
Charaxinae	Charaxini		*Polyura athamas*	SA-2-7	AF246605	Malaysia: Sabah, Luasong	AMNH
	Preponini		*Prepona* sp.	RB256	AF246554	Brazil: Rondonia, Ariquemes	AMNH
	Anaeini	Anaeina	*Hypna clytemnestra*	RB317	AF246557	Brazil: Rondonia, Ariquemes	AMNH
			Memphis sp.	RB226	AF246539	Brazil: Rondonia, Ariquemes	AMNH
		Zaretidina	*Zaretis itys*	RB261	AF246611	Brazil: Rondonia, Ariquemes	AMNH
Apaturinae			*Asterocampa clyton*	F20	AF246556	USA: Florida, Gainesville	AMNH
			Doxocopa sp.	RB273	AF246555	Brazil: Rondonia, Ariquemes	AMNH
			Eulaceura osteria	NP95-Y227	AF246588	Malaysia: (ex. Naomi Pierce)	MCZ
Morphinae	Morphini	Morphina	*Morpho helenor*	RB295	AF014144	Brazil: Rondonia, Ariquemes	AMNH
		Antirrhaeina	*Caerois chorinaeus*	RB318	AF246575	Brazil: Rondonia, Ariquemes	AMNH
			Antirrhaea sp.	RB355	AF246550	Brazil: Rondonia, Ariquemes	AMNH
	Amathusiini		*Amathusia phidippus*	SA-2-4	AF246583	Malaysia: Sabah, Luasong	AMNH
Brassolinae	Brassolini		*Caligo idomeneus*	RB247	AF246544	Brazil: Rondonia, Ariquemes	AMNH
			Opsiphanes cassina	RB259	AF246545	Brazil: Rondonia, Ariquemes	AMNH
Satyrinae	Haeterini		*Haetera piera*	RB353	AF246549	Brazil: Rondonia, Ariquemes	AMNH
	Biini	Biina	*Bia actorion*	RB306	AF246568	Brazil: Rondonia, Ariquemes	AMNH
		Melanitina	*Melanitis leda*	QL8	AF246578	Australia: Queensland, Brisbane	AMNH
	Elymniini	Lethina	*Lethe mekara*	NP95-Y235	AF246596	Malaysia: (ex. Naomi Pierce)	MCZ
		Mycalesina	*Mycalesis fusca*	SA-2-8	AF246600	Malaysia: Sabah, Luasong	AMNH
	Satyriini	Hypocystina	*Tisiphone abeona*	NSW3	AF246546	Australia: NSW, Broadwater	AMNH
		Euptychiina	*Megisto cymela*	F26	AF246547	USA: Florida, Gainesville	AMNH
			Oressinoma sorata	PE-6-1	AF246602	Peru: Cuzco, Alfamayo	AMNH
			Taygetis sp.	RB294	AF246548	Brazil: Rondonia, Ariquemes	AMNH
		Maniolina	*Cercyonis pegala*	NY13	AF014145	USA: New York, Ulysses	AMNH

(*Cont.*)

Table 1. (*Cont.*)

subfamily	tribe	subtribe	genus species	sample code	GenBank accession	locality	voucher
		Pronophilina	*Corades cistene*	PE-5-10	AF246587	Peru: Cuzco, Quebrada San Luis	AMNH
			Lymanopoda umbratilis	PE-5-9	AF246597	Peru: Cuzco, Quebrada San Luis	AMNH
			Pedaliodes sp.	PE-5-1	AF246604	Peru: Cuzco, Quebrada San Luis	AMNH
			Steroma sp.	PE-14-3	AF246608	Peru: Cuzco, Rio Lucumayo	AMNH
Danainae	Danaini	Amaurina	*Ideopsis gaura*	NP95-Y318	AF246592	Malaysia: (ex. Naomi Pierce)	MCZ
			Parantica agleoides	SA-1-1	AF246603	Malaysia: Sabah, Gum-Gum	AMNH
		Danaina	*Danaus plexippus*	C-3-8	AF246564	Colombia: Meta, Villavicencio	CUIC
			Tirumala hamata	NSW1	AF246565	Australia: Queensland, Brisbane	AMNH
	Euploeini	Euploeina	*Euploea core*	QL7	AF246566	Australia: Queensland, Brisbane	AMNH
		Itunina	*Anetia briarea*	CU1	AF246579	Cuba: Santiago de Cuba	AMNH
			Lycorea cleobaea	RB241	AF246567	Brazil: Rondonia, Ariquemes	AMNH
Tellervinae	Tellervini		*Tellervo zoilus*	QL1	AF246563	Australia: Queensland, Kirrama State Forest	AMNH
Ithomiinae	Tithoreini		*Elzunia pavonii*	E-12-1	AF246562	Ecuador: Macara	AMNH
			Tithorea harmonia	V20	AF246561	Venezuela: Delta Amacuro, Barrancas	AMNH
	Methonini		*Methona* sp.	RB229	AF246599	Brazil: Rondonia, Ariquemes	AMNH
	Melinaeini		*Athyrtis mechanitis*	RB328	AF246584	Brazil: Rondonia, Ariquemes	AMNH
			Melinaea maenius	RB288	AF014146	Brazil: Rondonia, Ariquemes	AMNH
	Mechanitini		*Mechanitis polymnia*	RB233	AF246560	Brazil: Rondonia, Ariquemes	AMNH
			Scada theaphia	RB238	AF246607	Brazil: Rondonia, Ariquemes	AMNH
			Thyridia psidii	RB314	AF014147	Brazil: Rondonia, Ariquemes	AMNH
	Oleriini		*Oleria aquata*	RB321	AF246558	Brazil: Rondonia, Ariquemes	AMNH
	Napeogenini		*Hyaliris* sp.	PE-10-14	AF246589	Peru: Cuzco, Quebrada Chaupimayo	AMNH
			Hypothyris daphnis	RB237	AF246591	Brazil: Rondonia, Ariquemes	AMNH
			Napeogenes pheranthes	RB235	AF246601	Brazil: Rondonia, Ariquemes	AMNH
	Dircennini		*Dircenna dero*	RB305	AF246571	Brazil: Rondonia, Ariquemes	AMNH
	(new tribe)		*Ceratinia nise*	RB324	AF246559	Brazil: Rondonia, Ariquemes	AMNH
			Pteronymia tucuna	PE-10-12	AF246606	P: Cuzco, Quebrada Chaupimayo	AMNH
	Godyrini		*Hypoleria* sp.	RB358	AF246576	Brazil: Rondonia, Ariquemes	AMNH
Libytheinae	Libytheini		*Libytheana carinenta*	RB276	AF246551	Brazil: Rondonia, Ariquemes	AMNH
OUTGROUP							
Pieridae			*Anthocharis midea*	NY15	AF246543	USA: Pennsylvannia, Holtwood	AMNH
			Pieris rapae	NY12	AFO14158	USA: New York, Ithaca	AMNH
			Delias sp.	NSW2	AF246577	Australia: NSW, Lismore	AMNH

placement of Riodininae will be addressed elsewhere (Campbell *et al.* 2000).

(c) *DNA extraction*

DNA was extracted from individual butterflies following the protocol presented in Brower (1994). Briefly, specimens were macerated in liquid nitrogen, digested with proteinase K in a Tris–EDTA lysis buffer and phenol–chloroform extracted. DNA was precipitated with isopropanol, washed with 70% ethanol and resuspended in water. The DNA stocks were stored at -20°C. The wings and abdomens of each specimen were preserved as vouchers (Brower 1996) and deposited at the American Museum of Natural History, Cornell University Insect Collection or the Museum of Comparative Zoology at Harvard (see table 1).

(d) *PCR and sequencing*

Polymerase chain reaction (PCR) amplifications were performed with the primers and standard conditions presented in Brower & DeSalle (1998). The PCR products were gene cleaned, cycle sequenced in a Perkin-Elmer 9600 and run on an ABI 373 automated sequencer from sense and anti-sense strands. The automated sequence outputs were edited manually and aligned by eye. The aligned data matrix is available on the World Wide Web (http://www.ent.orst.edu/browera/datasets/dataset7.htm) and individual sequences have been submitted to GenBank (see table 1 for the accession codes).

(e) *Data analyses*

Cladistic analyses were performed using PAUP 3.1 (Swofford 1991). All nucleotide sites were weighted equally and gaps were coded as missing data. Tree-bisection-reconnection (TBR) branch swapping was performed on five separate sets of 100 random addition sequences. The results of these searches were pooled and duplicate trees were culled. The branch support (Bremer 1988, 1994; Davis 1995) for selected nodes was calculated using anti-constraints with ten random additions. To assess the ability of the data to imply a self-consistent topology, successive approximations weighting (SAW) (Farris 1969) was performed in PAUP based on the maximum rescaled consistency index for each character among the most parsimonious cladograms from the equal-weighted analyses. Unless SAW yields a subset of the MP cladograms, the results of the respective analyses are not comparable by quantitative means because the two weighting schemes rely on different philosophical interpretations of the parsimony criterion. The most parsimonious (MP) and SAW trees differed in this study and are compared qualitatively here by assessment of topological congruence (Judd 1998).

3. RESULTS

One hundred and three nymphalid taxa representing 12 of the 13 subfamilies and 30 of the 38 tribes recognized by Harvey (1991) and three pierid outgroups were compiled for the *wingless* region. Nine of the sequences have been published previously in Brower & Egan (1997), 12 have been published previously in Brower & DeSalle (1998) and the remaining 85 are new. The resulting aligned data matrix of 106 taxa $\times$ 378 characters contains only three gap regions: two separate autapomorphic 3-base deletions in *Euptoieta* and *Lethe*, respectively and a single 3-base insertion (a putative synapomorphy) present in the three members of Melitaeini sampled (*Phyciodes*, *Eresia* and *Euphydryas*). The dynamics of the sequence divergence in the *wingless* gene are addressed in Brower & DeSalle (1998) and Campbell *et al.* (2000) and will not be discussed further here. In this matrix, 99 nucleotide sites are invariant, 59 vary in a single taxon and the remaining 220 are cladistically informative (potential synapomorphies within the Nymphalidae).

The equal-weighted parsimony analysis yielded 336 equally parsimonious cladograms of 2760 steps. The strict consensus (figure 3) implies monophyly of the family Nymphalidae, the subfamilies Heliconiinae, Nymphalinae, Brassolinae, Danainae, Ithomiinae, Charaxinae and Apaturinae and numerous less inclusive clades identified in Harvey's (1991) classification. The branch support values for many of these clades are indicated in figure 3. The Limenitidinae and Satyrinae appear as unresolved paraphyletic groups. Individual clades will be discussed further below.

SAW resulted in a single stable topology after nine iterations (figure 4). This cladogram is 2803 steps long when the character weights are restored to 1 (43 steps longer than the MP cladograms under equal weights). Eighty-one of the 102 nodes are congruent between the SAW cladogram and the MP consensus tree ('congruent' meaning that the SAW tree either exhibits the same clades as the MP consensus tree or provides resolution to its polytomies). With the exception of two basal nodes, the incongruent patterns of grouping are localized within three clades: Heliconiina, Danainae plus Ithomiinae and Satyrinae.

4. DISCUSSION

(a) *Quality*

The average informative character changes state more than 12 times on the most parsimonious trees and some change up to 35 times. Theoretically, such rampant homoplasy suggests that these data are not valuable as a source of evidence for inferring the patterns of relationship among the groups represented (Sanderson & Donoghue 1996). Hillis (1996) concluded from a simulation study that, although larger sets of taxa can resolve relationships with shorter sequences, approximately an order of magnitude more sequence per taxon than was included in this matrix is required to infer the simulated branching pattern accurately. These data offer an empirical refutation of those claims.

The historical course of evolutionary divergence is unknowable and empirical systematists eschew inductive summary measures of accuracy or confidence in favour of qualitative assessment of the stability and corroboration of prior hypotheses. The results presented here are stable to the extent that the inferred MP topology is largely self-consistent under the implied weights (Judd 1998). In general, SAW reinforced the groups implied by the data in the MP tree. The incongruence between the MP and SAW trees within Heliconiina occurs among weakly supported clades and neither of those implied branching orders matches the pattern resulting when taxa are sampled more intensively at the same locus (Brower & Egan 1997). Within the Ithomiinae plus Danainae clade, three instances of incongruence result from the instability of

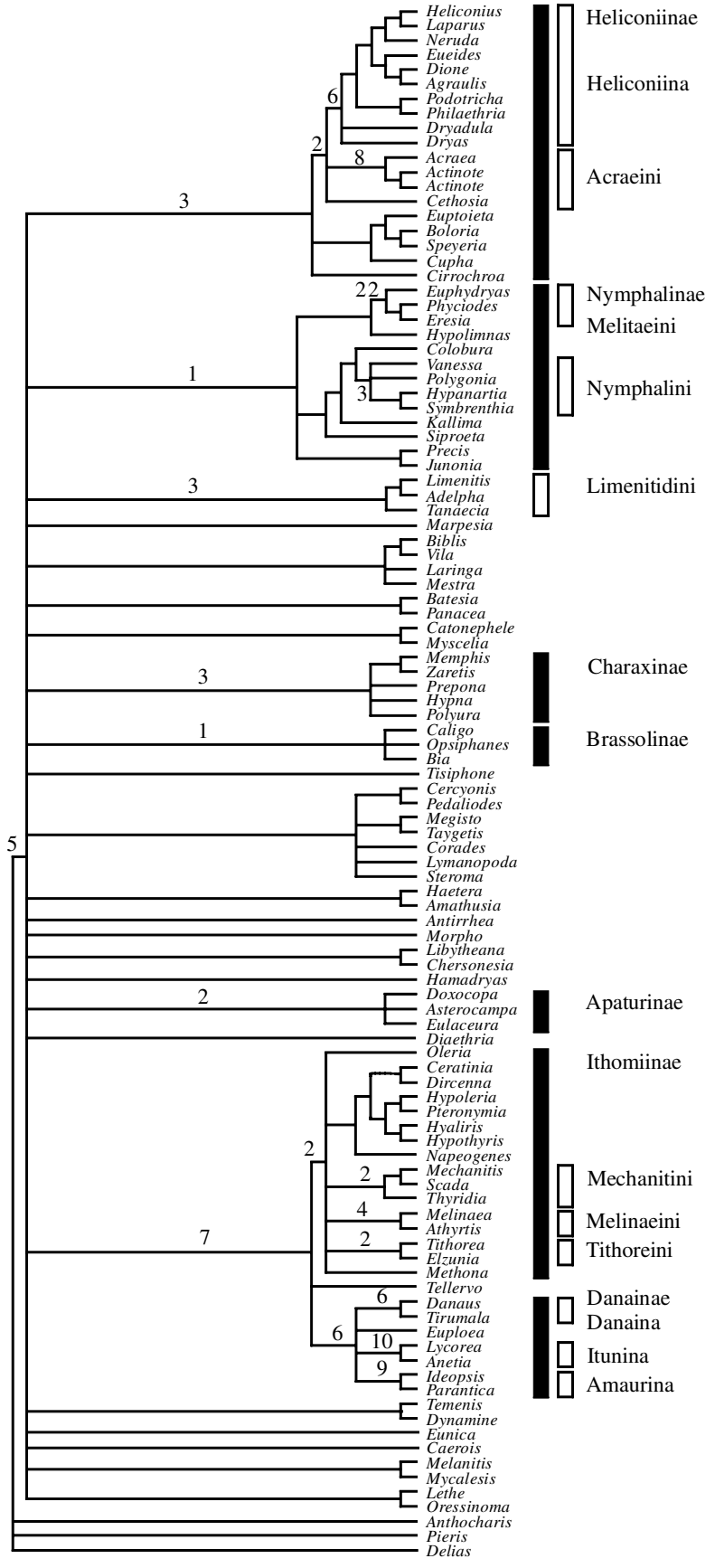

Figure 3. Strict consensus of 336 equally parsimonious cladograms for the *wingless* data matrix with equal weights (length = 2760, CI = 0.189 and RI = 0.531). Traditionally recognized subfamilies and tribes (or subtribes) appearing as clades here are indicated by black and white bars, respectively. The branch support values are indicated for these groups.

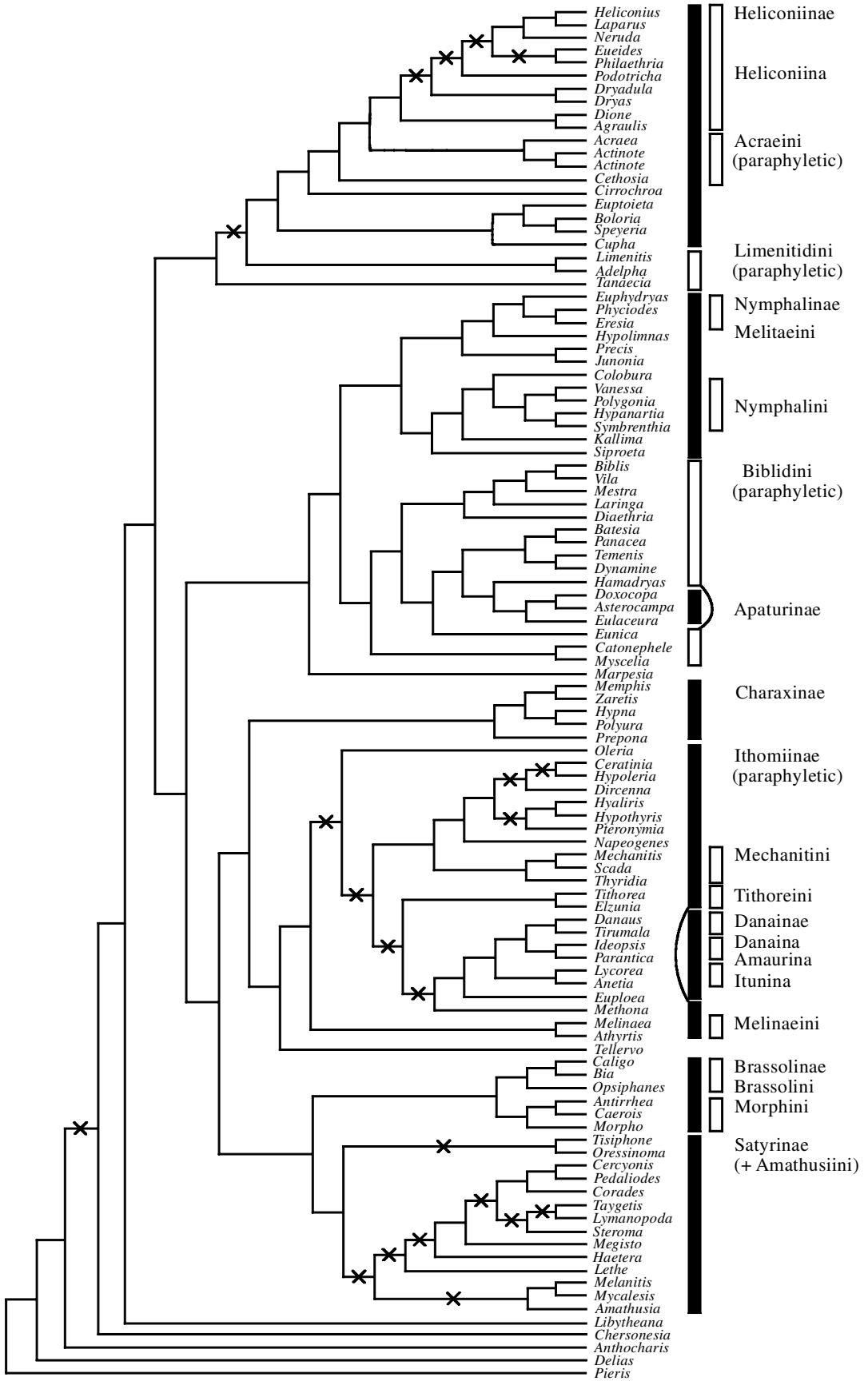

Figure 4. The most parsimonious cladogram resulting from SAW (with weights restored to unity) (length = 2803, CI = 0.18 and RI = 0.521). Traditionally recognized subfamilies and tribes (or subtribes) appearing as clades are indicated by black and white bars, respectively. Three paraphyletic grades are marked as well: arches link disjunct sections of the Biblidini and Ithomiinae. Crosses mark the nodes incongruent with the most parsimonious trees discovered with equal weighting.

Hypoleria and the remaining four result from the nesting of the Danainae between the basal and derived Ithomiinae in the SAW tree. The incongruence in the Satyrinae is not attributable to the instability of specific taxa or clades and may instead result from insufficient sampling of this extremely diverse group (which represents nearly one-third of all Nymphalidae) (Ackery *et al.* 1999).

The corroboration of prior systematic hypotheses also confers some degree of plausibility to the current results. The MP consensus implies monophyly in seven out of ten subfamilies (Tellervinae and Libytheinae with a single representative each included are also trivially monophyletic). The traditional subfamilies not supported here have long been considered artificial (e.g. Limenitidinae) or are not diagnosed by morphological synapomorphies (e.g. Satyrinae to the exclusion of Brassolinae) (Ackery *et al.* 1999). Thus, without the 'aid' of special process models and mathematical corrections, this short gene region generally corroborates higher taxa supported by evidence from other character systems and fails to corroborate those which are not so supported.

(b) *Taxonomic implications*

The sister relationship between the Heliconiina and Acraeini implied here necessitates reinterpretation of the female abdominal 'stink clubs' (Müller 1886) from the synapomorphy uniting the Heliconiini *sensu* Harvey (1991) to a synapomorphy uniting the Heliconiinae but lost in the Acraeini. D. Harvey (personal communication) suggested that the loss may be associated with the simplified courtship performed by acraeines, which exhibit sperm priority enforced by a sphragis (Smith 1984). The *ad hoc* invocation of stink club homoplasy is compensated for by the more parsimonious occurrence of the capacity to sequester or synthesize cyanoglucosides (Nahrstedt & Davis 1981), which, according to this hypothesis, is uniquely derived among the Heliconiina plus Acraeini.

The large and diverse 'subfamily Limenitidinae' has been long and almost unanimously recognized as an unnatural assemblage (Müller 1886; Scott & Wright 1990; Harvey 1991; Ackery *et al.* 1999), a view corroborated by the *wingless* data. The SAW tree suggests affinities between the Limenitidini and Heliconiinae, and between the Neotropical Biblidini and Nymphalinae. Oddly, the apaturines sampled nest well inside the Biblidini, although morphological features imply their closer affinity to the Charaxinae (Ackery *et al.* 1999). This is the least orthodox result from the *wingless* data and suggests that more detailed sampling of Old World representatives of both the Apaturinae and 'Limenitidinae' is necessary.

Although no unique morphological synapomorphy has been found to unite the Danainae s. l. (= Danainae plus Ithomiinae plus Tellervinae), most authors have considered them to form a monophyletic group (Bates 1861; Clark 1947; Ehrlich 1958; Ackery 1984, 1988). Ackery *et al.* (1999) argued that the use of pyrrolizidine alkaloids for courtship pheromone synthesis (and possibly for chemical defence) (Brower 1984) is a more promising shared trait than anything yet described from morphology. The Danainae s.l. is the most strongly supported major clade in this study, corroborating Ackery *et al.*'s (1999) ranking of the Ithomiinae plus Tellervinae plus Danainae as a single subfamily. The MP tree implies reciprocal monophyly of the Ithomiinae s. s. and Danainae s. s., but the Ithomiinae become paraphyletic in the SAW tree. It is intriguing that the Apocynaceae-feeding *Tithorea* and *Elzunia* are basal to the Apocynaceae and Asclepiadaceae-feeding 'Danainae' in the latter hypothesis. Both cladograms support the hypothesis that Apocynaceae-feeding, aposematic larvae are primitive characteristics of the Danainae s. l. (Edgar *et al.* 1974; Brown 1987).

Ackery *et al.* (1999) resurrected Ehrlich's (1958) inclusive Morphinae, encompassing both the Palaeotropical Amathusiinae and the Neotropical Brassolinae and Morphinae of various authors. Among the morphine and satyrine lineages sampled, only the monophyly of the Brassolinae (which clearly includes the formerly enigmatic genus *Bia*) (*vide* Miller 1968; DeVries *et al.* 1985) is supported by the MP cladogram. However, the SAW tree supports the monophyly of numerous traditionally recognized clades, including the Brassolinae, Morphini and the sister relationship of the Morphinae s. l. and Satyrinae, but *Amathusia* (the only amathusiine sampled) joins a basal clade with Old World satyrines. This result implies the parallel evolution of features associated with large size in the Neotropical and Palaeotropical realms. However, given the diversity of this clade, these patterns are best viewed merely as intriguing hypotheses to stimulate further research.

The snout butterflies (Libytheinae or Libytheidae) are often viewed as the sister taxon of all other Nymphalidae due to the plesiomorphic presence of functional forelegs in the females (Ehrlich 1958; Scott & Wright 1990; Ackery *et al.* 1999). The SAW tree implies a basal position for *Libytheana*, with *Chersonesia* (Cyrestidini = Marpesiini) as its immediate outgroup and the sister taxon to the Libytheinae plus all other nymphalids. The other cyrestidine, *Marpesia*, is sister to the Nymphalinae plus Biblidini, suggesting that the tribe may represent a polyphyletic basal assemblage. Both *Libytheana* and *Chersonesia* exhibit enlarged labial palpi (see Ackery *et al.* (1999) for comments on the range of variation in this character among nymphalid taxa).

(c) *Prospects*

This study supports the hypothesis (Brower & DeSalle 1998; Campbell *et al.* 2000) that the small fragment of the *wingless* gene examined provides a useful source of characters for cladistic inference among butterfly subfamilies, tribes and genera. Given the relatively small number of gene regions that have been employed with success in the molecular systematics of insects to date (Caterino *et al.* 2000), the empirical success of the *wingless* gene represents a significant addition to the battery of markers currently available. Although phylogenetic hypotheses based on data from single sources are potentially subject to bias or 'inaccuracy', the dense taxonomic sampling employed here appears to permit synapomorphy to overcome high levels of homoplasy and corroborate clades well supported by data from morphology. The initial phylogenetic structure presented here provides a framework for subsequent investigation of particular nymphalid clades in greater detail. The taxa in particular need of more intensive sampling or improved resolution are the Limenitidini, Satyrinae, Amathusiini and Ithomiini.

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