

## Molecular Phylogeny, Historical Biogeography, and Divergence Time Estimates for Swallowtail Butterflies of the Genus *Papilio* (Lepidoptera: Papilionidae)

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**Abstract.**— Swallowtail butterflies are recognized as model organisms in ecology, evolutionary biology, genetics, and conservation biology but present numerous unresolved phylogenetic problems. We inferred phylogenetic relationships for 51 of about 205 species of the genus *Papilio* (sensu lato) from 3.3-Kilobase (kb) sequences of mitochondrial and nuclear DNA (2.3 kb of cytochrome oxidases I and II and 1.0 kb of elongation factor 1 $\alpha$ ). Congruent phylogenetic trees were recovered within *Papilio* from analyses of combined data using maximum likelihood, Bayesian analysis, and maximum parsimony bootstrap consensus. Several disagreements with the traditional classification of *Papilio* were found. Five major previously hypothesized subdivisions within *Papilio* were well supported: *Heraclides*, *Pterourus*, *Chilasa*, *Papilio* (sensu stricto), and *Eleppone*. Further studies are required to clarify relationships within traditional “*Princeps*,” which was paraphyletic. Several biologically interesting characteristics of *Papilio* appear to have polyphyletic origins, including mimetic adults, larval host associations, and larval morphology. Early diversification within *Papilio* is estimated at 55–65 million years ago based on a combination of biogeographic time constraints rather than fossils. This divergence time suggests that *Papilio* has slower apparent substitution rates than do *Drosophila* and fig-pollinating wasps and/or divergences corrected using best-fit substitution models are still being consistently underestimated. The amount of sequence divergence between *Papilio* subdivisions is equivalent to divergences between genera in other tribes of the Papilionidae, and between genera of moths of the noctuid subfamily Heliiothinae. [Character evolution; fossils; mimicry; molecular systematics; swallowtail butterflies; substitution rates.]

The utility of DNA sequence comparisons for inferring phylogenetic relationships has been demonstrated thoroughly (e.g., Hasegawa and Yano, 1984; Gielly and Taberlet, 1994; Simon et al., 1994). However, their use for dating divergences has been much more controversial. Early hopes for a molecular clock (Zuckermandl and Pauling, 1962, 1965) via evolution in accordance with neutral theory (Kimura, 1983) have generally not been satisfied. Rather, most studies have revealed substantial variation in rates of evolution across genes and lineages (Britten, 1986; Gillespie, 1986; Avise, 1994; Li, 1997; Pawlowski et al., 1997; Page and Holmes, 1998; Hebert et al., 2002; Soltis et al., 2002), and methods of divergence time estimation must account for this variation (Rambaut and Bromham, 1998; Thorne et al., 1998; Sanderson, 2002).

Inferring absolute divergence dates for a given tree requires an accurate phylogenetic reconstruction including model-corrected branch lengths and reasonably well-established calibration points for the group in question. Accurate estimation of phylogeny remains perhaps the greatest challenge for systematists. Among methods commonly employed, maximum likelihood (ML) has been a consistent and efficient way to estimate phylogenies under a variety of simulated conditions where maximum parsimony (MP) and distance methods are expected to fail (Felsenstein, 1978, 1981; Huelsenbeck, 1995). ML has been remarkably resistant to variations in models and model parameters (Yang, 1996). However, robust ML analyses of large data sets are computationally limited (Sanderson and Kim, 2000), whereas MP and the various distance-based methods are less hindered by large numbers of taxa (Hillis, 1996). Bayesian inference of phylogeny has the benefit of a parametric statistical framework for analyzing DNA sequence data and, con-

trary to standard ML analysis, requires fewer computational resources because it does not necessarily attempt to find the globally optimal ML score (Huelsenbeck et al., 2001). The estimation of branch support in Bayesian analysis accompanies tree estimation, thus eliminating the need for separate time-intensive nonparametric bootstrapping (Larget and Simon, 1999).

Calibrating local molecular clock(s) for a given phylogenetic hypothesis may be accomplished via fossils or vicariance events, the latter chosen to represent one or more nodes in a tree (Jacobs and Pilbeam, 1980; Smith and Coss, 1984; Beerli et al., 1996). Calibration of a molecular clock based on vicariance events presents a major problem for absolute dating when the selected event separates previously contiguous areas under a variety of scenarios, e.g., gradual vicariances that occurred over extended periods of time or multiple sequential events in which the same areas were separated and reunited repeatedly over time. Although fossils are often considered reliable for calibration purposes, they pose other hidden dangers, such as incorrect systematic placement. Fossils are also constrained to the minimum age and cannot fix dates of internal nodes (Smith et al., 1992; Sanderson, 1997).

Here, we present a study of swallowtail butterflies of the genus *Papilio* sensu lato (Lepidoptera: Papilionidae), which includes about 206 species and represents more than one third of all Papilionidae, which has about 551 recognized species (Häuser et al., 2002). As one of the most well-known and broadly studied insect groups, swallowtails are recognized as model organisms in evolutionary biology, ecology, genetics, and conservation biology (Collins and Morris, 1985; Scriber et al., 1995). Yet despite numerous relevant recent studies (e.g., Ae, 1979; Hancock, 1983; Igarashi, 1984; Miller, 1987; Tyler et al., 1994; Scriber et al., 1995; Aubert et al., 1999; Caterino and

Sperling, 1999; Reed and Sperling, 1999; Yagi et al., 1999; Caterino et al., 2001), the phylogeny of *Papilio* is far from resolved, and many coevolutionary and biogeographic hypotheses founded on poorly supported phylogenetic reconstructions hang in limbo. Answers to many of these questions rely not only on accurate phylogenetic resolution but also on approximate dates for some of the more significant phylogenetic events (e.g., host switches, origins of mimicry). In this study, we made an attempt to establish some of these dates. Divergence dates were estimated for nodes of the tree, and these dates were used along with phylogenetic pattern to explore historical biogeography and the evolution of morphological and ecological traits of *Papilio*.

A summary of major classifications of *Papilio* is presented in Table 1. Munroe (1961) divided *Papilio* into

five sections, which he did not designate as subgenera because of the lack of any simple system of adult characters. Hancock (1983), in his explicitly cladistic estimation of relationships within *Papilio*, recognized six genera based on phylogenetic evidence and their inferred evolutionary antiquity, but that phylogeny still suffered from lack of character justification. Another classification was proposed by Igarashi (1984) based on morphology of immature stages, but this work was not complete in representing all of Hancock's genera. Igarashi recognized seven genera, with numerous discrepancies between this treatment and that of Hancock (1983). Hancock's classification was criticized by Miller (1987), who did not find justification for elevating *Papilio* subdivisions to the generic level. However, some elevation is seen in the latest widely available checklist of swallowtail butterflies

TABLE 1. Classification of *Papilio* by different authors.

Species group (Hancock, 1983)	Munroe (1961)	Hancock (1983)	Igarashi (1984)	Häuser et al. (2002)
<i>P. clytia</i> ( <i>clytia</i> )	Section I	<i>Chilasa</i> ( <i>Chilasa</i> )	<i>Chilasa</i>	<i>Chilasa</i> ( <i>Chilasa</i> )
<i>P. agestor</i> ( <i>epycides</i> )	-	-	-	-
<i>P. veiovis</i>	-	-	-	-
<i>P. laglazei</i>	-	-	-	-
<i>P. elwesi</i>	Section II(A)	<i>Chilasa</i> ( <i>Agehana</i> )	<i>Agehana</i>	<i>Chilasa</i> ( <i>Agehana</i> )
<i>P. anactus</i> ( <i>anactus</i> )	-	<i>Eleppone</i>	<i>Chilasa</i>	<i>P.</i> ( <i>Eleppone</i> )
<i>P. machaon</i> ( <i>alexanor</i> , <i>indra</i> , <i>machaon</i> , <i>hippocrates</i> , <i>polyxenes</i> , <i>zelicaon</i> , <i>hospiton</i> )	-	<i>Papilio</i>	<i>Papilio</i>	<i>P.</i> ( <i>Papilio</i> )
<i>P. xuthus</i> ( <i>xuthus</i> )	-	<i>Princeps</i> ( <i>Sinoprinceps</i> )	-	<i>P.</i> ( <i>Sinoprinceps</i> )
<i>P. demolion</i>	-	<i>Princeps</i> ( <i>Menelaides</i> )	<i>Papilio</i> , <i>Euchenor</i>	<i>P.</i> ( <i>Menelaides</i> )
<i>P. helenus</i> ( <i>helenus</i> )	-	-	<i>Menelaides</i> ; <i>Papilio</i>	-
<i>P. protenor</i> ( <i>protenor</i> , <i>macilentus</i> )	-	-	<i>Menelaides</i>	-
<i>P. bootes</i>	-	-	-	-
<i>P. memnon</i> ( <i>memnon</i> , <i>rumanzovia</i> )	-	-	-	-
<i>P. polytes</i> ( <i>polytes</i> )	-	-	-	-
<i>P. nephelus</i> ( <i>nephelus</i> )	-	-	-	-
<i>P. gambrisius</i> ( <i>aegeus</i> )	-	-	-	-
<i>P. amyntor</i>	-	-	-	-
<i>P. fuscus</i> ( <i>hipponous</i> )	-	-	<i>Achillides</i>	-
<i>P. paris</i> ( <i>paris</i> , <i>bianor</i> , <i>maackii</i> )	-	<i>Princeps</i> ( <i>Princeps</i> )	-	<i>P.</i> ( <i>Achillides</i> )
<i>P. palinurus</i>	-	-	-	-
<i>P. peranthus</i>	-	-	-	-
<i>P. ulysses</i>	-	-	-	-
<i>P. demoleus</i> ( <i>demoleus</i> , <i>demodocus</i> )	-	-	<i>Papilio</i>	<i>P.</i> ( <i>Princeps</i> )
<i>P. menesteus</i>	-	-	-	-
<i>P. delalandei</i> ( <i>delalandei</i> )	Section II(B)	-	?	-
<i>P. phorcas</i> ( <i>phorcas</i> , <i>dardanus</i> , <i>constantinus</i> )	-	-	-	-
<i>P. hesperus</i> ( <i>nobilis</i> )	-	-	-	-
<i>P. leucotaenia</i>	-	-	-	-
<i>P. cynorta</i>	-	<i>Princeps</i> ( <i>Druryia</i> )	-	<i>P.</i> ( <i>Druryia</i> )
<i>P. nireus</i> ( <i>oribazus</i> , <i>epiphorbas</i> )	-	-	<i>Achillides</i>	-
<i>P. zalmoxis</i>	-	-	?	-
<i>P. antimachus</i>	-	-	-	-
<i>P. rex</i> ( <i>rex</i> )	-	-	-	-
<i>P. zenobia</i>	-	-	-	-
<i>P. glaucus</i> ( <i>glaucus</i> , <i>canadensis</i> , <i>rutulus</i> , <i>multicaudatus</i> )	Section III	<i>Pterourus</i> ( <i>Pterourus</i> )	<i>Papilio</i>	<i>P.</i> ( <i>Pterourus</i> )
<i>P. troilus</i> ( <i>troilus</i> , <i>palamedes</i> , <i>pilumnus</i> , <i>esperanza</i> )	-	-	<i>Pterourus</i>	-
<i>P. thoas</i> ( <i>thoas</i> , <i>crephontes</i> , <i>astyalus</i> )	Section IV(A)	<i>Heraclides</i>	<i>Papilio</i>	<i>P.</i> ( <i>Heraclides</i> )
<i>P. torquatus</i> ( <i>torquatus</i> , <i>hectorides</i> )	Section IV(B)	-	?	-
<i>P. anchisiades</i> ( <i>anchisiades</i> , <i>erostratus</i> )	-	-	<i>Chilasa</i>	-
<i>P. zagreus</i>	Section V	<i>Pterourus</i> ( <i>Pyrrhosticta</i> )	?	<i>P.</i> ( <i>Pterourus</i> )
<i>P. scamander</i> ( <i>birchalli</i> , <i>scamander</i> )	-	-	-	-
<i>P. homerus</i> ( <i>garamas</i> )	-	-	-	-

Note: Species groups shown in bold are represented in this study (by the species listed in brackets).

(Häuser et al., 2002), where *Chilasa* is treated as a genus.

Successful hand-pairing of swallowtail butterflies by Clarke (1952) gave rise to studies of experimental hybridization in *Papilio* (e.g., Clarke and Sheppard, 1955, 1956, 1957; Ae, 1960, 1962, 1990; Remington, 1960). Data on egg viability, sex ratio in hybrid progeny, and fertility of F<sub>1</sub>'s were summarized in the form of a biological incompatibility index used to evaluate *Papilio* relationships (Ae, 1979, 1995; Scriber, 1995a).

Partly because of the limitations of traditional approaches and with the development of new systematic techniques, the classification of *Papilio* has received significant attention in recent years. Relationships among species within the *P. machaon* and *P. glaucus-troilus* species groups were studied based on allozyme variation (Sperling, 1987; Hagen and Scriber, 1991). Restriction fragment length polymorphism of mitochondrial DNA (mtDNA) was used to compare taxa within the same species groups in later studies (Sperling, 1991, 1993a, 1993b; Sperling and Harrison, 1994, Tyler et al., 1994). Phylogenetic relationships within *Papilio* have also been analyzed using DNA sequences of a variety of genes (28S, *cyt b*, *EF-1 $\alpha$* , *ND1*, *ND5*, *COI*, *COII*), but these studies have been confined to single species groups (Vane-Wright et al., 1999) or local geographic areas (Yagi et al., 1999) or have included limited sampling across *Papilio* subdivisions (Aubert et al., 1999; Caterino and Sperling, 1999; Reed and Sperling, 1999).

Although Munroe's (1961) hypothesis of relationships within *Papilio* has been widely used for classification, numerous details have been challenged. We focused on five areas that include species that are taxonomically problematic key species for testing biogeographic hypotheses (e.g., *P. anactus*), evolution of mimicry (e.g., *Chilasa*, *P. nobilis*), and host-plant associations (e.g., *P. alexanor*), and species that are rare and insufficiently studied (e.g., *P. esperanza*). Prior to the discovery of its larva, the enigmatic Mexican swallowtail, *Papilio esperanza*, was variously placed in four different species groups in two *Papilio* subgenera (*Pterourus* or *Heraclides*). Larval characters suggest yet a different species group of *Pterourus* (Beutelspacher, 1975; Hancock, 1983; Tyler et al., 1994), and the issue remains unresolved. The systematic position of *Papilio anactus* is similarly unclear. It is presently placed in a monotypic subgenus (*Eleppone*), with relationships somewhere "between the subgenera *Heraclides* and *Chilasa*" (Hancock, 1970: 53). A third species, *Papilio nobilis*, has wavered between the *phorcas* and *hesperus* groups (Munroe, 1961, and Hancock, 1983, respectively). Its placement is important for the understanding of mimicry evolution in the *P. phorcas* group (Vane-Wright et al., 1999). Fourth, the monophyly and rank of the danaid-mimicking *Chilasa* are uncertain. Munroe (1961) split its members among two *Papilio* subunits, but Hancock placed them together in a single genus, considered to be the sister taxon of *Eleppone*. We also revisited the question of *Papilio alexanor*. Although the relationships of this odd European Apiaceae feeder have been examined several times (Aubert et al., 1999; Caterino and Sperling,

1999; Reed and Sperling, 1999), no strong resolution has been obtained, and denser sampling was used to help refine its placement.

## MATERIALS AND METHODS

### *Taxonomic Sampling*

We added sequences for 31 species of *Papilio* sensu lato to the 23 available previously (Caterino and Sperling, 1999; Reed and Sperling, 1999; Caterino et al., 2001). Overall, we include sequences for 54 specimens from 51 *Papilio* species, which represent almost 25% of all species in this large genus. Species were selected to give more complete representation of major species groups and subgenera within *Papilio*. Representatives of 26 of 42 species groups (sensu Hancock, 1983) were included. The sampled species represent all genera recognized by Munroe (1961), Hancock (1983), and Häuser et al., (2002) and all except two genera (*Agehana* and *Euchenor*) recognized by Igarashi (1984). Outgroups were 16 species of Papilionidae from tribes other than Papilionini, and *Pieris napi* from the putative sister family, Pieridae. Newly sampled taxa, sources of material, and GenBank accession numbers for new materials are given in Table 2.

### *Genes*

We sequenced about 2.3 kilobases (kb) of the mitochondrial genes cytochrome oxidase subunit I (*COI*), tRNA-leucine, and cytochrome oxidase subunit II (*COII*) and about 1.0 kb of the nuclear protein-coding gene elongation factor 1 alpha (*EF-1 $\alpha$* ). Phylogenetic utility of both *COI-COII* and *EF-1 $\alpha$*  has been widely demonstrated (e.g., Simon et al., 1994; Cho et al., 1995; Mitchell et al., 1997), and a substantial database of lepidopteran sequences already exists for these genes (Sperling, 2003). These sequences are valuable sources for studies of the evolution of these genes and for reconstruction of the global phylogeny for Lepidoptera. We followed previous work on swallowtail phylogeny that utilized both *COI-COII* and *EF-1 $\alpha$*  (Caterino and Sperling, 1999; Reed and Sperling, 1999; Caterino et al., 2001) to expand the amount of analyzed data available for global analyses.

### *Molecular Techniques*

Total genomic DNA was extracted using a Qiagen DNeasy tissue kit. Polymerase chain reactions (PCRs) were performed with a Biometra TGradient thermal cycler using a hot start in which *Taq* Polymerase was added at the end of an initial 2-min denaturation at 94°C. This step was followed by 35 cycles of 1 min at 94°C, 1 min at 45–52°C (depending on primer combinations), and 1 min at 72°C and then a 7-min final extension at 72°C. PCR products were cleaned using the Qiagen QIAquick PCR purification kit when only a single DNA band was visible in a gel or using a combination of gel separation and subsequent purification with the Qiagen QIAEX II gel extraction kit when more than one band was observed. Sequencing reactions were carried out using a DYEnamic

TABLE 2. List of new material examined in present study.

Taxon <sup>a</sup>	Locality	GenBank nos.	
		COI-COII	EF-1 $\alpha$
Papilionidae			
Papilioninae			
Papilionini			
<i>Papilio (Princeps) bianor</i>	Taiwan: Taipei	AY457572	AY457603
<i>P. (Pr.) maackii</i>	Japan: Gifu Pref.	AY457573	AY457604
<i>P. (Pr.) paris</i>	China: Guangzhou	AY457574	AY457605
<i>P. (Pr.) helenus</i>	Japan: Gifu Pref.	AY457575	AY457619
<i>P. (Pr.) hipponous</i>	Philippines: Marinduque	AY457576	AY457620
<i>P. (Pr.) macilentus</i>	Japan: Mt. Takao	AY457577	AY457622
<i>P. (Pr.) memnon</i>	Japan: Gifu Pref.	AY457578	AY457623
<i>P. (Pr.) nephelus</i>	Malaysia: Penang	AY457579	AY457624
<i>P. (Pr.) polytes</i>	Malaysia: Penang	AY457580	AY457627
<i>P. (Pr.) protenor</i>	Japan: Aichi Pref.	AY457581	AY457628
<i>P. (Pr.) rumanzovia</i>	Philippines	AY457582	AY457631
<i>P. (Pr.) aegaeus</i>	Australia: Queensland	AY457583	AY457611
<i>P. (Pr.) dardanus</i>	Madagascar: Fianarantsoa	AY457584	AY457612
<i>P. (Pr.) delalandei</i>	Madagascar: Fianarantsoa	AY457585	AY457613
<i>P. (Pr.) rex</i> (two specimens)	Kenya: Kakamega	AY457586	AY457630
	Kenya: Kakamega	AY457587	AY457629
<i>P. (Pr.) demodocus</i>	Madagascar: Radriarmasy	AY457588	AY457614
<i>P. (Pr.) epiphorbas</i>	Madagascar: Radriarmasy	AY457589	AY457615
<i>P. (Pr.) nobilis</i> <sup>b</sup>	Kenya: Nairobi	AY457590	AY457625
<i>P. (Pr.) oribazus</i>	Madagascar: Fianarantsoa	AY457591	AY457626
<i>P. (Eleppone) anactus</i>	Australia: Queensland	AY457592	AY457608
<i>P. (Papilio) hippocrates</i>	Japan: Gifu Pref.	AY457593	AY457621
<i>P. (Chilasa) clytia</i>	Malaysia: Penang	AY457594	AY457606
<i>P. (Ch.) epycides</i>	Taiwan: Taoyuan: Gapyi	AY457595	AY457607
<i>P. (Pterourus) birchalli</i>	Costa Rica: Guatuso de Alajuela	AY457596	AY457610
<i>P. (Pt.) esperanza</i>	Mexico: Oaxaca	AY457597	AY457617
<i>P. (Heraclides) astyalus</i>	Brazil: Campinas	AY457598	AY457609
<i>P. (H.) erostratus</i>	El Salvador	AY457599	AY457616
<i>P. (H.) hectorides</i>	Brazil: Campinas	AY457600	AY457618
<i>P. (H.) thoas</i>	French Guiana: Pointe Macouria	AY457601	AY457632
<i>P. (H.) torquatus</i>	Brazil: Campinas	AY457602	AY457633

<sup>a</sup>Subgeneric names correspond to genera of Hancock (1983).

<sup>b</sup>Same specimen used by Vane-Wright et al. (1999).

ET terminator cycle sequencing kit (Amersham Pharmacia Biotech, Cleveland, OH). Sequenced products were filtered through Sephadex-packed columns, dried, resuspended, and fractionated on an ABI 377 automated sequencer. Part of the sequences were obtained following molecular procedures described previously (Caterino et al., 2001). All fragments were sequenced in both directions. Nucleotide sequences of the primers have been described previously (Caterino and Sperling, 1999; Reed and Sperling, 1999; Caterino et al., 2001). Sequences were assembled into contiguous arrays using Sequencher 4.1 (GeneCode Corp., Ann Arbor, MI).

#### Alignment of Sequence Data

Sequences of COI-COII genes were aligned against the published sequence from *Drosophila yakuba* (Clary and Wolstenholme, 1985) with multiple sequence alignment using ClustalX 1.81 (Thompson et al., 1997) with the default settings (gap opening = 10, gap extension = 0.20) followed by adjustment by eye. EF-1 $\alpha$  gene sequences did not contain any introns and were aligned against sequence from *Bombyx mori* (Kamiie et al., 1993) by eye

with the aid of SeAl 2.0 (Rambault, 2002). To determine codon positions, we used MacClade 4.0 (Maddison and Maddison, 2000).

#### Phylogenetic Analysis

PAUP\* 4.0b8-b10 (Swofford, 1998) was used for all parsimony, ML, bootstrap, and decay analyses. To get an accurate estimation of phylogenetic relationships in *Papilio* sensu lato, we followed the following strategies.

*MP analyses.*—To check the data for nucleotide bias among taxa, we used the test of homogeneity of base composition implemented in PAUP\* using all characters and using parsimony-informative sites alone. To reveal possible incongruence among different genes, we performed an incongruence length difference (ILD) test (Farris et al., 1994) referred to in PAUP\* as a partition homogeneity test. We implemented the test under parsimony with 100 random addition sequences of taxa and used 500 replicates to generate the null distribution. The utility of the ILD test has been questioned (Graham et al., 1998; Barker and Lutzoni, 2002; Darlu and Lecointre, 2002). Therefore, to determine whether

the data partitions carried substantially different phylogenetic signals, we also analyzed combined and partitioned data separately.

To evaluate the effect of different outgroup and ingroup combinations, we performed several MP analyses for the combined data set: (1) with *Pieris napi* as an outgroup, and the 70 remaining taxa as the ingroup; (2) with *Pieris napi* excluded from the analysis, two sequences from *Baronia brevicornis* as an outgroup, and the 68 remaining taxa in the ingroup; and (3) with only *Papilio* sensu lato in the ingroup, two outgroups (*Pachliopta neptunus* and *Eurytides marcellus*) used in previous studies (Caterino and Sperling, 1999; Reed and Sperling, 1999), and all other taxa excluded from the analyses.

All parsimony analyses utilized heuristic searches: starting trees determined by 100 random taxon additions, tree bisection–reconnection (TBR) branch swapping, gaps treated as missing data, multistate characters treated as polymorphisms, and all characters equally weighted. Robustness of the parsimony hypothesis was tested with bootstrap analyses (Felsenstein, 1985) with 500 repetitions and 10 random taxon additions but otherwise under the same conditions as for initial parsimony searches. Constraint searches were carried out for each data set to determine the number of additional steps needed to accommodate alternative phylogenetic hypotheses (Bremer, 1988), and decay indices were extracted using the program Autodecay (Eriksson, 1998).

**ML analyses.**—Prior to ML phylogenetic reconstruction, we applied a hierarchical likelihood ratio test to determine how well competing substitution models fit the data. We tested models ranging from the simple Jukes–Cantor model to the most parameter-rich general time reversible (GTR) model. Using the program Modeltest (Posada and Crandall, 1998), we calculated the test statistic  $\delta = 2 \log \Lambda$ , where  $\Lambda$  is the ratio of the likelihood of the null model divided by the likelihood of the alternative model (Huelsenbeck and Crandall, 1997). Tests were performed for both combined and partitioned data sets. All model parameters were estimated from corresponding (partition) MP trees and then fixed during ML searches. MP trees were used as starting trees for TBR branch swapping under the best model supported by Modeltest. To obtain a measure of support for ML trees, we ran 1,000 bootstrap replicates under minimum-evolution criteria based on the same model used in the ML analysis.

**Bayesian analyses.**—Bayesian phylogenetic analyses were conducted for combined and partitioned data sets with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) under the same model that was selected for ML (GTR model with gamma shape parameter and proportion of invariable sites: GTR+ $\Gamma$ +I). Specific nucleotide substitution model parameter values were estimated as part of the analysis, and each gene in the combined analysis was allowed to have its own estimates. We ran four chains simultaneously, three heated and one cold. Each Markov chain was started from a random tree and run for  $10^6$  generations, sampling the chains every 100th cycle. The log-likelihood scores of sample points were plotted against generation time to determine when the chain became sta-

tionary. All sample points prior to reaching stationarity (2,000–3,000 trees) were discarded as burn-in samples. To reduce bias in our results, we ran each partitioned analysis twice and combined analyses three times, each beginning with different starting trees, and compared their apparent stationarity levels for convergence (Huelsenbeck and Bollback, 2001). Data remaining after discarding burn-in samples were used to generate a majority rule consensus tree, where percentage of samples recovering any particular clade represented the clade's posterior probability (Huelsenbeck and Ronquist, 2001). Probabilities of  $\geq 95\%$  were considered indicative of significant support. The mean, variance, and 95% credibility interval were calculated from the set of substitution parameters. Because only a single outgroup taxon is allowed in MrBayes, we used *Eurytides marcellus* (Graphiini) to root the trees. For dating analyses, the produced basal trichotomy had to be resolved, and *E. marcellus* was pruned from the trees leaving *Pachliopta neptunus* (Troidini) as the only sister taxon to the ingroup (*Papilio*).

#### Character Evolution

Selected morphological and ecological traits (including host-plant associations, mimicry, presence of iridescent patches on wings, female secretion deposited on egg surface, shape of larval minute body hairs, resting larval behavior, and number of crochet rows on larval prolegs) were scored based on literature or personal observations. Character states were optimized on the ML phylogeny using the program Mesquite (Maddison and Maddison, 2003). Both MP and ML character optimizations were applied to reconstruct the ancestral states. Characters were treated as unordered in MP reconstructions. ML optimizations were done using the Markov k-state one-parameter model (Lewis, 2001). Because of methodological restrictions, ML optimization of larval feeding habits was done only for primary host plants, and polymorphic traits (use of multiple plant families) were reconstructed using MP optimizations.

Other uncertainties with reconstruction of the ancestral states are related to incomplete information about resting larval behavior and proleg structures. Optimization of larval feeding habits may be tentative in the absence of a well-supported phylogeny for *Papilio* outgroups. The tribe Troidini was believed to be the sister group for Papilionini, i.e., *Papilio* sensu Miller (1987), and the tribe Leptocircini was considered their sister group (Hancock, 1983; Caterino et al., 2001). However, some studies have suggested a basal position of the genus *Meandrusa* within Papilionini (Aubert et al., 1999). Although we were not able to sample this genus, we performed alternative character optimizations placing *Meandrusa* as a sister taxon of *Papilio* to test different hypothesis. Differences in ancestral state reconstructions due to choice of sister taxa are outlined in the discussion.

#### Divergence Time Estimation

The hypothesis of rate constancy among taxa was tested by comparing the likelihoods of the data, using a

likelihood ratio test (Felsenstein, 1988), given the ML tree topology under the best model selected with and without the constraint of a molecular clock. We tested clocklike behavior in both combined and partitioned data sets. To estimate divergence times, semiparametric rate smoothing using a penalized likelihood approach was applied to the inferred phylogeny reconstruction with the aid of the computer program r8s (Sanderson, 2002). Penalized likelihood combines the likelihood term for a saturated model with a different rate on every branch and the nonparametric penalty function that keeps those rate estimates from varying excessively across the tree. The relative contribution of the two terms is controlled by a smoothing parameter. Cross validation provides an objective method for model selection and choice of optimal smoothing value (Sanderson, 2002).

To obtain SDs for estimated divergence times, the data set was bootstrapped 100 times using the seqboot module from PHYLIP 3.6 (Felsenstein, 1989), and branch lengths were reestimated for each node under the constrained initial topology in PAUP\*. The dating analyses were then repeated for each tree, and node statistics were summarized using the program r8s.

Calibration of the molecular clock was not an easy task because fossil data are scarce for swallowtail butterflies. *Praepapilio colorado* and *P. glacialis*, known from the middle Eocene and dated at about 48 million years ago (MYA), are two of the oldest known undisputed butterfly fossils (Durdon and Rose, 1978). Resembling *Baronia brevicornis*, these two species were considered the most primitive swallowtails and were placed into their own subfamily Praepapilioninae. However, their affinity to swallowtails has been disputed (Scott, 1986). Other unidentified papilionids have been found in Europe (dated at 24 MYA) and Japan (dated at 1.6 MYA) (Emmel et al., 1992).

We tried to estimate divergence dates based on molecular clock rates of 0.02 substitutions per site per million years, as calibrated for *COI* and *COII* of other insects (DeSalle et al., 1987; Brower, 1994). Thus, with an average number of 0.336 substitutions per site (calculated under GTR+ $\Gamma$ +I model) between ingroup taxa and outgroups, the estimated age of the subfamily Papilioninae was approximately  $16.8 \pm 2.7$  MY. The origin of the family Papilionidae, estimated under the same assumption, was calculated to be no more than  $26 \pm 7$  MYA based on the average number of 0.52 substitutions per site. Neither standard rates of molecular evolution nor fossil dates for Papilionidae accord well with the present day geographic distribution of swallowtails. Patterns exhibited by the group strongly suggest the effects of continental drift (Holloway and Nielsen, 1999), which indicate much older dates.

Although at least 18 species of the family Papilionidae have been recorded as migrating (Williams, 1930), few butterflies are capable of passing over 1,000-mile water barriers (Drake and Gatehouse, 1995). Because the fragmentation of Gondwanaland was completed well before 48 MYA (Dietz and Holden, 1970), the best explanation for the worldwide distribution of *Papilio* is an older age

for the group. The hypothesis of an older age for *Papilio* is supported by a recent study by Gaunt and Miles (2002), who presented an insect molecular clock calibration estimating the date for the most recent common ancestor of *Papilio* and its sister taxa (*Pachliopta neptunus* and *Eurytides marcellus*) as 82.5–89.1 (MYA). We used these dates as an external calibration point to date the root (i.e., the most recent common ancestor) of the *Papilio* tree. Several additional age constraints for the internal nodes that could be assumed from phylogenetic pattern and geological history were also applied.

*Papilio* can be considered to fall into two major clades: one (with few exceptions), restricted to the New World and the other comprising mostly Old World species. This fact, along with the suggested center of the origin for *Papilio* in the North America–Europe landmass (Hancock, 1983), gives reason to assume that initial diversification was related to disconnection between the present Nearctic and Palearctic. This event was almost completed by the end of the Cretaceous about 65 MYA (Dietz and Holden, 1970). The latest direct land connection between Europe and eastern North America, known as the Thulean route, was apparently available into the Miocene and persisted up to as recently as approximately 20 MYA (Tiffney, 1985). However, because climatic cooling after the Eocene/Oligocene boundary apparently rendered the Thulean route unsuitable for many warm temperature-adapted groups, the vicariance event may have been approximately 35 MYA (Noonan, 1988) or even earlier (Chapco et al., 2001). Based on this geological time frame, we constrained the maximum and minimum time of the initial split within *Papilio* to 65 and 35 MYA, respectively.

The distribution of *P. anactus*, which is confined to Australia, and its common ancestry with African and Malagasy species groups of *P. phorcas*, *P. rex*, and *P. delalandei* suggest that the species may have evolved during a long period of isolation, since separation of Australia from the remains of Gondwanaland. Australia had close contact with Antarctica before approximately 45 MYA when its northward drifting speed increased rapidly (Raven and Axelrod, 1972). Australia is thought to have been linked with Antarctica by an island chain before approximately 35 MYA, when circumpolar currents became established and triggered glaciation of Antarctica (Cook, 1990; Li and Powell, 2001). We used the later date (35 MYA) to constrain the minimum age of the common ancestor for *P. anactus* and its sister clade.

The next calibration point was selected for another species with a highly restricted distribution, *P. hospiton*, which is endemic to the islands Corsica and Sardinia. The separation to the Corsica–Sardinia microplate from the Iberian Peninsula is consistently dated at approximately 29 MYA (Alvarez, 1972), although this date seems to be too old and rather implausible to explain endemism of *P. hospiton*. A more recent event, recession of the Mediterranean sea for about 1500 years and successive re-isolation of the islands by the waters from the Atlantic ocean, took place about 5 MYA (Hsu, 1972) and provides a more

reasonable date to constrain the split between *P. hospiton* and *P. machaon*.

*Papilio memnon* and *P. rumanzovia* are sister species with allopatric distributions (Collins and Morris, 1985). *Papilio memnon* occurs widely in southeast Asia, from India to southern Japan and Indonesia, but not in the Philippines. *Papilio rumanzovia* has a more restricted geographic range and occurs in the Philippines (except Palawan) and on Batu, Talaud, and Sangihe islands. Around 5 MYA, the Philippine platform approached its present position in the immediate vicinity of East Malaysia and Indonesia (Hall, 1996, 2001), allowing the ancestral species to expand its range and eventually evolve into two distinct species. Based on this assumption, we constrained the maximum age of the *P. memnon*–*P. rumanzovia* split at 5 MY.

To provide some more recent divergences, we constrained the minimum age of the common ancestor of *P. hippocrates* and *P. machaon* to 0.01 MY. Known from Japan, *P. hippocrates* is generally regarded as a subspecies of *P. machaon* but was accepted as a full species by Hancock (1983). Speciation in the *P. machaon* complex has been attributed to Pleistocene climatic changes (Sperling, 1987; Sperling and Harrison, 1994). Pleistocene glaciation was similarly implicated as a driving force in the speciation of *Parnassius stubbendorfi* (which inhabits a large area of continental Asia) and *P. glacialis* (which occurs only in the Japan archipelago) in East Asia (Yagi et al., 2001). *Papilio hippocrates* may have been isolated in Japan before the last glacial maximum, which affected connections between the mainland and the Japan archipelago approximately 0.01 MYA (Oshima, 1990; Matsui et al., 1998). To constrain a very recent age of siblings with close to zero amount of sequence divergence, we arbitrarily fixed the maximum age of the *P. rex* clade at 100 years.

To obtain estimates for a possible range of divergence dates, the selected calibration points were applied in different combinations. We fixed the time of the tree root at 82.5 MYA (first) and 89.1 MYA (second), with no other constraints enforced. Then we applied all discussed constraints for internal nodes without constraining the time of the root. We then applied all age constraints at the same time.

## RESULTS

### *Alignment and Data Description*

Alignment of *EF-1 $\alpha$*  sequences was unambiguous because of the absence of indels. Total length of the aligned *EF-1 $\alpha$*  region was 995 bp. Some length differences were found in the mtDNA sequence, primarily in the *COI* initiation region (Caterino and Sperling, 1999). Seventeen sites here were deleted from the alignment, including six nucleotides corresponding to the first two codon positions of the *COI* gene (Clary and Wolstenholme, 1985; Caterino and Sperling, 1999). As Caterino and Sperling (1999) noted, a few taxa demonstrated 1-bp indels (mostly phylogenetically uninformative) in the tRNA-leucine gene. There were also a 3-bp insertion immediately following the *COI* termination codon in

*P. zelicaon* and an insertion of one codon (AAT: arginine) in *P. dardanus* between positions equivalent to 3,471 and 3,472 of *Drosophila yakuba*. We found that *P. oribazus* had a 3-bp insertion (AAA: lysine) in the same position as in *P. dardanus*. The final aligned sequences included 2,293 nucleotides for the mtDNA partition and 3,288 nucleotides in the combined data set. Previously published *EF-1 $\alpha$*  sequence for *Atrophaneura alcinous* (Caterino et al., 2001) was determined to be incorrect at the 5' end and has been updated for this study and in GenBank.

No base composition heterogeneity was found for any gene partition among ingroup taxa (*COI*:  $\chi^2 = 61.6$ ,  $df = 159$ ,  $P = 1.0$ ; *COII*:  $\chi^2 = 56.6$ ,  $df = 159$ ,  $P = 1.0$ ; *EF-1 $\alpha$* :  $\chi^2 = 30.7$ ,  $df = 159$ ,  $P = 1.0$ ) and across all species including outgroup taxa (*COI*:  $\chi^2 = 103.2$ ,  $df = 213$ ,  $P = 1.0$ ; *COII*:  $\chi^2 = 74.6$ ,  $df = 213$ ,  $P = 1.0$ ; *EF-1 $\alpha$* :  $\chi^2 = 124.9$ ,  $df = 213$ ,  $P = 0.99$ ). However, after all noninformative characters were excluded, base composition heterogeneity was revealed in the *COI* partition both for ingroup only ( $\chi^2 = 237.9$ ,  $df = 159$ ,  $P < 0.005$ ) and for the full set of taxa ( $\chi^2 = 390.7$ ,  $df = 213$ ,  $P < 0.005$ ). However, analysis of the corresponding MP tree did not reveal any grouping of species with similar nucleotide frequencies. Analysis of partitioned and combined data using LogDet distances resulted in trees with topologies almost identical to those of the corresponding ML trees.

Based on the results of the IILD test, partitions of the data into *COI*, *COII*, and *EF-1 $\alpha$*  were homogeneous (sum of gene tree length = 8,407;  $P = 0.114$ ). *COI* and *COII* gene partitions alone were homogeneous (sum of gene tree length = 6,789;  $P = 0.492$ ) and partitions between *COI* and *COII* together and *EF-1 $\alpha$*  were also homogeneous (sum of tree lengths = 8,505;  $P = 0.774$ ). We analyzed both partitioned and combined data to gain insight into any distinctively different phylogenetic results due to data partitions and to obtain a phylogenetic reconstruction based on a maximum number of informative characters. In all cases, when all the sequences were combined for the analysis resolution and node support in the tree improved markedly. Many other studies have demonstrated improved resolution and increased bootstrap supports in combined analyses (e.g., Baker and DeSalle, 1997; Vogler and Welsh, 1997; Crespi et al., 1998; Remsen and DeSalle, 1998; Klompen et al., 2000; Chapco et al., 2001).

### *Phylogeny Inference*

*MP analyses*.—The total number of informative characters for the combined data set with all outgroups was 1,129 (34.3%), with 847 sites in the mtDNA partition and 282 sites in the *EF-1 $\alpha$*  data set. Number of informative characters was greatest for third-codon positions (601 sites [71% of all informative characters] for *COI*–*COII* and 260 sites [92.2%] for *EF-1 $\alpha$* ). With all outgroups excluded, number of informative characters was reduced to 933 (28.4%), with 725 informative sites in the mtDNA partition and 208 sites in the *EF-1 $\alpha$*  data set. Proportion of

informative characters for third-codon positions increased and was 75% (547 sites) of all informative characters for *COI-COII* and 94% (196 sites) for *EF-1 $\alpha$* .

Parsimony analysis of the combined data set resulted in a single tree (8,474 steps; consistency index [CI] = 0.242; retention index [RI] = 0.492) shown in Figure 1. We used this topology as an initial estimate of the phylogeny and compared it with those obtained under alternative optimality criteria. The comparison of all topologies revealed that the monophyly of subclades corresponding to Hancock's (1983) genera and subgenera was mostly consistent among trees recovered from different data partitions. Thus, we labeled those clades as individual nodes and illustrated alternative topologies in the form of simplified trees (Fig. 2). Because the issue of phylogenetic relationships outside of *Papilio* is beyond the scope of this study, all outgroup taxa were excluded from the illustration of these simplified trees. The number of outgroups used in the phylogenetic analyses also was reduced because the topology within the *Papilio* subtree was not affected by the exclusion of distant outgroups (data not shown). Two outgroups, *Eurytides marcellus* and *Pachliopta neptunus*, were used for further analyses. There are few disagreements between phylogenies inferred from mtDNA and nuclear partitions, but the conflicts are primarily in nodes that are weakly supported in the combined MP tree and in bootstrap analyses (e.g., basal position of *Heraclides* in *COI + COII* and combined tree vs. a sister relationship with the *P. alexanor + Chilasa + Pterourus* lineage).

**ML analyses.**—Modeltest supported use of the GTR model (Lanave et al., 1984), with invariable sites (I) and gamma-distributed rates ( $\Gamma$ ), as the best fit for all data sets (including the combined data set) except for a reduced *EF-1 $\alpha$*  data set (*Papilio* sensu lato plus two outgroup taxa), for which the best model was TrN (Tamura and Nei, 1993) + I +  $\Gamma$ . Estimated parameters used in ML analyses are given in Table 3. The C-T substitution rate was substantially higher than other substitution types, and substitution rates for A-C and C-G were 2–2.5 times higher for the 71-taxon data set within the *COI-COII* partition.

Heuristic searches performed for three data sets under estimated parameters produced the trees shown in Figure 2. Analyses based on only mtDNA data produced a tree with the same relationships as illustrated in the MP bootstrap consensus tree in *Priniceps* and *Papilio* sensu stricto but with alternative groupings in the *Heraclides + Pterourus + Chilasa* clade. Analysis from the *EF-1 $\alpha$*  data set resulted in a tree with several polytomies.

The ML analysis for combined data revealed a single tree with a negative log-likelihood score ( $-\ln L$ ) of 31023.619 that was identical to the combined MP bootstrap consensus tree (Fig. 2). In the expanded ML tree for combined data that shows all species (Fig. 3), grouping within the major terminal subclades (corresponding to species groups and subgenera) is usually congruent with the relationships inferred by the MP combined data tree (Fig. 1). However, relationships among many major clades remain weakly supported.

**Bayesian analyses.**—Both independent analyses for *COI-COII* reached stationarity well before 300,000 cycles. We discarded 3,000 trees as burn-in samples and computed the consensus tree from the remaining 14,000 trees. The branching pattern is identical to that of the ML tree and resembles the MP tree based on only mtDNA data (Fig. 2), where *Heraclides* (see Fig. 1) is a sister group to the rest of *Papilio*. Also, as in the ML tree, *Pterourus* appeared to be paraphyletic with respect to *Papilio alexanor + Chilasa*, but there is low support for this relationship. Many nodes on the tree are well supported, and the overall posterior probability of the tree was 0.93. Of the 53 total nodes, 32 had a significance level of 1.0 and 42 had support >0.9.

Bayesian analyses performed with two independent runs on the *EF-1 $\alpha$*  data subset yielded only 2,000 burn-in trees per run. A consensus tree was constructed from the remaining 16,000 trees and resulted in a resolution very similar to that of the tree from the corresponding partitioned ML analysis (Fig. 2). The overall probability of the tree was only 0.85, and many nodes reflecting phylogenetic relationships between major lineages in the *Priniceps + Papilio* (sensu stricto) clade had lower posterior probabilities.

Three independent analyses for the combined data set converged on similar log-likelihood scores and reached stationarity before generation 300,000. For the *COI-COII* data, the initial 3,000 trees from each analysis were discarded. A consensus tree was constructed from the combined set of 21,000 trees (Fig. 3). The branching pattern of the tree is completely identical to that of the MP bootstrap consensus and ML trees from combined analyses. The average posterior probability for the inferred phylogeny was 0.97. Thirty-eight ingroup nodes (of 53) had posterior probabilities of 1.0, and 46 nodes were supported with significance level >0.9. Bayesian analyses supplied higher values for posterior probabilities compared with bootstrap supports from ML and MP analyses.

#### Divergence Time Estimation

Likelihood ratio tests rejected clocklike behavior of sequences ( $P < 0.001$ ) for combined and partitioned (*COI-COII* vs. *EF-1 $\alpha$* ) data and also for full (71 taxa) and reduced (56 taxa) taxon sets. Tests for all six permutations of partitions (three) versus taxon sets (two) gave  $P$  values of  $\ll 0.001$ . Application of the selected calibration points in the penalized likelihood procedure provided us with a range of dates (with SDs). Initial results were obtained with the default settings for dating analyses in the r8s program, with the cross-validation function enforced. The rate smoothing parameter with the lowest cross-validation scores was selected, and the dating procedure was then repeated. Age estimates (with SDs) for all internal nodes numbered in Figure 4 are shown in Table 4.

#### Character Evolution

Character evolution optimized on the phylogeny of *Papilio* is illustrated in Figure 4. Based on the inferred

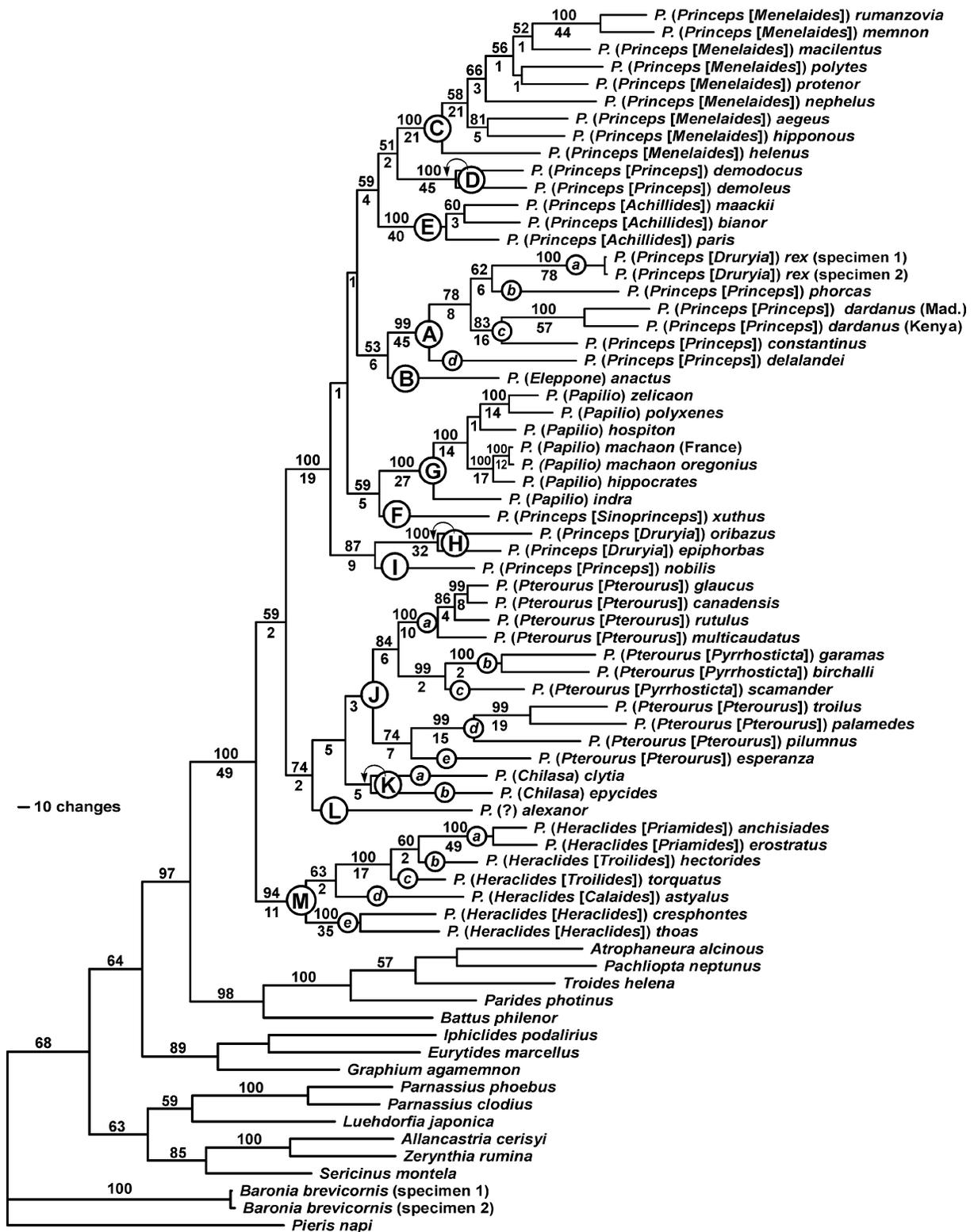


FIGURE 1. Maximum parsimony tree from combined equally weighted data. Numbers above branches indicate bootstrap support (values >50 shown). Bremer support (decay index) is given under branches for nodes within *Papilio* sensu lato. Circled letters label subclades and nodes to indicate terminal nodes on trees in Figure 2.

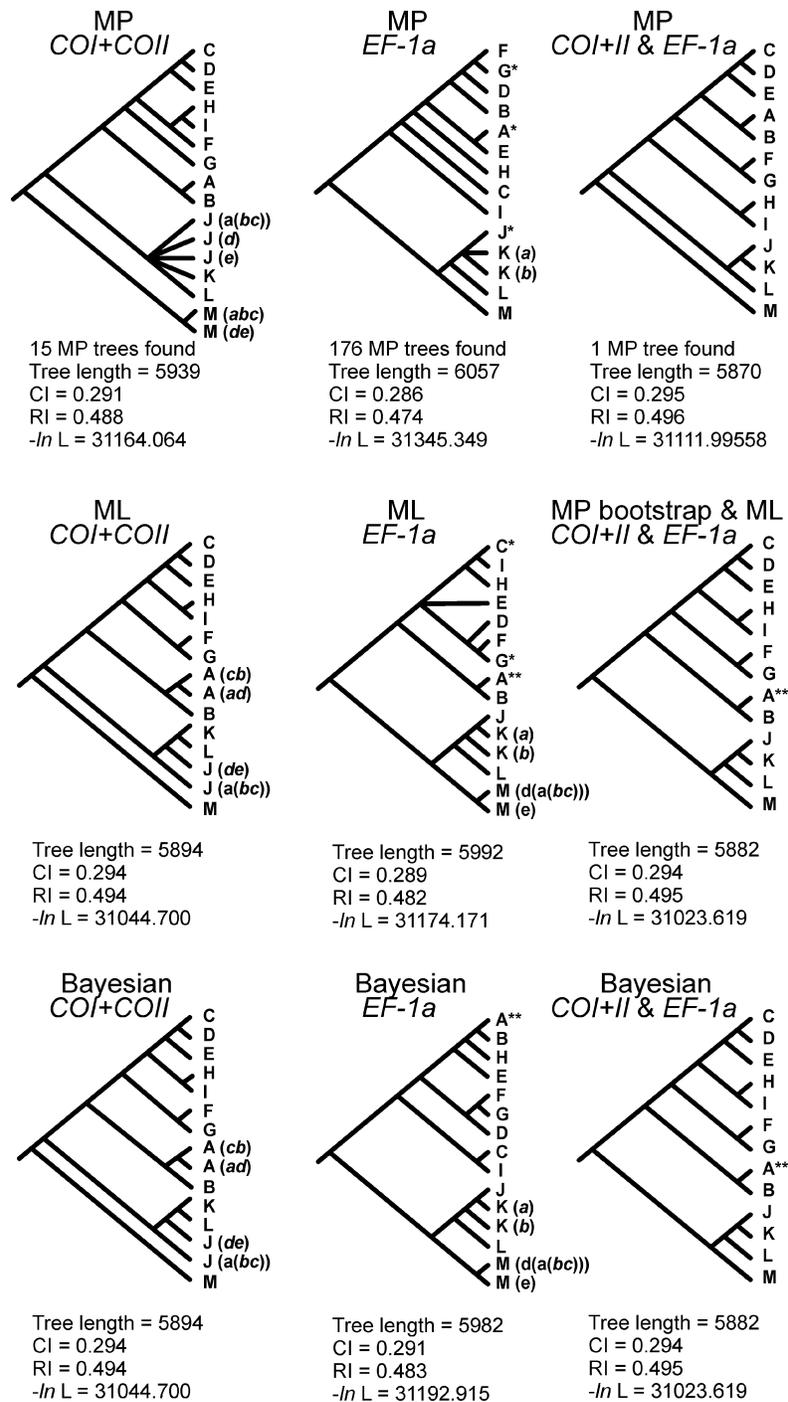


FIGURE 2. Summarized tree topologies inferred from MP, ML, and Bayesian analyses for partitioned and combined equally weighted data sets with two outgroup taxa and only ingroup topologies shown. Terminal node names correspond to subclades and nodes labeled in Figure 1. Lowercase letters indicate nodes and subclades different from those on the tree in Figure 1. A = subgenera *Druryia* and *Princeps* (a = *Papilio rex*; b = *P. phorcas*; c = *P. dardanus* and *P. constantinus*; d = *P. delalandei*); B = *Eleppone anactus*; C = subgenus *Menelaides*; D = *P. demoleus* species group; E = subgenus *Achillides*; F = *P. xuthus*; G = subgenus *Papilio*; H = *P. oribazus* and *P. epiphorbas*; I = *P. nobilis*; J = subgenus *Pterourus* (a = *Papilio glaucus* species group; b = *P. garmas* and *P. birchalli*; c = *P. scamander*; d = *P. troilus* species group; e = *P. esperanza*); K = subgenus *Chilasa* (a = *P. clytia*; b = *P. epycides*); L = *P. alexanor*; M = subgenus *Heraclides* (a = *P. torquatus* and *P. anchisiades* species groups plus *P. astyalus*; b = *P. thoas* and *P. cresphontes*). When more than one MP tree was found in a particular analysis, topology of the strict consensus tree is shown. Asterisks beside taxa on the *EF-1a* MP consensus tree indicate that all nodes within these clades are collapsed with three exceptions: clade G (*machaon gorganus* + *machaon oregonius*); clade A has an unresolved tritomy (*dardanus* + *phorcas* + *constantinus*); and clade J has an unresolved tritomy (*glaucus* + *canadensis* + *multicaudatus*). Asterisks beside taxa on the *EF-1a* ML tree indicate that these clades have polytomies: clade G (*indra* + *hippocrates* + *hospiton*) and clade C (*nepheleus* + *macilentus* + the remaining species of *Menelaides*, except *helenus*). Node A with two asterisks represents an alternative position of *P. phorcas* as sister taxon to *P. dardanus* + *P. constantinus*. The MP bootstrap consensus tree has a branching pattern identical to that of the combined ML tree but different from that of the single most-parsimonious tree.

TABLE 3. Substitution model parameters<sup>a</sup> estimated under ML from combined and partitioned data sets.

Data set	Model	Base frequencies				Substitution rates						$\Gamma$	I
		A	C	G	T	A-C	A-G	A-T	C-G	C-T	G-T		
mtDNA, 71 taxa	GTR+ $\Gamma$ +I	0.3775	0.0746	0.1092	0.4387	42.6477	23.0684	13.2155	12.3900	494.9504	1.0000	0.3733	0.4277
<i>EF-1<math>\alpha</math></i> , 71 taxa	GTR+ $\Gamma$ +I	0.2817	0.2340	0.2200	0.2643	0.7154	6.4703	2.2787	0.8428	9.4152	1.0000	1.3979	0.6431
Combined 71 taxa	GTR+ $\Gamma$ +I	0.3290	0.1079	0.1417	0.4214	13.1789	18.6198	13.5734	10.0175	136.6209	1.0000	0.5502	0.5048
mtDNA, 56 taxa	GTR+ $\Gamma$ +I	0.3525	0.870	0.1145	0.4460	20.5719	19.4641	9.2873	4.9981	292.5973	1.0000	0.4016	0.4637
<i>EF-1<math>\alpha</math></i> , 56 taxa	TrN+ $\Gamma$ +I	0.2688	0.2486	0.2343	0.2483	1.0000	5.6763	1.0000	1.0000	9.5215	1.0000	1.2584	0.6503
Combined 56 taxa	GTR+ $\Gamma$ +I	0.3022	0.1376	0.1562	0.4039	6.2882	15.9335	11.3400	5.0694	84.5150	1.0000	0.5856	0.5258

<sup>a</sup> $\Gamma$  =  $\alpha$ , estimated shape parameter; I = proportion of invariable sites.

phylogeny, the available morphological and ecological data, and *Pachliopta neptunus* (a member of Troidini) as the sister taxon, MP reconstructions indicated that the ancestral states for the selected characters were larval feeding on Rutaceae, nonmimetic wing pattern, lack of iridescent patches on wings, lack of female secretion deposits on egg surface, straight larval minute body hairs, one row of crochets on larval prolegs, and lack of a silk pad on the larval resting site (Table 5).

When a different outgroup was chosen, no substantial differences were observed for reconstruction of ancestral states of any characters except larval host plant. When *Meandrusa* was placed as the closest *Papilio* outgroup, an uncertain MP reconstruction of the ancestral host plant was obtained at the root of *Papilio* (node 88), indicating equal probabilities for larval feeding on Rutaceae and Lauraceae. In ML reconstructions, with *Meandrusa* as a sister taxon for *Papilio*, all changes except larval hosts at nodes 88 and 101 (Fig. 4) had the same transitions but with slightly higher or equal log-likelihood support (data not shown). Inclusion of *Meandrusa* decreased the likelihood (from 97.3% to 87.1% out of total likelihood) that Rutaceae feeding was the primitive trait for *Papilio* larvae. As the ancestral larval host, Lauraceae had less than 0.6% of total likelihood. Inclusion of *Meandrusa* increased this estimate up to 11.6%. Uncertainty remains for the ancestral state of node 101, where none of the reconstructions gave a strong indication for larvae feeding on any one of Rutaceae, Apiaceae, or Lauraceae as the primitive trait. Incomplete data did not allow ML character optimizations for presence or absence of female secretion on eggs, shape of larval body hairs, number of crochet rows on larval prolegs, and presence or absence of a silk pad on larval resting sites.

## DISCUSSION

### *Phylogenetic Relationships*

No previous molecular phylogenetic studies on *Papilio* have resulted in a single robust or relatively comprehensive phylogenetic reconstruction. The improved resolution obtained here allowed us to revisit several inadequately resolved issues. In congruence with previous studies of *Papilio* phylogeny (Aubert et al., 1999; Caterino et al., 1999; Reed and Sperling, 1999), our results in the ML and Bayesian analyses on combined data show that

the genus *Papilio* comprises two lineages (Fig. 5): one includes Hancock's genera (here treated as subgenera) *Heraclides*, *Pterourus*, and *Chilasa* and *Papilio alexanor*, and the other consists of *Princeps*, *Papilio* (sensu stricto), and the monotypic *Eleppone*. A potential alternative basal topology, with *Heraclides* as the first clade to diverge from the remainder of *Papilio* sensu lato (Fig. 1), suggests the existence of three major *Papilio* lineages: *Heraclides*, *Pterourus* + *Chilasa* + *Papilio alexanor*, and *Princeps* + *Papilio* (sensu stricto) + *Eleppone*. However, this branching pattern has weaker support in most analyses, and therefore we relied on the relationships obtained from the combined MP bootstrap, ML, and Bayesian analyses.

Our most significant taxonomic finding is the strong placement of *Papilio esperanza*. Several other researchers, although recognizing some uncertainty, concluded that this species was a member of *Heraclides* (Hancock, 1983). We find strong support for the countervailing position, that the species is instead related to (and in our analysis basal to) the *troilus* species group (see Collins and Morris, 1985).

Another significant result is the strongly supported placement of *P. anactus* at the base of part of the *Princeps* lineage. This species had previously been placed in its own subgenus, close to *Chilasa* (in the other major clade of Papilionini) (Hancock, 1979). The placement was based in part on shared mimicry, although the specific mimetic patterns differ as do larval morphology and host plants. Larvae of *Chilasa* develop on Lauraceae and Magnoliaceae, whereas those of *P. anactus*, like members of *Princeps*, feed on Rutaceae (Hancock, 1979).

Several findings add further support to recent suggested taxonomic changes. In agreement with Scriber et al. (1991) and Caterino and Sperling (1999), we found *Pterourus* sensu Hancock (1983) to be paraphyletic with respect to *Pyrrhosticta*. Monophyly of *Heraclides*, and relationships within it, were strongly supported in all analyses. There is some evidence against division of *Heraclides* into four subgenera, *Heraclides*, *Calaidides*, *Troilides* and *Priamides*, as suggested in Tyler et al. (1994). At least for *Troilides*, we found relatively strong support for paraphyly. However, additional samples are needed. Our results support the previously weak placement of *Papilio alexanor* near *Pterourus*, as found by Caterino and Sperling (1999) and Reed and Sperling (1999). Previous suggestions that this species belongs near the *P. machaon* species group (Munroe, 1961; Hancock, 1983) can now



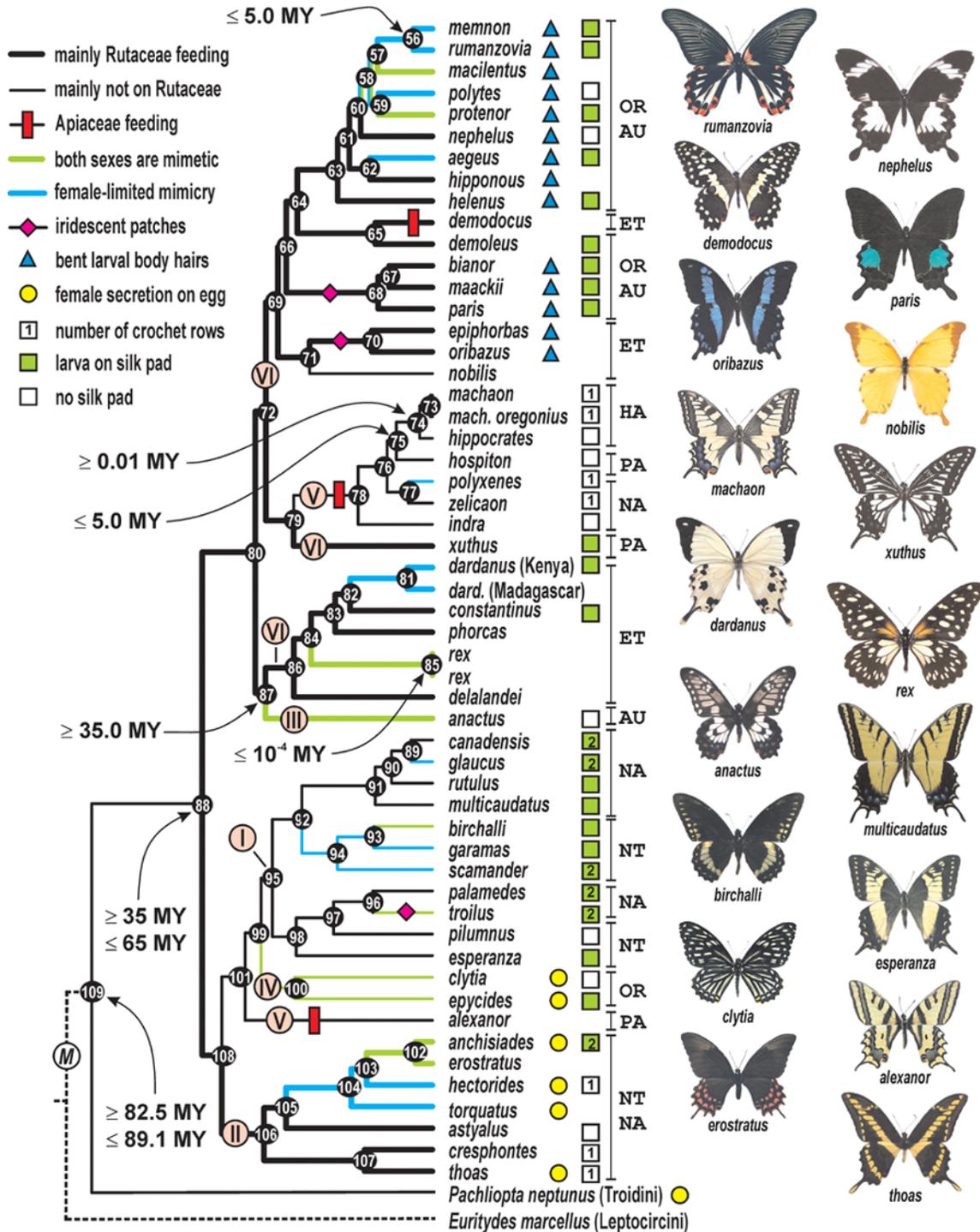


FIGURE 4. Calibration points and evolution of ecological and morphological traits in *Papilio*. Penalized likelihood procedure (Sanderson, 2002) was applied to the combined Bayesian tree using constrained age of the nodes indicated by arrows. Dating information for all internal nodes (shown in black circles) is provided in Table 4. Roman numerals correspond to Hancock's genera: I = *Pterourus*; II = *Heraclides*; III = *Eleppone*; IV = *Chilasa*; V = *Papilio*; VI = *Princeps*. Branches corresponding to major Rutaceae feeding groups are heavier (Tyler et al., 1994; Scriber et al., 1995). Independent origin of Apiaceae feeding is shown as red bars on the tree. Branches for mimetic species are shown in blue (if only female is mimetic) or green (if both sexes are mimetic) (Hancock, 1983). Presence of metallic iridescent scales on large areas of the wings is shown as mauve diamonds (Munroe, 1961). Blue triangles beside taxon names indicate that minute body hairs in last-instar larva are bent backwards, whereas they are straight in other species (Igarashi, 1984). Green squares beside taxon names indicate the ability of larvae to spin a silk pad on leaf upper surface (where data are available; Hagen, 1999). Open squares indicate that larvae rest on twigs or stems of the host plant without spinning a silk pad. Numerals inside squares indicate number of crochet rows, if known (Hagen, 1999). Yellow circles indicate species that have eggs with visible granular female secretion attached to the chorion (Igarashi, 1984; Scriber et al., 1995). Presence of species in zoogeographic regions (Collins and Morris, 1985) is shown on the right of the tree: OR = Oriental; AU = Australasian; ET = Ethiopian; PA = Palearctic; HA = Holarctic; NA = Nearctic; NT = Neotropical. Circled *M* indicates probable phylogenetic position of *Meandrusa*.

TABLE 4. Age estimates ( $\pm$ SD) in millions of years of internal nodes in *Papilio* tree using calibration points shown in Figure 4 and model-corrected branch lengths.

Node (Fig. 4)	Combined data set					
	Root fixed at 82.5 MY	Root fixed at 89.1 MY	Root relaxed, six nodes constrained	Root and six nodes constrained	Root and six nodes constrained <sup>a</sup>	
					<i>COI+COII</i>	<i>EF-1<math>\alpha</math></i>
56	4.73 $\pm$ 2.62	5.11 $\pm$ 2.81	5.00 $\pm$ 1.67	4.70 $\pm$ 1.65	5.00 $\pm$ 2.40	4.38 $\pm$ 8.50
57	13.36 $\pm$ 6.55	14.50 $\pm$ 7.04	14.52 $\pm$ 5.46	13.27 $\pm$ 5.62	15.99 $\pm$ 7.90	11.11 $\pm$ 9.10
58	15.14 $\pm$ 7.20	16.43 $\pm$ 7.75	16.49 $\pm$ 6.05	15.03 $\pm$ 6.23	18.18 $\pm$ 8.76	
59	13.17 $\pm$ 6.33	14.30 $\pm$ 6.79	14.35 $\pm$ 5.49	13.07 $\pm$ 5.53	16.09 $\pm$ 7.36	10.01 $\pm$ 9.68
60	17.46 $\pm$ 8.36	18.94 $\pm$ 9.06	19.05 $\pm$ 7.28	17.33 $\pm$ 7.37	21.31 $\pm$ 10.33	
61	20.22 $\pm$ 9.44	16.50 $\pm$ 10.18	22.10 $\pm$ 8.26	20.07 $\pm$ 8.48		12.00 $\pm$ 8.19
62	15.20 $\pm$ 7.72	21.94 $\pm$ 8.39	16.63 $\pm$ 6.78	15.08 $\pm$ 7.11	20.16 $\pm$ 10.11	7.35 $\pm$ 9.57
63	23.04 $\pm$ 10.44	25.01 $\pm$ 11.34	25.24 $\pm$ 9.29	22.87 $\pm$ 9.46	25.24 $\pm$ 12.94	20.22 $\pm$ 9.14
64	32.77 $\pm$ 13.49	35.60 $\pm$ 14.65	36.04 $\pm$ 12.83	32.51 $\pm$ 12.86	31.67 $\pm$ 15.53	40.09 $\pm$ 11.43
65	13.88 $\pm$ 6.99	15.07 $\pm$ 7.55	15.26 $\pm$ 6.50	13.77 $\pm$ 6.64	13.69 $\pm$ 6.91	16.88 $\pm$ 7.67
66	35.43 $\pm$ 14.35	38.50 $\pm$ 15.58	39.01 $\pm$ 13.82	35.15 $\pm$ 13.71	33.26 $\pm$ 16.71	
67	10.42 $\pm$ 6.00	11.35 $\pm$ 6.47	11.52 $\pm$ 5.91	10.34 $\pm$ 5.79	11.53 $\pm$ 6.35	9.66 $\pm$ 8.39
68	13.12 $\pm$ 7.01	14.28 $\pm$ 7.58	14.49 $\pm$ 6.71	13.02 $\pm$ 6.73	13.59 $\pm$ 7.97	13.19 $\pm$ 10.43
69	37.47 $\pm$ 15.28	40.72 $\pm$ 16.56	41.28 $\pm$ 14.56	37.17 $\pm$ 14.70	36.02 $\pm$ 18.70	
70	14.69 $\pm$ 7.34	15.98 $\pm$ 7.75	16.20 $\pm$ 7.10	14.58 $\pm$ 6.99	14.88 $\pm$ 7.86	11.87 $\pm$ 8.79
71	29.77 $\pm$ 12.36	32.36 $\pm$ 13.35	32.80 $\pm$ 12.01	29.53 $\pm$ 11.92	28.36 $\pm$ 14.28	34.79 $\pm$ 10.17
72	40.37 $\pm$ 15.99	43.86 $\pm$ 17.38	44.51 $\pm$ 15.32	40.04 $\pm$ 15.58	39.88 $\pm$ 20.97	45.10 $\pm$ 9.39
73	0.52 $\pm$ 0.72	0.56 $\pm$ 0.77	0.36 $\pm$ 1.20	0.35 $\pm$ 1.65	0.20 $\pm$ 0.27	4.36 $\pm$ 5.92
74	3.08 $\pm$ 2.55	3.35 $\pm$ 2.78	1.97 $\pm$ 2.74	1.94 $\pm$ 2.73	1.50 $\pm$ 1.18	
75	8.73 $\pm$ 5.61	9.50 $\pm$ 6.19	5.00 $\pm$ 5.46	5.00 $\pm$ 5.62	5.00 $\pm$ 3.37	
76	10.96 $\pm$ 6.69	11.91 $\pm$ 7.28	8.31 $\pm$ 6.42	7.90 $\pm$ 6.72	7.90 $\pm$ 5.80	12.44 $\pm$ 7.02
77	5.86 $\pm$ 4.35	6.37 $\pm$ 4.64	4.61 $\pm$ 4.26	4.37 $\pm$ 4.34	4.45 $\pm$ 4.00	7.51 $\pm$ 5.37
78	18.02 $\pm$ 9.26	19.59 $\pm$ 10.04	16.58 $\pm$ 8.85	15.33 $\pm$ 9.10	17.76 $\pm$ 10.36	15.64 $\pm$ 7.98
79	33.60 $\pm$ 13.79	36.53 $\pm$ 15.00	35.70 $\pm$ 13.13	32.27 $\pm$ 13.39	35.48 $\pm$ 18.42	33.58 $\pm$ 9.84
80	42.92 $\pm$ 16.83	46.61 $\pm$ 18.28	47.86 $\pm$ 16.18	42.98 $\pm$ 16.53	41.87 $\pm$ 22.30	49.67 $\pm$ 7.41
81	6.12 $\pm$ 3.53	6.62 $\pm$ 3.73	6.76 $\pm$ 3.42	6.11 $\pm$ 3.47	6.93 $\pm$ 4.27	4.43 $\pm$ 6.08
82	20.04 $\pm$ 8.97	21.75 $\pm$ 9.70	22.21 $\pm$ 8.77	19.98 $\pm$ 8.95	23.05 $\pm$ 11.82	19.95 $\pm$ 6.98
83	23.69 $\pm$ 10.63	25.71 $\pm$ 11.60	26.26 $\pm$ 10.22	23.61 $\pm$ 10.49	27.87 $\pm$ 14.38	21.11 $\pm$ 7.42
84	29.55 $\pm$ 12.39	32.10 $\pm$ 13.60	32.79 $\pm$ 11.82	29.45 $\pm$ 12.09	32.84 $\pm$ 16.51	29.96 $\pm$ 8.46
85	0.26 $\pm$ 1.53	0.29 $\pm$ 3.80	0.00 $\pm$ 0.35	0.00 $\pm$ 0.35	10 <sup>-4</sup> $\pm$ 2.12	
86	33.67 $\pm$ 13.75	36.58 $\pm$ 15.07	37.42 $\pm$ 13.07	33.62 $\pm$ 13.33	33.52 $\pm$ 18.29	38.90 $\pm$ 8.36
87	40.50 $\pm$ 16.05	43.99 $\pm$ 17.49	45.10 $\pm$ 15.40	40.52 $\pm$ 15.70	40.18 $\pm$ 21.52	47.08 $\pm$ 10.73
88	55.75 $\pm$ 20.07	60.42 $\pm$ 21.67	65.00 $\pm$ 20.06	57.89 $\pm$ 20.12	56.05 $\pm$ 27.35	65.00 $\pm$ 6.37
89	5.08 $\pm$ 3.90	5.64 $\pm$ 4.22	6.17 $\pm$ 3.86	5.34 $\pm$ 3.93	5.65 $\pm$ 3.65	8.25 $\pm$ 14.71
90	9.71 $\pm$ 5.77	10.74 $\pm$ 6.29	11.70 $\pm$ 5.83	10.18 $\pm$ 5.98	11.67 $\pm$ 6.51	
91	13.66 $\pm$ 7.53	15.00 $\pm$ 8.16	16.27 $\pm$ 7.66	14.26 $\pm$ 7.76	16.83 $\pm$ 9.15	10.04 $\pm$ 5.73
92	31.56 $\pm$ 12.47	34.26 $\pm$ 13.50	36.79 $\pm$ 12.94	32.76 $\pm$ 13.06	36.61 $\pm$ 16.40	20.85 $\pm$ 8.80
93	14.29 $\pm$ 6.51	15.44 $\pm$ 7.01	16.52 $\pm$ 6.66	14.80 $\pm$ 6.91	20.14 $\pm$ 9.19	4.63 $\pm$ 3.91
94	23.00 $\pm$ 9.58	24.92 $\pm$ 10.31	26.72 $\pm$ 9.83	23.86 $\pm$ 10.17	25.83 $\pm$ 11.83	16.59 $\pm$ 7.13
95	38.73 $\pm$ 14.60	42.01 $\pm$ 15.76	45.08 $\pm$ 15.10	40.19 $\pm$ 15.01	45.49 $\pm$ 19.78	25.91 $\pm$ 9.65
96	14.43 $\pm$ 6.47	15.65 $\pm$ 7.06	16.80 $\pm$ 6.74	14.98 $\pm$ 6.75	20.54 $\pm$ 9.42	5.75 $\pm$ 5.10
97	23.94 $\pm$ 9.52	25.95 $\pm$ 10.23	27.83 $\pm$ 9.75	24.83 $\pm$ 9.86	29.11 $\pm$ 12.84	14.75 $\pm$ 7.50
98	32.97 $\pm$ 12.54	35.74 $\pm$ 13.54	38.34 $\pm$ 13.01	34.21 $\pm$ 12.94	39.56 $\pm$ 16.70	21.95 $\pm$ 8.84
99	41.44 $\pm$ 15.50	44.95 $\pm$ 16.76	48.24 $\pm$ 15.96	43.00 $\pm$ 15.74	46.70 $\pm$ 21.08	29.94 $\pm$ 10.55
100	33.29 $\pm$ 12.88	36.13 $\pm$ 13.97	38.79 $\pm$ 13.26	34.56 $\pm$ 13.36	37.09 $\pm$ 16.39	25.21 $\pm$ 10.32
101	45.31 $\pm$ 16.61	49.14 $\pm$ 18.00	52.75 $\pm$ 16.88	47.02 $\pm$ 16.77	49.58 $\pm$ 22.51	34.28 $\pm$ 9.96
102	4.15 $\pm$ 2.94	4.46 $\pm$ 2.95	4.76 $\pm$ 2.80	4.29 $\pm$ 2.76	4.68 $\pm$ 3.27	3.13 $\pm$ 6.08
103	15.93 $\pm$ 7.77	17.27 $\pm$ 8.49	18.52 $\pm$ 8.25	16.53 $\pm$ 7.90	17.41 $\pm$ 9.28	15.60 $\pm$ 7.82
104	19.62 $\pm$ 8.97	21.30 $\pm$ 9.73	22.86 $\pm$ 9.33	20.36 $\pm$ 9.02	21.60 $\pm$ 11.11	17.55 $\pm$ 8.75
105	35.35 $\pm$ 13.73	38.42 $\pm$ 14.92	41.27 $\pm$ 14.17	36.71 $\pm$ 13.92	38.91 $\pm$ 18.35	28.32 $\pm$ 10.01
106	40.68 $\pm$ 15.68	44.19 $\pm$ 16.87	47.46 $\pm$ 15.76	42.23 $\pm$ 15.76	43.24 $\pm$ 20.95	33.01 $\pm$ 9.50
107	16.37 $\pm$ 8.97	17.82 $\pm$ 9.05	19.18 $\pm$ 8.55	17.01 $\pm$ 8.81	18.13 $\pm$ 10.52	12.25 $\pm$ 8.71
108	50.94 $\pm$ 18.50	55.24 $\pm$ 19.94	59.34 $\pm$ 18.51	52.87 $\pm$ 18.54	54.37 $\pm$ 25.07	42.24 $\pm$ 9.12
109	82.50 $\pm$ 0.00	89.10 $\pm$ 0.00	102.78 $\pm$ 11.59	89.10 $\pm$ 2.29	89.10 $\pm$ 1.17	89.10 $\pm$ 2.15

<sup>a</sup>No age estimation could be obtained for nodes that were collapsed in partitioned phylogenies.

the combined ML tree and the high posterior probability of the corresponding clade in the Bayesian phylogeny.

Another significant finding is the support for parphyly of *Princeps* with respect to *Papilio* sensu stricto and *Eleppone*. Hancock (1983) divided *Princeps* into four subgeneric lineages. Only one, *Menelaides*, is supported

here as a monophyletic taxonomic unit; a second group, *Sinoprinceps*, is represented here by a single species. Hancock's other two subgenera, *Druryia* and *Princeps* sensu stricto, were found to be paraphyletic. Some clades within *Princeps*, however, are strongly supported: (1) *Menelaides* (sensu Hancock, 1983); (2) the *P. demoleus*

TABLE 5. Reconstructed ancestral states and major gains and losses (excluding character changes in individual terminal taxa) for selected characters in *Papilio* using *Pachliopta neptunus* (Troidini) as its sister taxon.

Node (Fig. 4)	MP	ML <sup>a</sup>
Host plant families		
88	Rutaceae (Rutaceae or Lauraceae) <sup>b</sup>	Rutaceae (46.97; 0.973)
101	Rutaceae or Apiaceae or Lauraceae	Apiaceae (48.05; 0.33)
		Lauraceae (48.07; 0.33)
		Rutaceae (48.09; 0.32)
99	Lauraceae	Lauraceae (46.95; 0.991)
91	Rosaceae, Oleaceae, Salicaceae, Betulaceae	Rosaceae (46.96; 0.988)
78	Apiaceae	Apiaceae (46.96; 0.988)
106 <sup>b</sup>	Rutaceae	
80 <sup>b</sup>	Rutaceae	
Mimicry in imago		
88	Nonmimetic	Nonmimetic (50.55; 0.992)
104	Female	Female limited (50.83; 0.75), nonmimetic (52.55; 0.18)
102	Both sexes	Both sexes (50.63; 0.917)
100	Both sexes	Both sexes (50.63; 0.912)
94	Female	Female limited (50.83; 0.748), nonmimetic (52.26; 0.171)
85	Both sexes	Both sexes (50.62; 0.92)
81	Female	Female limited (50.63; 0.911)
58	Female or both sexes	Both sexes (51.49; 0.386), female limited (51.52; 0.374), nonmimetic (51.97; 0.240)
56	Female	Female limited (50.60; 0.942)
Iridescent patches on wings		
88	Absent	Absent (13.59; 0.999)
70	Present	Present (13.62; 0.973)
100	Present	Present (13.62; 0.972)
Female secretion on eggs		
88	Not present	Not computable (missing data)
106	Equivocal	
104	Present	
100	Present	
Larval body hair		
88	Straight	Not computable (missing data)
69	Bent	
65	Straight	
Number of crochet rows on larval prolegs		
88	1	Not computable (missing data)
102	1 or 2	
101	1 or 2	
95	2	
Silk pad on a resting site		
88	Not present	Not computable (missing data)
72	Equivocal	
66	Present	
78	Not present	
102	Equivocal	
99	Equivocal	
95	Present	

<sup>a</sup>Estimates of marginal ancestral state probabilities are given as character state (negative log likelihood; proportion of a total likelihood).

<sup>b</sup>Alternative transition when *Meandrusa* was placed as a sister taxon to *Papilio*.

species group; (3) *Achillides* (sensu Shimogori, 1997); (4) *P. nireus* species group; and (5) the ((*rex*, *phorcas*)*delalandei*) species groups from subdivisions *Druryia* and *Princeps*. In congruence with another study (Vane-Wright et al., 1999), *P. nobilis* is strongly associated with this latter group. The present results offer the strongest support

yet for relationships among the three species of the *phorcas* group; our finding of ((*dardanus*, *constantinus*)*phorcas*) contrasts with the ((*dardanus*, *phorcas*)*constantinus*) resolution suggested by Vane-Wright et al. (1999).

#### Molecular Clock Predates Fossil Records of Papilionidae

The estimate of  $26 \pm 7$  MYA for the origin of the family Papilionidae calculated from standard rates of  $20 \times 10^{-9}$  substitutions per site per year is substantially less than the minimum age of 48 MY indicated by fossils. One explanation for this mismatch is that despite efforts to correct them, divergences have been substantially underestimated because of high saturation in relatively rapidly evolving *COI* and *COII* genes. Standard substitution rates have generally been applied to recently evolved taxa separated by sequence divergence of <5–7% (Harrison and Bogdanowicz, 1995). The approximately twofold difference in GTR+I+ $\Gamma$ -corrected relative substitution rates in *COI*-*COII* sequences between the data sets with and without basal species of Papilionidae (Table 3) may indicate that at least the mtDNA sequences are badly saturated and that even the best-fit model gives corrected divergences that are a substantial underestimate. At the same time, proportionally less difference in rates was observed in *EF-1 $\alpha$*  sequences for data with complete versus reduced numbers of outgroup taxa. Substitution rates at third codon positions are roughly 18–28 times greater in *COI* and *COII* than in *EF-1 $\alpha$* , which is comparable to estimates from previous studies (e.g., Reed and Sperling, 1999). Nevertheless, the selected substitution model was designed to incorporate significant rate differences among taxa, and thus the divergence estimates should have accounted for some of this variation.

Our proposed dating suggests slow rates of evolution in mtDNA in the genus *Papilio*. Substitution rates for the mtDNA genes *COI* and *COII* and the nuclear gene *EF-1 $\alpha$*  are  $7.8$ – $10.2 \times 10^{-9}$  and  $1.32$ – $2.0 \times 10^{-9}$  per site per year, respectively. These rates are 2–4 (for mtDNA) and up to 30 (for *EF-1 $\alpha$* ) times slower than the “standard” substitution rates for *COI* in *Drosophila* ( $20.0$ – $29.0 \times 10^{-9}$  substitutions per site per year; Beckenbach et al., 1993) and fig-pollinating wasps ( $19.0 \times 10^{-9}$ ; Machado et al., 2001). However, there is now abundant evidence for a diversity of mtDNA rates in insects. Low transformation rates have been reported for carabid beetles ( $2.8 \times 10^{-9}$  substitutions per site per year; Su et al., 1998), *Halobates* sea skaters ( $4$ – $7 \times 10^{-9}$  per year; Andersen et al., 2000), *Bacillus* phasmids ( $3$ – $4.7 \times 10^{-9}$  per year; Mantovani et al., 2001), and *Limnoporus* water-striders ( $4$ – $4.5 \times 10^{-9}$  per year; Sperling et al., 1997). Accelerated substitution rates have been reported for the honeybee *Apis mellifera* (approximately  $34.0$ – $49.0 \times 10^{-9}$ ; Crozier et al., 1989) and parasitic lice of the genus *Dennyus* (up to  $90 \times 10^{-9}$ ; Page et al., 1998).

Our estimate of an age for major lineages within *Papilio* of around 30–50 MY (with the age of the split of *Papilio* from Troidini up to 100 MY) would also increase the time of the origin of the family Papilionidae to

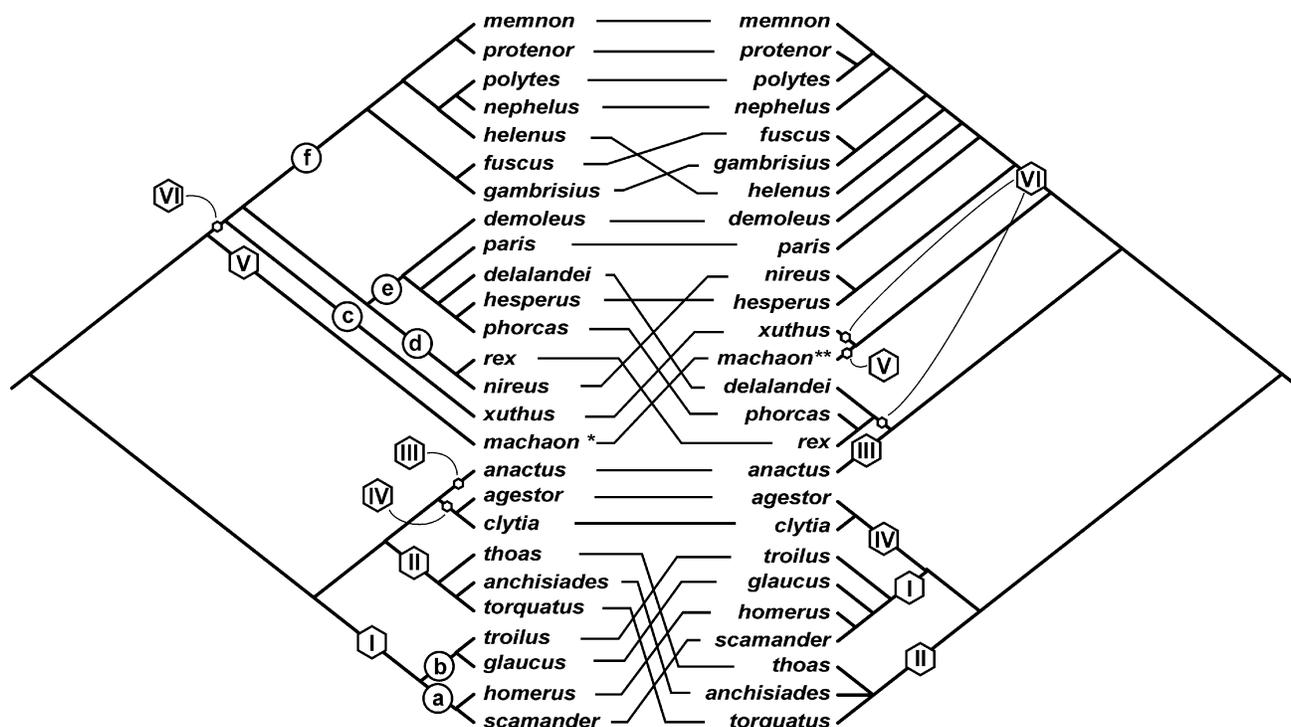


FIGURE 5. Phylogenetic relationships of species groups and major subdivisions of *Papilio* sensu lato: Left: tree according to Hancock (1983); right: tree based on data presented here. Roman numerals correspond to Hancock's genera: I = *Pterourus*; II = *Heraclides*; III = *Eleppone*; IV = *Chilasa*; V = *Papilio*; VI = *Princeps*. Letters refer to Hancock's subgenera: a = *Pyrrhosticta*; b = *Pterourus*; c = *Simoprinceps*; d = *Druryia*; e = *Princeps*; f = *Menelaides*. Single asterisk indicates inclusion of *Papilio alexanor* as per Hancock (1983). Double asterisks indicate exclusion of *P. alexanor* based on our data.

the lower Cretaceous, substantially predating the earliest fossils. However, this type of observation is increasingly common in studies that attempt to ascertain dates of taxonomic origin based on molecular data (Pellmyr and Leebens-Mack, 1999; Machado et al., 2001). Because of their fragile nature, delicate-wing butterflies are rarely preserved as fossils. Although at least 44 butterfly fossils are known worldwide (Emmel et al., 1992), very few swallowtail fossils have been reported, with the oldest putative swallowtail, *Praepapilio*, dated as 48 MY old, and few other *Papilio* representatives from more recent periods. The richest fossil butterfly fauna in the world is known from 36–34-MY Florissant shales, and this assemblage indicates that all major families of butterflies had already evolved well before this date (Emmel et al., 1992). Some fossils from the Lower Oligocene have been described and placed within extant genera of the family Nymphalidae, e.g., *Vanessa amerindica*, which has a strong affinity to *V. indica* (see Emmel et al., 1992). Thus, at least some butterflies very similar to modern genera in Rhopalocera were already present at least 35 MYA. The family Papilionidae is certainly among the oldest Papilionoidea, so the origin of swallowtail butterflies in the Cretaceous seems plausible.

#### Vicariance Biogeography

Previous authors have outlined relatively concrete biogeographic scenarios for Papilionini (Munroe, 1961;

Hancock, 1983; Miller, 1987; Scriber, 1995b). The ancestor of the Papilionini, from a center of origin in eastern North America, is considered to have dispersed first to the Palearctic and later to South America before the end of the Cretaceous, leaving *Pterourus* in North America. The Palearctic ancestor spread to China (and subsequently to Southeast Asia, Africa, and Australia) as *Princeps*, leaving *Papilio* sensu stricto in western Asia. The South American ancestor spread via Gondwanaland to Australia as *Eleppone* and from there to Southeast Asia as *Chilasa*, leaving *Heraclides* in South America.

The inferred pattern of phylogenetic relationships within *Papilio* as found here at least partly conflicts with these previously proposed vicariance hypotheses. The major conflict stems from the origin of *Papilio alexanor*, subgenus *Chilasa*, and subgenus *Pterourus* from a common ancestor and the distant relationship between New World branches of *Papilio* and the Old World *P. (Eleppone) anactus*. The origin of *Chilasa* from the common ancestor with *Eleppone* via Gondwanaland to Australia and Asia, as was suggested based on a traditional view of *Papilio* phylogeny (Scriber, 1995b), is not supported by our results.

The best currently available evidence indicates that the common ancestor of *Papilio* apparently produced two descendant lineages in the North America–Europe block about 55–65 MYA (see Fig. 4, Table 4), well before the complete disjunction of the two continents. This scenario is not dependent on the use of this vicariance event in

establishing divergence rates, because several other calibration points gave similar dates. One lineage was the ancestor of *Papilio* (sensu stricto), *Princeps*, and *Eleppone*, and another was the common ancestor of *Heraclides*, *Pterourus*, *Chilasa*, and *Papilio alexanor*. Before the end of the Eocene (around 53–59 MYA, or even 42 MYA according to *EF-1 $\alpha$*  data), New World swallowtails were established in South America, and modern *Heraclides* evolved. Around 60 MYA, North and South America were temporarily and loosely connected by an island arc (Donnelly, 1988), providing a possible avenue for this dispersal. Diversification of *P. alexanor*, *Chilasa*, and *Pterourus* is dated within 30–48 MY, at the time of favorable climatic conditions across the Thulean route (Bowen et al., 2002). *Chilasa* may have then spread to Asia soon after the Turgai Strait, which separated Europe and Asia until 45 MYA, had dried, opening the Turgai route from Europe to Asia (Kurtén, 1971). The ancestral lineage that was left in North America evolved *Pterourus* millions of years before the Thulean route was severed by climate changes around 35 MYA (Noonan, 1988).

The common ancestor of Old World swallowtails might have produced three major lineages. The oldest one may have spread to Africa and Madagascar sometime between 42 and 50 MYA and left as descendants some extant groups currently placed in *Princeps*. Based on our phylogenetic results, this lineage should somehow have contributed to the origin of the Australian endemic *Papilio* (*Eleppone*) *anactus*. Traditional views on the origin of this species (Hancock, 1979) suggested instead a South American ancestry, with migration through Antarctica. *Eleppone* could have reached the Australian continent through the Tasmanian land bridge around 40–47 MYA. That hypothesis requires some connection between Africa–Madagascar and Antarctica around that time. A possible explanation comes from a late Cretaceous connection between Australia/Antarctica and Indo-Madagascar through the Kerguelen Plateau (Sampson et al., 1998; Hay et al., 1999) and Eocene–Oligocene uplift of archipelagos in the Indian Ocean and Mozambique Strait that apparently served as stepping stones for faunal exchange (McKenzie and Sclater, 1973; Haq et al., 1988).

A second lineage of Old World *Princeps*-like swallowtails underlies extant Asian *Sinoprinceps* and Holarctic *Papilio* (sensu stricto), which apparently diverged around 33–36 MYA. Speciation in *Papilio* (sensu stricto) has been attributed to several subsequent dispersals across Beringia before and during the Pleistocene (Sperling, 1987).

The third lineage of Old World swallowtails produced a diverse group of modern *Princeps*, which would have been distributed throughout southern Eurasia, Africa, and Australia sometime between 32 and 41 MYA. Around that time India collided with Eurasia, and Australia established a connection with Southeast Asia via intervening islands (Raven and Axelrod, 1972). Geographic proximity of the continents would favor numerous dispersals, resulting in the present day mixed distribution pattern with numerous distinctive popula-

tions appearing to be taxonomically isolated (Wallace, 1864).

#### *Evolution of Morphological and Ecological Traits*

Species of the genus *Papilio* are highly diverse in adult wing pattern and immature stage morphology. They also have highly varied larval host plant utilization, ranging from restricted use of one or two host species to broad polyphagy of up to eight different plant families (see Bossart and Scriber, 1995). However, about 80% of species of *Papilio* (*Eleppone* and almost all species in *Heraclides* and *Princeps*) are primarily Rutaceae feeders. The main hosts of most *Papilio* (sensu stricto) species are Apiaceae. Larvae of *Chilasa* usually develop on Lauraceae. Basal species of *Pterourus* also feed on members of the families Lauraceae and Magnoliaceae, but some species utilize Rosaceae and other hosts (see Tyler et al., 1994; Scriber et al., 1995). The lack of a stable *Papilio* phylogeny and its relationships with other swallowtails hindered previous attempts to resolve host use evolution and to determine ancestral host plant relationships (Miller, 1987).

Larvae of *Pachliopta neptunus* and all other Troidini, a generally accepted sister group of Papilionini, develop on species of Aristolochiaceae, whereas the primary hosts for Leptocircini (the sister group for Troidini and Papilionini) are Annonaceae. Some species of Leptocircini also develop on Lauraceae, Hernandiaceae, and Rosaceae. However, according to a recently published molecular phylogeny of the genus *Graphium* (Makita et al., 2003), basal lineages of Leptocircini are those whose larva develop on Hernandiaceae and Rosaceae. Thus, none of the presumed close relatives of the genus *Papilio* indicate Lauraceae as the ancestral host plants.

Based on the possible sister relationship of *Papilio* and *Meandrusa*, whose larvae develop on Lauraceae (Igarashi, 1984), it was hypothesized that use of these plants should be regarded as a primitive character (Aubert et al., 1999). Based on this assumption, there were at least two shifts to Rutaceae, once in *Heraclides* and once in the Old World lineage of *Papilio*, with subsequent shifts in the *P. machaon* group to Apiaceae and in some African *Princeps* back to Lauraceae or close relatives (Ackery et al., 1995). However, the reliability of morphological characters that were used to justify the affinity of *Meandrusa* to the tribe Papilionini is questionable (Häuser, 1993), and molecular evidence for this relationship (Aubert et al., 1999) is tentative.

The phylogenetic relationships within *Papilio* obtained here (Fig. 4) suggest that ancestral feeding on Rutaceae is at least equally plausible based on MP character optimization when *Meandrusa* was included as the sister taxon of *Papilio*. Reconstruction of the ancestral larval feeding habits under the ML model supported Rutaceae feeding as a primitive trait even when *Meandrusa* was included in character optimization. We have estimated the first split within *Papilio* at approximately 55–65 MYA, fitting the proposed age of the initial diversification within Rutales at about 67 MYA (Magallón et al., 1999). Under

this hypothesis, there would be only one major shift to Lauraceae, in the stem lineage of *Chilasa* and *Pterourus* (after the divergence of *P. alexanor*). Another shift to Lauraceae in the African *P. hesperus* group (Ackery et al., 1995) is not associated with major diversification and can be compared with the shift from Rutaceae to Fabaceae feeding in the Australian subspecies of *P. demoleus*, *P. d. sthenelus* (Braby, 2000). Another observation supporting Rutaceae as the ancestral host is that even those species whose larvae normally develop on members of other plant families possess the ability to feed on Rutaceae, both in the wild and in captivity (Tyler et al., 1994).

The pattern of relationships within *Papilio* and the exclusion of *P. alexanor* from the *P. machaon* group suggest at least three independent origins of Apiaceae feeding. This shift may be associated with changes in larval coloration. The distinctive larval coloration of *P. alexanor* appears to be convergent with that of the *machaon* group (Caterino and Sperling, 1999). Some populations of *P. demodocus* in South Africa have switched from Rutaceae to Apiaceae and have evolved a similarly cryptic pattern (Clarke et al., 1963), whereas other *P. demodocus* on Apiaceae have retained the green *Princeps*-like larval coloration. A shift to Apiaceae feeding is seen in some populations of *P. paeon*, a member of the *P. thoas* group (subgenus *Heraclides*) that usually develops on Rutaceae (Tyler et al., 1994; Sperling and Feeny, 1995); however, there appears to be no change in larval color pattern in these populations (Walker, 1882). Evolution of host plant use in *Papilio* also has been affected by recent speciation in temperate latitudes. *Papilio machaon*, with its Holarctic distribution and larvae feeding primarily on Apiaceae, has further evolved to use Asteraceae in western and northern North America and parts of central Asia (Sperling, 1990). Nearctic species of the *P. glaucus* group breed on Lauraceae, Rutaceae, Tiliaceae, or Magnoliaceae in the southern parts of their ranges but develop to the north on a wide range of temperate deciduous species in the Betulaceae, Oleaceae, Salicaceae, and Rosaceae (Bossart and Scriber, 1995).

About half of all *Papilio* species exhibit mimetic wing patterns (Fig. 4). However, nonmimetic wing pattern is very likely the ancestral state for *Papilio*, and the direction of evolution among mimetic forms is unclear. Mimetic phenotypes have evolved independently in *Heraclides*, *Chilasa*, *Princeps* (*Menelaides*), *Eleppone*, and *Papilio* sensu stricto and at least twice in *Pterourus* and African *Princeps*. Most *Princeps* (except several African representatives) and *Papilio* (sensu stricto) are nonmimetic. Many examples are restricted to female-limited Batesian mimicry, and many of the mimetic forms are polymorphic. Among the most famous is the African *P. dardanus*, which may have three sympatric forms of females that mimic different species of the genus *Amauris* (Danainae) (Clarke and Sheppard, 1960). In Southeast Asia, *P. memnon* also mimics three or more different models of troidine Papilionidae (Clarke and Sheppard, 1971). For the most part, the mimetic phenotype in both sexes seems to be a derived character state with males occasionally

developing mimetic wing patterns in species with originally female-limited mimicry. However, both sexes in *Chilasa* and *Eleppone* appear to have evolved mimetic phenotypes directly from their respective sister nonmimetic species. This may also be true of *P. rex*, but phylogenetic study of more African mimetic species of *Papilio* (apparent relatives of the *phorcas*, *rex*, and *delalandei* groups) is needed to explore this possibility.

Many other morphological characteristics of *Papilio* may have polyphyletic origins (Fig. 4). Some of these have been used as significant taxonomic characters justifying *Papilio* subdivisions. For example, a granular female secretion attached to the egg surface, which is well known in Troidini, is also reported from a number of species of *Papilio* (Igarashi, 1984). In the context of our phylogeny there is no consistent pattern for this character among species groups, although it had been hypothesized previously as a synapomorphy of *Heraclides* + *Chilasa* (Igarashi, 1984). This character instead appears to be either symplesiomorphic or derived in two separate lineages (*Chilasa* and *Heraclides*) in *Papilio*.

Extensive metallic iridescent scales have evolved independently in three different lineages of *Papilio*: *Achillides*, known as gloss-papilios; the *P. nireus* group from African *Princeps*; and *Papilio troilus*, a mimic of *Battus philenor*. This trait appears convergent with iridescent scales in some Troidini (e.g., *Trogonoptera*).

Species with curved minute hairs on the body of the last instar larvae form a monophyletic group, as was suggested by Igarashi (1984), with two exceptions: the *P. demoleus* group and *P. nobilis*. More species of *Princeps* should be studied to clarify the evolution of this trait.

Some morphological traits of *Papilio* larvae (such as the morphology of prolegs) are tightly related to their behavior. The number of crochet rows in abdominal prolegs is related to the ability of the larva to attach to the host plant (Hagen, 1999). Larvae with clasping prolegs, with only a single row of crochets, can cling more firmly to thin twigs, whereas larvae with gripping prolegs and two rows of crochets usually produce a silk pad on the leaf surface and hold on to this pad. Twig-clasping prolegs seem to be plesiomorphic in *Papilio*. Although data on proleg morphology are incomplete, there are several examples of apparently independent gains or losses of the two-row gripping prolegs and the ability to spin a silk pad (Fig. 4).

These examples demonstrate that both adult and immature morphological characters are highly labile both within and among species. Experimental hybridization of swallowtails indicates that in some cases wing and body coloration in *Papilio* is controlled by a single gene or set of tightly linked loci in a supergene, providing phenotypic plasticity (Clarke and Sheppard, 1960, 1971; Fisher, 1977; Nijhout, 1991; Loeliger and Karrer, 2000; Ffrench-Constant and Koch, 2003). Additional genetic data pertaining to the extent to which subsets of the genome maintain their genetic integrity would be very helpful for understanding the contribution of genetic architecture to speciation (Sperling, 2003).

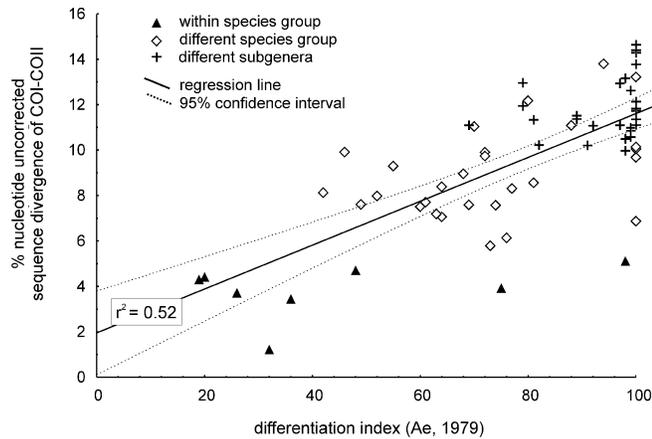


FIGURE 6. Comparison of Ae's (1979) differentiation index versus *COI-COII* uncorrected percentage nucleotide sequence divergence for *Papilio* species pairs from the same species groups, different species groups, and different subgenera.

#### Taxonomic Implications: Is *Papilio* a Single Genus?

*Papilio* remains a taxonomic enigma. Few if any *Papilio* subdivisions can be delineated precisely using adult or larval color pattern or other morphological characters (Munroe, 1961; Hancock, 1983; Igarashi, 1984; Miller, 1987). No consistent conclusions on taxonomic rank and species relationships can be derived from the comparison with hybrid incompatibility index (Ae, 1979). Although distantly related taxa with up to 15% nucleotide sequence divergence usually have high incompatibility scores and rare cases of successful matings between species from different subgenera may produce sterile hybrids (generally in the heterogametic sex; Sperling, 1990, 2003), crosses between species within the same species group may either completely fail or result in viable and fertile hybrids (Fig. 6). Although there is much overlap between Ae's differentiation index and groupings based on comparisons within species groups, between species groups, and between subgenera, there is much better correspondence between mtDNA divergence and these taxonomic groupings. An uncorrected mtDNA sequence divergence of about 5% seems to provide a clear boundary between comparisons within versus between species groups.

An alternative method for evaluating the taxonomic rank of *Papilio* subdivisions would be to compare their sequence divergence with known intergeneric differences in other Lepidoptera. The close relatives of *Papilio*, the Troidini, include three genera of birdwing butterflies, *Trogonoptera*, *Ornithoptera*, and *Troides*, that apparently have the same evolutionary ages as major *Papilio* subdivisions, around 40 MY (Morinaka et al., 1999, 2000), and exhibit comparable nucleotide sequence divergence. Average values for intra- and intergeneric divergences among accepted Graphiini and Troidini genera (Parsons, 1996a, 1996b; Morinaka et al., 1999, 2000) and putative genera of *Papilio* are presented in Figure 7, based on the taxa included in Figure 1. Average divergences between

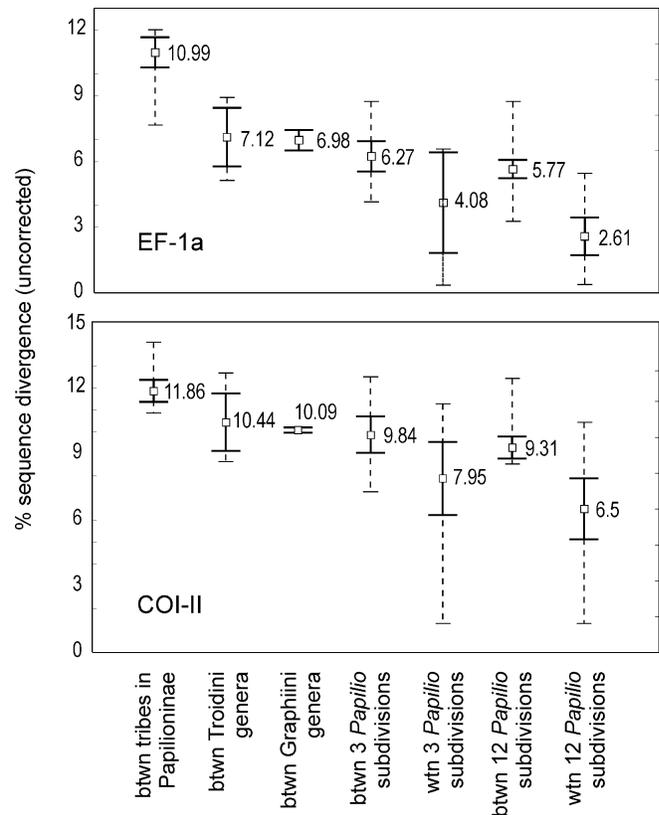


FIGURE 7. Average values of uncorrected percentage sequence divergence in two mitochondrial genes and one nuclear gene compared at different taxonomic levels within the Papilionidae. Three putative genera in *Papilio* represent *Heraclides*, *Pterourus* + *Chilasa* + *Papilio alexanor*, and *Princeps* + *Eleppone* + *Princeps* sensu stricto. Twelve putative genera constitute all major lineages from Figure 1 as A, B, C, D, E, F, G, H + I, J, K, L, and M. Solid bars indicate 95% confidence interval; dashed bars show ranges of values.

putative genera within *Papilio* are comparable to intergeneric distances in Troidini and Graphiini, whereas divergences within the putative genera are substantially lower. Average divergences in *EF-1α* at different taxonomic levels within Papilioninae are comparable to pairwise divergences in Noctuidae (primarily Heliethinae) that fall within the range of 10.1–13.5% for intertribal and 6.5–9.0% for intergeneric distances (Mitchell et al., 1997). However, although these data can be considered to support the elevation of various *Papilio* subdivisions to generic level, we will not endorse specific changes based on sequence divergences until a more thorough sampling of species can be included.

#### CONCLUSIONS

Mitochondrial (*COI-COII*) plus nuclear (*EF-1α*) DNA sequence data have provided considerable new resolution for the phylogeny of *Papilio*, a large and important genus for research in ecology, genetics, and evolutionary and conservation biology. This phylogeny provides a number of insights into the evolutionary history of the group.

However, estimation of divergence times remains a difficult task that involves substantial uncertainties due to a lack of reliable fossil records or well-dated vicariance events. In this study, we relied on several calibration points that were applied in different combinations. The inferred estimate for the divergence of *Papilio* from the Troidini (up to 100 MYA, when all other calibration points were enforced with no constraint age at the tree root) and the age of subdivisions within *Papilio* (around 30–50 MYA) fit well with the results of one other insect molecular clock calibration (Gaunt and Miles, 2002). These estimates agree with dating of the diversification of the major group of host plants in swallowtails, the Rutaceae (Magallón et al., 1999). With few exceptions, the dates conform with likely vicariant dispersal patterns in the family (see Scriber et al., 1995) and are consistent with known fossil records (see Emmel et al., 1992).

*Papilio* is relatively old and is a highly complex and diverse taxonomic unit. Many characteristics of *Papilio* have a polyphyletic origin, with multiple gains and losses of particular morphological or ecological traits. Although no definitive morphological synapomorphies for generic subdivisions are broadly recognized, the evolutionary age and degree of genetic divergence between monophyletic groups within *Papilio* would support elevation of at least five taxa, *Heraclides*, *Pterourus*, *Chilasa*, *Papilio*, and *Eleppone*, to generic rank. A new genus name would also be required for *P. alexanor*. Further studies are required to clarify the composition of *Princeps* (sensu Hancock, 1983), which in contrast to traditional taxonomy is paraphyletic and seems to include at least three divergent groups. However, we refrain from offering formal taxonomic conclusions until evolutionary patterns are clarified more consistently across *Papilio*.

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