# Interaction of Process Partitions in Phylogenetic Analysis: An Example from the Swallowtail Butterfly Genus *Papilio*

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In this study, we explored how the concept of the process partition may be applied to phylogenetic analysis. Sequence data were gathered from 23 species and subspecies of the swallowtail butterfly genus *Papilio*, as well as from two outgroup species from the genera *Eurytides* and *Pachliopta*. Sequence data consisted of 1,010 bp of the nuclear protein-coding gene *elongation factor-1α* (*EF-1α*) as well as the entire sequences (a total of 2,211 bp) of the mitochondrial protein-coding genes *cytochrome oxidase I* and *cytochrome oxidase II* (*COI* and *COII*). In order to examine the interaction between the nuclear and mitochondrial partitions in a combined analysis, we used a method of visualizing branch support as a function of partition weight ratios. We demonstrated how this method may be used to diagnose error at different levels of a tree in a combined maximum-parsimony analysis. Further, we assessed patterns of evolution within and between subsets of the data by implementing a multipartition maximum-likelihood model to estimate evolutionary parameters for various putative process partitions. *COI* third positions have an estimated average substitution rate more than 15 times that of *EF-1α*, while *COII* third positions have an estimated average substitution rate more than 22 times that of *EF-1α*. Ultimately, we found that although the mitochondrial and nuclear data were not significantly incongruent, homoplasy in the fast-evolving mitochondrial data confounded the resolution of basal relationships in the combined unweighted parsimony analysis despite the fact that there was relatively strong support for the relationships in the nuclear data. We conclude that there may be shortcomings to the methods of ''total evidence'' and ''conditional combination'' because they may fail to detect or accommodate the type of confounding bias we found in our data.

## Introduction

It has been recognized since the early days of molecular systematics that the estimation of organismal phylogenies may benefit from the analysis of multiple genetic data sets (e.g., Goodman et al. 1982; Gouy and Li 1989). This realization, coupled with improvements in DNA sequencing technology, has provided incentive for workers to include sequences from two or more genes in any given study (a few of many recent examples include Fang et al. [1997], Lutzoni [1997], and Whiting et al. [1997]). It is often perceived that combining data sets that have been evolving at different rates may help clarify relationships at different levels of a phylogeny (Hillis 1987; Pennington 1996). Although this may seem like a relatively simple concept, in practice it has proven to be complex. Three sources of bias that may confound combined analyses have been described by de Queiroz, Donoghue, and Kim (1995) and Swofford et al. (1996): random error (sampling error), systematic error (different stochastic processes), and different partition histories. In order to avoid or accommodate these sources of error, it is important to understand the patterns of evolution in the data of interest.

In systematic studies in which the ultimate goal is the estimation of an organismal phylogeny, potentially problematic characters are often identified and discarded before the commencement of phylogenetic analysis. It is the purpose of this stage of character ''sieving'' to avoid the bias that can be caused by uninformative or overly homoplastic data that may cause error in an analysis (Brower and DeSalle 1994). Although for many years this paradigm of character analysis has served morphology-based systematics well, for practical and theoretical reasons it may be of limited use to those who work with large, heterogeneous molecular data sets. Phylogenetic problems that span great periods of time and are concerned with relationships at many different levels may not necessarily benefit from expunging characters that cause error at one level of divergence but are informative at another level of divergence. Where is the line drawn between a ''good'' and a ''bad'' character? A character may be both ''good'' and ''bad,'' depending on what level of divergence it is being used to resolve.

In modern systematics, in which sizable character matrices are common, it is often most effective to work with groups of characters after they have been divided into sets known as ''process partitions.'' Process partitions are subsets of data that have ''evolved according to rules that are demonstrably different from those in other subsets'' (Bull et al. 1993). One of the primary assumptions of maximum-parsimony analysis is that characters have been evolving under similar evolutionary processes (Felsenstein 1978). If process partitions are appropriately identified, the potential bias caused by the violation of the assumptions of maximum-parsimony (that is, systematic error) may be avoided or compensated for. Likewise, maximum-likelihood estimation has been observed to suffer from inconsistency when data are analyzed using a model that does not explicitly account for different evolutionary processes (Gaut and Lewis 1995; Chang 1996). The proper identification of

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process partitions allows more accurate models to be implemented.

Huelsenbeck et al. (1994) noted that ''data sets could differ in their ability to accurately reconstruct relationships in different regions or levels of the tree but nonetheless agree upon a compatible set of groupings.'' In cases like this, the method of phylogenetic estimation is robust to the violation of assumptions inherent in combining process partitions and the combined analysis results for a more confident estimate of relationships. Ideally, this would be the case during the simultaneous analysis of fast- and slow-evolving characters, permitting recent, as well as older, relationships to be resolved in a simultaneous analysis. However, as we will demonstrate with our data, for such cooperation to take place, error caused by the fast-evolving characters may need to be compensated for.

Simulations have demonstrated that extreme rate heterogeneity between process partitions may increase the chance of an inaccurate estimate of phylogeny in a combined maximum-parsimony analysis (Bull et al. 1993; Chippindale and Wiens 1994). Workers have sometimes dealt with this phenomenon by downweighting or expunging fast-evolving characters such as third positions (e.g., Mitchell et al. 1997) or the hypervariable regions of RNA genes (e.g., Friedrich and Tautz 1997). Recognizing factors such as rate heterogeneity between process partitions allows an exploration of data that makes easier the identification of possible sources of phylogenetic error, helping to clarify the causes, as opposed to the symptoms (such as topological incongruence), of that error.

Process partitions are not necessarily incongruent partitions, which are subsets of data that result in significantly conflicting topologies when analyzed separately. Topological incongruence may be caused by phylogenetic error (primarily systematic error; see Swofford et al. 1996 for discussion) or by partitions having truly different histories (Maddison 1997). Given that the method of phylogenetic estimation may influence the likelihood of systematic error (Cunningham 1997), topological incongruence is not necessarily an inherent biological characteristic of partitions. Differing evolutionary processes, the hallmark of process partitions, are quantifiable biological characteristics of which topological incongruence (via phylogenetic error) may be a symptom.

Our present study had two main concerns: (1) to examine the interaction of partitions in phylogenetic analysis and (2) to consider sources of bias in a combined-data analysis. To examine the interaction of independent process partitions in phylogenetic analysis, we analyzed two independent process partitions both separately and simultaneously. Incongruence was tested for and bias in the simultaneous analysis was identified. A hypothesis of relationships was inferred using maximum-parsimony and maximum-likelihood tree estimation procedures. To consider the sources of bias in the combined-data analysis, we broke the data into a series of nonindependent process partitions and analyzed them using various methods. We present several lines of evidence to speculate on the sources of bias in the combined parsimony analysis.

**Table 1**
**List of Species Examined**

| Tribe | Genus | Group[a] | Species[b] | *EF-1α* GenBank No.[c] |
|---|---|---|---|---|
| Graphiini . . . . | *Eurytides* | *marcellus* | *marcellus* | AF044815 |
| Troidini . . . . . | *Pachliopta* | *coon* | *neptunus* | AF044829 |
| Papilionini . . . | *Papilio* | *glaucus* | *glaucus* | AF044826 |
| | | | *canadensis* | AF044816 |
| | | | *multicaudatus* | AF044831 |
| | | | *rutulus* | AF044839 |
| | | *troilus* | *troilus* | AF044820 |
| | | | *pilumnus* | AF044834 |
| | | | *palamedes* | AF044817 |
| | | *scamander* | *scamander* | AF044818 |
| | | *homerus* | *garamas* | AF044833 |
| | | *thoas* | *cresphontes* | AF044832 |
| | | *anchisiades* | *anchisiades* | AF044822 |
| | | *machaon* | *machaon* | AF044819 |
| | | | *ma. oregonius* | AF044828 |
| | | | *zelicaon* | AF044827 |
| | | | *polyxenes* | AF044823 |
| | | | *alexanor* | AF044821 |
| | | | *indra* | AF044824 |
| | | | *hospiton* | AF044830 |
| | | *xuthus* | *xuthus* | AF044838 |
| | | *demoleus* | *demoleus* | AF044825 |
| | | *phorcas* | *phorcas* | AF044837 |
| | | | *constantinus* | AF044836 |
| | | | *dardanus* | AF044835 |

[a] Species groups are based on Hancock (1983).
[b] See Caterino and Sperling (1999) for localities and voucher numbers.
[c] See Caterino and Sperling (1999) for *COI*/*COII* accession numbers.

The sources of our data were 23 species and subspecies of the swallowtail butterfly genus *Papilio*, as well as two outgroup species from the genera *Eurytides* and *Pachliopta*. Data consisted of 1,010 bp of the nuclear protein-coding gene *elongation factor-1α* (*EF-1α*), as well as the entire sequences (a total of 2,211 bp) of the protein-coding mitochondrial genes *cytochrome oxidase I* and *cytochrome oxidase II* (*COI* and *COII*).

## Materials and Methods

### Taxonomic Sampling

The taxonomic sampling in this study corresponds to that of Caterino and Sperling (1999), and the same specimens were used to generate sequences in both their study and ours. These taxa reflect a diversity of divergence times that allow a test of the utility of different subsets of data for resolving relationships at different levels of divergence.

A total of 23 species and subspecies of *Papilio* (of approximately 220 extant species; Munroe 1961) were examined (table 1). The *machaon* and *glaucus* species groups were especially well represented in order to assess the utility of certain data for resolving relationships between closely related species. Sampling of additional species from lineages likely to be basal within *Papilio* provided an opportunity to assess the utility of the more slowly evolving characters for resolving older relationships. Hancock's (1983) preliminary phylogenetic hy-

**Table 2**
**Papilionid-Specific *EF-1α* Primers**

| Name[a] | Primer Sequence | Reference Position[b] |
|---|---|---|
| E15f . . . . . | 5′-CGGACACGTCGACTCCGG | −3–15 |
| E234f . . . . . | 5′-GTCACCATCAT(C/T)GACGC | 218–234 |
| E456f . . . . . | 5′-CC(G/A)CCATACAGCGA(G/A)TCCCG | 437–456 |
| E600rc . . . . . | 5′-CTCCTTACGCTCAACATTCC | 600–619 |
| E801rc . . . . . | 5′-GTGGTGAT(G/A)TT(G/A)GCAGGCG | 801–819 |
| E1025rc . . . . . | 5′-TCCAA(T/C)ACTGGTGT(A/G)TAACC | 1025–1044 |

NOTE.—The same primers were used for amplification and sequencing.
[a] f = forward, rc = reverse complement.
[b] Positions are based on the *Heliothodes diminutivus* sequence (Cho et al. 1995).

pothesis of major lineages within *Papilio* was used to select putatively basal taxa. Two species that have traditionally been problematic in *Papilio* systematics, *P. alexanor* and *P. xuthus*, were included in the hope that resolving their phylogenetic positions would offer insight for later investigations into the evolution of the morphology, geographical distribution, and host plant associations of the genus. Thus, our sampling of extant taxa is uneven, with a few heavily sampled groups and a handful of ''long-branch'' taxa.

Outgroup taxa were chosen from Graphiini (*Eurytides marcellus*) and Troidini (*Pachliopta neptunus*), two of the three tribes considered to be most closely related to Papilionini in a morphological treatment by Miller (1987). The tribe Teinopalpini was considered by Miller (1987) to be the sister tribe to Papilionini; however, representatives from this group were not available at the time of this study.

All specimens were collected alive and immediately stored at −70°C. Abdomens and wings of the specimens are stored at Cornell University's Museum of Entomology as voucher lot 1204. The locality and voucher code for each specimen may be found in Caterino and Sperling (1999).

## Molecular Protocols

Whole genomic DNA was extracted from wing muscle tissue of the specimens using the protocols outlined by Harrison, Rand, and Wheeler (1987) as modified by Sperling and Harrison (1994). The extracted DNA was stored at −20°C or −70°C. PCR amplification of *EF-1α* from each specimen was first attempted using general primers described by Cho et al. (1995). These primers succeeded in amplifying *EF-1α* in most taxa; however, primers more specific to *Papilio* were needed to amplify this gene from some taxa (table 2). We ultimately sequenced the first 1,010 bp of the *EF-1α* coding region for every specimen. Primers used for PCR amplification were also used for sequencing. *COI* and *COII* sequences are from Caterino and Sperling (1999).

Polymerase chain reactions (PCRs) were run on either an Ericomp Twinblock EasyCycler (using the profile: 1 cycle of 2.5 min at 95°C; then 30 cycles of 30s at 95°C, 30s at 55°C, and 2 min at 72°C; and finally 1 cycle of 8 min at 72°C), or an Invitrogen Robocycler Gradient (using the profile: 1 cycle of 5 min at 95°C, 5 min at 55°C, and 3 min at 72°C; then 30 cycles of 1.5 min at 95°C, 2.5 min at 55°C, and 3 min at 72°C; and, finally, 1 cycle of 7 min at 72°C). All PCRs were run as ''hot starts,'' where *Taq* was added at the end of the initial denaturation step. PCR products were purified using either Millipore Ultrafree MC tubes or the Qiagen PCR Purification Kit. PCR cloning to test for heterozygosity was done using an Invitrogen TA Cloning Kit.

Cycle sequencing was done using a Perkin Elmer/ABI Dye Terminator Cycle Sequencing Kit or an Amersham Thermosequenase Cycle Sequencing Kit. Reactions were run on an MJ Research PTC200 thermocycler using the profiles recommended by the kit manufacturers. Cycle sequencing products were cleaned using Centrisep columns. Cleaned cycle sequencing products were sequenced with an ABI 377 automated sequencer. All samples were sequenced in both directions. GenBank accession numbers for *EF-1α* sequences are listed in table 1.

All sequences were aligned by eye using Sequence Navigator (Applied Biosystems, Inc.). Alignment was unambiguous due to the absence of indels in the region sequenced. MacClade, version 3.06 (Maddison and Maddison 1997), was used to determine codon positions (assuming the reading frame of Cho et al. 1995) and to generate NEXUS files (Maddison, Swofford, and Maddison 1997).

## Data Partitioning

The primary goal of our study was to detect the presence and nature of bias in a simultaneous parsimony analysis of multiple data sets. To achieve this end, we tested for incongruence between process partitions. However, to extract meaningful information from the incongruence test, it is first important to select meaningful partitions. DeSalle and Brower (1997) described a case in which different partitioning strategies lead to different inferences of incongruence, a finding that underlies the fact that assumptions surrounding the selection of partitions must be considered carefully when interpreting the results of an incongruence test.

Within a data set, there can be anywhere from a single partition to a number of partitions equal to the number of characters. It is tempting to divide a data set into smaller and smaller partitions in an attempt to increase the resolution of process inference, but the smaller a partition is, the more likely it is that random error or paucity of information will become a factor in separate analyses of partitions (de Queiroz, Donoghue, and Kim 1995; Swofford et al. 1996). Bull et al. (1993) rec-

ommended that partitions be recognized based on a criterion of process whereby partitions are not necessarily independent but have evolved through different processes. This criterion would allow partitioning based on codon positions, genes, coding versus noncoding regions, and so on. Miyamoto and Fitch (1995) recommended that partitions be recognized based on a criterion of independence whereby ''character sets may be recognized as independent process partitions when (1) their genes are not genetically linked, (2) the products of their genes do not interact with each other, (3) the genes do not specify the same function, (4) the gene products are not components of a common pathway (e.g., electron transport system), and (5) the gene products do not regulate the expression of loci in other partitions.''

Significant incongruence between partitions may be attributed to either systematic error or different partition histories (de Queiroz 1993; Swofford et al. 1996). If independent process partitions are not incongruent, one cannot reject the null hypotheses that (1) the partition histories are the same and (2) levels of systematic error are insignificant. However, if nonindependent process partitions are tested for incongruence, the null hypothesis regarding partition histories may remain insufficiently tested. Because we were interested in testing for the possibility of error due to different partition histories, we found it preferable to divide our data into process partitions based on Miyamoto and Fitch's (1995) criterion of independence. By dividing the data into the two partitions, *EF-1α* sequences and *COI*/*COII* sequences, the five requirements described by Miyamoto and Fitch (1995) were met. Under the independence criterion, it would not have been appropriate to break *COI* and *COII* into separate partitions, because they are linked and their products are components of a common pathway (Harrison 1989). For the sake of consistency, we also used this two-partition scheme for the separate analyses and for differential weighting in the combined analysis.

For our multipartition maximum-likelihood analysis, we adopted a partitioning scheme based on the criterion of process. The analysis was implemented to estimate evolutionary parameters of various putative process partitions of our data, and not to test specifically for error or to estimate phylogeny. Thus, we were free of the responsibility to avoid violation of the independence criterion. We divided our data into nine partitions: three codon positions for each of three genes.

### Incongruence Test

To test for incongruence between the two partitions, *EF-1α* and *COI*/*COII*, we implemented the incongruence length difference (ILD) test as described by Farris et al. (1994) and discussed by Mason-Gamer and Kellogg (1996) and DeSalle and Brower (1997). The ILD test works by first finding the sum of the lengths of the most parsimonious trees of the partitions in question. Partitions of sizes equal to the original partitions are then generated by random sampling (without replacement) from the entire data set. By summing tree lengths from multiple random partitions, a null distribution of tree length sums may be generated. By comparing the original tree length sum to the null distribution, it is possible to determine if the data partitions in question are significantly incongruent. In our study, we used 1,000 replicates to generate the null distribution. We implemented the ILD test in PAUP* 4.0d64, where it is referred to as the ''partition homogeneity test.''

### Separate Analyses

PAUP* 4.0d56–58 (written by D. L. Swofford) was used for all parsimony, bootstrap, and decay index analyses of the separate independent process partitions. All parsimony analyses were heuristic searches with starting trees determined by 10 replicate random stepwise additions. Gaps were treated as missing information, and polymorphic sites were treated as polymorphisms. The tree-bisection-reconnection (TBR) algorithm (Swofford 1991) was used for branch swapping. *Eurytides marcellus* and *Pac. neptunus* were defined as the outgroups in all parsimony analyses. Accelerated transformation (ACCTRAN) of characters was assumed for all character state reconstructions. Maximum-parsimony bootstrap values based on 500 replicates were calculated for the separate partitions. Heuristic search options for the parsimony bootstrap analyses were the same as those for the tree searches. Bootstrap values provide an effective measure of support for groups within a phylogeny (Sanderson 1989), although they should not be used to compare support for groups between trees (Hillis and Bull 1993). We used bootstrap values as rough indicators of hierarchical signal within data partitions.

PAUP* 4.0d56–58 was used to calculate uncorrected and corrected pairwise divergences as well as mean node-to-present distances in order to diagnose sequence saturation and homoplasy in a range of process partitions. Corrected pairwise divergences were calculated using the model of Tamura and Nei (1993), which we chose as a compromise between computability and accuracy. We found that more general model-based measures of genetic distance were incapable of estimating the pairwise divergences of the highly homoplastic third positions in our mitochondrial data.

### Combined Analyses

For the combined analysis, the branch support metrics implemented were maximum-parsimony bootstrap values based on 250 replicates (with heuristic search options as described above). The tree presented is a strict consensus of 20 separate bootstrap consensus trees, each generated using a different partition weight ratio. Above each branch is a histogram that represents the bootstrap values from each weighted analysis. Weight ratios of *EF-1α*:*COI*/*COII* were chosen to be between 1 and 5, because that is the interval that best illustrates the interaction between the partitions in simultaneous analysis. Microsoft Excel was used to generate bootstrap histograms for the combined analysis.

Weighting in this context serves as a non-arbitrary method of analyzing the interaction between two data partitions. It was not our intention to implement this

weighting scheme to compensate for bias or error, but rather to identify bias or error. The issue of how to construct compensatory weighting schemes is beyond the scope of this study.

In addition to the combined parsimony analyses, we also implemented a combined-data maximum-likelihood heuristic tree search using PAUP* 4.0d64. We utilized the HKY85 model of nucleotide substitution (Hasegawa, Kishino, and Yano 1985), along with a discrete gamma distribution (d$\Gamma$), to account for rate variation among sites (Wakeley 1993; Yang 1993, 1994). Our model had four rate categories, $\Gamma$ shape parameter ($\alpha$) set to 0.5, and transition/transversion ratio ($\kappa$) set to 2. For the heuristic search, the starting trees were generated using stepwise addition, with starting branch lengths approximated using the Rogers-Swofford method. Branch swapping was accomplished using the TBR algorithm. A molecular clock was not enforced.

### Multipartition Maximum-Likelihood Analysis

We implemented a multipartition maximum-likelihood (MPML) model to evaluate putative process partitions within genes by estimating evolutionary parameters of those partitions. A version of PAML v1.3 (Yang 1997), modified to account for extreme between-partition parameter heterogeneity, was used for our analysis. The MPML analysis we implemented differs from a series of separate maximum-likelihood analyses of partitions in that some parameters were shared between partitions, while others were estimated separately for each partition. This allowed quantitative data to be generated for relative comparisons between partitions. For our purposes, we were most interested in comparing average substitution rates. This was achieved by assuming proportional branch lengths between partitions and estimating a separate branch length proportionality parameter for each partition. This branch length proportionality parameter was then considered to be that partition's relative substitution rate.

Our MPML model utilized the HKY85+d$\Gamma$ model of nucleotide substitution, which was the most general model we could implement with the available software. The multipartition log-likelihood function was

$$l = \log\left[\prod_p \text{prob}(D_p \mid T, \pi_p, \kappa_p, \alpha_p, t, c_p)\right]$$

with the known parameters being the data $D_p$ (nucleotide sequences) of partition $p$, the lineage-specific tree $T$, and $\pi_p = \{\pi_T, \pi_C, \pi_A, \pi_G\}_p$, the relative nucleotide frequencies of partition $p$ as determined by empirical observation. $\kappa_p$ is the transition/transversion rate ratio parameter of partition $p$, and $\alpha_p$ is the shape parameter of the 15-class discrete gamma distribution of partition $p$.

Let $n$ be the number of branches in tree $T$, such that $t = \{t_1, t_2, \ldots, t_n\}$ represents the branch lengths of tree $T$. Let $c_p$ be the relative substitution rate parameter of partition $p$, such that $c_p t_i$ represents the length of branch $i$ for partition $p$. In essence, $c$ is a branch length proportionality coefficient that allows partition-specific branch lengths $c_p t$ to be derived from the lineage-specific branch lengths $t$ (Yang 1996). This method eliminates the need to estimate branch lengths separately for each partition, thereby allowing a drastic decrease in the number of parameters to be estimated. Because $c$ is a relative-rate parameter, $c_1 = 1$.

For our MPML analysis, we divided our data into nine partitions: three codon positions for each of three genes. There are a total of 73 parameters in our model: 47 branch lengths ($t$), 9 transition/transversion rate ratios ($\kappa$), 9 $\Gamma$-distribution shape parameters ($\alpha$), and 8 relative substitution rate parameters ($c$). Polymorphic sites attributed to heterozygosity in the *EF-1α* third-position partition were excluded from the analysis.

## Results and Discussion

### Heterozygosity in *EF-1α*

*EF-1α* sites that consistently displayed double-peaks on electropherograms after multiple sequencing runs were assumed to be heterozygous. Cloning of PCR fragments from several suspected heterozygotes verified this interpretation. In the *EF-1α* data matrix, a total of 26 out of 25,250 character states were scored as polymorphic. These polymorphic states were presumed to be attributable to the presence of two different alleles in the individuals from which sequences were taken.

Fifteen of the 25 individuals showed evidence of heterozygosity, with 8 individuals having one polymorphic site, 4 individuals having two polymorphic sites, 2 individuals having three polymorphic sites, and 1 individual having four polymorphic sites. Seventeen of the 26 states were recorded as T/C polymorphisms. All polymorphisms were apparently synonymous and occurred at third positions.

### Incongruence Test

The ILD test results suggest that the *EF-1α* and *COI*/*COII* partitions were not significantly incongruent (fig. 1). The sum of the most parsimonious tree lengths for the *EF-1α* and *COI*/*COII* data was 2,860 steps, with the median of the null tree length sum distribution being 2,862 steps. A significance level of $P = 0.315$ suggests that the data were not even close to rejecting the null hypotheses that the partitions in question (1) have the same history and (2) cause insignificant levels of systematic error.

### Separate Analyses

Heuristic maximum-parsimony tree searches produced 40 most-parsimonious trees for the *EF-1α* data and 3 most-parsimonious trees for the *COI*/*COII* data. Arbitrarily selected most-parsimonious trees from each partition are shown, along with branch lengths, in figure 2. Strict-consensus trees for each partition are shown in figure 3.

The *COI*/*COII* data resolved more recent relationships rather well, while older relationships remained unresolved or poorly supported. In these data, most hierarchical signal is present at the tips of the tree, as evidenced by resolved topology and high bootstrap values on the strict-consensus tree (fig. 3*A*). In the *COI*/*COII* strict-consensus tree (fig. 3*A*), several relationships at

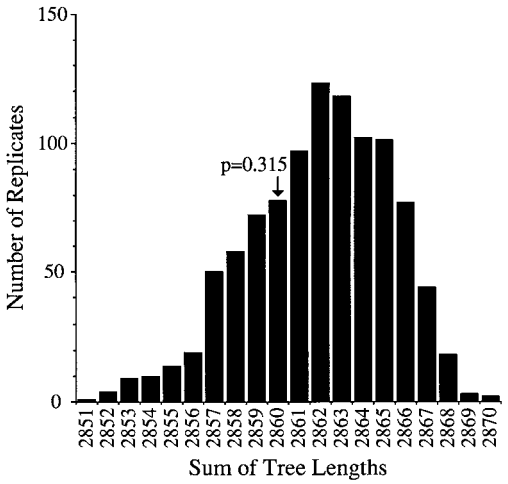


FIG. 1.—Results of an incongruence length difference test on two partitions consisting of *COI*/*COII* sequence data and *EF-1α* sequence data, respectively. The histogram represents the null distribution of the sum of partition tree lengths. When the actual sum of partition tree lengths was compared with the null distribution, significant incongruence was not detected ($P = 0.315$).

the base of the tree receive relatively low support (three bootstrap values ≤50% and decay indices ≤2). The relatively lengthy branches at the base of the *COI*/*COII* tree (in the range of 20 to 30 steps, see fig. 2*A*) suggest that the hierarchical signal in this region of the tree may be compromised by character conflict, as opposed to a paucity of information. It has been recognized that one of the shortcomings of the maximum-parsimony method is its inability to detect multiple changes on long branches, a phenomenon that may cause bias or inconsistency in phylogeny estimation (Felsenstein 1978; Swofford et al. 1996). That there is a lack of hierarchical signal associated with relatively long branches makes it reasonable to speculate that information concerning older relationships has been replaced or obscured by homoplasy in the *COI*/*COII* data.

With the *EF-1α* data, older relationships were generally well resolved, while more recent relationships were ambiguous or poorly supported (bootstrap values <50%; see fig. 3*B*). With the *EF-1α* data, most of the hierarchical structure appears to be localized at the base of the tree. The short branch lengths at the tips of the *EF-1α* tree (fig. 2*B*) suggest that the lack of resolution may be due to a paucity of information, as opposed to character conflict.

## Combined Analysis

We implemented a method of combining data that provides information regarding the effects of differential partition weighting on statistical metrics of branch support in a maximum-parsimony analysis. By viewing branch support as a function of partition weights, we were able to visualize not only which partitions caused bias, but also where in the tree the bias occurred. When increasing the weight of a partition causes an overall decrease in branch support in a combined analysis, a bias is being detected.

In figure 4, the three histograms with sharp increasing trends indicate that the *COI*/*COII* partition was responsible for a bias at those respective branches. A comparison with the separate *COI*/*COII* strict-consensus tree

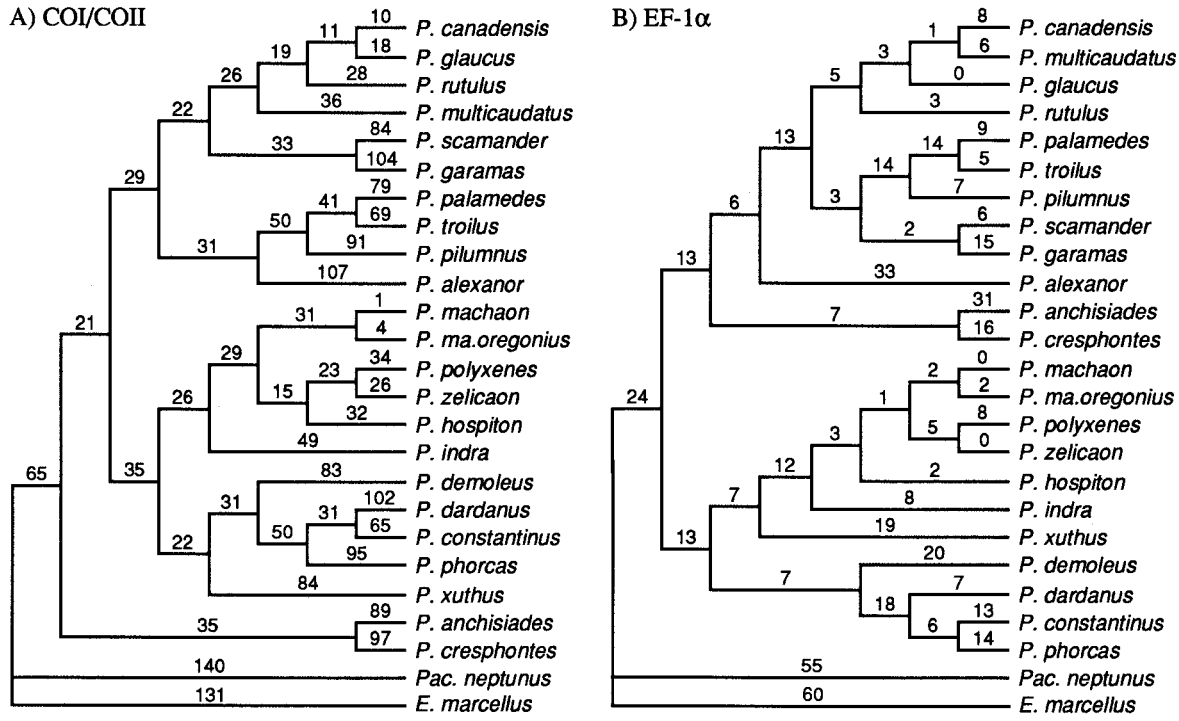


FIG. 2.—Separate maximum-parsimony analyses of partitions. Branch lengths are reported over branches. *A*, *COI*/*COII* tree: one of three most-parsimonious trees; 761 variable sites, with 551 being parsimony-informative; length = 2,334 steps; consistency index (CI) = 0.4332. *B*, *EF-1α* tree: one of 40 most-parsimonious trees; 242 variable sites, with 160 being parsimony-informative; length = 526 steps; CI = 0.5760.

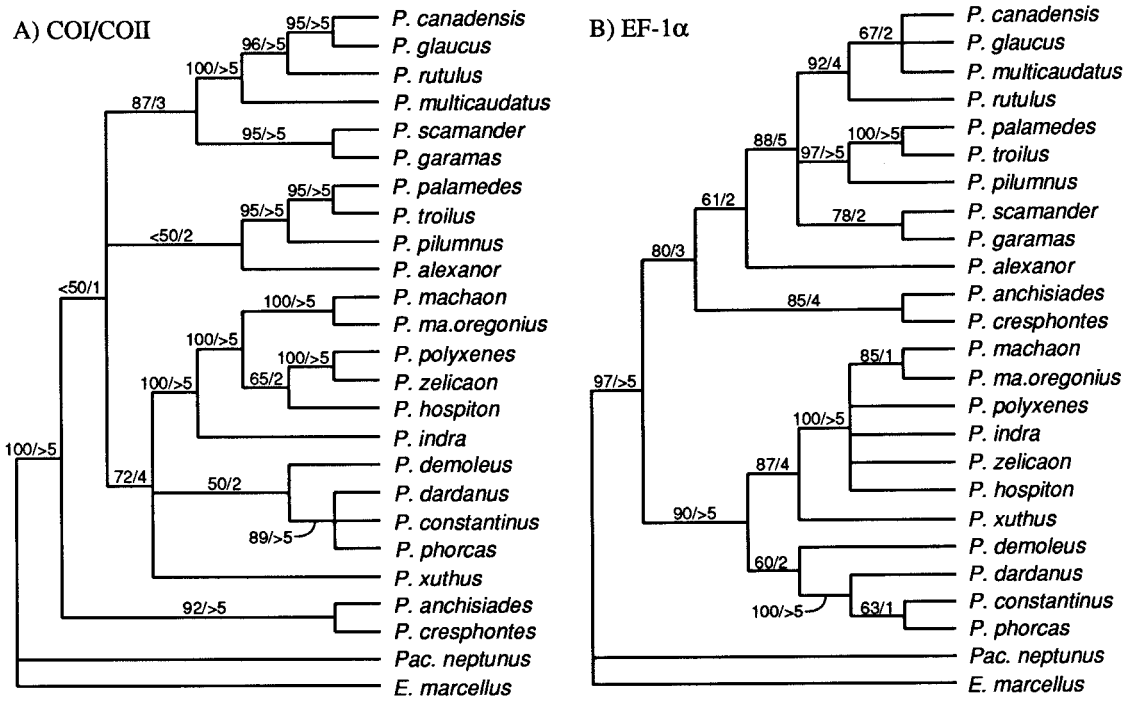


FIG. 3.—Separate maximum-parsimony analyses of partitions. Bootstrap values/decay indices are reported over branches. *A, COI/COII* tree: strict consensus of three most-parsimonious trees. *B, EF-1α* tree: strict consensus of 40 most-parsimonious trees.

(fig. 3*A*) suggests that these are branches for which there is little or no hierarchical signal in the *COI/COII* data (bootstrap values ≤50%, decay indices ≤2), despite the fact that branches are relatively long (in the range of 20 to 30 steps; fig. 2*A*). These same branches had bootstrap values of less than 50% in an unweighted combined analysis (fig. 4). This bias was strong enough to overcome the relatively clear hierarchical signal in the *EF-1α* data (for which these same branches all have bootstrap values ≥80% and decay indices ≥3). The error that confounded resolution of these branches in the separate *COI/COII* analysis is apparently associated with the obscuring bias found in the combined analysis.

The maximum-likelihood phylogeny estimate from the combined data is the same as the tree in figure 4 (except for the *P. dardanus* species group, which we discuss below). In the maximum-likelihood tree, the branches that were confounded by the bias in the *COI/COII* data were resolved. The maximum-likelihood model we implemented is designed to account for multiple changes on long branches and is much more resistant to error caused by homoplasy than is maximum-parsimony (Kuhner and Felsenstein 1994; Huelsenbeck 1995). That the combined maximum-likelihood analysis apparently was unaffected by the obscuring bias of the *COI/COII* data is consistent with the hypothesis that the bias was due to the maximum-parsimony method's limited ability to accommodate homoplasy on long branches.

The histograms with decreasing trends are indicative of the *EF-1α* partition causing bias at particular branches. With one important exception, this appears only as a slight trend on a few branches toward the tips of the tree, where the separate analysis of *EF-1α* indicates a lack of hierarchical signal in the data. It is plausible that histograms with decreasing trends were indicative of cases in which a paucity of informative substitutions amplified the error caused by the presence of small amounts of noise (a random error effect).

The histogram with sharply decreasing bootstrap values over the branch leading to the (*P. canadensis, P. glaucus, P. rutulus*) group suggests that *EF-1α* strongly biased phylogenetic signal. The separate analysis of *EF-1α* suggested the relationship (*P. rutulus* (*P. multicaudatus, P. glaucus, P. canadensis*)), with a 67% bootstrap value for the monophyly of the (*P. multicaudatus, P. glaucus, P. canadensis*) group and a 92% bootstrap value for the monophyly of all four species. The separate analysis of *COI/COII* suggested the relationship (*P. multicaudatus* (*P. rutulus* (*P. glaucus, P. canadensis*))), with all bootstrap values being 95% or higher. That the partitions conflict and both possess some hierarchical signal for the *glaucus* group suggests the possibility that the partitions may have different histories. The *glaucus* group is a complex of wide-ranging species found throughout North America. Within the complex are a number of hybrid zones, interspecies clines, and other phenomena that would make the observation of real conflict between mitochondrial and nuclear phylogenies unsurprising and even expected (Sperling 1993). However, the conflicting bias caused by the *EF-1α* data may also be attributed to random error; within *EF-1α,* there is a dearth of informative characters in the *glaucus* group, as evidenced by the short branch lengths in figure 2*B* (the mean branch length in the *glaucus* group is less than 4).

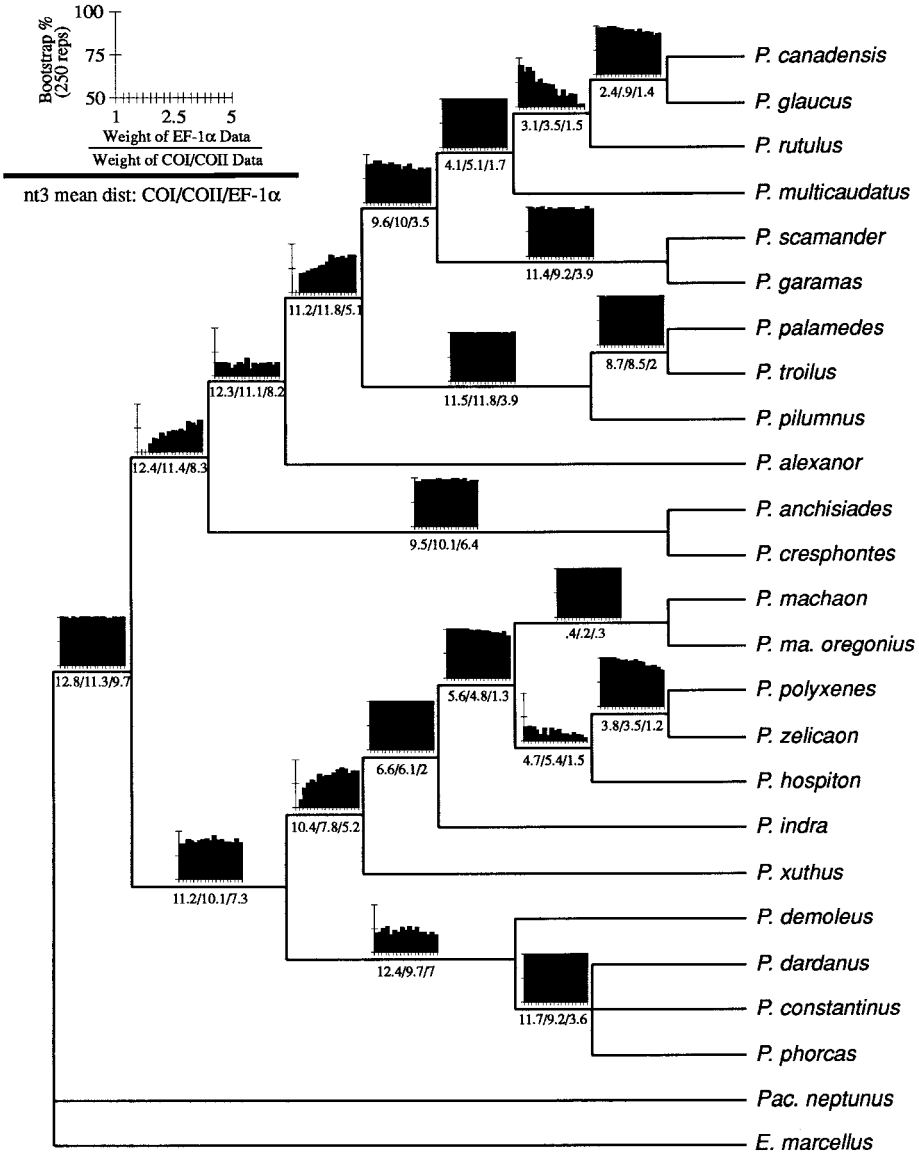


FIG. 4.—Combined analysis of all sequence data. The tree presented is a strict consensus of 20 maximum-parsimony bootstrap consensus trees. The histograms over the branches represent bootstrap support as a function of the *EF-1α*:*COI*/*COII* weight ratio. The values under the branches are third-position mean distances of *COI*, *COII*, and *EF-1α* from the subsequent node to the present.

The relationships among *P. phorcas*, *P. constantinus*, and *P. dardanus* remain unresolved in the combined parsimony analysis. These three species, known collectively as the *phorcas* group, have been the source of much debate in papilionid systematics, and no consensus concerning their relationships to each other has been reached (Clarke et al. 1991; Vane-Wright and Smith 1991). All three species exist sympatrically over much of sub-Saharan Africa and have been shown to hybridize both in nature and in the laboratory (Vane-Wright and Smith 1992). Although the combined-data maximum-likelihood tree estimates the relationship as (*P. phorcas* (*P. dardanus*, *P. constantinus*)), a likelihood ratio test (not shown) suggests that this outcome is not significant, and various partitions of the data, when analyzed separately using maximum-likelihood, will support any of the three possible topologies, none of which is significantly preferred. The fact that we were unable to resolve relationships within the *phorcas* group, despite the fact that the third-position mean distance for the species (refer to discussion on sequence saturation) is well within the range of utility of all three genes examined, suggests that the model of the dichotomously branching tree may not be applicable to this problem.

## Sequence Saturation and Homoplasy

Two approaches were taken to diagnose sequence saturation and homoplasy in the data. The first approach was to calculate the mean third-position uncorrected distances between each node and the present (Mitchell et al. 1997). Values for each gene are presented under the branches preceding each node in figure 4. Stabilization of these values at nodes increasingly deeper in the tree is indicative of saturation: most sites free to change have

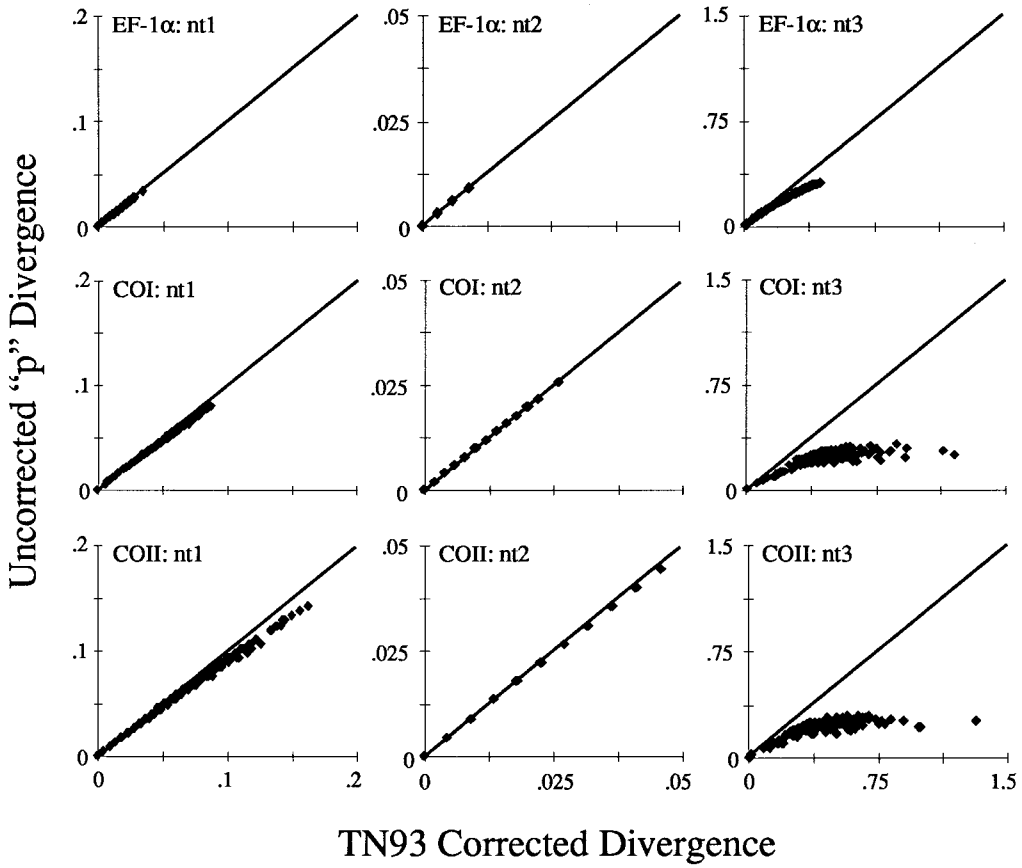


FIG. 5.—Scatter plots of uncorrected pairwise divergences versus pairwise divergences corrected using the model of Tamura and Nei (1993). Deviation of points from the x = y line suggests the degree of homoplasy for each data partition.

already done so. A steady increase in these values at nodes increasingly deeper in the tree suggests that the point of saturation has not been reached. The values calculated from our data suggest that *COI* third positions may be near saturation at 12.3%–12.8% divergence, that *COII* third positions may be near saturation at 11.1%–11.8% divergence, and that *EF-1α* third positions have not yet reached saturation at 8.3% divergence.

Another qualitative test for saturation and homoplasy is to compare uncorrected pairwise divergences with corrected pairwise divergences. When a scatter plot of uncorrected versus corrected divergences is made, the degree to which points deviate from the x = y line indicates saturation and the degree of homoplasy (Villablanca 1993; Zamudio, Jones, and Ward 1997). We implemented this type of analysis for each codon position of each gene (fig. 5). *COI* and *COII* third positions appear to be saturated and have relatively high levels of homoplasy, a fact that is further supported by the observation that *COI* third positions had 43 uncorrectable pairwise comparisons and *COII* third positions had 98 uncorrectable pairwise comparisons. Uncorrectable pairwise comparisons are those that exceed the maximum calculable value of the correction method being used. No other partitions had uncorrectable pairwise comparisons. *COII* first positions, *EF-1α* third positions, and, to a lesser extent, *COI* first positions also appear to be saturated, although they apparently are less affected by homoplasy than are *COI* and *COII* third positions.

### Multipartition Maximum-Likelihood Analysis

Evidence for differing patterns of evolution was detected both within and between genes (table 3). Process partitions within loci were attributable to the well-understood differences in evolutionary process between codon positions (Li 1997), with no striking departures from the trends one would expect given the constraints of the genetic code.

Perhaps the most intriguing differences between process partitions were between the mitochondrial and nuclear genes. The estimated transition/transversion rates for *COI* and *COII* third positions ($\kappa = 90.897$ and $\kappa = 126.765$, respectively) are much greater than those for *EF-1α* third positions ($\kappa = 7.713$). The Γ-distribution shape parameters for *COI* and *COII* third positions ($\alpha = 0.507$ and $\alpha = 0.395$, respectively) suggest that high substitution rates are restricted to relatively few sites compared with *EF-1α* third positions ($\alpha = 1.169$), which have rate variation spread fairly evenly among sites.

As would be expected from our combined data analyses and saturation analyses, the average substitution rates of *COI* and *COII* (summarized in table 3) are high. Although the rates differ between *COI* and *COII*, it is

**Table 3**
**Partition Parameters Estimated Using the 73-Parameter HKY85+dΓ Multipartition Maximum-Likelihood Model Described in the Text**

| Gene | Position | κ | α | *c* | $\pi_T$ | $\pi_C$ | $\pi_A$ | $\pi_G$ | No. of Sites |
|---|---|---|---|---|---|---|---|---|---|
| *EF-1α*..... | nt1 | 13.937 | 0.006 | 0.073 | 0.1405 | 0.1744 | 0.2965 | 0.3886 | 337 |
| | nt2 | 3.197 | <0.005 | 0.009 | 0.2708 | 0.2411 | 0.3255 | 0.1626 | 336 |
| | nt3 | 7.713 | 1.169 | 1.000 | 0.2219 | 0.3955 | 0.1594 | 0.2233 | 313 |
| *COI* ..... | nt1 | 13.304 | 0.112 | 0.345 | 0.3056 | 0.1500 | 0.3162 | 0.2282 | 511 |
| | nt2 | 2.237 | <0.005 | 0.045 | 0.4190 | 0.2337 | 0.1915 | 0.1557 | 511 |
| | nt3 | 90.897 | 0.507 | 15.922 | 0.4929 | 0.0642 | 0.4310 | 0.0119 | 511 |
| *COII*..... | nt1 | 18.610 | 0.158 | 0.545 | 0.2809 | 0.1538 | 0.3965 | 0.1688 | 226 |
| | nt2 | 2.237 | 0.077 | 0.079 | 0.4103 | 0.1653 | 0.3044 | 0.1200 | 226 |
| | nt3 | 126.765 | 0.395 | 22.329 | 0.5665 | 0.0621 | 0.3619 | 0.0094 | 226 |

NOTE.—Relative nucleotide frequencies ($\pi$) were empirically derived from the data. κ = transition/transversion rate ratio; α = Γ shape parameter; *c* = relative substitution rate with *EF-1*α nt3 arbitrarily set to 1. The assumed topology is the tree presented in figure 4. *EF-1*α codon partition lengths are not equal due to the exclusion of polymorphic sites from the analysis.

not clear if these differences are statistically significant. The fact that the estimated substitution rates of *COI* and *COII* third positions are, respectively, 15.922 and 22.329 times greater than that of *EF-1α* third positions supports our argument that homoplasy in the *COI*/*COII* partition is the main cause of error in the combined analysis with *EF-1α*.

## Conclusion

As greater amounts of more varied sequence data become available to systematists, it is apparent that special precautions must be taken when combining process partitions in phylogenetic analyses. Understanding the nature of the process partitions in question is important for identifying possible sources of phylogenetic error. With our data, we found that homoplastic characters in a partition not only may lack useful phylogenetic information, but also may actively obscure phylogenetic information in another partition during a simultaneous analysis.

Our own results generally confirm the conclusion of Bull et al. (1993) that the inclusion of fast-evolving characters in a combined analysis may obscure information from slower-evolving characters. In addition, our results confirm the findings of Chippindale and Wiens (1994) that downweighting fast-evolving characters may improve tree resolution in a combined maximum-parsimony analysis. In our unweighted combined parsimony analysis, three basal branches remained unresolved, while downweighting the partition with the fast-evolving characters better resolved these branches.

Prior to any combined analyses, our data passed the ''conditional combination'' test, in that significant conflict between partitions was not detected. Although there was no significant incongruence, rate heterogeneity still compromised the effectiveness of the combined analysis. This finding may call into question the usefulness of the ''total evidence'' and ''conditional combination'' approaches as they are currently conceived (see Huelsenbeck, Bull, and Cunningham 1996 for a review). Phylogenetic error may cause confounding bias in combined analyses without causing actual incongruence between partitions. Under the paradigms of ''total evidence'' and ''conditional combination,'' the type of confounding bias we found in our study would not have been detected or accommodated. Unweighted combined parsimony analyses may be especially susceptible to the type of systematic error we found; however, weighted parsimony methods and model-based methods such as maximum likelihood should generally fare better (as they did with our data). In any case, refusal to take evolutionary process into account in phylogenetic analyses may compromise efforts to find ''the true tree.''

Future studies would benefit from the ability to apply statistical rigor to the identification of putative process partitions. The ability to determine if patterns of evolution in different data partitions are indicative of significantly different evolutionary processes would be of great utility in molecular character analysis. Identifying potentially problematic data partitions using some criterion besides incongruence would make it easier to prevent or accommodate more insidious forms of phylogenetic error. Parametric bootstraping based on MPML-estimated parameters may be useful in determining whether estimated parameters are significantly different between partitions.

Another issue that deserves more thorough exploration is the diagnosis of systematic error. Phylogenetic error caused by the violation of method assumptions may be difficult to detect. While obvious results of systematic error such as long-branch attraction are becoming fairly well understood, the more subtle effects of systematic error on maximum-likelihood parameter estimates and tree metrics such as maximum-parsimony bootstrap scores are less well understood.

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