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Molecular phylogeny and systematics of the Pieridae (Lepidoptera: Papilionoidea): higher classification and biogeography

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The systematic relationships of the butterfly family Pieridae are poorly understood. Much of our current understanding is based primarily on detailed morphological observations made 50–70 years ago. However, the family and its putative four subfamilies and two tribes, have rarely been subjected to rigorous phylogenetic analysis. Here we present results based on an analysis of molecular characters used to reconstruct the phylogeny of the Pieridae in order to infer higher-level classification above the generic level and patterns of historical biogeography. Our sample contained 90 taxa representing 74 genera and six subgenera, or 89% of all genera recognized in the family. Three complementary approaches were employed: (1) a combined analysis of a 30 taxon subset for sequences from four gene regions, including elongation factor-1 alpha (*EF-1α*), *wingless*, cytochrome oxidase subunit I (*COI*), and *28S* (3675 bp, 1031 parsimony-informative characters), mainly to establish higher-level relationships, (2) a single-gene analysis of the 90 taxon data set for sequences from *EF-1α* (1066 bp, 364 parsimony-informative characters), mainly to establish lower-level relationships, and (3) an all available data analysis of the entire data set for sequences from the four genes, to recover both deep and shallow nodes. Analyses using maximum parsimony, maximum likelihood and Bayesian inference provided similar results. All supported monophyly for the four subfamilies but not for the two tribes, with the Anthocharidini polyphyletic and the Pierini paraphyletic. The combined and all available data analyses support the following relationships among the subfamilies: ((Pseudopontiinae + Dismorphiinae) + (Coliadinae + Pierinae)), corroborating Ehrlich's 1958 phenetic hypothesis. On the basis of these analyses, and additional morphological and life history evidence, we propose a reclassification of the subfamily Pierinae into two tribes (Anthocharidini *s.s.*, Pierini *s.s.*) and two informal groups (*Colotis* group, *Leptosia*), with the tribe Pierini *s.s.* subdivided into three subtribes (Appiadina, Pierina, Aporiina) and three genera (*Elodina*, *Dixeia*, *Belenois*) of uncertain status (*incertae sedis*). The combined and all available data analyses support the following relationships among the Pierinae: (*Colotis* group + Anthocharidini *s.s.* + *Leptosia* + (*Elodina* + ((*Dixeia* + *Belenois*) + Appiadina + Pierina + Aporiina))). Application of a molecular clock calibrated using fossil evidence and semiparametric rate smoothing suggests that divergence between the Pierina and Aporiina occurred no later than the Palaeocene (> 60 Myr). The minimum estimate for the age of the crown-group of the Pieridae was 112–82 Myr, with a mean of 95 Myr. A historical biogeographical hypothesis is proposed to explain the present-day distribution of the clade Pseudopontiinae + Dismorphiinae, which argues for an origin of the two subfamilies in western Gondwana (Africa + South America) during the Late Cretaceous. © 2006 The Linnean Society of London, *Zoological Journal of the Linnean Society*, 2006, **147**, 239–275.

ADDITIONAL KEYWORDS: Coliadinae – Cretaceous – Dismorphiinae – Dispersal – Gondwana – Pierinae – Pseudopontiinae – Vicariance.

INTRODUCTION

The Pieridae are among the most poorly understood butterfly families within the Papilionoidea in terms of their higher-level systematics and classification. Indeed, almost 20 years ago Robbins & Henson (1986)

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emphasized that '. . . there is glaring need for a worldwide treatment of the pierines'. Yet pierids have played an important role in evolutionary studies (e.g. Courtney, 1986; Watt, Donohue & Carter, 1996; Brunton, 1998; Stavenga *et al*., 2004; Kemp, Rutowski & Mendoza, 2005) and include species of major economic significance, such as the cabbage whites (*Pieris*) and sulphurs (*Colias*). Unlike several other families, or subfamilies/tribes within other families, such as the Papilionidae (Miller, 1987; Caterino *et al*., 2001; Braby, Trueman & Eastwood, 2005), Nymphalidae (Ackery & Vane-Wright, 1984; Brower, 2000; Penz & Peggie, 2003; Wahlberg, Weingartner & Nylin, 2003; Freitas & Brown, 2004) and Riodinidae (Harvey, 1987; Campbell, Brower & Pierce, 2000), the Pieridae and its putative subfamilies and tribes have rarely been subjected to rigorous phylogenetic analyses of morphological or molecular data. Although Janz & Nylin (1998; pers. comm) published a phylogeny of the Pieridae based on 39 terminal taxa (with five taxa each comprising the combination of two genera), their cladistic analysis of previously published morphological data dealt with only two subfamilies (Coliadinae and Pierinae) and provided little resolution within the major clades.

Although the monophyly of the Pieridae is well established (Kristensen, 1976; de Jong, Vane-Wright & Ackery, 1996; Ackery, de Jong & Vane-Wright, 1999; Wahlberg *et al*., 2005), the phylogenetic position of pierids in relation to the other butterfly families is uncertain (Robbins, 1988; Vane-Wright, 2003; Wahlberg *et al*., 2005). The Pieridae are considered to be either the sister family to the Papilionidae (Ehrlich, 1958; Scott, 1985), or, more probably, sister to Nymphalidae + (Riodinidae + Lycaenidae) (Kristensen, 1976; de Jong *et al*., 1996; Weller, Pashley & Martin, 1996; Ackery *et al*., 1999; Wahlberg *et al*., 2005). However, unlike some other families (e.g. Nymphalidae, Lycaenidae), the integrity of the Pieridae as a natural group has never been in dispute. Synapomorphies supporting monophyly include wing scales with pterin pigments, foretarsus with distinctly bifid claws, outer edge of forewing third axillary with tooth, and lateral plates of pronotum not fused medially (Ackery *et al*., 1999; Vane-Wright, 2003). The family is worldwide in distribution, and contains approximately 1100 species (Robbins, 1982; Ackery *et al*., 1999; Vane-Wright, 2003) currently arranged in 98 lower taxa (83 genera, 15 subgenera) (Braby, 2005). The two most speciose genera are *Delias* Hübner and *Catasticta* Butler, but putative radiations have also occurred in *Colias* Fabricius, *Eurema* Hübner, *Colotis* Hübner, *Mylothris* Hübner and the *Tatochila* Butler group of genera. The adult butterflies are of medium size, typically white, orange or yellow in colour, and the pupal morphology is highly distinctive (Chapman, 1895; Talbot, 1939; Mosher, 1969). Many species migrate and/or exhibit seasonal phenotypic variation.

Much of our current understanding of the higher classification and interrelationships of the Pieridae has been based on detailed morphological work conducted 50–70 years ago (Klots, 1933; Ehrlich, 1958). The family is currently arranged in four subfamilies (Pseudopontiinae, Dismorphiinae, Coliadinae, Pierinae), with the Pierinae usually divided into two tribes (Pierini, Anthocharidini) (Ackery, 1984; Bridges, 1988; de Jong *et al*., 1996; Ackery *et al*., 1999; Vane-Wright, 2003). The Pseudopontiinae are monotypic, containing the single monobasic genus *Pseudopontia* Plötz from central and western Africa. The Dismorphiinae are relatively small, comprising approximately 60 species in seven genera and, with the exception of the single disjunct genus *Leptidea* Billberg in the Palaearctic, are found predominantly in South America, with a smaller representation in Central America. The Coliadinae comprise approximately 220 species in 18 genera, and are cosmopolitan, although the greater proportion of species occurs in tropical latitudes. The Pierinae, also cosmopolitan, are by far the largest subfamily, containing approximately 840 species in 57 genera (Ackery *et al*., 1999; Braby, 2005), and thus make up between two-thirds and three-quarters of the total species and generic diversity of the family.

Although the four subfamilies have remained relatively stable in terms of their composition, considerable uncertainties exist in the systematics and phylogenetic relationships among the higher taxa (Fig. 1). Klots (1933), building on his own earlier work (Klots, 1929) as well as that of Butler (1870), Scudder (1875b), Dixey (1894, 1932), Grote (1900), Röber (1908–09), and Aurivillius (1910) among others, recognized three subfamilies, with one of these, the Pierinae, consisting of three tribes (Euchloini, Rhodocerini, Pierini). The Rhodocerini and Euchloini have since proven to be subjective synonyms of the Coliadini (Talbot, 1935) and Anthocharidini (Bridges, 1988), respectively. Klots' intuitive phylogeny (Fig. 1A) showed that the Dismorphiinae and Pseudopontiinae were closely related and formed the sister group to the Pierinae. Clench (1955) followed Klots and recognized the same three subfamilies, but noted that the Pseudopontiinae were 'intermediate' between the two other subfamilies.

Ehrlich's (1958) phenetic tree (Fig. 1B) was similar to that of Klots, except that the tribe Coliadini was treated as a distinct subfamily, the Coliadinae, in accordance with Talbot (1935), and phylogenetically removed from, and sister to, the Pierinae. Scott (1985) reached the same conclusion as Ehrlich (1958) with regard to the classification and relationships of the pierid subfamilies. In both Ehrlich's and Scott's classifications, the Pierinae were not further subdivided

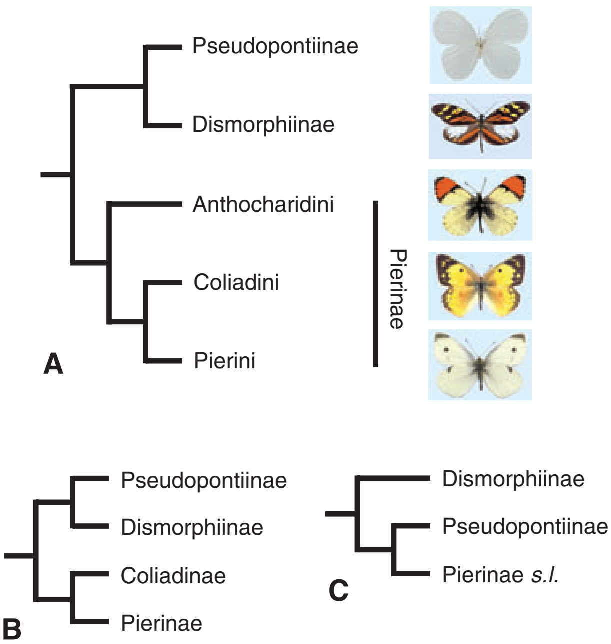


Figure 1. Three different phylogenetic hypotheses of the higher classification of the Pieridae. A, Klots (1933). B, Ehrlich (1958). C, Venables (1993).

into tribes. The only other broad-based study of the higher classification of the Pieridae is the work of Venables (1993), who made a preliminary cladistic analysis of a larger data set (43 genera) and incorporated Klots' morphological characters. Her cladogram showed that the Coliadinae were paraphyletic, whereas the Pierinae were largely monophyletic except they contained the coliadine taxon *Nathalis* Boisduval. On this basis, Venables (1993) tentatively subsumed the Coliadinae within the Pierinae so that her classification of higher taxa was essentially similar to that of Klots (1933) and Clench (1955), except that the Dismorphiinae were sister to Pseudopontiinae + Pierinae *s.l.* (Fig. 1C).

Several other studies have dealt with the higher systematics of the Pieridae, but these are more limited in scope, as only small numbers of taxa or characters were analysed. Ehrlich & Ehrlich (1967) analysed five taxa within their broader phenetic study of the Papilionoidea. The relationships among pierid subfamilies were found to be variable, and the family grouped inconsistently with the Papilionidae. Geiger (1981) studied phenetic relationships among 24 European taxa using enzyme electrophoretic data. His results showed a clear biochemical distinction between the subfamilies Dismorphiinae, Coliadinae, and Pierinae. However, separation of the tribes Pierini and Anthocharidini within the Pierinae was much weaker. In a study of butterflies and their host plants, Janz & Nylin (1998) published a simplified version of the phylogeny of the Papilionoidea, based on the data sets of Ehrlich & Ehrlich (1967) and Geiger (1981) for the Pieridae. Their cladistic analysis of 39 terminal taxa in the Coliadinae and Pierinae recovered the two subfamilies as reciprocally monophyletic, although their study did not include *Pseudopontia*, and the Dismorphiinae were used as a single outgroup taxon. Cheong (1990) studied the female genitalia from 90 species representing 23 genera. However, too few independent morphological characters (a total of 15) were available to infer phylogenetic relationships. Lukhtanov (1991) studied chromosome relationships and noted that the Dismorphiinae, Coliadinae, and Anthocharidini, but not Pierini, all had the same basic number ($n = 31$). de Jong *et al*. (1996) and Ackery *et al*. (1999) included seven exemplar pierid species in their higher-level cladistic analysis of morphological characters of the butterflies. They provisionally maintained the four subfamilies but noted that relationships between them were uncertain. The Dismorphiinae (*Dismorphia*) were sister to the six other species, but the Pierinae appeared to be paraphyletic as the exemplar genera (*Pieris*, *Delias*, *Euchloe*) rarely grouped together and the subfamily included both the Coliadinae (*Eurema* + *Colias*) and Pseudopontiinae as subordinate taxa. Moreover, they were unable to find any convincing synapomorphies for either the Coliadinae or the Pierinae. Pollock *et al*. (1998) sequenced a small fragment of mitochondrial cytochrome oxidase subunit I (*COI*) and ribosomal *12S* + *16S* genes for 21 taxa (mainly *Colias*) representing eight pierid genera. In contrast to de Jong *et al*. (1996), their molecular phylogenetic analysis recovered the Coliadinae (four genera) as a strongly supported monophyletic group and sister to the four other genera (*Anthocharis*, *Euchloe*, *Pieris*, *Pontia*). Similarly, T. Yamauchi, O. Yata & A. Venables (unpubl. data), conducted a phylogenetic analysis of adult morphological characters representing all genera and also recovered the Coliadinae as a monophyletic group.

Part of the uncertainty and lack of agreement among workers in the interpretation of phylogenetic relationships and systematic status of higher taxa of the Pieridae may lie in the fact that Klots' (1933) original systematic classification and ideas of evolutionary relatedness were incongruent with one another. In Figure 2, we have attempted to reconstruct Klots' intuitive phylogeny as a cladogram, according to his generic revision and systematic framework, and hypothetical chart of evolution of the higher taxa (subfamilies, tribes, and generic groups). Klots proposed several 'natural' groupings based on the examination of numerous morphological characters of the male genitalia and wing venation. However, he expressed considerable uncertainty about the placement of 12

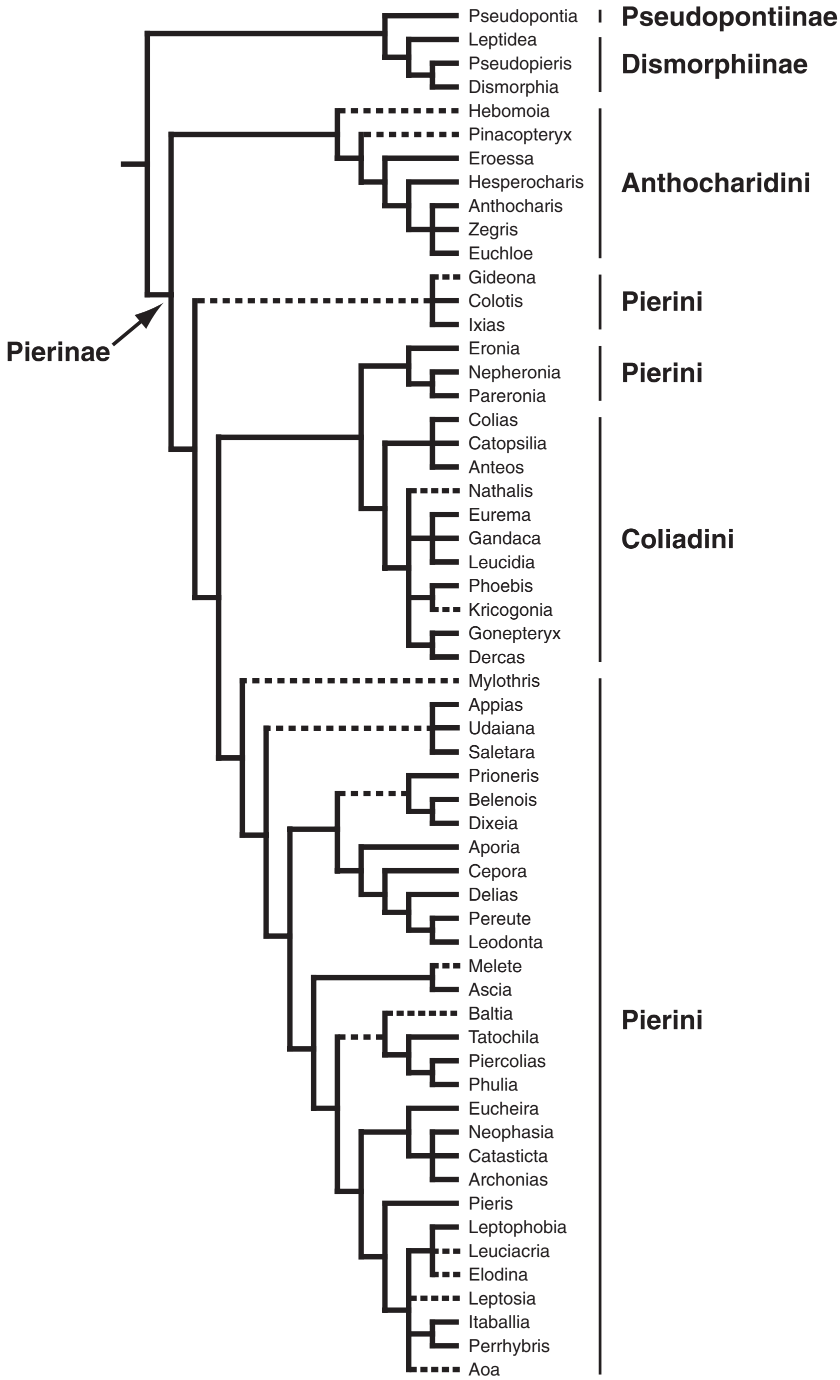


Figure 2. Klots' (1933) intuitive phylogeny of the Pieridae, reconstructed from his generic revision and systematic classification, and hypothetical chart of evolution of the subfamilies and main stock of the Pierinae. Dashed lines indicate uncertainty in the phylogenetic position of genera or groups of genera.

genera, namely *Hebomoia* Hübner and *Pinacopteryx* Wallengren in the Anthocharidini; *Nathalis* and *Kricogonia* Reakirt in the Coliadini; and *Gideona* Klots, *Mylothris*, *Melete* Swainson, *Baltia* Moore, *Leuciacria* Rothschild & Jordan, *Elodina* Felder & Felder, *Leptosia* Hübner and *Aoa* de Nicéville in the Pierini. Moreover, the Pierini were not envisaged as a monophyletic entity. Klots (1933) regarded the genera *Colotis* and *Ixias* Hübner to be 'derived' from the Anthocharidini in an evolutionary sense, but nonetheless classified them with the Pierini; he also considered the *Eronia* group of genera (*Eronia*, *Nepheronia*, *Pareronia*) to be more closely related to the Coliadini than to the Pierini, in which he placed them.

The goal of this study was to reconstruct the phylogeny of the Pieridae using molecular characters and exemplar species representing nearly all of the currently recognized lower taxa (genera, subgenera). We employed three complementary approaches to investigate the monophyly and relationships of the extant taxa in the family: (1) a combined analysis of fragments of four genes, namely nuclear elongation factor-1 alpha (*EF-1α*), nuclear *wingless*, mitochondrial *COI*, and ribosomal 28S (*28S*), of a 30 taxon data set to establish higher-level phylogenetic patterns at deeper nodes (subfamilies, tribes); (2) a single-gene (*EF-1α*) analysis of a 90 taxon data set to infer lower-level phylogenetic patterns at more shallow nodes (genera, subgenera); and (3) an all available data analysis of the entire 90 taxon data set using all four genes to recover both deep and shallow nodes. The phylogenetic hypothesis based on the combined and all available data analyses was then used as a framework to revise the higher classification of the family and to explore patterns of historical biogeography. We also estimated the age of divergence events calibrated with fossil evidence for the *EF-1α* analysis.

MATERIAL AND METHODS

Molecular markers

Currently, there are relatively few genes available for reconstructing arthropod divergences of Mesozoic age (Caterino, Cho & Sperling, 2000). *EF-1α* is a nuclear protein-encoding gene involved in the translation of mRNA to protein, specifically the binding of aminoacyl-tRNA to the ribosome (Kamie *et al*., 1993; Palumbi, 1996). It evolves relatively slowly, insertion/deletions are absent and it provides relatively unambiguous alignment (Cho *et al*., 1995; Caterino *et al*., 2000; Sperling, 2003). For Lepidoptera that have been studied, most of the phylogenetic information lies in the third codon position, most substitutions are synonymous, pairwise differences between closely related taxa are small, and saturation levels (transversion/transition ratios) tend be low (Cho *et al*., 1995; Mitchell *et al*., 1997; Roger *et al*., 1999). Because first and second positions are highly conserved, nonsynonymous changes are rare, but third positions frequently show saturation. These properties render the gene as a useful marker for resolving the more recent divergence events (e.g. mid-Tertiary) of insects, especially Lepidoptera, which have lost all introns and which have only a single copy of the gene (i.e. there are no paralogous copies) (Cho *et al*., 1995; Danforth & Shuqing, 1998). In this group, *EF-1α* has proven useful in reconstructing phylogenies at the 'intermediate' systematic levels, such as genus and tribe (Cho *et al*., 1995; Mitchell *et al*., 1997; Mitchell, Mitter & Regier, 2000; Friedlander *et al*., 1998; Reed & Sperling, 1999; Caterino *et al*., 2001; Monteiro & Pierce, 2001; Morinaka, Miyata & Tanaka, 2002; Wahlberg *et al*., 2003).

Wingless is another protein-encoding gene involved in wing pattern formation. It belongs to the *wnt* gene family, whose paralogs are easily distinguishable, and shows a relatively rapid rate of substitution. In Lepidoptera, it has been used successfully for resolving phylogenetic relationships at both higher and lower systematic levels (Brower & Egan, 1997; Brower & DeSalle, 1998; Brower, 2000; Campbell *et al*., 2000; Wahlberg *et al*., 2003).

COI is a widely used mitochondrial protein-encoding gene. Because it is faster evolving, third codon positions quickly become saturated at the deeper levels of divergence, but it is relatively conserved compared with other mitochondrial genes (e.g. Simon *et al*., 1994; Hillis *et al*., 1996a; Palumbi, 1996). In molecular phylogenetic studies of Lepidoptera it has shown great utility for resolving shallow (recent) divergence events (Caterino *et al*., 2000; Sperling, 2003).

The rDNA *28S* marker has been used successfully for reconstructing phylogenetic relationships among many invertebrate taxa (Caterino *et al*., 2000). In Lepidoptera, the unambiguously aligned regions are highly conserved, rendering the gene especially useful for recovering deeper (old) divergence levels (Weller *et al*., 1992, 1994, 1996; Weller & Pashley, 1995).

Because the four genes evolve at different rates, combining all of them will probably increase the phylogenetic estimation and resolution of most, if not all, nodes, provided the data partitions are congruent (Caterino *et al*., 2000). In Lepidoptera, several recent studies have demonstrated improved resolution based on standard measures of nodal support at both deep and shallow levels of divergence in a combined analysis of nuclear, mitochondrial or ribosomal genes (Monteiro & Pierce, 2001; Caterino *et al*., 2001; Wahlberg *et al*., 2003; Kandul *et al*., 2004; Zakharov, Caterino & Sperling, 2004).

Taxon sampling

For the combined analysis, 26 exemplars (Table 1) were sampled, representing the entire systematic and phylogenetic diversity of the family, based on previous systematic hypotheses and the results from the *EF-1α* larger taxon data set. This data set also included four taxa (*Leptosia*, *Elodina*, *Dixeia*, *Belenois*) of uncertain status. For the single-gene (*EF-1α*) analysis, 86 exemplar species of Pieridae (Table 1) were sampled from

Table 1. Exemplar taxa used in this study, with collection localities and GenBank accession numbers. Voucher numbers refer to those specimens deposited in the Museum of Comparative Zoology (MCZ), Harvard University, USA. The nomenclature of pierid taxa follows Braby (2005)

			GenBank accession no.			
Taxon	MCZ voucher no.	Locality	*EF-1α*	*wingless*	*COI*	*28S*
Pieridae						
Anteos clorinde	MFB-00-P188	COSTA RICA. La Guacima	AY870570			
Anthocharis belia	RV-00-T884	SPAIN. Vallgrassa, Parc del Garraf, Barcelona	AY870560	AY954604	AY954574	AY954544
Aoa affinis	MFB-00-P469	INDONESIA. Karaentha, S. Sulawesi	AY870585	AY954608	AY954578	AY954548
Aphrissa statira	MFB-00-P199	COSTA RICA. Río Poas, La Garita	AY870572			
Aporia (Aporia) crataegi	RV-00-T758	SPAIN. Universitat Autònoma de Barcelona, Cerdanyola del Vallés, Barcelona	AY870529			
Aporia (Metaporia) agathon	MFB-00-P481	CHINA. Near Lijang, Yunnan Province	AY870590			
Appias (Catophaga) paulina	MFB-00-P159	AUSTRALIA. Buderim Forest Park, Buderim, Qld	AY870588			
Appias (Glutophrissa) drusilla	MFB-00-P195	COSTA RICA. Río Poas, La Garita	AY870525	AY954609	AY954579	AY954549
Appias (Phrissura) aegis	MFB-00-P491	PHILIPPINES. Camiguin Island, near Luzon.	AY870589			
Archonias brassolis	MFB-00-P263	COSTA RICA. Río Orosi, Cartago Province	AY870534			
Ascia monuste	MFB-00-P170	COSTA RICA. Colón	AY870582			
Baltia butleri	MFB-00-P492	INDIA. Tagalanga-la, Kashmir	AY870583			
Belenois java	DL-00-Q546	AUSTRALIA. Waikeri, SA	AY870593	AY954617	AY954587	AY954557
Catasticta teutila	MFB-00-P247	COSTA RICA. Copey	AY870532	AY954614	AY954584	AY954554
Catasticta cerberus	MFB-00-P266	COSTA RICA. Cerro de la Muerte, Villa Mills	AY870533			
Catopsilia pomona	MFB-00-P059	PAPUA NEW GUINEA. WEI, Wau, Morobe Province	AY870569			
Cepora perimale	MFB-00-P131	AUSTRALIA. Brisbane Forest Park, Qld	AY870524			
Charonias eurytele	MFB-00-P395	PERU. 9 km south of Tingo María	AY870531			
Colias eurytheme		CANADA. Ontario	AF173400*			
Colias philodice	NP-98-U268	USA. Harvard Forest, MA	AY870565	AY954600	AY954570	AY954540
Colotis hetaera	SC-01-T434	KENYA. Kibwezi	AY870520	AY954601	AY954571	AY954541
Cunizza hirlanda	MFB-00-P398	PERU. 9 km south of Tingo María	AY870556			
Delias aganippe	MFB-97-U344	AUSTRALIA. Mt. Ainslie, ACT	AY870511			
Delias belladonna	DL-01-N104	THAILAND. Doi Inthanon NP, Chiang Mai	AY870510			
Dercas gobrias	MFB-00-P475	MALAYSIA. Mt. G Serapi, Sarawak	AY870512			
Dismorphia zathoe	MFB-00-P231	COSTA RICA. Monteverde	AY870578	AY954596	AY954566	AY954536
Dixeia charina	SC-01-T400	KENYA. Kibwezi	AY870523	AY954619	AY954589	AY954559
Elodina angulipennis	MFB-00-P133	AUSTRALIA. Brisbane Forest Park, Qld	AY870522	AY954618	AY954588	AY954558
Enantia lina	MFB-00-P405	PERU. 9 km south of Tingo María	AY870576			
Eroessa chiliensis	MFB-00-P437	CHILE. Parque Nacional Puyehue, Los Lagos Región	AY870554	AY954605	AY954575	AY954545
Eronia cleodora	SC-01-T424	KENYA. Kibwezi	AY870551			
Eucheira socialis	MFB-00-P284	MEXICO. Kilometer 64 on Highway 40, town of El Madrono, Durango	AY870535			
Euchloe ausonides	AF-94-A022	USA. Naked Hills, Gothic, CO	AY870558			

Eurema (Eurema) mexicana	DW-92-Z084	USA. South fork of Cave Creek, Chiricahua Mtns., Cochise Co., AZ	AY870563	AY954598	AY954568	AY954538
Eurema (Terias) hecabe	MFB-00-P036	PAPUA NEW GUINEA. WEI, Wau, Morobe Province	AY870587			
Gandaca harina	MFB-00-R059	BORNEO. Sungai Turan, north-east of Kalimantan	AY870514			
Ganyra josephina	MFB-00-P201	COSTA RICA. Río Poas, La Garita	AY870539			
Gideona lucasi	GA-93-Z024	MADAGASCAR. Kirindy Forest	AY870521			
Gonepteryx rhamni	RV-00-R023	SPAIN. Cànoves (Vallés Oriental), Barcelona	AY870568			
Hebomoia glaucippe	NP-95-Y258	MALAYSIA. Fraser's Hill, Pahang	AY870581			
Hesperocharis crocea	MFB-00-P268	COSTA RICA. Alajuela	AY870555	AY954606	AY954576	AY954546
Hypsochila wagenknechti	MFB-00-P432	CHILE. Farellones, Región Metropolitana	AY870544			
Infraphulia ilyodes	MFB-00-P416	CHILE. Parinacota, Parque Nacional Lauca, Tarapaca	AY870542			
Itaballia demophile	MFB-00-P277	COSTA RICA. Near Río Virilla, 6 km north-west of Colón	AY870584			
Ixias pyrene	DL-00-Q581	THAILAND. Bang Khantak, Samut Songkram	AY870552	AY954602	AY954572	AY954542
Kricogonia lyside	MFB-00-P215	COSTA RICA. Parque Nacional Santa Rosa	AY870566			
Leodonta tellane	MFB-00-P265	COSTA RICA. Río Orosi	AY870537			
Leptidea sinapis	RV-00-T760	SPAIN. Vallgrassa, Parc del Garraf, Barcelona	AY870573	AY954595	AY954565	AY954535
Leptophobia aripa	MFB-00-P189	COSTA RICA. La Guacima	AY870546			
Leptosia nina	NP-95-Y248	MALAYSIA. Fraser's Hill, Pahang	AY870519	AY954616	AY954586	AY954556
Leuciacria acuta	MFB-00-P468	INDONESIA. Pass Valley Wamena, Papua.	AY870591			
Leuciacria olivei	MFB-00-S095	PAPUA NEW GUINEA. Schleinitz Mts, New Ireland	AY870592			
Leucidia brephos	PDV-94-B004	ECUADOR. Prov. Sucumbios, Garza Cocha – Anangu, 175 km east-south-east of Coca	AY870561			
Lieinix nemesis	MFB-00-P234	COSTA RICA. Monteverde	AY870579			
Mathania leucothea	MFB-00-P428	CHILE. Farellones, Región Metropolitana	AY870557			
Melete lycimnia	MFB-00-P316	PERU. 10 km south-west of San Ramón, Chanchamayo	AY870530			
Moschoneura pinthous	MFB-00-P403	PERU. 9 km south of Tingo María	AY870575			
Mylothris agathina	NP-99-T486	SOUTH AFRICA. Muizenberg, Western Cape	AY870528	AY954615	AY954585	AY954555
Mylothris bernice	SC-01-M002	TANZANIA. Mufindi, Kihansi	AY870513			
Nathalis iole	DC-98-U711	USA. Ft. Huadrua field, Garden Canyon, AZ	AY870562	AY954599	AY954569	AY954539
Neophasia menapia	AMS-00-R052	USA. Ice Spring Road, Glenn Co, CA	AY870536			
Nepheronia thalassina	SC-01-T403	KENYA. Kibwezi	AY870518			
Pareronia valeria	MFB-00-P442	INDIA. Madras, Tamil Nadu	AY870517	AY954603	AY954573	AY954543
Patia orize	MFB-00-P353	PERU. 10 km south-west of San Ramón, Chanchamayo	AY870577			
Pereute charops	MFB-00-P283	COSTA RICA. Río Macho, Cartago Province	AY870538			
Perrhybris pamela	MFB-00-P202	COSTA RICA. Río Poas, La Garita	AY870545			
Phoebis sennae	MFB-00-P197	COSTA RICA. Río Poas, La Garita	AY870571			
Phulia nymphula	MFB-00-P375	PERU. 22 km south-south-east of Junín	AY870541	AY954610	AY954580	AY954550
Pieriballia viardi	MFB-00-P221	COSTA RICA. Río Alondra, San Luís	AY870547	AY954612	AY954582	AY954552
Pieris napi		USA. CA	AF173401*			
Pieris rapae	NM-95-Y381	USA. Nora Murphy culture	AY870550	AY954611	AY954581	AY95451

Table 1. *Continued*

Taxon	MCZ voucher no.	Locality	GenBank accession no.			
			EF-1α	*wingless*	*COI*	*28S*
Pierphulia rosea	MFB-00-P410	CHILE. Parque Nacional Lauca, Tarapaca Region	AY870543			
Pinacopteryx eriphia	GA-93-Z028	MADAGASCAR. Kirindy Forest	AY870553			
Pontia (Pontia) helice	NP-99-T476	SOUTH AFRICA. Swartberg Pass, north of Oudtshoorn, Western Cape	AY870549			
Pontia (Synchloe) callidice	AF-94-A003	USA. Gold Basin, Gunnison, CO	AY870548			
Prioneris philonome	DL-00-Q610	THAILAND. Gaw Chan Waterfall, Ratchaburi	AY870527	AY954613	AY954583	AY954553
Pseudopieris nehemia	MFB-00-P312	PERU. 10 km south-west of San Ramón, Chanchamayo	AY870574	AY954597	AY954567	AY954537
Pseudopontia paradoxa	SC-01-T380	ZAMBIA. Zambesi Bridge, Ikelenge	AY870580	AY954594	AY954564	AY954534
Pyrisitia proterpia	MFB-00-P211	COSTA RICA. Parque Nacional Santa Rosa	AY870586			
Saletara liberia	NP-95-Y239	MALAYSIA. Fraser's Hill, Pahang	AY870526	AY954607	AY954577	AY954547
Talbotia naganum	MFB-00-P467	LAOS. Laksao	AY870516			
Tatochila autodice	MFB-00-P306	PERU. San Mateo	AY870540			
Teriocolias zelia	MFB-00-P419	CHILE. Socoroma, Tarapaca Region	AY870564			
Theochila maenacte	MFB-00-P449	ARGENTINA. Escobar, Buenos Aires Province	AY870515			
Zegris (Zegris) eupheme	RV-00-Q026	SPAIN. Mequinenza, N.211 Km 293, Aragón	AY870559			
Zerene cesonia	MFB-00-P204	COSTA RICA. Parque Nacional Santa Rosa	AY870567			
Papilionidae						
Papilio rutulus	AF-94-A002	USA. Gold Basin, Gunnison, CO	AY954620	AF233563†	AY954560	AY954530
Nymphalidae						
Vanessa virginiensis	CA-94-N006	USA. Charles River, Middlesex County, MA	AY954621	AY954591	AY954561	AY954531
Lycaenidae						
Lycaena helloides	NP-99-W131	USA. Lost Man Creek, Pitkin County, CO	AY954622	AY954592	AY954562	AY954532
Riodinidae						
Uraneis hyalina	PDV-94-T013	ECUADOR. 175 km east-south-east of Coca, Prov. Sucumbíos Garza Cocha – Anangu	AY954623	AY954593	AY954563	AY954533

EF-1α, elongation factor-1 alpha; *COI*, cytochrome oxidase subunit I.
*Sequences for these taxa are those published by Caterino *et al*. (2001).
†The sequence for this taxon is that published by Campbell *et al*. (2000).

74 genera plus six subgenera (i.e. a total of 80 lower taxa) representing all the higher systematic groups (four subfamilies, two tribes). This sample represents 89% of all genera and 82% of all lower taxa (genera and subgenera) currently recognized within the Pieridae (Braby, 2005).

Four species from the butterfly families Papilionidae, Nymphalidae, Riodinidae, and Lycaenidae were chosen as outgroup taxa (Table 1). The final data set for the combined analysis thus comprised 30 taxa (26 Pieridae, four outgroups), whereas that for the *EF-1α* analysis comprised 90 taxa (86 Pieridae, four outgroups). The Pieridae are considered to be either the sister group to the Papilionidae (Ehrlich, 1958; Scott, 1985) or the sister group to Nymphalidae + (Riodinidae + Lycaenidae) (Kristensen, 1976; de Jong *et al*., 1996; Weller *et al*., 1996; Ackery *et al*., 1999). A recent combined molecular and morphological study of all the butterfly families and superfamilies by Wahlberg *et al*. (2005) suggested that the latter hypothesis is more probable.

Nine pierid genera [*Abaeis*, *Prestonia*, *Rhabdodryas*, *Glennia*, *Reliquia*, *Piercolias*, *Calopieris*, *Udaiana*, *Appias* (*Appias*)] were not sampled, in some cases because of their rarity or occurrence in inaccessible/remote locations. The relationships of most of these taxa have been reasonably well hypothesized based on morphology and their absence was assumed probably not to affect overall tree topology and hence the higher-level systematic relationships at the tribal and subfamily level, although their inclusion in future research will help elucidate relationships among the lower levels (e.g. genera). A further three subgenera [*Zegris* (*Microzegris*), *Aporia* (*Mesapia*), *Appias* (*Hiposcritia*)], and the putative subgenera of *Colias*, were not sampled in the present study, although the genera to which all of these taxa belong were included in our study, in some cases by more than one species.

To improve our sampling, and to test for potential nonmonophyly, two exemplar species (sometimes representing different subgenera) were included in each of the following ten genera: *Eurema*, *Colias*, *Pieris*, *Pontia*, *Aporia*, *Delias*, *Leuciacria*, *Catasticta*, *Mylothris*, and *Appias* Hübner. Previous molecular phylogenetic studies have confirmed the monophyly of at least three genera: *Colias* (Brunton, 1998; Pollock *et al*., 1998), *Gonepteryx* [Leach] (Brunton & Hurst, 1998), and *Delias* (Morinaka *et al*., 2002; Braby & Pierce, 2006).

Molecular techniques

The following protocol was adopted to obtain DNA sequences of *EF-1α*, *wingless*, *COI*, and *28S*. Three additional sequences were obtained from GenBank based on the published work of Campbell *et al*. (2000) and Caterino *et al*. (2001) (see Table 1).

Specimen preparation

Specimens were collected as fresh adults from the field using a hand net and killed by pinching the thorax. Wings were immediately excised and stored in paper envelopes as vouchers for identification and the bodies were preserved in plastic vials containing 100% ethyl alcohol. The specimens were temporarily stored at –20 C for laboratory use and then ultimately transferred to –80 C for permanent storage. A few of the specimens were collected and preserved (from a few months to several years) as dried adults before the wings and body were stored and preserved as for the fresh specimens. All specimens are deposited in the DNA and tissues collection at the Museum of Comparative Zoology, Harvard University, USA.

DNA extraction

For the freshly preserved specimens, gDNA was extracted from the metathorax, homogenized manually in a 1.5 ml microcentrifuge tube containing 200–400 l buffer solution [2% sodium dodecyl sulphate, 50 mM Tris-HCl, 20 mM ethylene diamene tetra acetic acid (EDTA) at pH 8.0], digested with Proteinase K (Gibco BRL/Life Technologies) 20 g l^{-1} for 2–3 h at 55 C, and then purified to separate the nucleic acids from the cellular debris through successive salt solution and ethanol precipitation at low temperature. The purified gDNA was dried and then resuspended in 110 l of TE buffer (10 mM Tris, 0.1 M EDTA at pH 8) and stored at –20 C. For dried specimens, gDNA was extracted from a leg; the tissue was first rehydrated in 200 l of buffer solution in a 1.5 ml Eppendorf tube for approximately 1 week at 4 C before homogenization, digestion, and precipitation. The precipitation steps were similar to the method used for fresh material but adjusted to maximize extraction of the degraded DNA fragments.

DNA amplification

The primers used for the amplification of the four genes in this study are given in Table 2. Approximately 1.1 kb of the *EF-1α* gene was amplified in one or two fragments using different sets of primers. We used both published (Cho *et al*., 1995; Monteiro & Pierce, 2001) and original primers for polymerase chain reaction (PCR) amplifications and sequencing. For *wingless*, an approximately 420 bp fragment was amplified using the single set of primers published in Brower & DeSalle (1998). For mitochondrial *COI*, an approximately 1.2 kb fragment was amplified using

Table 2. Primers used for the amplification and sequencing of the four genes. Position numbers correspond to the following reference sequences: elongation factor-1 alpha (*EF-1α*), *Bombyx mori* (GenBank D13338); *wingless*, *Drosophila melanogaster* (GenBank M17230); cytochrome oxidase subunit 1 (*COI*), *Drosophila yakuba* (GenBank X03240); *28S*, *Drosophila melanogaster* (GenBank M21017)

Gene	Primer name (forward or reverse)	Positions (5′→3′)	Sequence (5′→3′)
EF-1α	EF44 (fwd)	240–262	GCYGARCGYGARCGTGGTATYAC
	EF46.1I (fwd)	549–567	GAGGAAATYAARAAGGAAG
	EF46.1III (fwd)	548–567	CGAGGAAATCAARAARGAAG
	EF46.1IV (fwd)	549–567	GAAGAAATCAAAAARGAAG
	EF51.9 (fwd)	798–817	CARGACGTATACAAAATCGG
	EF77I (fwd)	816–835	GGTGGTATTGGAACAGTRCC
	EF77II (fwd)	816–835	GGTGGTATTGGAACAGTSCC
	EF51r (rev)	650–631	CATGTTGTCGCCGTGCCAAC
	EF52.6r (rev)	940–921	GCTTCGTGGTGCATTTCAAC
	EFrcM4 (rev)	1351–1329	ACAGCVACKGTYTGYCTCATRTC
wingless	LepWG1 (fwd)	1111–1136	GARTGYAARTGYCAYGGYATGTCTGG
	LepWG2 (rev)	1775–1750	ACTICGCRCACCARTGGAATGTRCA
COI	LCO1490 (fwd)	1490–1514	GGTCAACAAATCATAAAGATATTGG
	Ron (fwd)	1729–1751	GGATCACCTGATATAGCATTCCC
	Tonya (fwd)	2191–2216	GAAGTTTATATTTTAATTTTACCGGG
	Nancy (rev)	2216–2191	CCCCGTAAAATTAAAATATAAACTTC
	Hobbes (rev)	2756–2735	AAATGTTGNGGRAAAAATGTTA
28S	Mo6 (fwd)	3318–3337	CCCCCTGAATTTAAGCATAT
	D2B (fwd)	3549–3568	GTCGGGTTGCTTGAGAGTGC
	S3660 (fwd)	3668–3690	GAGAGTTMAASAGTACGTGAAAC
	D3A (fwd)	4046–4065	GACCCGTCTTGAAACACGGA
	D2B-r (rev)	3568–3549	GCACTCTCAAGCAACCCGAC
	D3A-r (rev)	4065–4046	TCCGTGTTTCAAGACGGGTC
	A335 (rev)	4394–4375	TCGGARGGAACCAGCTACTA
	D3B (rev)	4414–4395	TCGGAAGGAACCAGCTACTA

standard primers (Folmer *et al*., 1994; Monteiro & Pierce, 2001). For ribosomal *28S*, approximately 1.2 kb was amplified according to the primers published in Schmitz & Moritz (1994), Sequeira, Normark & Farrell (2000), and Saux, Fisher & Spicer (2004), although for approximately half the taxa only the 'downstream' 800 bp was amplified and sequenced.

Fragments were amplified according to standard PCR techniques using a thermal cycler and Qiagen PCR kit. For *EF-1α*, *wingless*, and *COI*, standard PCR reactions, with a total volume of 25 l, were prepared using 0.5 l of gDNA template at various dilutions, with 2.5 l of buffer (100 mM Tris-HCl solution with 50 mM KCI), 0.5 l $MgCl_2$ (25 mM), 0.125 l of each dNTP (2.5 mM), 1.25 l of each primer (10 M), and 0.125 l of *Taq* polymerase (5 units l^{-1}). For *28S*, 25 l reactions were prepared using 0.25 l of gDNA template at various dilutions, with 2.5 l of buffer (100 mM Tris-HCl solution with 50 mM KCI), 2 l $MgCl_2$ (25 mM), 1 l dimethyl sulphoxide, 0.25 l of each dNTP (2.5 mM), 1.2 l of each primer (10 M), and 0.2 l of *Taq* polymerase (5 units l^{-1}).

For *EF-1α*, samples were initially denatured at 95 C for 2 min followed by 30 cycles of amplification (denaturation at 95 C for 60 s, annealing at 55–51 C for 60 s, extension at 72 C for 2 min) with a final extension at 72 C for 10 min; three or four cycles were used at each successive annealing temperature. If faint or no DNA bands were detected in the gel, PCRs were repeated and the concentrations of the template and/or the magnesium optimized. For dried specimens, a second amplification of the PCR product was necessary. The conditions for the amplification of *wingless* and *COI* followed the protocols reported in Campbell *et al*. (2000), and Rand *et al*. (2000) and Monteiro & Pierce (2001), respectively. For *28S*, samples were initially denatured at 95 C for 2 min followed by 35 cycles of amplification (denaturation at 95 C for 60 s, annealing at 52 C for 60 s, extension at 72 C for 2 min) with a final extension at 72 C for 4 min. Negative controls were included in all PCRs to check for possible contamination. The PCR products of each template were combined and separated by electrophoresis on a 1 or 2% low-melting temperature agarose gel. The portion of the gel containing DNA fragments was excised and the gel-extracted PCR products then purified using QIAquick gel extraction kit columns.

DNA sequencing and alignment

Both strands of purified DNA fragments for each gene were reamplified and sequenced with a range of forward and reverse primers (see Table 2) using ABI Dye Terminator or Big Dye cycle sequencing kits. Half cycle sequence reactions (10 l) were prepared and denatured at 96 C for 3 min followed by 25 cycles (Dye Terminator: 96 C for 30 s, 50 C for 15 s, 60 C for 4 min; Big Dye: 96 C for 10 s, 50 C for 10 s, 60 C for 4 min). Samples were loaded on to a polyacrylamide gel and sequenced on an ABI 370 or 377 automated sequencer, or loaded into a 3100 ABI genetic analyser capillary sequencer. Sequence contigs generated from each reaction were edited manually and then aligned for each sample using SEQUENCHER version 3.0 (Sequencher, 1995) or version 4.1.2 (Sequencher, 2000) software. Ambiguities and gaps (typically at the ends of a sequence) were treated as missing data.

For *EF-1α*, the consensus sequence of each sample was aligned against the published sequence for *Bombyx mori* (Kamie *et al*., 1993) and primer ends were removed, resulting in 1066 bp (corresponding to positions 263–1328). For *wingless*, sequences (403 bp after the removal of primer ends) were aligned against other published Lepidoptera sequences (Brower & DeSalle, 1998; Campbell *et al*., 2000). For *COI*, the consensus sequences were aligned against the published reference sequence for *Drosophila yakuba* (Clary & Wolstenholme, 1985) and/or other Lepidoptera sequences on GenBank; the final fragment was 1220 bp (corresponding to positions 1515–2734). Aligning *EF-1α* and *COI* sequences did not require any indels, but in *wingless* one sample (*Mylothris agathina*) had a one-codon deletion, and another sample (*Phulia nymphula*) had three-codon deletions. Codon positions were either analysed in SEQUENCHER 3.0 or exported into MacClade version 3.08a (Maddison & Maddison, 1999) or version 4.03 (Maddison & Maddison, 2001) and translated to amino acids. For *28S*, sequences were initially aligned against the published reference sequence for *Drosophila melanogaster* (Tautz *et al*., 1988); improved alignment was obtained using CLUSTALX version 1.81 (Thompson *et al*., 1997) and then manually using MacClade 4.03 (Maddison & Maddison, 2001). Ambiguous regions were removed, resulting in a final character set of 986 bp, which included internal gaps as well as nonsequenced terminal regions for some taxa. GenBank accession numbers for all sequences are given in Table 1.

Phylogenetic analysis

Maximum (cladistic) parsimony (MP), maximum likelihood (ML) (using PAUP), ML (using PHYML), and Bayesian inference (BI) were carried out for the smaller taxon data set of the four genes combined, as well as for the larger taxon data set of the *EF-1α* gene. We also ran an 'all available data' analysis, using MP and ML, of the entire data by combining the 30 taxon data set of the four genes with the 90 taxon data set of *EF-1α*. The final data matrix of this data set thus consisted of 90 taxa, that is, 30 taxa with sequences from *EF-1α*, *wingless*, *COI*, and *28S*, plus 60 taxa with sequences from *EF-1α* only, with the remaining three genes coded as 'missing' data.

MP

Phylogenetic trees were reconstructed using unweighted and weighted MP as the optimality criterion, as implemented in PAUP* version 4.0b10 (Swofford, 2002). Tree estimation involved heuristic searches with the tree-bisection-reconnection (TBR) branch-swapping algorithm, stepwise addition with up to 1000 random starts to check for islands of trees, and 'MulTrees' option in effect. Searches of large data sets that still recovered numerous islands of trees after approximately 100 random additions were repeated using PAUPRat (Sikes & Lewis, 2001). Strict consensus trees were computed where there was more than one equally parsimonious tree. Results based on MP analyses of each codon position, as well as those obtained from other methods (e.g. neighbour joining), were compared to establish that there was no conflict of signal within each data set. Bootstrap analyses (Felsenstein, 1985, 1988), based on a full heuristic search of 1000 pseudoreplicates using TBR branch swapping and simple stepwise addition, were carried out for each analysis to determine the level of support of each node (clades with bootstrap values < 50% were collapsed). In order to ascertain the extent of saturation, transition : transversion ratios were plotted against the observed or uncorrected pairwise 'p' distance for each codon position. Various weighting schemes were also explored, including weighting transversions over transitions (2 : 1 or 3 : 1).

For the smaller taxon data set of the four genes combined, each gene partition was first analysed separately using unweighted MP and the topology of the resulting trees compared for congruence before combining the data. Clade robustness was also evaluated using Bremer support (decay index) (Bremer, 1988, 1994) using the program TreeRot version 2 (Sorenson, 1999). Partitioned Bremer support was calculated to assess the contribution of each data partition to the total Bremer support values in the combined analysis.

ML

Phylogenetic trees were estimated using ML tree-building methods, as implemented in PAUP* version 4.0b10. Analyses based on the ML optimality criterion were performed according to the general time reversible substitution model (Lanave *et al*., 1984; Rodríguez *et al*., 1990) with among-site rate variation (invariable sites and gamma distribution) (i.e. GTR + I + Γ). Model selection was determined according to the hierarchical likelihood ratio test (hLRT) as implemented in ModelTest 3.06 (Posada & Crandall, 1998) with the starting trees obtained by MP. Models that best fitted the observed data were then used to generate an ML tree under a heuristic search using the TBR branch-swapping algorithm with as-is stepwise addition. Minor variations in estimates of model parameters were found not to affect the final tree topology. ML trees were also reconstructed using PHYML version 2.4.3 (Guindon & Gascuel, 2003). The model used was GTR + I + Γ, according to hLRT, with model parameters optimized automatically. Starting trees were distance based (BIONJ) according to the default option. Nonparametric bootstrap analyses based on 2000 pseudoreplicates were carried out to determine the approximate level of support for all branching events, with support percentages computed by majority rule consensus.

BI

Finally, we ran BI partitioned by codon position (first and second; third) in MrBayes 3.0b4 (Ronquist & Huelsenbeck, 2003), with the HKY85 + I + Γ model of sequence evolution for first and second positions, and GTR + I + Γ for third positions. Unlinked model parameters were preset as starting values for all partitioned analyses. Three independent Bayesian runs at temperature settings from 0.2 to 0.4 were performed on the data using metropolis-coupled Markov chain Monte Carlo simulations, from one to 10 million generations each, and tree sampling every 100 generations. Bayesian topology and branch posterior probabilities were computed by majority rule consensus after deleting as 'burn in' all preasymptotic tree scores.

Age of divergence estimations

In order to estimate the approximate age of divergence events within the Pieridae, the evolutionary rate of substitution for the molecular data set was calibrated using dated fossils, rather than ages of vicariance events inferred from biogeography and geological data, to estimate minimum divergence times of lineages within the framework of our phylogenetic hypothesis (Hillis, Mable & Moritz, 1996b; Arbogast *et al*., 2002; Hedges & Kumar, 2003; Magallón, 2004). Although butterflies are rarely preserved as fossils, several have been discovered and described from the Pieridae (Scudder, 1875a, 1889; Zeuner, 1942; Brown, 1976; Shields, 1976; Carpenter, 1992; Emmel, Minno & Drummond, 1992). Four of these fossils are recorded from the Tertiary (Table 3), the oldest being two species from the Florissant Formation, Colorado, dated Late Eocene (34.07 ± 0.10 Myr) (Evanoff, McIntosh & Murphey, 2001). Because the nearest relatives of these fossils have been determined with some degree of certainty, the fossils served as useful calibration points.

To calibrate the rate of substitution, we first assessed if the rate was constant (i.e. clock-like) by comparing the likelihood scores of our best ML model of the *EF-1α* data set with and without enforcing a molecular clock, using a LRT in PAUP. The LRT test rejected the null hypothesis that the data were clock-

Table 3. Summary of known pre-Quaternary fossils recorded for the Pieridae*. Data collated from Scudder (1875a), Zeuner (1942), Shields (1976), Brown (1976), Emmel *et al*. (1992), and Carpenter (1992)

Taxon	Closest relative(s)	Locality	Deposit	Epoch (Myr)
Stolopsyche libytheoides Scudder, 1889	*Pieris*	USA (Colorado)	Lacustrine shales	Upper Eocene (34)
Oligodonta florissantensis F.M. Brown, 1976	*Catasticta* group (possibly *Leodonta*)	USA (Colorado)	Lacustrine shales	Upper Eocene (34)
Coliates proserpina Scudder, 1875	*Delias–Prioneris* group (possibly *Aporia*)	France (Aix-en-Provence)	Calcareous marls of gypsum quarries	Lower Oligocene (33.5–30)
Miopieris talboti Zeuner, 1942	*Pontia* group (possibly *Pontia*)	Germany (Randecker Maar)	Dysodil shales	Upper Miocene (10.5–5.5)

**Mylothrites pluto* (Heer, 1849), recorded from marls of lacustrine beds of Lower Miocene age (23.5–16.5) from Radoboj, Yugoslavia, is excluded from the list because of doubt over its correct identity. Zeuner (1942) considered that it belonged to the Nymphalidae, but Carpenter (1992) treated it as a pierid, noting that its wings were similar in venation to *Mylothris* but similar in shape to the distantly related *Hebomoia*.

like ($\delta = 196$, d.f. $= 88$, $P < 0.0001$). However, inspection of the topology and branch lengths of our phylogram showed that rates of change within and between two major clades of interest were reasonably homogeneous (i.e. clock-like), relative to the rest of the clades in the tree. We therefore applied two methods: (1) Sanderson's semiparametric rate smoothing using a penalized likelihood method, as implemented in the r8s program (Sanderson, 2002), to correct rate heterogeneity across the entire tree; and (2) the quartet method (Cooper & Penny, 1997), which assumes that the data are ultrametric and clock-like, but does allow for rate variation among lineages (i.e. nonclock-like subsets of the data). The latter method involves determining the average genetic divergence between a pair of related taxa (i.e. between the lineages of two fossils), and calculating the rate of substitution using the age of the oldest fossil. The calculation is then repeated for another pair of related taxa. The two distantly related pairs of taxa are then combined into a quartet, and the two substitution rates averaged to give a calibrated rate for the gene. The minimum divergence time of the two pairs forming the quartet is then estimated based on the average corrected pairwise distance between the two pairs of fossils.

RESULTS

Combined analysis

The smaller (30 taxon) data set was assembled primarily to investigate patterns of higher-level relatedness within the Pieridae (e.g. subfamilies, tribes) by combining four independent markers. The final data set comprised a total of 3675 bp, of which 1031 bp (28%) were parsimony informative (Table 4).

The results of MP, ML (PHYML), and BI of the combined data set of the four genes are summarized in Figure 3. The results of the ML (PAUP) analysis were identical to those for ML (PHYML) (tree not shown). Tree topologies generated by each method of analysis were broadly similar, the major differences being in the placement of *Pareronia*, *Leptosia*, and *Elodina*. Eleven major clades within the Pieridae (labelled A–K) were identified. With the exception of clade E, these clades were consistently recovered under the different methods of analysis and with a high level of support (Fig. 3, Table 5). MP and ML analyses of the individual data partitions showed no deep phylogenetic structure, and hence little conflict, among the basal nodes (trees not shown). Partitioned Bremer support of the combined data set under MP revealed a high level of congruence between the four genes for most nodes (Table 5). The only substantial source of conflict was with the mitochondrial gene *COI*, which contributed negatively to the total support value in two major clades (C and G) (Table 5), probably as a result of saturation due to high A–T bias.

In terms of the current higher-level classification, relationships within the Pieridae showed good agreement with those based on morphology (Klots, 1933; Ehrlich, 1958; Bridges, 1988). There was strong support for the monophyly of the three larger subfamilies in the combined analyses: Dismorphiinae (clade B: bootstrap 100% MP, ML, and BI), Coliadinae (clade C: bootstrap 73% MP, 100% ML and BI), and Pierinae (clade D: bootstrap 76% MP, 97% ML, 100% BI) (Fig. 3). A sister relationship between the Dismorphiinae and the Pseudopontiinae was evident in two analyses with reasonable support (bootstrap 76% MP, 80% ML), and in the two ML trees this clade formed the sister group to the rest of the Pieridae (Fig. 3B). The Coliadinae and Pierinae were sister taxa in two analyses (bootstrap 85% ML, 97% BI). The Pierinae was found to consist of three major clades (E–G), with clade G composed of four smaller subclades (H–K). The tribe Pierini, however, was paraphyletic as it included the three exemplars from the Anthocharidini (clade F).

EF-1α analysis

The *EF-1α* analysis of the larger (90 taxon) data set was used primarily to investigate patterns of lower-level relatedness within the Pieridae (e.g. genera,

Table 4. Character summary for the combined data set, with numbers of sites for each codon position for each gene partition

Gene partition	*EF-1α*				*wingless*				*COI*					
Codon position	1st	2nd	3rd	All	1st	2nd	3rd	All	1st	2nd	3rd	All	28S	Total
Number parsimony informative	22	10	271	303	40	11	127	178	74	15	320	409	141	1031
Number variable but parsimony uninformative	19	6	42	67	21	26	3	50	34	15	47	96	87	300
Number constant	314	339	43	696	73	97	5	175	299	376	40	715	758	2344
Total characters	355	355	356	1066	134	134	135	403	407	406	407	1220	986	3675

EF-1α, elongation factor-1 alpha; *COI*, cytochrome oxidase subunit I.

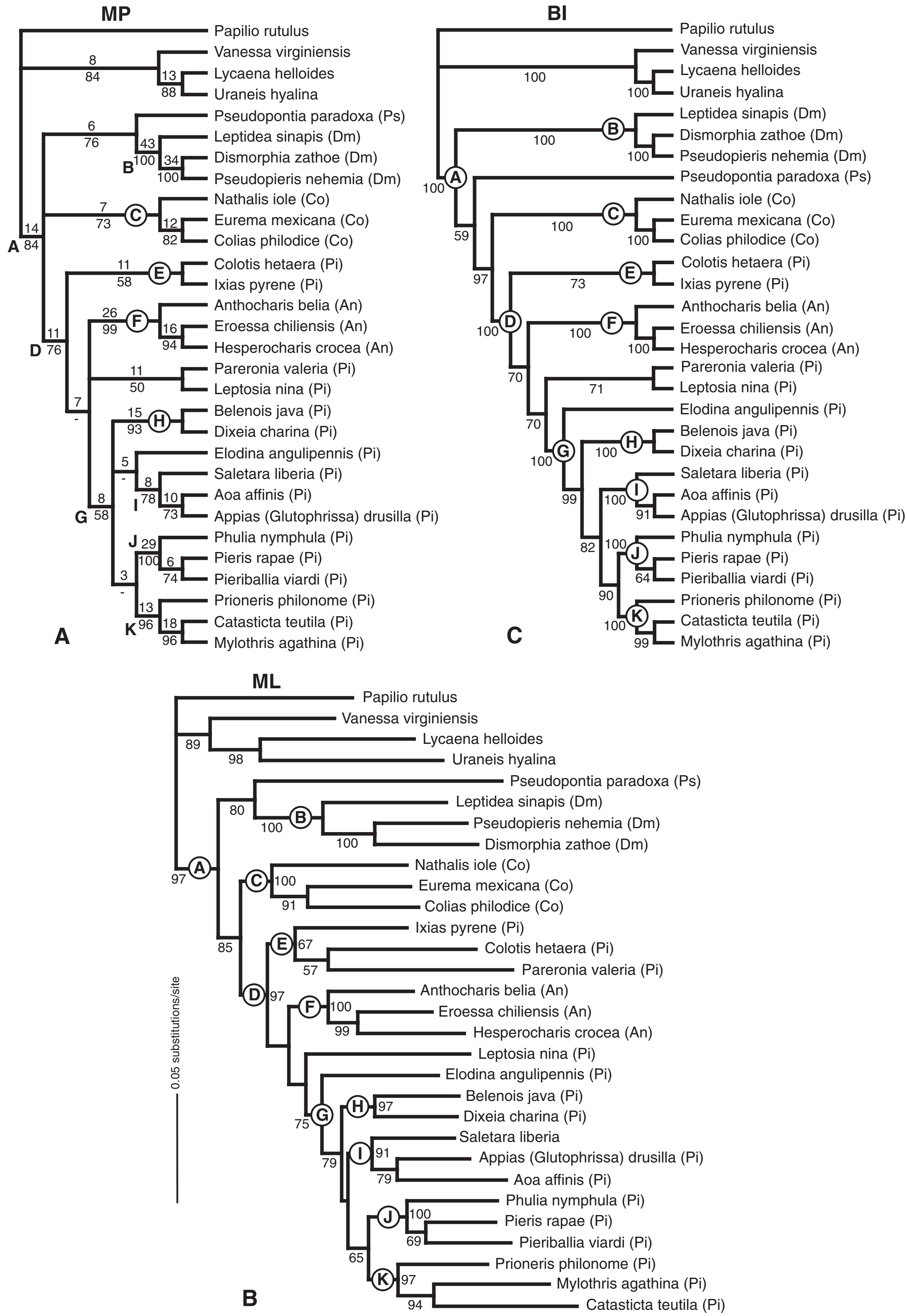

MP
Papilio rutulus
Vanessa virginiensis
Lycaena helloides
Uraneis hyalina
Pseudopontia paradoxa (Ps)
Leptidea sinapis (Dm)
Dismorphia zathoe (Dm)
Pseudopieris nehemia (Dm)
Nathalis iole (Co)
Eurema mexicana (Co)
Colias philodice (Co)
Colotis hetaera (Pi)
Ixias pyrene (Pi)
Anthocharis belia (An)
Eroessa chiliensis (An)
Hesperocharis crocea (An)
Pareronia valeria (Pi)
Leptosia nina (Pi)
Belenois java (Pi)
Dixeia charina (Pi)
Elodina angulipennis (Pi)
Saletara liberia (Pi)
Aoa affinis (Pi)
Appias (Glutophrissa) drusilla (Pi)
Phulia nymphula (Pi)
Pieris rapae (Pi)
Pieriballia viardi (Pi)
Prioneris philonome (Pi)
Catasticta teutila (Pi)
Mylothris agathina (Pi)
A
BI
Papilio rutulus
Vanessa virginiensis
Lycaena helloides
Uraneis hyalina
Leptidea sinapis (Dm)
Dismorphia zathoe (Dm)
Pseudopieris nehemia (Dm)
Pseudopontia paradoxa (Ps)
Nathalis iole (Co)
Eurema mexicana (Co)
Colias philodice (Co)
Colotis hetaera (Pi)
Ixias pyrene (Pi)
Anthocharis belia (An)
Eroessa chiliensis (An)
Hesperocharis crocea (An)
Pareronia valeria (Pi)
Leptosia nina (Pi)
Elodina angulipennis (Pi)
Belenois java (Pi)
Dixeia charina (Pi)
Saletara liberia (Pi)
Aoa affinis (Pi)
Appias (Glutophrissa) drusilla (Pi)
Phulia nymphula (Pi)
Pieris rapae (Pi)
Pieriballia viardi (Pi)
Prioneris philonome (Pi)
Catasticta teutila (Pi)
Mylothris agathina (Pi)
C
ML
Papilio rutulus
Vanessa virginiensis
Lycaena helloides
Uraneis hyalina
Pseudopontia paradoxa (Ps)
Leptidea sinapis (Dm)
Pseudopieris nehemia (Dm)
Dismorphia zathoe (Dm)
Nathalis iole (Co)
Eurema mexicana (Co)
Colias philodice (Co)
Ixias pyrene (Pi)
Colotis hetaera (Pi)
Pareronia valeria (Pi)
Anthocharis belia (An)
Eroessa chiliensis (An)
Hesperocharis crocea (An)
Leptosia nina (Pi)
Elodina angulipennis (Pi)
Belenois java (Pi)
Dixeia charina (Pi)
Saletara liberia
Appias (Glutophrissa) drusilla (Pi)
Aoa affinis (Pi)
Phulia nymphula (Pi)
Pieris rapae (Pi)
Pieriballia viardi (Pi)
Prioneris philonome (Pi)
Mylothris agathina (Pi)
Catasticta teutila (Pi)
0.05 substitutions/site
B

Figure 3. Phylogenetic trees for 26 pierid taxa based on the combined data set of four genes (3675 bp, 1031 informative characters). A, strict consensus of three equally most parsimonious trees [length = 6572, consistency index (CI) = 0.321, retention index (RI) = 0.316]; values below the branches are bootstraps (1000 full heuristic search replicates) for nodes with ≥50% support, those above the branches are total Bremer support indices. B, maximum likelihood (ML–PHYML) tree according to the GTR + I + Γ substitution model [log likelihood score = –32254.152: relative rate matrix A↔C 2.2381, A↔G 7.7859, A↔T 7.5951, C↔G 2.3345, C↔T 14.0782, G↔T 1.0; base frequencies A = 0.2482, C = 0.2307, G = 0.2095, T = 0.3116; proportion of invariable sites (I) = 0.5423; shape parameter (α) of gamma distribution (Γ) = 0.8036], with bootstrap values (2000 pseudoreplicates) shown below the branches or adjacent to nodes with ≥50% support. C, Bayesian inference (BI) tree partitioned by gene and codon position; the unlinked partition model is HKY85 + I + Γ for first and second codon positions for elongation factor-1 alpha (*EF-1α*), *wingless*, and cytochrome oxidase subunit I (*COI*) partitions and for all positions for *28S* partition, GTR + I + Γ for third codon positions for each gene partition, at a sampling temperature of 0.4; values below the nodes are posterior branch supports estimated from majority rule consensus of 90 000 trees (10^7 generations, 10^6 burned). The two letters in parentheses after each taxon name are the currently recognized subfamily/tribe, abbreviated as follows: Ps, Pseudopontiinae; Dm, Dismorphiinae; Co, Coliadinae; Pi, Pierini (Pierinae); An, Anthocharidini (Pierinae). Bold capitalized letters (A–K) denote major clades evident in the analysis. *Papilio*, *Vanessa*, *Lycaena*, and *Uraneis* are outgroup taxa.

Table 5. Total Bremer support and partitioned Bremer support for each gene for nodes in the strict consensus maximum parsimony (MP) cladogram of the combined data set (Fig. 3A). Rows in bold refer to nodes/clades that comprise the higher taxa recognized in this work (see Discussion)

Node	Clade	Higher taxon	Total	*EF-1α*	*wingless*	*COI*	*28S*
1			8	–0.7	3.0	–1.3	7.0
2			13	3.3	1.0	3.7	5.0
3	A	Pieridae	14	3.3	3.0	0.7	7.0
4		Pseudopontiinae + Dismorphiinae	6	0.3	3.0	–2.3	5.0
5	**B**	**Dismorphiinae**	**43**	**11.3**	**16.0**	**3.7**	**12.0**
6			34	8.3	11.0	4.7	10.0
7	**C**	**Coliadinae**	**7**	**3.3**	**2.0**	**–3.3**	**5.0**
8			12	11.3	–5.0	–3.3	9.0
9	**D**	**Pierinae**	**11**	**5.3**	**3.0**	**0.7**	**2.0**
10	E		11	2.3	2.0	3.7	3.0
11			7	2.3	–2.0	7.7	–1.0
12	**F**	**Anthocharidini *s.s.***	**26**	**3.7**	**6.2**	**7.7**	**8.4**
13			16	6.8	6.5	3.7	–1.0
14			11	0.3	5.0	2.7	3.0
15	**G**	**Pierini *s.s.***	**8**	**1.3**	**10.5**	**–3.3**	**–0.5**
16	H	*Dixeia* + *Belenois*	15	6.3	–2.0	9.2	1.5
17			5	–0.7	–2.0	5.7	2.0
18	**I**	**Appiadina**	**8**	**3.3**	**4.0**	**–0.3**	**1.0**
19			10	3.3	5.0	–0.3	2.0
20			3	2.3	0.0	1.2	–0.5
21	**J**	**Pierina**	**29**	**10.3**	**10.3**	**2.0**	**6.4**
22			6	–2.7	0.0	7.7	1.0
23	**K**	**Aporiina**	**13**	**1.3**	**3.0**	**8.7**	**0.0**
24			18	5.8	1.5	8.7	2.0

EF-1α, elongation factor-1 alpha; *COI*, cytochrome oxidase subunit I.

subgenera), that is, relationships among the shallow nodes or tips of the tree. Of the 1066 bp sequenced for the gene, 364 sites (34%) were parsimony informative. As expected, most of the variable sites were in the third codon position. First and second positions were highly conserved, with a total of only 46 sites (4%) parsimony informative. Mean base frequencies were similar and not significantly different across bases (A = 0.26263, C = 0.25572, G = 0.23161, T = 0.25004). A plot of the transition/transversion ratio against uncorrected pairwise 'p' distance for all codon positions, third codon positions, and first and second positions inferred under MP, revealed that the gene was significantly saturated after approximately 10% divergence (not shown). Saturation was limited to third positions, being most pronounced among the deeper-level divergences (> 30%), and was not evident in first and second positions.

Figure 4 shows the strict consensus of nine equally MP trees based on unweighted analysis. The analysis recovered the Dismorphiinae (clade B), Coliadinae (clade C), and two clades within the Pierinae (clades J and K), with moderate to high support (bootstrap 64–100%), plus a number of smaller, less well-supported groups (clades E–I) that were evident in the combined analysis (Fig. 3). BI gave a similar tree to MP (tree not shown). ML (PHYML) yielded a single tree with similar topology in terms of shallow relationships among the exemplar taxa (Fig. 5). The same four well-supported clades were again evident (bootstrap 75–100%), and there was increased support for the monophyly of another large clade (F). However, the deeper nodes lacked support and, although the supported clades were concordant with those obtained in the combined smaller data set, their relationships were not resolved.

Of the ten genera where multiple species were examined, seven (70%) were monophyletic and three (*Eurema*, *Catasticta*, *Appias*) were not (Figs 4, 5). However, only in *Eurema* was there significant evidence (bootstrap 75% MP, 87% ML) in support of paraphyly: the *Eurema* clade included the genera *Leucidia*, *Teriocolias*, and *Pyrisitia*. Relationships within the two other genera, *Catasticta* and *Appias*, were essentially unresolved.

Dismorphiinae

The exemplars of the subfamily Dismorphiinae formed a tightly structured, well-supported monophyletic group (clade B: bootstrap 98% MP, 99% ML) (Figs 4, 5). Phylogenetic relationships within the Dismorphiinae were extremely well resolved. The Palaearctic *Leptidea* was sister to the remaining genera, all from the Neotropical region. In the latter clade, both MP and ML analyses yielded the following topology: *Pseudopieris* + (*Moschoneura* + ((*Enantia* + *Patia*) + (*Dismorphia* + *Lieinix*))).

Coliadinae

Members of the subfamily Coliadinae (the sulphurs) formed a well-supported monophyletic group (clade C: bootstrap 70% MP, 90% ML) (Figs 4, 5). Deeper-level splits within the Coliadinae were not well supported and provided only a polytomy. However, tree topologies generated by MP and ML methods were in general agreement, with both analyses suggesting that *Nathalis* and *Kricogonia* from the New World were sister taxa to all other genera in the Coliadinae. In all analyses, *Zerene* and *Colias* were recovered as sister taxa and were well supported by MP and ML (bootstrap 71–76%).

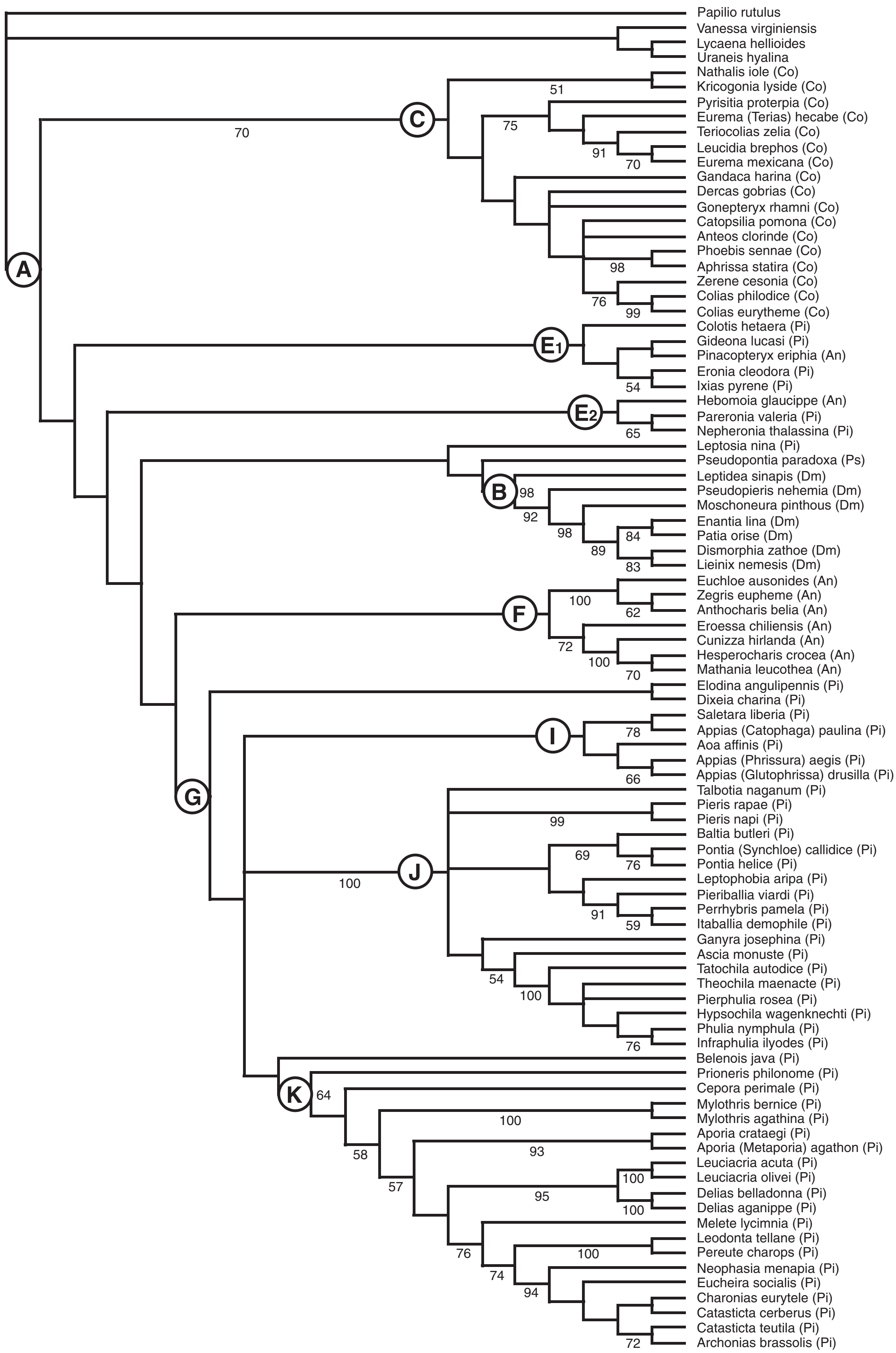
Pierinae

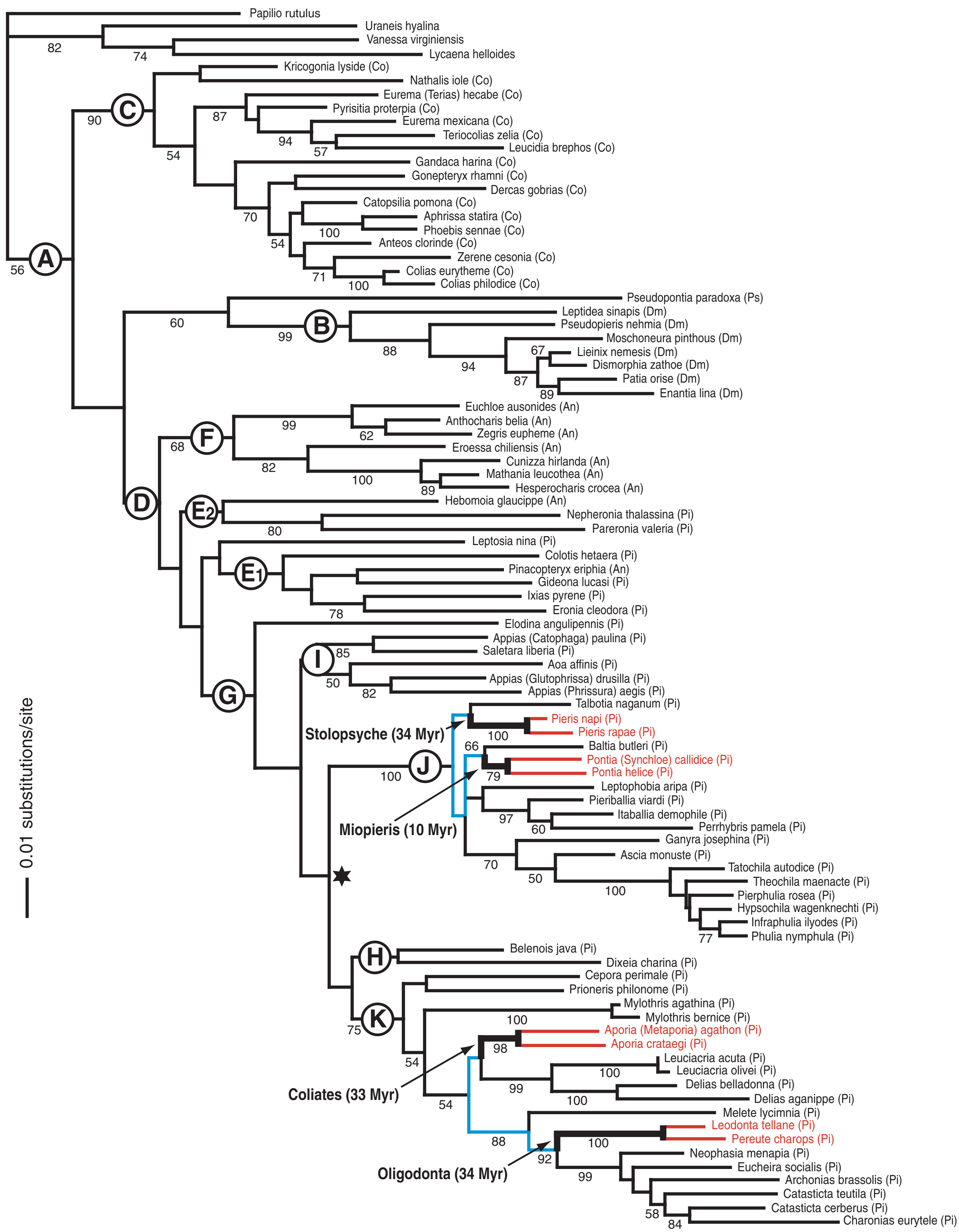
The Pierinae (clade D) were recovered as a monophyletic group under ML but without support. The Anthocharidini and Pierini were both nonmonophyletic, with two genera (*Hebomoia*, *Pinacopteryx*) traditionally placed in the Anthocharidini (clade F) and seven genera (*Colotis*, *Gideona*, *Ixias*, *Eronia*, *Pareonia*, *Nepheronia*, *Leptosia*) normally associated with the Pierini (clade G) comprising three separate groups (clades E_1 and E_2, *Leptosia*) outside these two tribes (Figs 4, 5).

The seven other genera from the Anthocharidini [*Euchloe*, *Anthocharis*, *Zegris* (*Zegris*), *Eroessa*, *Cunizza*, *Hesperocharis*, *Mathania*] appeared to form a clade, but support for their monophyly was weak (clade F: bootstrap < 50% MP, 68% ML) (Figs 4, 5). This clade, however, consisted of two well-supported subclades: the Holarctic *Anthocharis* group, commonly known as the 'orange tips', and the Neotropical *Hesperocharis* group. The *Anthocharis* group (bootstrap 100% MP, 99% ML) comprised the genera *Euchloe*, *Anthocharis*, and *Zegris* (*Zegris*) in an unresolved trichotomy; whereas the *Hesperocharis* group (bootstrap 72% MP, 82% ML) consisted of *Eroessa*, *Cunizza*, *Hesperocharis*, and *Mathania*. The latter three taxa formed a well-supported monophyletic group (bootstrap 100% MP and ML) sister to *Eroessa* with the following topology: *Cunizza* + (*Hesperocharis* + *Mathania*).

Of the other taxa within the Pierini (clade G), one group of taxa, comprising the genera *Appias* (*Catophaga*), *Appias* (*Glutophrissa*), *Appias* (*Phrissura*), *Saletara*, and *Aoa*, appeared to form a cluster (clade I),

Figure 4. Strict consensus of nine equally maximum parsimony (MP) trees for the family Pieridae based on 1066 bp elongation factor-1 alpha (*EF-1α*) [364 informative characters: length = 3777, consistency index (CI) = 0.190, retention index (RI) = 0.548]. Bootstrap values (1000 full heuristic search replicates) are shown below the branches or adjacent to nodes with ≥50% support. Letters in parentheses after each taxon name are as per Figure 3. Bold capitalized letters (A–K) denote the major clades evident in the combined analysis of Figure 3. *Papilio*, *Vanessa*, *Lycaena*, and *Uraneis* are outgroup taxa.


A
C
E1
E2
B
F
G
I
J
K
Papilio rutulus
Vanessa virginiensis
Lycaena hellioides
Uraneis hyalina
Nathalis iole (Co)
Kricogonia lyside (Co)
Pyrisitia proterpia (Co)
Eurema (Terias) hecabe (Co)
Teriocolias zelia (Co)
Leucidia brephos (Co)
Eurema mexicana (Co)
Gandaca harina (Co)
Dercas gobrias (Co)
Gonepteryx rhamni (Co)
Catopsilia pomona (Co)
Anteos clorinde (Co)
Phoebis sennae (Co)
Aphrissa statira (Co)
Zerene cesonia (Co)
Colias philodice (Co)
Colias eurytheme (Co)
Colotis hetaera (Pi)
Gideona lucasi (Pi)
Pinacopteryx eriphia (An)
Eronia cleodora (Pi)
Ixias pyrene (Pi)
Hebomoia glaucippe (An)
Pareronia valeria (Pi)
Nepheronia thalassina (Pi)
Leptosia nina (Pi)
Pseudopontia paradoxa (Ps)
Leptidea sinapis (Dm)
Pseudopieris nehemia (Dm)
Moschoneura pinthous (Dm)
Enantia lina (Dm)
Patia orise (Dm)
Dismorphia zathoe (Dm)
Lieinix nemesis (Dm)
Euchloe ausonides (An)
Zegris eupheme (An)
Anthocharis belia (An)
Eroessa chiliensis (An)
Cunizza hirlanda (An)
Hesperocharis crocea (An)
Mathania leucothea (An)
Elodina angulipennis (Pi)
Dixeia charina (Pi)
Saletara liberia (Pi)
Appias (Catophaga) paulina (Pi)
Aoa affinis (Pi)
Appias (Phrissura) aegis (Pi)
Appias (Glutophrissa) drusilla (Pi)
Talbotia naganum (Pi)
Pieris rapae (Pi)
Pieris napi (Pi)
Baltia butleri (Pi)
Pontia (Synchloe) callidice (Pi)
Pontia helice (Pi)
Leptophobia aripa (Pi)
Pieriballia viardi (Pi)
Perrhybris pamela (Pi)
Itaballia demophile (Pi)
Ganyra josephina (Pi)
Ascia monuste (Pi)
Tatochila autodice (Pi)
Theochila maenacte (Pi)
Pierphulia rosea (Pi)
Hypsochila wagenknechti (Pi)
Phulia nymphula (Pi)
Infraphulia ilyodes (Pi)
Belenois java (Pi)
Prioneris philonome (Pi)
Cepora perimale (Pi)
Mylothris bernice (Pi)
Mylothris agathina (Pi)
Aporia crataegi (Pi)
Aporia (Metaporia) agathon (Pi)
Leuciacria acuta (Pi)
Leuciacria olivei (Pi)
Delias belladonna (Pi)
Delias aganippe (Pi)
Melete lycimnia (Pi)
Leodonta tellane (Pi)
Pereute charops (Pi)
Neophasia menapia (Pi)
Eucheira socialis (Pi)
Charonias eurytele (Pi)
Catasticta cerberus (Pi)
Catasticta teutila (Pi)
Archonias brassolis (Pi)



Papilio rutulus
Uraneis hyalina
Vanessa virginiensis
Lycaena helloides
Kricogonia lyside (Co)
Nathalis iole (Co)
Eurema (Terias) hecabe (Co)
Pyrisitia proterpia (Co)
Eurema mexicana (Co)
Teriocolias zelia (Co)
Leucidia brephos (Co)
Gandaca harina (Co)
Gonepteryx rhamni (Co)
Dercas gobrias (Co)
Catopsilia pomona (Co)
Aphrissa statira (Co)
Phoebis sennae (Co)
Anteos clorinde (Co)
Zerene cesonia (Co)
Colias eurytheme (Co)
Colias philodice (Co)
Pseudopontia paradoxa (Ps)
Leptidea sinapis (Dm)
Pseudopieris nehmia (Dm)
Moschoneura pinthous (Dm)
Lieinix nemesis (Dm)
Dismorphia zathoe (Dm)
Patia orise (Dm)
Enantia lina (Dm)
Euchloe ausonides (An)
Anthocharis belia (An)
Zegris eupheme (An)
Eroessa chiliensis (An)
Cunizza hirlanda (An)
Mathania leucothea (An)
Hesperocharis crocea (An)
Hebomoia glaucippe (An)
Nepheronia thalassina (Pi)
Pareronia valeria (Pi)
Leptosia nina (Pi)
Colotis hetaera (Pi)
Pinacopteryx eriphia (An)
Gideona lucasi (Pi)
Ixias pyrene (Pi)
Eronia cleodora (Pi)
Elodina angulipennis (Pi)
Appias (Catophaga) paulina (Pi)
Saletara liberia (Pi)
Aoa affinis (Pi)
Appias (Glutophrissa) drusilla (Pi)
Appias (Phrissura) aegis (Pi)
Talbotia naganum (Pi)
Pieris napi (Pi)
Pieris rapae (Pi)
Stolopsyche (34 Myr)
Baltia butleri (Pi)
Pontia (Synchloe) callidice (Pi)
Pontia helice (Pi)
Leptophobia aripa (Pi)
Pieriballia viardi (Pi)
Itaballia demophile (Pi)
Perrhybris pamela (Pi)
Miopieris (10 Myr)
Ganyra josephina (Pi)
Ascia monuste (Pi)
Tatochila autodice (Pi)
Theochila maenacte (Pi)
Pierphulia rosea (Pi)
Hypsochila wagenknechti (Pi)
Infraphulia ilyodes (Pi)
Phulia nymphula (Pi)
Belenois java (Pi)
Dixeia charina (Pi)
Cepora perimale (Pi)
Prioneris philonome (Pi)
Mylothris agathina (Pi)
Mylothris bernice (Pi)
Aporia (Metaporia) agathon (Pi)
Aporia crataegi (Pi)
Leuciacria acuta (Pi)
Leuciacria olivei (Pi)
Delias belladonna (Pi)
Delias aganippe (Pi)
Coliates (33 Myr)
Melete lycimnia (Pi)
Leodonta tellane (Pi)
Pereute charops (Pi)
Neophasia menapia (Pi)
Eucheira socialis (Pi)
Archonias brassolis (Pi)
Catasticta teutila (Pi)
Catasticta cerberus (Pi)
Charonias eurytele (Pi)
Oligodonta (34 Myr)
A
B
C
D
E2
E1
F
G
H
I
J
K
0.01 substitutions/site

Figure 5. Maximum likelihood (ML–PHYML) tree for the family Pieridae based on 1066 bp elongation factor-1 alpha (*EF-1α*) according to the GTR + I + Γ substitution model [log likelihood score = –17727.75: relative rate matrix A↔C 2.1063, A↔G 9.3033, A↔T 3.9410, C↔G 1.4829, C↔T 13.0754, G↔T 1.0; base frequencies A = 0.2729, C = 0.2222, G = 0.1946, T = 0.3102; proportion of invariable sites (I) = 0.5903; shape parameter (α) of gamma distribution (Γ) = 1.0686]. Bootstrap values are given below the branches or adjacent to nodes with ≥ 50% support (2000 pseudoreplicates). Letters in parentheses after each taxon name are as per Figure 3. Bold capitalized letters (A–K) denote the major clades evident in the combined analysis of Figure 3. *Papilio*, *Vanessa*, *Lycaena*, and *Uraneis* are outgroup taxa. Extinct taxa based on fossils are indicated by thick lines at various nodes and along internal branches according to their putative relative(s) (taxa and external branches are highlighted in red) (see Table 3). Internal branches for each set of pairwise comparisons are shown in blue. The minimum age of divergence between clades J and K (node indicated by an asterisk) is estimated to be approximately 60 Myr.

but support for monophyly was not evident (Figs 4, 5).

Another 17 genera/subgenera within the Pierini (clade G) comprised an extremely well-supported monophyletic group in our analysis (clade J: bootstrap 100% MP and ML) (Figs 4, 5). This clade included the familiar *Pieris* and allied taxa often referred to as the typical 'whites'. MP and ML analyses revealed three well-resolved subclades within clade J: the Neotropical *Itaballia* group, the largely Holarctic *Pontia* group, and the Neotropical *Tatochila* group. Relationships among these groups, however, were unresolved and the positions of five taxa (*Ascia*, *Ganyra*, *Leptophobia*, *Pieris*, *Talbotia*) were uncertain. The *Itaballia* group (bootstrap 91% MP, 97% ML) included three taxa: *Itaballia*, *Pieriballia*, and *Perrhybris*. The *Pontia* group (bootstrap 69% MP, 66% ML) included the taxa *Pontia* (*Pontia*), *Pontia* (*Synchloe*), and *Baltia*. The *Tatochila* group comprised a well-supported monophyletic group (bootstrap 100% MP and ML) of six genera from South America (*Tatochila*, *Hypsochila*, *Theochila*, *Pierphulia*, *Phulia*, *Infraphulia*) but not the Palaearctic *Baltia*. *Ascia* and *Ganyra* from the New World appeared to comprise sister taxa to the *Tatochila* group, although evidence for the associations were weak.

A further 15 genera/subgenera within the Pierini (clade G) formed a second major monophyletic group in our analysis (clade K: bootstrap 64% MP, 75% ML) (Figs 4, 5). This clade included the large and speciose genera *Delias* and *Catasticta*, as well as the Palaearctic *Aporia*, Afrotropical *Mylothris*, and the predominantly Oriental *Cepora* and *Prioneris*, both of which were sister to the remaining taxa. Both MP and ML analyses revealed strikingly similar topologies and significant structure within clade K, with several major subclades evident, including the Australian–Oriental *Delias* group, and the predominantly Neotropical *Catasticta* group. The *Delias* group comprised two Old World genera, *Delias* and the Australian *Leuciacria*, the monophyly of which was extremely well supported (bootstrap 95% MP, 99% ML). The *Catasticta* group comprised a well-supported monophyletic group of eight genera (bootstrap 76% MP, 88% ML), all from the New World but predominantly from Central and South America (i.e. *Melete*, *Leodonta*, *Pereute*, *Neophasia*, *Eucheira*, *Catasticta*, *Archonias*, *Charonias*).

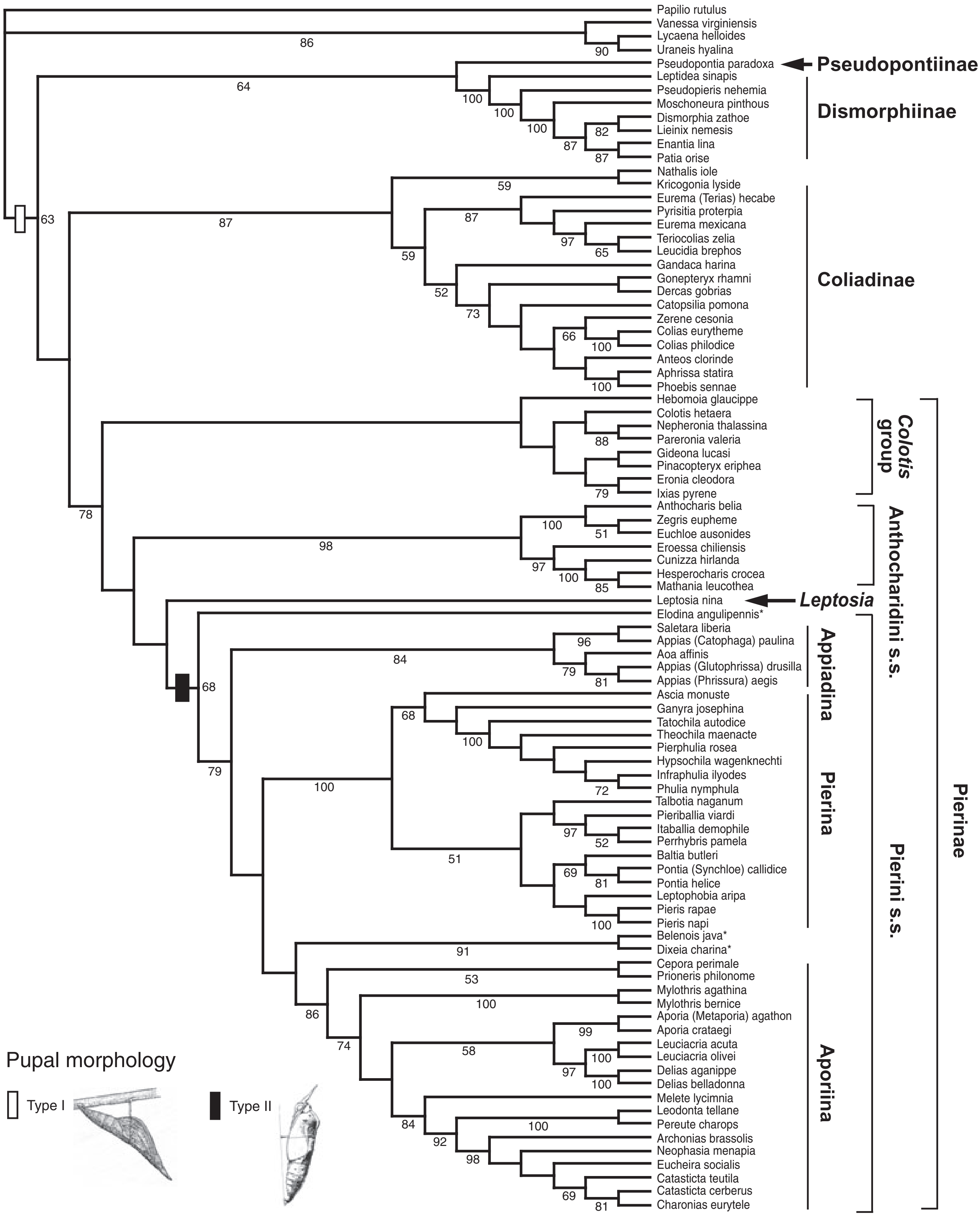
The remaining 12 taxa currently recognized from the Pierini did not belong to the four clades (F, I, J, K) outlined above (Figs 4, 5). Eight genera (*Colotis*, *Eronia*, *Ixias*, *Gideona*, *Pinacopteryx*, *Hebomoia*, *Pareronia*, *Nepheronia*), predominantly from the Afrotropical–Oriental regions, appeared to form two closely related clades (E_1, E_2), but there was no support for their monophyly. Four genera (*Leptosia*, *Elodina*, *Dixeia*, *Belenois*) were scattered across the topology of the trees, but their phylogenetic positions were inconsistent and hence their systematic relationships unresolved.

All available data analysis

The all available data analysis of the entire 90 taxon data set (i.e. 30 taxa for *EF-1α*, *wingless*, *COI*, and *28S*, plus 60 taxa for *EF-1α*) summarized well the topologies generated by the smaller and larger data sets, with high support for both shallow and deep nodes (Fig. 6). The deeper branching events were concordant with those generated by most trees in the combined analysis (Fig. 3), whereas the tips of the tree showed the same basic structure as that generated by the *EF-1α* analysis (Figs 4, 5). However, the level of support for the basal nodes and for the monophyly of some clades was not as high as that recovered in the smaller combined data set.

Age of divergence estimations

The wings, or parts thereof, of the four fossils used as calibration points were sufficiently well preserved to determine their broad systematic relationships within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Vanessa virginiensis
Lycaena helloides
Uraneis hyalina
Pseudopontia paradoxa
Pseudopontiinae
Leptidea sinapis
Pseudopieris nehemia
Moschoneura pinthous
Dismorphia zathoe
Lieinix nemesis
Enantia lina
Patia orise
Dismorphiinae
Nathalis iole
Kricogonia lyside
Eurema (Terias) hecabe
Pyrisitia proterpia
Eurema mexicana
Teriocolias zelia
Leucidia brephos
Gandaca harina
Gonepteryx rhamni
Dercas gobrias
Catopsilia pomona
Zerene cesonia
Colias eurytheme
Colias philodice
Anteos clorinde
Aphrissa statira
Phoebis sennae
Coliadinae
Hebomoia glaucippe
Colotis hetaera
Nepheronia thalassina
Pareronia valeria
Gideona lucasi
Pinacopteryx eriphea
Eronia cleodora
Ixias pyrene
Colotis group
Anthocharis belia
Zegris eupheme
Euchloe ausonides
Eroessa chiliensis
Cunizza hirlanda
Hesperocharis crocea
Mathania leucothea
Anthocharidini s.s.
Leptosia nina
Leptosia
Elodina angulipennis*
Saletara liberia
Appias (Catophaga) paulina
Aoa affinis
Appias (Glutophrissa) drusilla
Appias (Phrissura) aegis
Appiadina
Ascia monuste
Ganyra josephina
Tatochila autodice
Theochila maenacte
Pierphulia rosea
Hypsochila wagenknechti
Infraphulia ilyodes
Phulia nymphula
Talbotia naganum
Pieriballia viardi
Itaballia demophile
Perrhybris pamela
Baltia butleri
Pontia (Synchloe) callidice
Pontia helice
Leptophobia aripa
Pieris rapae
Pieris napi
Pierina
Belenois java*
Dixeia charina*
Cepora perimale
Prioneris philonome
Mylothris agathina
Mylothris bernice
Aporia (Metaporia) agathon
Aporia crataegi
Leuciacria acuta
Leuciacria olivei
Delias aganippe
Delias belladonna
Melete lycimnia
Leodonta tellane
Pereute charops
Archonias brassolis
Neophasia menapia
Eucheira socialis
Catasticta teutila
Catasticta cerberus
Charonias eurytele
Aporiina
Pierini s.s.
Pierinae
Pupal morphology
Type I
Type II

Figure 6. Cladogram for the Pieridae based on the all available data analysis of the 90 taxon data set of the four genes [30 taxa for elongation factor-1 alpha (*EF-1α*), *wingless*, and cytochrome oxidase subunit I (*COI*), and *28S,* plus 60 taxa for *EF-1α*] (3675 bp, 1091 informative characters). Tree topology according to the GTR + I + Γ substitution model inferred under maximum likelihood (ML–PHYML) [log likelihood score = –41028.64: relative rate matrix A↔C 2.6276, A↔G 8.7266, A↔T 6.7753, C↔G 2.3732, C↔T 14.7215, G↔T 1.0; base frequencies A = 0.2518, C = 0.2191, G = 0.2023, T = 0.3265; proportion of invariable sites (I) = 0.5663; shape parameter (α) of gamma distribution (Γ) = 0.2260]. Bootstrap values (500 pseudoreplicates) are shown below the branches for nodes with ≥ 50% support. *Papilio*, *Vanessa*, *Lycaena*, and *Uraneis* are outgroup taxa. To the right of the tree are the formal and informal names of the higher taxa recognized in this work (see Discussion and Appendix). Asterisks denote taxa of uncertain status. The two main pupal forms (types I, II) are mapped on the tree (see Discussion).

the family with some degree of confidence (Table 3). *Stolopsyche libytheoides* is considered to be the ancestor or sister taxon of *Pieris* (Scudder, 1889; Carpenter, 1992), whereas the venation of *Miopieris talboti* is very similar to *Pontia* and its allies (Zeuner, 1942), especially *Baltia*. *Oligodonta florissantensis* shows features reminiscent of the *Catasticta* group (Brown, 1976), particularly *Leodonta* and *Catasticta*: according to our estimate of the phylogeny it could be the ancestor of either *Leodonta* + *Pereute* or *Neophasia* + *Eucheira* + *Catasticta* + *Archonias* + *Charonias*. The forewing of *Coliates proserpina* is similar to *Delias* in shape, the form of the anterior end of the discal cell, and in having vein R_2 absent, but the venation is unusual with veins R_3 and R_4 stalked, $R_3 + R_4$ long-stalked with R_5 (in *Delias* and allied genera, R_4 is fused with R_5 into a single vein), and vein M_2 forming a straight line with the discocellular vein at its point of origin. Scudder (1875a) placed *Coliates* in the *Prioneris–Delias* group; according to our phylogeny, it could belong either with *Prioneris*, *Cepora*, *Mylothris*, *Aporia* or *Delias* + *Leuciacria*. We provisionally placed it with *Aporia* on biogeographical grounds, although we acknowledge that the forewing discal cell (which has the anterior end neatly truncated) and radial venation of *Coliates* are quite distinct from *Aporia*. According to our phylogeny, two fossil genera (*Miopieris*, *Stolopsyche*) belong to clade J, whereas the two other genera (*Oligodonta*, *Coliates*) belong to clade K. We mapped the approximate positions of the four fossils on the nodes and internal branches of the phylogram of our ML model that best fitted the observed data according to their nearest sister taxa (Fig. 5). From the phylogenetic distribution of these fossils, and their known age, it should be possible to estimate the approximate minimum ages of clades J and K, and their immediate common ancestor, to which the four extinct taxa belong. Although there was little support for the basal nodes in the tree generated by the *EF-1α* analysis, the topology did not contradict that estimated in both the combined and all available data analyses for the nodes of interest. That is, clades J and K are either sister taxa (Fig. 3) or comprise a monophyletic group with clade H (Fig. 6).

Penalized likelihood method

From the distribution of the four fossils (Fig. 5), it is clear that, for each fossil, two calibration points can be made depending upon which node is selected along the internal branches. In order to provide a conservative estimate of the substitution rate (i.e. fastest rate) and hence minimum age, we selected the most basal node for each fossil. From these four calibration points, the ages of various nodes were estimated in r8s with the value of the smoothing parameter λ set to 1000 and 3000 (i.e. two reconstructions were performed). The smoothing parameter was optimized using the cross-validation method, which minimizes the square and chi-square error terms. The high estimates of the smoothing parameter suggest that the data were in fact behaving in a clock-like manner. Confidence intervals were calculated for each estimate in r8s based on two (95%) and four (99.9%) standard deviations (SD) of the mean, with the four calibration points fixed and not free to vary.

The average rate of substitution for the *EF-1α* gene was estimated to be 0.1277 ± 0.0024% (SD) per site per million years, which is equivalent to a divergence rate of 1% in 7.83 Myr. This substitution rate seems reasonable given that the average substitution rate for mtDNA (*COI*), which is much faster evolving than nuclear *EF-1α,* is approximately 1.5% Myr^{-1} (i.e. 1% in 0.667 Myr) for arthropods (Quek *et al.*, 2004). In other words, our estimate of substitution for the nuclear gene is approximately 12 times slower than that for mitochondrial *COI* of other arthropods. The estimated minimum age of divergence for the putative split between clades J and K varied from 62.3 Myr (λ = 1000) to 60.7 Myr (λ = 3000). Errors in these estimates were small, with the confidence interval varying from 66.4–55.8 Myr (2 SD) to 68.8–54.1 Myr (4 SD) for the latter estimate. The minimum mean estimate for the crown-group of clade J was 40.6 Myr [λ = 3000; confidence interval (4 SD) = 46.2–36.9 Myr], whereas that for the crown-group of clade K was 50.1 Myr [λ = 3000; confidence interval (4 SD) = 56.7–44.9 Myr]. The minimum mean estimate for the crown-group of the Pieridae was 95.5 Myr [λ = 3000; confidence interval (4 SD) = 111.6–82.5 Myr], although we are cautious about the wisdom of extrapolating too far beyond the calibration points to nodes deeper in the tree.

Quartet method

The mean corrected pairwise distance for each pair of fossil taxa, based on their nearest extant relatives, and their respective evolutionary rates are given in Table 6. The substitution rates for each pair varied greatly, with the *Coliates*–*Oligodonta* split (0.327% Myr^{-1}) being almost twice that of the *Miopieris*–*Stolopsyche* split (0.178% Myr^{-1}). Averaging the two rates gave an overall mean rate of evolution within clades J–K of 0.252 ± 0.106% Myr^{-1} (SD) (i.e. 1% = 4.0 Myr). The mean corrected pairwise distance between the *Miopieris*–*Stolopsyche* lineage and the *Coliates*–*Oligodonta* lineage, that is, between (*Pontia* (*Pontia*) *callidice* + *Pontia* (*Synchloe*) *helice*) and (*Pieris rapae* + *P. napi*), and (*Aporia* (*Aporia*) *crataegi* + *Aporia* (*Metaporia*) *agathon*) and (*Leodonta tellane* + *Pereute charops*), was calculated to be 14.67%. According to the average rate of substitution (0.252% Myr^{-1}), and assuming a molecular clock, this level of genetic divergence between the two pairs of fossil lineages extrapolates to an average divergence time of 58 Myr (Table 6). In other words, the minimum age of the common ancestor of clades J and K, to which the fossil taxa belong, is 58 Myr.

DISCUSSION

Higher classification

Our study represents the first rigorous phylogenetic analysis of the Pieridae, and indeed the first comprehensive phylogenetic study of a higher butterfly taxon at the familial level to date. The monophyly of the four currently recognized subfamilies is well supported (Table 5), corroborating several previous studies that recognize these taxa as distinct lineages (Ehrlich, 1958; Geiger, 1981; Scott, 1985; Janz & Nylin, 1998; Wahlberg *et al*., 2005). However, it is clear that the largest subfamily, the Pierinae, contains far greater within-phylogenetic diversity than hitherto recognized. We therefore use these results in combination with previously published hypotheses to propose a revised higher-level systematic classification of the Pieridae (see the Appendix; also shown to the right of terminal taxa in Fig. 6 and in Table 5). In this classification, we provisionally place the 83 genera in the conventional four subfamilies in order to maintain the nomenclatural stability of the present classification (Knapp *et al*., 2004), but divide the Pierinae into two tribes (Anthocharidini *s.s.*, Pierini *s.s.*) and two informal groups (*Colotis* group, *Leptosia*). The Pierini *s.s.* are further subdivided into three subtribes (Appiadina, Pierina, Aporiina) and two informal groups comprising three genera (*Elodina*, *Dixeia*, *Belenois*) of uncertain status (*incertae sedis*). We discuss the status, monophyly and phylogenetic relationships of these higher taxa in more detail below.

A revised estimate of the phylogeny for the Pieridae, summarizing the interrelationships of the higher taxa and major clades recovered in the smaller combined

Table 6. Estimated time of divergence between clades J and K according to the phylogeny of Figure 5 using the quartet method (see Cooper & Penny, 1997). The estimate is based on the minimum divergence time for each pair of related taxa (lineages) according to their oldest known fossils. The fossils *Miopieris talboti* and *Stolopsyche libytheoides* belong to clade J, and *Oligodonta florissantensis* and *Coliates proserpina* belong to clade K. Pairwise distances are the average corrected pairwise distances according to a GTR + I + Γ substitution model (see Fig. 5). The nearest relative of *Coliates* is provisionally placed with *Aporia* on biogeographical grounds

						Split between clades J and K	
Fossil taxon	Nearest related taxa (lineage)	Pairwise distance (%)	Minimum divergence time (Myr)	Average substitution rate (% Myr^{-1})		Pairwise distance (%)	Estimated age (Myr)
Miopieris	*Pontia (Synchloe) callidice + Pontia helice*	6.0415	34	0.1777	0.2523	14.668	58
Stolopsyche	*Pieris rapae + P. napi*						
Coliates	*Aporia crataegi + A. (Metaporia) agathon*	11.1148	34	0.3269			
Oligodonta	*Leodonta + Pereute*						

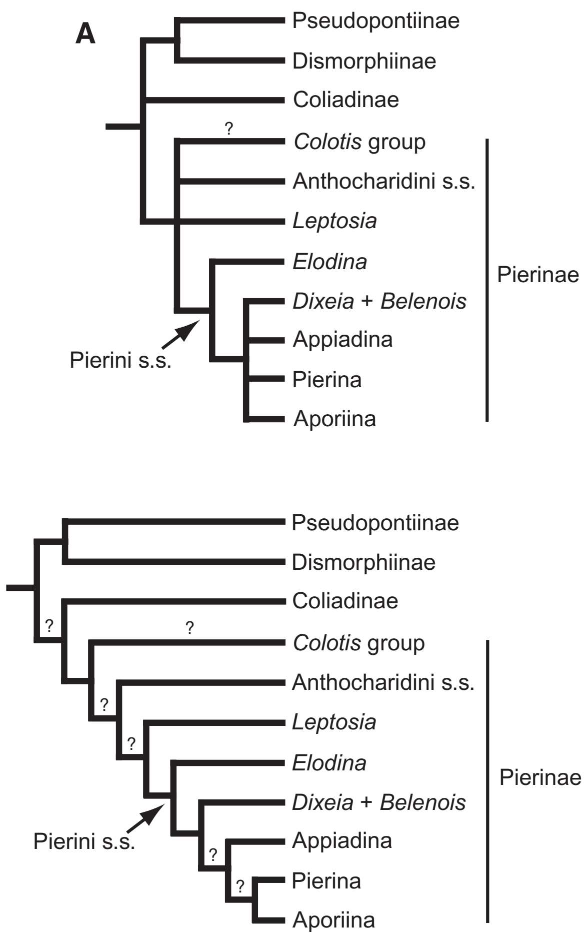

Figure 7. Higher classification of the Pieridae, showing two possible phylogenetic hypotheses according to the combined and all available data analyses of this study (Figs 3, 6). A, consensus tree summarizing nodes that are well supported or that are consistently recovered under different methods of analysis (maximum parsimony, maximum likelihood, Bayesian inference), with a question mark denoting uncertainty in the monophyly of the *Colotis* group. B, fully resolved tree, with question marks denoting uncertainty among nodes and in the monophyly of the *Colotis* group. Four subfamilies are recognized, with the subfamily Pierinae comprising four major lineages (two tribes, two informal groups); the tribe Pierini is subdivided into five lineages (three subtribes, two subclades of uncertain status).

analysis (Fig. 3) and the larger all available data analysis (Fig. 6), is presented in Figure 7A. In this tree, only nodes that are well supported or consistently recovered under different analytical methods (Giribet, 2003) are shown. Figure 7B portrays a fully resolved hypothesis and higher classification of the Pieridae according to the branching order of our ML tree (Fig. 3B), with question marks denoting uncertainty of nodes that are not well supported. Although there is uncertainty in the phylogenetic position of the Coliadinae, the sister relationship between the Pseudopontiinae and the Dismorphiinae is well supported, and this lineage is almost certainly the sister group to the rest of the Pieridae. The most probable hypothesis for the subfamilial relationships is therefore (Pseudopontiinae + Dismorphiinae) + (Coliadinae + Pierinae) (Fig. 7B). This topology is identical to that originally proposed by Ehrlich (1958) (Fig. 1B) and will serve as our best estimate until further evidence is obtained to indicate a contrary pattern. Resolution of some of the uncertainties among the deeper-level divergences may only be overcome by addition of further genetic markers and/or integration of morphological characters from both adult and early stages (Wahlberg & Nylin, 2003).

An interesting feature of our all available data analysis (Fig. 6) is the close agreement with Klots' (1933) intuitive phylogeny based on morphology (Fig. 2). With the exception of the phylogenetic placement of the Anthocharidini and the Coliadinae, there is remarkable concordance of the two trees in terms of the higher-level structure. In relation to the lower-level structure, there is also strong concordance between groups of genera at the tips of the trees. Indeed, most of the 'shallow' differences lie among the 12 genera whose relationships Klots himself was uncertain about. Quite striking are parallels in the evolutionary relationships of the Pierini *s.l.* In a phylogenetic sense, Klots' concept of the Pierini was paraphyletic as it included the Coliadinae. Moreover, he envisaged two clades comprising six genera (*Gideona* + *Colotis* + *Ixias*) and (*Eronia* + (*Nepheronia* + *Pareronia*)), as each having separate origins from the main stock of Pierini *s.l.* (Fig. 2). These taxa show a similar pattern in our analyses, and comprise an assembly, termed the *Colotis* group, phylogenetically removed from the remaining Pierini *s.s.* (Fig. 6).

Our results also show a strong association between the morphology of the pupal stage and higher taxa. Pierid pupae approximately fall into two major groups according to differences in their morphology and habits (Talbot, 1939). In the Pseudopontiinae, Dismorphiinae, Coliadinae, and some groups in the Pierinae (i.e. *Colotis* group, Anthocharidini, *Leptosia*), the pupa (type I) is smooth, the wings are often strongly curved ventrally to form a prominent 'keel', and the head is tapered apically, often forming a prominent point or spine. The pupa is suspended loosely by the central girdle, usually horizontally or sometimes slightly upwards or downwards, but always with the ventral surface facing uppermost, similar to that of many Papilionidae. In contrast, the pupae of all members of the Pierini *s.s.* (i.e. Appiadina, Pierina, Aporiina, *Elodina*, *Dixeia*, *Belenois*) are characterized by having markedly different morphology and habits. In these taxa, the pupa (type II) is more elongate with the ventral surface flat, the head has a horn or spine-like process anteriorly, which may be very prominent, the thorax has a pronounced dorsal ridge, the anterior abdominal segments (usually two to four) frequently have a series of dorsolateral spines or projections, and sometimes the abdomen has a series of dorsal projections on each segment. A central girdle secures the pupa close to the substrate, usually vertically or horizontally, but nearly always with the dorsal surface facing uppermost. These two pupal forms are mapped on the topology of our cladogram for the all available data analysis (Fig. 6). From the phylogeny it is clear that pupal form type I is the ancestral (plesiomorphic) form and that pupal form type II is a derived trait, having evolved once within the family and after the origin of the Pierinae. Pupal form type II is thus a synapomorphy for the Pierini *s.s.*

Pseudopontiinae and Dismorphiinae

The close relationship between the Pseudopontiinae and the Dismorphiinae (Table 5, Figs 3–6) supports previous conclusions drawn by Klots (1933) and Ehrlich (1958) (Fig. 1A, B), and Ackery *et al*. (1999) based on morphological evidence, particularly the male genitalia. *Pseudopontia* was placed in a separate subfamily because of its peculiar venation and other features. The lineage is long-branched (Figs 3B, 5) and comprises a single terminal taxon, indicating that either substantial evolutionary change has occurred since it diverged from the ancestor of Dismorphiinae + Pseudopontiinae (possibly due to a population bottleneck in the past), or that there have been considerable extinction events in the lineage. Yoshimoto (2000) suggested that *Leptidea* was unrelated to the *Dismorphia* group, but our analysis refutes this and recovers the Dismorphiinae as a well-supported monophyletic group, with *Leptidea* sister to the remaining six genera.

Coliadinae

In contrast to the preliminary analysis based on morphology by Venables (1993), our results (Table 5, Figs 3–6) support the conclusion of a number of other studies (Klots, 1933; Ehrlich, 1958; Geiger, 1981; Janz & Nylin, 1998; Pollock *et al*., 1998; T. Yamauchi, O. Yata & A. Venables, unpubl. data) that recognize the Coliadinae as a natural or monophyletic group. de Jong *et al*. (1996) and Ackery *et al*. (1999) were unable to identify clear synapomorphies for their exemplar taxa, although T. Yamauchi, O. Yata & A. Venables (unpubl. data) noted two characters (patagia sclerotized; valvenansatz a short, narrow lobe) that appear to be diagnostic. The subfamily is almost certainly the sister group to the Pierinae. Of the 18 genera currently recognized in the subfamily (Braby, 2005), only three (*Abaeis*, *Prestonia*, *Rhabdodryas*) were not included in our study. *Abaeis* Hübner contains two species restricted to North and Central America (Lamas, 2004); it was previously considered to be a subgenus of *Eurema* and may well belong to the New World *Eurema* and allied taxa (Klots, 1933). *Prestonia* Schaus is a monotypic genus, with type species *clarki* Schaus, from Mexico (Lamas, 2004); it was previously treated as a synonym of *Phoebis* Hübner (Klots, 1933). *Rhabdodryas* Godman & Salvin includes the single species *trite* (Linnaeus) from Central and South America (Lamas, 2004); Klots (1933) treated it as a subgenus of *Phoebis*.

Pierinae

The analyses of the combined and all available data provide strong support for the monophyly of the Pierinae (Table 5, Fig. 6). Of the 57 genera currently recognized in the subfamily (Braby, 2005), 51 were included in our study. On the basis of our analyses, together with morphological evidence (Klots, 1933), we propose a reclassification of the Pierinae and divide the subfamily into four rather than two main lineages. These lineages comprise two tribes (Anthocharidini *s.s.*, Pierini *s.s.*) and two informal groups (*Colotis* group, *Leptosia*), the interrelationships of which are unresolved (Fig. 7A).

Colotis *group*

We place eight genera, previously included in the Pierini *s.l.* (i.e. *Colotis*, *Eronia*, *Ixias*, *Gideona*, *Pareronia*, *Nepheronia*) or Anthocharidini *s.l.* (i.e. *Hebomoia*, *Pinacopteryx*), into an informal group termed the *Colotis* group. *Calopieris* Aurivillius, although not included in this study, presumably belongs here. It is a monotypic genus, with type species *eulimene* (Klug), restricted to areas adjacent to the Red Sea of Africa (Chad, Sudan, Arabia) (Ackery, Smith & Vane-Wright, 1995); it was previously regarded as a subgenus of *Colotis*, to which it is probably closely related. These nine taxa may well comprise a separate lineage sister to the rest of the Pierinae, but evidence for their monophyly is currently lacking (Fig. 6). As noted earlier, Klots (1933) regarded the first six genera to be phylogenetically unrelated to the other Pierini *s.l.* (Fig. 2), and this pattern is also evident in our analysis. The higher taxon name Teracolini, introduced by Reuter (1896) for the genus *Colotis*, is available (Bridges, 1988), but for the present we prefer not to recognize the group as a formal tribe without further evidence.

Klots (1933) described the monotypic genus *Gideona* endemic to Madagascar on the basis of its distinct genitalia, noting that it was probably related to *Colotis* or possibly *Eronia* and *Nepheronia* + *Pareronia*. However, Lees, Kremen & Raharitsimba (2003), following Bernardi (1954), treated *Gideona* as a subgenus of *Colotis*. In our all available data analysis, *Gideona* appears to be more closely related to *Pinacopteryx* and *Eronia* + *Ixias*, although evidence supporting this arrangement is not convincing. Although the monophyly of *Nepheronia* + *Pareronia* is well supported, reinforcing Klots' view (Fig. 2) of a close relationship between these taxa, the systematic relationship of *Colotis* is not resolved.

Anthocharidini s.s.

In our *EF-1α* (Figs 4, 5) and all available data (Fig. 6) analyses, the Anthocharidini, as delimited and classified by Klots (1933), are polyphyletic, with two Old World genera, the Afrotropical *Pinacopteryx* and the predominantly Oriental *Hebomoia*, falling outside the remaining genera. Indeed, Klots (1933: 174–175) stated that he 'does not regard the Euchloini as here delineated as being an entirely natural group' by inclusion of *Pinacopteryx* and *Hebomoia*. We thus narrow the concept of the Anthocharidini to include only seven genera (*Euchloe*, *Anthocharis*, *Zegris*, *Eroessa*, *Cunizza*, *Hesperocharis*, *Mathania*), the monophyly of which is extremely well supported in the combined and all available data analyses (Table 5, Fig. 6). Interestingly, the broad, straight subapical orange band present on the forewing in the Holarctic *Anthocharis* and *Zegris* (*Zegris*) of the *Anthocharis* group, also occurs in *Eroessa* of the Neotropical *Hesperocharis* group, and may be a synapomorphy for the tribe, with independent losses in *Euchloe* and the *Hesperocharis* subclade.

Leptosia *group*

Klots (1933) was uncertain about the phylogenetic position of *Leptosia*, noting that 'In none of its characters does it show any close relationship to any other modern Pieridae, but stands alone.' He went on further to state that '*Leptosia* appears to have no close relatives. It probably represents a derivative of a stock that split off far back on the Pierine line of development.' Klots' sentiments are clearly borne out in our all available data analysis in which the genus shows no close relatives other than belonging somewhere in the subfamily Pierinae (Fig. 6). The taxon possibly comprises a distinct lineage and may well warrant formal tribal status. However, for the moment we recognize it as an informal group within the Pierinae, pending further study of its exact relationships.

Pierini s.s.

We remove ten genera (in the *Colotis* group, and *Leptosia*) from the Pierini *s.l.*; otherwise the tribe is non-monophyletic in the broad sense. The monophyletic Pierini *s.s.* (Table 5, Fig. 6) is distinguished from all other pierids by possession of pupal type II morphology. Five lineages are recognized in the tribe: three subtribes (Appiadina, Pierina, Aporiina) and two groups of uncertain status (*Elodina*, *Dixeia* + *Belenois*). The interrelationships of these lineages are largely unresolved, although *Elodina* is almost certainly sister to the remaining taxa (Fig. 7A).

Appiadina

We provisionally place four genera (*Saletara*, *Appias*, *Udaiana*, *Aoa*) in the subtribe Appiadina, introduced by Kusnezov (1921), based on strong evidence of monophyly in the combined and all available data analyses (Table 5, Fig. 6). Klots (1933) considered *Saletara*, *Appias* and *Udaiana* to be very closely related on morphological grounds (Fig. 2), and Yata (1985: 359) noted that *Saletara* 'should be phylogenetically included in the comprehensive genus *Appias*'. *Udaiana* Distant, not included in this study, contains a single species, *cynis* (Hewitson), known only from a restricted area in south-east Asia. *Aoa* was thought to be unrelated to these genera, although Klots (1933: 223) commented that 'Its exact relationships are . . . very obscure' and Yata (1985) noted that the butterfly is similar to *Appias* and *Cepora*. The genitalia of *Aoa* are remarkably similar to *Appias*, and our molecular evidence suggests a close relationship between these two taxa. The genus *Appias*, which includes five subgenera (*Appias*, *Hiposcritia*, *Catophaga*, *Phrissura*, *Glutophrissa*), is almost certainly paraphyletic. *Appias* (*Appias*), not included in this study, includes seven species restricted to the Oriental and Australian regions (Yata, 1985; Vane-Wright & de Jong, 2003). Although not well supported, the Appiadina may be sister to Pierina + Aporiina.

Pierina

We place 19 genera in the subtribe Pierina. Of these, 16 genera were included in our *EF-1α* and all available data analyses, all of which formed an extremely well-supported monophyletic group (Figs 4–6). The three genera (*Glennia*, *Reliquia*, *Piercolias*) not included in our study presumably belong here according to morphological evidence. The monotypic genus *Glennia* Klots contains the species *pylotis* Godart from southern Brazil, but its systematic position is problematic, having affinities with either *Pieris* Schrank or *Pontia* Fabricius (Robbins & Henson, 1986). *Reliquia* Ackery is another monotypic genus, containing the species *santamarta* Ackery restricted to Sierra Nevada de Santa Marta of north-eastern Colombia (above 3000 m) (Ackery, 1975; Shapiro, 1978b). It has affinities with the *Pieris* and *Tatochila* groups of genera, and is considered to have phylogenetic and biogeographical importance in understanding the evolution and radiation of the high Andean and Patagonian pierine fauna. *Piercolias* Staudinger contains three rare species from the high Andes of southern Peru and Bolivia, and belongs to the *Tatochila* group of genera; it is probably the sister genus of *Pierphulia* Field (Field, 1958; Field & Herrera, 1977).

Klots (1933: 218–219) was uncertain about the position of *Baltia* from the Himalayas, stating that, on the one hand it 'probably represents a group, originally derived from *Synchloe* or some closely related stock', but on the other hand 'Whether there is a real relationship between *Baltia* and *Phulia* or whether the resemblances are merely to be regarded as similar developments, in the same type of environment is a matter of doubt'. Field (1958) placed *Baltia* in the *Tatochila* group of genera, the members of which predominantly inhabit the high Andes of South America. Although our analysis strongly supports the monophyly of the South American genera of the *Tatochila* group, it does not support a close relationship between these otherwise disjunct taxa, with *Baltia* being more closely related to *Pontia* (*Pontia*) + *Pontia* (*Synchloe*) from the Holarctic, as originally suggested by Klots. We tentatively conclude that the striking similarities in morphology between some members of *Baltia* (Central Asia) and the *Tatochila* group *s.s.* (South America) are due to convergence of living at extreme altitudes and not to common ancestry.

Aporiina

We place 14 genera (embracing around 480 species) in the subtribe Aporiina, first introduced by Chapman (1895) as a subfamily to distinguish genera such as *Aporia* and *Delias* from *Pieris*, but expand the concept of the taxon to include a larger number of genera. The monophyly of the group is well supported (Table 5, Fig. 6). Most of the genera are morphologically distinct (especially the early stages; M.F. Braby & K. Nishida, unpubl. data), biologically peculiar with the larvae of the vast majority of species feeding gregariously and producing considerable quantities of silk, and phylogenetically removed from the Pierina. Indeed, more than 25 years ago, the late John Eliot (in Corbet & Pendlebury, 1978, 1992) suggested that *Delias* together with the African *Mylothris* and South American *Catasticta*, *Archonias*, *Pereute*, and *Leodonta* probably form a distinct tribe. Chapman's (1895) higher divisions of the Pieridae were based primarily on fundamental differences in pupal morphology, including the structure, shape (especially wing cases), and motility of segments. In the Aporiina, he noted that both abdominal segments 5 and 6 are movable (when molested the abdomen twitches violently), whereas only abdominal segment 5 is movable in the Coliadinae and the Pierina, but no segments are movable in the Anthocharidini. Our combined analysis suggests that the well-supported Aporiina (Table 5, Fig. 6) may be the sister taxon to the Pierina.

Six major lineages are evident in the Aporiina: *Cepora*, *Prioneris*, *Mylothris*, *Aporia*, *Delias* group, and the *Catasticta* group. The last four taxa/subclades are very closely related (Braby, Pierce & Vila, 2006); they share a number of larval and adult morphological features, and the majority of species for which life histories are known feed as larvae on mistletoes in the order Santalales. It is not clear whether *Cepora* and *Prioneris* form a monophyletic group sister to (*Mylothris* + *Aporia* + *Delias* group + *Catasticta* group), or represent two independent lineages that diverged early in the evolution of the Aporiina.

Incertae sedis

The phylogenetic positions of three genera (*Elodina*, *Dixeia*, *Belenois*) are uncertain in our combined and all available data analyses (Figs 3, 6) in that they do not belong to any of the higher taxa recognized above. Klots (1933) was equally uncertain about the relationships of the Australian *Elodina*, which appears to be the sister lineage to the rest of the Pierini. Klots (1933) suggested that the African *Dixeia* and *Belenois* were closely related and probably allied to *Prioneris* (Fig. 2). The monophyly of *Dixeia* and *Belenois* is corroborated in our combined analysis (Table 5), and these two genera probably constitute a separate subtribe within the Pierini (Fig. 7A).

BIOGEOGRAPHY

The age of divergence estimates generated by the two different methods based on fossils were approximate. Nevertheless, both estimates for the age of divergence between the Pierina (clade J) and Aporiina (clade K) were similar and around 60 Myr (Palaeocene): 61 Myr for penalized likelihood and 58 Myr for the quartet method. Moreover, the 99.9% confidence interval for the mean estimate under penalized likelihood was relatively small and precise (69–54 Myr). These observations indicate that the *EF-1α* data are robust, clock-like and, if potential sources of error are assumed to be small, the age estimates may be accurate. The main assumptions and potential sources of error in the estimates are that the topology of the phylogram accurately represents evolutionary relationships of the extant taxa, the age of the fossil deposits have been dated accurately, and that the fossils have been identified and placed on the tree correctly. Our all available data analysis suggests that our phylogenetic hypothesis of the Pieridae is reasonably robust (Fig. 6), although additional or independent data (e.g. from morphological characters) would be desirable. The age of the deposit from which the two oldest fossils were described (Florissant Formation) has been accurately dated using the 40Ar–39Ar decay method (Evanoff *et al*., 2001). The identity of the fossils has been determined with a high degree of certainty at the subclade level (i.e. generic groups) (Table 3), but with less certainty among the extant genera within those groups. Given that fossils provide only minimum estimates of age, and that our estimates are conservative in that we calculated the fastest possible rate under penalized likelihood, the common ancestor of the Pierina + Aporiina is more likely to have originated before than during the Palaeocene. Clearly the ancestor of the Pieridae must be older than 60 Myr. Extrapolation of our phylogram from the node uniting the Pierina and Aporiina (Fig. 5) indicates that the stem-group of the family must have arisen well before the Tertiary. Indeed, our extrapolated mean estimate of 95 Myr (99.9% confidence interval: 112–82 Myr) for the crown-group of the Pieridae under penalized likelihood is in close agreement with the maximum age of 94–91 Myr estimated from the analysis of larval host plant associations and reconstruction of the ancestral host in relation to the maximum age of the plants (Braby & Trueman, 2006). These findings suggest a possible maximum origin of the Pieridae in the Cenomanian-Turonian of the Late Cretaceous.

Pierids occur worldwide but are not evenly distributed throughout the major zoogeographical regions (Table 7). In terms of taxonomic richness at the generic and subgeneric level, the Neotropical region has by far the highest diversity (46 taxa), whereas the Australian region has the lowest diversity (13 taxa). The Afrotropical and Palaearctic regions have similar totals in richness, but considerably less than the Oriental and Nearctic regions. More than two-thirds of the Neotropical fauna (70%) is endemic to the region at the generic/subgeneric level, and more than one-third of the fauna in the Afrotropical region (42%) is endemic. In contrast, the large Holarctic region (Palaearctic and Nearctic) has a low level of endemism, with only five endemic taxa, of which two are restricted to the Himalaya (*Baltia*, *Aporia* (*Mesapia*)) and two to North America (*Eucheira*, *Neophasia*). The Australian region likewise has a very low level of endemism (15%), with *Leuciacria* and *Elodina* being the only taxa endemic to the region, with the latter genus extending as far west as Sulawesi in Wallacea. Although the Oriental region has a relatively high richness (second to the Neotropics), the level of endemism is comparatively low (29%), but much higher than that of the Australian, Nearctic, and Palaearctic. The Oriental and Australian regions, however, often share taxa because of frequent dispersal across Wallacea: combining the faunas for the two regions revealed a high level of endemism (55%), most of which is centred near the Old World tropics, although overall richness (29 taxa) is still low compared with the Neotropics.

Although the generic/subgeneric framework is incomplete for the Pieridae (Braby, 2005), it is unlikely that further improvements to the higher classification will affect the broad patterns enumerated in Table 7. South America clearly stands out as an area that has a highly distinctive fauna in terms of its composition, richness and endemism, whereas the Australian region has the most impoverished fauna. Moreover, four groups (Pseudopontiinae, Dismorphiinae, Anthocharidini, Pierina) are notably absent from Australia.

In terms of the higher taxa recognized in this work, the subfamilies, tribes, subtribes, and informal groups have markedly different distribution patterns among the six major zoogeographical regions. The Pseudopontiinae are endemic to Africa, whereas the Dismorphiinae are restricted mainly to the Neotropical region, with a disjunct occurrence in the Palaearctic. The Coliadinae, although especially well represented in the Oriental and New World faunas, are cosmopolitan. Indeed, two genera (*Eurema s.l.*,

Table 7. Comparison of taxonomic richness between the zoogeographical regions at the generic and subgeneric level (compiled from data listed in the Appendix)

Zoogeographical region	Number of taxa: Major occurrence	Minor occurrence	Total	No. of endemic taxa (%)
Neotropical	46	0	46	32 (70)
Oriental	20	8	28	8 (29)
Nearctic	15	8	23	2 (9)
Afrotropical	14	5	19	8 (42)
Palaearctic	14	4	18	3 (17)
Australian	8	5	13	2 (15)

Colias) are almost cosmopolitan. Within the Pierinae, the *Colotis* group and *Leptosia* occur in the Old World, mainly in the Afrotropical and Oriental regions, with weaker representations in the Australian and/or Palaearctic. The Anthocharidini *s.s.* are restricted geographically to the Neotropical and Holarctic regions (with a weak representation in Africa) and contain two monophyletic subclades, the *Hesperocharis* and *Anthocharis* groups, confined to each of these zoogeographical regions, respectively. Within the Pierini *s.s.*, the Appiadina are pantropical, but the higher taxa (genera, subgenera) are concentrated in the Oriental region and few species occur in the other regions. The Aporiina, the putative sister group of the Pierina, are more strongly represented in the southern hemisphere (Neotropical, Australian, and to a lesser extent Afrotropical) and south-east Asia (Oriental) than in the Holarctic. In contrast, the Pierina are strongly represented in the Neotropical and Holarctic regions, with a weaker representation in the Afrotropical and Oriental. *Elodina* is restricted to the Australian region, with a very small representation in submontane eastern Indonesia of Wallacea (Vane-Wright & de Jong, 2003), whereas *Dixeia* and *Belenois* are predominantly African.

Phylogenetic relationships within most of these higher taxa are, in general, too poorly resolved to interpret broad historical biogeographical patterns. Also, given the strong migratory tendencies in the family, ancient vicariant patterns have probably long been obscured by subsequent dispersal events, and levels of differentiation may only be sought among closely related genera or species within genera. Moreover, few higher taxa are restricted to areas of endemism. The Pseudopontiinae and Dismorphiinae, however, are an exception. The phylogenetic hypothesis concerning the generic relationships of the clade Pseudopontiinae + Dismorphiinae is well supported and the terminal taxa are restricted either to the Afrotropical, Neotropical, or Palaearctic regions. These two subfamilies are therefore the most amenable of the higher taxa within the Pieridae to historical biogeographical analysis.

Pseudopontiinae–Dismorphiinae

Scott (1985: 261) hypothesized that the ancestor of Pseudopontiinae + Dismorphiinae evolved in Western Gondwana before Africa and South America finally split apart (100–90 Myr); the two subfamilies then diverged vicariantly following the break-up of the two continents. However, the disjunct geographical distribution of the Dismorphiinae has remained an outstanding biogeographical enigma in the Pieridae. To explain the presence of the Dismorphiinae in the Palaearctic, Scott suggested that *Leptidea* dispersed across the Bering Strait to reach Eurasia. However, this scenario assumes at least four major biogeographical steps or costs [in the sense of Ronquist (1997)]: (1) dispersal from South America to North America, (2) extinction in South America, (3) dispersal from North America to Eurasia, and (4) extinction in North America. Dispersal is relatively easy to envisage in most pierid butterflies, but the extinction or range contraction of a whole genus from both North and South America is much harder to comprehend. Even if the ancestor of the Dismorphiinae expanded its range to the northern hemisphere before the split between *Leptidea* and the Neotropical Dismorphiinae, such that *Leptidea* and Neotropical Dismorphiinae diverged allopatrically in Eurasia and South America, respectively, this three-step hypothesis implies that the ancestor became extinct in North America but not in Europe.

An alternative vicariance hypothesis is that the ancestor of *Leptidea* reached Europe via northern Africa rather than via North America. According to our phylogeny, there are two major speciation events: (a) Pseudopontiinae (Africa) and Dismorphiinae (South America), and (b) Neotropical Dismorphiinae (northern South America) and *Leptidea* (northern Africa). The first speciation event may have occurred by vicariance, and the second event possibly through long-distance postspeciation dispersal. This hypothesis thus requires a minimum of three biogeographical steps: (1) long-distance dispersal of the ancestor of Dismorphiinae from northern South America to northern Africa followed by allopatric speciation of *Leptidea* in northern Africa; (2) dispersal (range expansion) of the ancestor of *Leptidea* from northern Africa to Eurasia; and (3) extinction (range contraction) of *Leptidea* in northern Africa (Fig. 8). *Leptidea* is currently not known from northern Africa, although Tennent (1996: 102) drew attention to the possibility that the genus may occur in the coastal regions. Step (2) probably involved simple range expansion following collision of the African plate with Eurasia during the early Tertiary (60 Myr), rather than long-distance dispersal. Step (3) probably involved range contraction following aridification of northern Africa with formation of the Sahara Desert after the Miocene. Subsequent differentiation of the Neotropical Dismorphiinae (at the generic level) in South America presumably represents a duplication event (sympatric speciation) within this area of endemism.

Scott's and our biogeographical hypotheses rest on the assumption that the ancestor of the Psuedopontiinae + Dismorphiinae originated in Western Gondwana, that is, when Africa and South America were still connected. Plate tectonic models show that the opening of the South Atlantic Ocean between Africa and South America started in the south in the

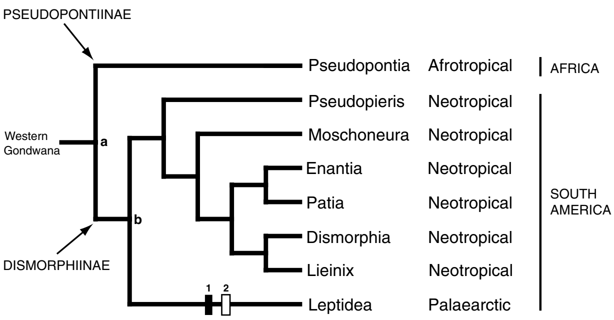

Figure 8. Historical biogeographical hypothesis of the Pseudopontiinae + Dismorphiinae, with dispersal and extinction events optimized to reconcile the area cladogram. Letters designate speciation events: a, vicariance between Pseudopontiinae (Africa) and Dismorphiinae (South America), following the final break-up of Western Gondwana (Late Cretaceous); b, long-distance dispersal of the ancestor of Dismorphiinae from northern South America to northern Africa (Late Cretaceous), followed by allopatric speciation of *Leptidea* in northern Africa (Late Cretaceous). Numbers designate major biogeographical events: 1, dispersal (range expansion) of the ancestor of *Leptidea* from northern Africa to Eurasia, following contact of Africa with Eurasia (early Tertiary); 2, extinction (range contraction) of *Leptidea* in northern Africa following formation of the Sahara Desert (Quaternary). Once *Leptidea* reached Eurasia it colonized much of the Palaearctic, the Neotropical Dismorphiinae subsequently spread into Central America, whereas the Pseudopontiinae contracted to central western Africa.

Early Cretaceous (from *c.* 135 to 130 Myr) and propagated northwards until the mid- to Late Cretaceous (*c.* 110–90 Myr) when a transform fault opened between Guinea and Brazil, so that northern Western Gondwana separated much later than southern Western Gondwana (Smith, Smith & Funnell, 1994; White, 1994; Cox & Moore, 2000; Scotese, 2001; Sanmartín & Ronquist, 2004). Thus, if the ancestor of Psuedopontiinae + Dismorphiinae occurred in Western Gondwana such that the two subfamilies evolved under a process of vicariance, then the two lineages would have diverged sometime in the Late Cretaceous (> 90 Myr) at the very latest. This implies an origin of the Pieridae around the Late Cretaceous, which agrees well with our approximate estimates based on fossils (95 Myr) and larval host plant associations (94–91 Myr). The alternative scenario is that the speciation event occurred more recently through long-distance dispersal from South America to Africa, or vice versa. Although possible, this hypothesis is less parsimonious, as it requires an extra biogeographical step.

Whatever the true sequence of events and mode of speciation, the molecular and morphological divergence between the Pseudopontiinae and Dismorphiinae is substantial (average corrected pairwise distance for *EF-1*α = 30.5%), and no doubt reflects a long period of isolation between the two subfamilies. Moreover, a recent molecular phylogeny of *Leptidea* (Martin, Gilles & Descimon, 2003) suggests that *L. duponcheli* (endemic to the Mediterranean) is the sister taxon to the remaining species, most of which occur widely in the Palaearctic, including Siberia (e.g. *L. sinapis*, *L. morsei*, *L. amurensis*). Such a biogeographical pattern is consistent with our hypothesis that *Leptidea* reached Europe from Africa and not from North America. We tentatively conclude that the Pseudopontiinae + Dismorphiinae originated in Western Gondwana, and that divergence of the two groups occurred by vicariance between South America and Africa, probably sometime during the Late Cretaceous.

Other taxa

Three other groups (Coliadinae, Anthocharidini *s.s.*, *Tatochila* group) show interesting biogeographical patterns that also point towards an origin in South America/southern hemisphere. Although relationships within the Coliadinae are not well resolved, it is curious that both *Nathalis* and *Kricogonia*, relictual taxa sister to the rest of the subfamily, are found only in the New World, especially Central and South America. Indeed, A. Shapiro (pers. comm.) has suggested that *Nathalis*, which has its main occurrence in the high altitudes of the Andes, probably originated in South America and colonized North America recently.

The Anthocharidini *s.s.* are restricted largely to the Neotropical and Holarctic regions. An origin of the tribe in South America would involve two major biogeographical steps: (1) long-distance dispersal or range expansion of the ancestor of the stem-group to North America, followed by differentiation of the *Anthocharis* and *Hesperocharis* groups in North and South America, respectively, and (2) dispersal (range expansion) of the *Anthocharis* group to Europe/northern Africa. However, a northern hemisphere origin in North America is equally parsimonious. The relictual distribution of *Eroessa*, which is limited to cool temperate rainforest (valdivian forest) of southern Chile–western Argentina (Shapiro, 1991; M.F. Braby & K. Nishida, unpubl. data), provides circumstantial evidence in favour of the first hypothesis. If correct, the timing of such events may date back to the early Tertiary (50–40 Myr) when North and South America joined and then separated again following formation of the Greater Antilles.

Within the subtribe Pierina (Pierini), the *Tatochila* group of genera (*Tatochila*, *Hypsochila*, *Theochila*, *Pierphulia*, *Phulia*, *Infraphulia*, and probably *Pierco-*

lias and *Reliquia*) comprises a well-supported monophyletic group. Theories concerning its origin and evolution in the high Andes of South America have long attracted attention (summarized by Shapiro, 1978a, 1994). It has generally been assumed that the ancestor of the *Tatochila* group dispersed from the Palaearctic/Holarctic to South America during the Great American Interchange 3–2 Myr, and then radiated explosively once it colonized the high Andes, which are young geologically. However, this hypothesis rests on the presumption that the *Tatochila* group is closely related to *Baltia* [which is limited to high altitudes (> 5000 m) in Central Asia] and/or *Pontia* from the northern hemisphere. Our molecular phylogeny shows that the nearest relatives of the *Tatochila* group are probably *Ascia* and *Ganyra*, and that the subclade *Baltia* + *Pontia* is more distantly related. *Ascia* and *Ganyra* are restricted to the New World, but have their major centre of distribution (i.e. in terms of both diversity and area of occurrence) in Central and South America. Hence, it is probable that lowland tropical Amazonia may have been the source for high-altitude colonization by the *Tatochila* group rather than stock from the northern hemisphere. Our phylogram (Fig. 5) supports the contention that the *Tatochila* group represents an example of rapid and probably recent radiation in the high Andes: the stem-group is subtended by a long branch and the crown-group shows little resolution, with the terminal taxa (six genera in our analysis) having very short branches, giving a long 'broom handle' pattern typical of explosive radiations (Crisp, Cook & Steane, 2004). The minimum mean estimate for the node (crown-group) of the *Tatochila* group under penalized likelihood was 10.4 Myr [$\lambda = 3000$; confidence interval (4 SD) = 14.5–7.3 Myr], which coincides with the time of initial uplift of the Andes.

DIRECTIONS FOR FUTURE WORK

Our study represents the first thorough phylogenetic study of the Pieridae and has provided a preliminary framework for higher-level classification of the family. However, many issues still require attention, and future systematic studies of the Pieridae might concentrate in the following three areas. (1) Among the higher taxa of the Pieridae, deep-level relationships are still poorly resolved, especially the relationship of the Coliadinae to the three other subfamilies. The Coliadinae are assumed to be sister to the Pierinae. (2) Within the subfamily Pierinae, the relationships of the four major lineages are not well understood, particularly the two informal groups (*Colotis* group, *Leptosia*), which may prove to constitute separate tribes. The *Colotis* group is envisaged to comprise nine genera (*Colotis*, *Calopieris*, *Eronia*, *Ixias*, *Gideona*, *Pareronia*, *Nepheronia*, *Hebomoia*, *Pinacopteryx*), but further investigation is required to establish monophyly. (3) Within the tribe Pierini *s.s.*, the relationships of the five major lineages are also poorly resolved, particularly the phylogenetic positions of the genus *Elodina* and the subclade *Dixeia* + *Belenois*.

Reconstructing deep-level relationships is often fraught with difficulty because ancient divergence times inevitably result in considerable noise (homoplasy) among characters so that the phylogenetic signal is weak. Some of these issues may only be resolved by inclusion of data from other gene regions that are able to recover deeper-level splits. The analysis and integration of morphological characters (e.g. Wahlberg & Nylin, 2003; Wahlberg *et al.*, 2005), especially from immature stages, may also improve resolution and aid in the recognition of synapomorphies to help diagnose clades and further define the higher taxa proposed in this work.

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APPENDIX

Zoogeographical distributions of the Pieridae [higher classification according to this study; lower classification according to Braby (2005)]. Zoogeographical distributions are divided into two categories: 'major' and 'minor', the latter category refers to taxa that are poorly represented in the region in terms of numbers of species relative to other 'major' region(s) and/or that have a very small area of occurrence relative to other region(s). The eastern boundary of the Oriental region is set along Wallace's Line, whereas the western boundary of the Australian region is set along Lydekker's Line. *Aoa*, the only taxon endemic to Wallacea, the intervening area between Wallace's Line and Lydekker's Line, was categorized as 'Oriental' because of its restricted occurrence in Sulawesi, close to Wallace's Line. Distributions are based primarily on D'Abrera (1971, 1980, 1981, 1982, 1990), supplemented with other regional faunistic works for specific continents.

	Zoogeographical distribution	
Taxon	Major	Minor
Subfamily Pseudopontiinae		
Pseudopontia	Afrotropical	
Subfamily Dismorphiinae		
Leptidea	Palaearctic	
Pseudopieris	Neotropical	
Moschoneura	Neotropical	
Dismorphia	Neotropical	
Lieinix	Neotropical	
Enantia	Neotropical	Nearctic
Patia	Neotropical	

APPENDIX *Continued*

	Zoogeographical distribution	
Taxon	Major	Minor
Subfamily Coliadinae		
Nathalis	Neotropical, Nearctic	
Kricogonia	Neotropical, Nearctic	
Eurema	Neotropical, Nearctic, Oriental, Australian, Afrotropical	
Subgen. *Terias*	Oriental, Australian	Afrotropical
Abaeis	Neotropical, Nearctic	
Pyrisitia	Neotropical	Nearctic
Teriocolias	Neotropical	
Leucidia	Neotropical	
Gandaca	Oriental, Australian	
Gonepteryx	Palaearctic	Oriental
Dercas	Oriental	
Phoebis	Neotropical, Nearctic	
Prestonia	Neotropical	
Rhabdodryas	Neotropical	
Aphrissa	Neotropical	Nearctic
Catopsilia	Oriental, Australian	Afrotropical
Anteos	Neotropical	Nearctic
Colias	Palaearctic, Nearctic, Neotropical	Afrotropical, Oriental
Zerene	Nearctic, Neotropical	
Subfamily Pierinae		
Colotis group		
Colotis	Afrotropical	Palaearctic, Oriental
Calopieris	Afrotropical	
Eronia	Afrotropical	
Ixias	Oriental	
Pinacopteryx	Afrotropical	
Gideona	Afrotropical	
Hebomoia	Oriental	Palaearctic
Nepheronia	Afrotropical	
Pareronia	Oriental	Australian
Tribe Anthocharidini *s.s.*		
Euchloe	Palaearctic, Nearctic	Afrotropical
Anthocharis	Palaearctic, Nearctic	
Zegris	Palaearctic	Nearctic
Subgen. *Microzegris*	Palaearctic	
Eroessa	Neotropical	
Cunizza	Neotropical	
Hesperocharis	Neotropical	
Mathania	Neotropical	
Leptosia group		
Leptosia	Afrotropical, Oriental	Australian
Tribe Pierini *s.s.*		
Subtribe Appiadina		
Saletara	Oriental	Australian
Appias	Oriental	Australian
Subgen. *Catophaga*	Oriental, Australian	
Subgen. *Hiposcritia*	Oriental	
Subgen. *Glutophrissa*	Afrotropical, Neotropical	Nearctic
Subgen. *Phrissura*	Oriental	
Udaiana	Oriental	
Aoa	Oriental	

APPENDIX *Continued*

Taxon	Zoogeographical distribution: Major	Zoogeographical distribution: Minor
Subtribe Pierina		
Pieris	Palaearctic, Nearctic	Oriental, Afrotropical
Talbotia	Oriental	
Glennia	Neotropical	
Leptophobia	Neotropical	
Itaballia	Neotropical	
Pieriballia	Neotropical	
Perrhybris	Neotropical	
Pontia	Palaearctic, Nearctic, Afrotropical	
Subgen. *Synchloe*	Nearctic, Palaearctic	
Baltia	Palaearctic	
Ganyra	Neotropical	Nearctic
Ascia	Neotropical	Nearctic
Reliquia	Neotropical	
Tatochila	Neotropical	
Hypsochila	Neotropical	
Theochila	Neotropical	
Piercolias	Neotropical	
Pierphulia	Neotropical	
Phulia	Neotropical	
Infraphulia	Neotropical	
Subtribe Aporiina		
Cepora	Oriental	Australian
Prioneris	Oriental	
Mylothris	Afrotropical	
Aporia	Palaearctic	Oriental
Subgen. *Metaporia*	Palaearctic	Oriental
Subgen. *Mesapia*	Palaearctic	
Delias	Australian, Oriental	Palaearctic
Leuciacria	Australian	
Melete	Neotropical	
Pereute	Neotropical	
Leodonta	Neotropical	
Neophasia	Nearctic	
Eucheira	Nearctic	
Catasticta	Neotropical	
Archonias	Neotropical	
Charonias	Neotropical	
incertae sedis		
Elodina	Australian	
Dixeia	Afrotropical	
Belenois	Afrotropical	Oriental, Australian, Palaearctic