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A Maximum-Likelihood Analysis of Eight Phylogenetic Markers in Gallwasps (Hymenoptera: Cynipidae): Implications for Insect Phylogenetic Studies

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We assessed the utility of eight DNA sequence markers (5.8S rDNA, 18S rDNA, 28S rDNA, ITS regions, long-wavelength opsin, elongation factor 1-*α*, cytochrome *b*, and cytochrome oxidase I) in reconstructing phylogenetic relationships at various levels of divergence in gallwasps (Hymenoptera: Cynipidae), using a set of eight exemplar taxa. We report sequence divergence values and saturation levels and compare phylogenetic results of these sequences analyzed both separately and combined to a well-corroborated morphological phylogeny. Likelihood ratio tests were used to find the best evolutionary model fitting each of the markers. The likelihood model best explaining the data is, for most loci, parameter rich, with strong A-T bias for mitochondrial loci and strong rate heterogeneity for the majority of loci. Our data suggest that 28S rDNA, elongation factor 1-*α*, and long-wavelength opsin may be potentially useful markers for the resolution of cynipid and other insect within-family-level divergences (circa 50–100 mya old), whereas mitochondrial loci and ITS regions are most useful for lower-level phylogenetics. In contrast, the 18S rDNA marker is likely to be useful for the resolution of above-family-level relationships. © 2001 Elsevier Science (USA)

Key Words: **gallwasps; Cynipidae; Hymenoptera; likelihood; DNA sequence; rDNA; EF1*α* F1; opsin; cytochrome *b*; cytochrome oxidase I; insect systematics.**

INTRODUCTION

Gallwasps are a family of wasps (Hymenoptera: Cynipidae) that parasitize herb and tree species in the Palaearctic and Nearctic regions, inducing gall formation (Table 1) (Askew, 1984). A number of species within the family (members of the tribe Synergini) have lost the ability to induce galls themselves but instead develop inside the galls of other cynipids; these are called inquilines (Table 1) (Ronquist, 1994). Little is known about cynipid phylogenetic relationships and few studies have dealt with the issue (Ronquist, 1994; Liljeblad and Ronquist, 1998; Stone and Cook, 1998). There are 1369 described species within the Cynipidae, currently divided, on the basis of morphological data, into six tribes listed in Table 1 (Liljeblad and Ronquist, 1998). Biogeographic and fossil evidence suggest that cynipids originated at least as long ago as the mid Cretaceous (83 mya) (Ronquist, 1999). Fossil data also suggest that major groups in one tribe, the inquiline Synergini, diverged at least 45 mya (Fig. 1) (Ronquist, 1999; Z. Liu, pers. comm.). This dating also suggests that the woody rosid gallers (Fig. 1), as obligate hosts to the inquilines, must also have diverged at least 45 mya. The evolutionary age of Cynipidae makes them an appropriate model taxon for the testing of models of sequence evolution and the utility of molecular markers for family-level insect phylogenetics.

Models of nucleotide substitution are important for estimation of evolutionary trees and for understanding of the evolutionary processes of DNA sequences (Yang *et al.*, 1994; Swofford *et al.*, 1996). Whereas even the best currently available models do not describe the evolution of DNA sequences perfectly (Goldman, 1993), it is well documented that better models lead to more accurate estimates of the evolutionary history of the species concerned and to a better understanding of the forces and mechanisms that affected the evolution of the sequences (e.g., Yang *et al.*, 1994; Swofford *et al.*, 1996; Huelsenbeck and Rannala, 1997; Lewis, 1998). Therefore, correct estimation of the parameters involved in the construction of models of sequence evolution (such as rate heterogeneity among sites, base composition, and types of substitution) is an important task and statistical methods such as maximum-likelihood (ML) allow explicit evaluation of parameters involved in phylogenetic estimation (Swofford *et al.*, 1996; Lewis, 1998). On a more general note, the esti-

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TABLE 1

Overview of the Diversity of Gallwasps (Hymenoptera: Cynipidae)

Tribe	Genera	Species	Biology	Taxa used in this study
Synergini	7	171	Phytophagous inquilines[a] in galls of other cynipids	*Synergus gallaepomiformis* *Periclistus brandti*
Aylacini	21	156	Mostly gallers on eudicot herbs; this tribe is paraphyletic (indicated by the quotation marks in Fig. 1)	*Barbotinia oraniensis* *Panteliella bicolor*
Diplolepidini	2	63	Gallers on *Rosa*	*Diplolepis rosae*
Eschatocerini	1	3	Gallers on *Acacia* and *Prosopis* (Fabaceae)	—
Pediaspidini	2	2	Gallers on *Acer*	—
Cynipini	44	974	Gallers on Fagaceae and Nothofagaceae, mostly on *Quercus*	*Plagiotrochus quercusilicis* *Andricus kollari* *Andricus curvator*

Note. From Ronquist (1999).
[a] Inquilines are cynipids that have lost the ability to induce gall formation on their own but develop inside the galls of other cynipids.

mation of a molecular phylogeny depends on the achievement of a match between the mutation rate of the marker selected and the time for which the selected lineages have been diverging. A marker with a low mutation rate may evolve too slowly to resolve relationships in rapidly diversifying lineages. Similarly, a rapidly evolving marker will become mutationally saturated over larger time scales (due to the effect of multiple substitutions) and so will be a poor estimator of a phylogeny.

In groups in which sequence and genome evolution have been well studied (e.g., hominoids, *Drosophila*), the task of selecting loci for phylogeny reconstruction is relatively straightforward, since the evolutionary "behavior" of a number of loci is well characterized (e.g., Johns and Avise, 1998). Additionally, primer design for the exploration of new markers is easy, cost- and information-wise, since data from genome projects are already available. However, for the vast majority of less-studied taxonomic groups (such as gallwasps and arthropods more generally) such information is unavailable. When a molecular phylogeny for such a group is being reconstructed, it has become common practice to use one (or two) of a set of gene regions already applied with success in similar circumstances (for a review see Caterino *et al.*, 2000). This practice is generally justified because of its success in most cases (e.g., Hillis *et al.*, 1996b), owing to the existence of a large suite of conserved primers (Brower and DeSalle, 1994; Simon *et al.*, 1994; Palumbi, 1996) and the standardization of techniques associated with PCR amplification and sequencing (Hillis *et al.*, 1996b). In insect phylogenetics, the most frequently used loci have been the cytochrome oxidase subunits (e.g., Beckenbach *et al.*, 1993; Crespi *et al.*, 1998), 16S ribosomal DNA (e.g., Whitfield and Cameron, 1998), and cytochrome *b* (Cytb) (e.g., Jermiin and Crozier, 1994; Stone and Cook, 1998) from the mitochondrial genome and the ribosomal DNA array (Hillis and Dixon, 1991) and elongation factor 1-α (Cho *et al.*, 1995; Danforth and Ji, 1998) from the nuclear genome. Additionally, a number of researchers have identified further loci useful for arthropod systematics (Friedlander *et al.*, 1992, 1994; Cho *et al.*, 1995; Mardulyn and Cameron, 1999).

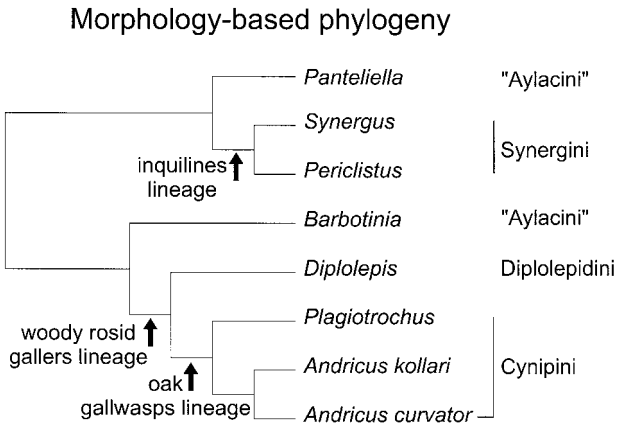

FIG. 1. The morphology-based phylogeny of the eight exemplar species used in the present analysis. The topology was generated by use of parsimony (Liljeblad and Ronquist, 1998). The tribes in which the taxa belong are also mentioned. Arrows indicate lineages discussed in the text.

Despite the success of this general approach, few studies have directly compared the utility of commonly used loci across a range of taxonomic levels in a single clade (e.g., Hillis and Dixon, 1991; Zardoya and Meyer, 1996). In this paper we attempt to identify which of the currently popular markers would be the best for the task of reconstruction of the gallwasp tree at various taxonomic levels. For this task we have sequenced eight markers for eight exemplar species. Our primary aim is not to create a robust phylogeny per se, but to identify which markers are potentially useful at a particular taxonomic level. This study may serve as a test case (i.e., identification of which genes should be tried first at a particular taxonomic level) for less-studied

groups of insects in general (such as gallwasps). We suggest that our results may be of interest to those workers on parasitoid wasps in the sister groups of cynipids, themselves the object of increasing numbers of evolutionary studies (e.g., Dowton and Austin, 1995; Mardulyn and Whitfield, 1999). For reasons outlined above, we decided to adopt a statistical approach (maximum-likelihood) for the analysis of our dataset. This is not the only way; for example, Graybeal (1994) employed a cladistic approach in an attempt to find genes informative about deep divergences in the vertebrate lineage. Caveats of our approach are also discussed.

All but one of the six nuclear and two mitochondrial loci employed here have been used frequently to reveal phylogenetic relationships for a wide variety of taxonomic groups (for reviews see Brower and DeSalle, 1994; Simon *et al.*, 1994; Caterino *et al.*, 2000). Specifically, we use the nuclear loci elongation factor 1-α and the ribosomal DNA array which includes 5.8S rDNA, 18S rDNA, 28S rDNA, and internal transcribed spacer regions (ITS1 and ITS2, treated as a single locus), in addition to the mitochondrial loci cytochrome *b* and cytochrome oxidase I (COI). Additionally, we sequenced a nuclear locus recently proposed to be useful in insect phylogenetics, long-wavelength opsin (LW *Rh*) (Mardulyn and Cameron, 1999). In total, we generated more than 7500 bp of aligned nucleotide sequence from eight gallwasp species which were selected for their positions in the existing wider morphological and molecular phylogenies of Cynipidae (Liljeblad and Ronquist, 1998; Stone and Cook, 1998). In this paper we attempt to (1) identify the best-fit evolutionary model for each locus, using a statistical framework (maximum-likelihood) and comparing the evolutionary "behavior" of each locus, and (2) assess the phylogenetic utility of markers by describing sequence divergence across different time scales and by comparing the phylogenetic results (obtained from the markers) to a morphology-based phylogeny.

MATERIALS AND METHODS

Selection of Species for Molecular Analysis

Our selection of cynipid species for molecular analysis was based primarily on the morphological character-based phylogeny of Liljeblad and Ronquist (1998), with additional information from the mitochondrial cytochrome *b* phylogeny of oak gallwasps from Stone and Cook (1998). Our criteria for selection of taxa were that selected taxa should (1) represent all the major clades of the gallwasp family and (2) exhibit a range of phylogenetic distances from each other. The selection of species is shown in Table 1 and their phylogeny, according to morphology, is shown in Fig. 1. We did not use members of two tribes (Eschatocerini and Pediaspidini) because of their low species richness (see Table 1). We did not use an outgroup of Cynipidae since the focus of this paper is more on the quality of the markers themselves than on the acquisition of the most correct phylogeny for this set of taxa.

Selection of Genes, Amplification, and Sequencing

Amplification and sequencing of all loci was done by PCR with primers previously published and/or of our design. Details of the primers used, PCR conditions, and fragments amplified are listed in the Appendix. In short, we amplified fragments of 18S rDNA, 28S rDNA, 5.8S rDNA, ITS1 and ITS2, elongation factor 1-α F1 (EF1αF1), long-wavelength opsin, cytochrome b, and cytochrome oxidase I. Either sequencing was performed directly on the PCR product or the PCR fragment was first cloned and subsequently sequenced with previously described methods (Rokas *et al.*, 2001; Stone *et al.*, 2001). Intraindividual variation for most genes was nonexistent. In the case of the ITS region, intraindividual variation was observed, but detailed analyses in a single gallwasp species suggest that this will not obscure interspecific relationships (Rokas *et al.*, 2001). All sequencing reactions were done twice to minimize PCR artifacts, ambiguities, and base-calling errors. Sequencing was carried out with Perkin–Elmer BigDye Terminator chemistry and an ABI 377 sequencer.

Sequence Analysis

Each locus was aligned with Clustal W (Thompson *et al.*, 1994) with the default settings options. The alignment generated a total sequence of 7685 bp per specimen. Because it was impossible to obtain an accurate alignment for certain parts of the ITS1 and ITS2 region, a dataset consisting only of the conserved regions was manually assembled for the ITS1–ITS2 fragment. We will refer to this fragment as ITS. After this adjustment, the complete aligned dataset consisted of 5908 bp. All the alignments used in this study are available electronically from TreeBASE (http://www.herbaria.harvard.edu/treebase/, TreeBASE Study Accession No. S645; see Appendix for matrix accession numbers of individual datasets).

Phylogenetic analysis was performed with maximum-likelihood algorithms, which allow hypothesis testing in a statistical framework (e.g., Huelsenbeck and Rannala, 1997; Lewis, 1998) and description of important aspects of sequence evolution, such as rate heterogeneity, transition/transversion (ti/tv) rate ratios, and compositional bias. These are important parameters widely used in phylogenetic reconstructions based on molecular data (for a review see Lewis, 1998).

Each locus was analyzed separately. Comparisons among mtDNA loci (COI and Cytb combined), ribosomal DNA loci (rDNA array: 18S, 28S, 5.8S, and ITS regions combined), nuclear loci (rDNA array, LW *Rh*, and EF1α F1), and all datasets combined (all loci) were

also made. Gaps were coded as missing. The best-fit ML model for each locus and for the complete dataset was identified with Modeltest 3.0 (Posada and Crandall, 1998). The parameters allowed to vary in model-fitting were base composition, substitution rates (which includes variation in transition/transversion ratio), and rate heterogeneity across sites (by both the invariable-sites model and the gamma-distributed rates model). Modeltest utilizes likelihood ratio tests (Huelsenbeck and Rannala, 1997; Lewis, 1998) to identify the ML model of sequence evolution on an initial, approximate tree (Posada and Crandall, 1998), since it has been shown that estimation of ML parameters is not very sensitive in regard to the tree topology on which they are estimated (Yang *et al.*, 1994). Tree reconstruction was performed with ML as implemented in the package PAUP* (Swofford, 2000). ML searches were performed with the branch and bound algorithm on 100 bootstrap replicates with the ML values suggested by Modeltest. All subsequent measures of sequence divergence were estimated with the same parameters.

The ML topologies generated from the "mtDNA loci" (COI and Cytb combined), "nuclear loci" (all the nuclear loci combined), and "all loci" (all the loci combined) datasets were compared with the morphological phylogeny with the SOWH test (Goldman *et al.*, 2000). The SOWH test is a likelihood-based test for comparison of tree topologies which are not specified *a priori* (as is the case for an alternative, the Kishino–Hasegawa test) (for discussion see Goldman *et al.*, 2000). The principle behind the SOWH test is the generation of a null distribution for the difference in likelihood scores in the two topologies (by use of parametric bootstrap analysis) and the testing of the observed data against this distribution (Goldman *et al.*, 2000). SOWH tests were performed with the software packages PAUP* (Swofford, 2000) and Seq-Gen (Rambaut and Grassly, 1997). Null distributions were generated with 100 simulations.

Mutational saturation was investigated with the simple visual method proposed by Philippe *et al.* (1994), which consists of the plotting of the proportion of the observed (uncorrected) differences between pairs of species as a function of the estimated (in this case the estimation made with ML) proportion of differences for the same species pairs. Observed differences initially increase linearly with estimated differences, but as the sequences under study become saturated, observed differences approach an asymptote and change little with increasing estimated differences. The transition from linear increase to asymptote indicates the onset of saturation; while additional substitutions are occurring (as indicated by the raise in the estimated proportion) they are not actually observed (as indicated by the asymptote being approached by the observed proportion). Beyond this point, estimated differences continue to increase substantially while observed differences increase very little.

RESULTS

Aspects of Molecular Evolution for the Eight Loci

Rate heterogeneity. Failure to account for rate heterogeneity (testing of whether substitution rates over all nucleotide sites are constant) can have serious effects on phylogenetic estimation (Yang *et al.*, 1994; Yang, 1996). The two most commonly used methods for explicitly dealing with rate heterogeneity are the invariable-sites model (Palumbi, 1989), in which some proportion of sites is assumed to never change, with all variable sites assumed to evolve at the same rate, and the gamma-distributed-rates model, in which the distribution of relative rates over sites is assumed to follow a gamma distribution (Yang, 1994) whose shape parameter α determines the strength of rate heterogeneity. Both models were tested (individually and combined) for statistically significant improvement in the likelihood score. Only the 5.8S locus showed no rate heterogeneity (Table 2). For most loci (with the exceptions of 18S, the rDNA array, and all data, which required both models) the gamma-distributed-rates model adequately explained rate heterogeneity along a locus and addition of the invariable-sites model did not significantly improve the ML model (Table 2).

Variation in base composition across loci. Individual loci of the rDNA array (18S, 28S, 5.8S, and ITS) are the only loci in which a model that assumes equal base frequencies is supported. For all the other loci (including the whole rDNA array dataset), models that allow unequal base frequencies provided a significantly better fit to the data. At the other extreme, mtDNA loci verify the general observation from insects and especially Hymenopterans of a strong A-T bias (Crozier and Crozier, 1993; Dowton and Austin, 1995; Whitfield and Cameron, 1998). The two mtDNA loci of Cynipidae are 74.85% A-T rich (32.87% A, 12.20% C, 12.96% G, 41.97% T). These frequencies agree with the cytochrome oxidase I data of Dowton and Austin (1995), showing a higher A-T content in parasitic wasps (Apocrita; A-T content: 74.0 $\pm$ 0.7%—gallwasps belong here) than in nonparasitic wasps (higher Symphyta; A-T content: 70.7 $\pm$ 0.7%).

Rates of different substitution types. In the simplest case (one-parameter model of Jukes and Cantor) all nucleotide substitutions occur at the same rate (see review by Swofford *et al.*, 1996). From the one-parameter model, more complex models can be constructed if rates of substitution are free to vary (e.g., by allowance of different rates for transitions and transversions, etc.). In the most general (parameter-rich) case (represented by the general time reversible model; GTR) all

TABLE 2

Best-Fitting ML Models and Their Estimated Parameter Values for Eight Loci and Their Combined Datasets

Locus	ML model	Base frequencies	No. of substitution rates[a]	Rate heterogeneity models		Sequence length
				Invariable sites	Gamma-distributed rates	
18S rDNA	TrNef+I+G	Equal	3 (2 ti, 1 tv)	0.9195	0.7167	1798
28S rDNA	K80+G	Equal	2 (1 ti, 1 tv)	0	0.0768	1073
5.8S rDNA	JC69	Equal	1	0	∞	122
ITS	TrNef+G	Equal	3 (2 ti, 1 tv)	0	0.4491	557[b]
Ef1α F1	TrN+G	Unequal	3 (2 ti, 1 tv)	0	0.27	367
LW *Rh*	GTR+G	Unequal	6 (2 ti, 4 tv)	0	0.4158	481
COI	TVM+G	Unequal	5 (1 ti, 4 tv)	0	0.2917	1077
Cytb	TVM+G	Unequal	5 (1 ti, 4 tv)	0	0.3844	433
rDNA array	TrN+I+G	Unequal	3 (2 ti, 1 tv)	0.7227	0.7664	3550
mtDNA loci	TVM+G	Unequal	5 (1 ti, 4 tv)	0	0.3131	1510
Nuclear loci	GTR+I+G	Unequal	6 (2 ti, 4 tv)	0.7024	0.6077	4398
All loci	GTR+I+G	Unequal	6 (2 ti, 4 tv)	0.4928	0.5281	5908

Note. The sequence length of each locus is also shown. Short-name descriptions of the ML models are according to Posada and Crandall (1998).

[a] ti, number of transition types, tv: number of transversion types.

[b] This fragment contains only the alignable regions.

six possible substitution types—two transitions (A ↔ G, T ↔ C) and four transversions (A ↔ T, A ↔ C, C ↔ G, G ↔ T)—are free to occur at different rates. The model and number of different substitution types utilized by each of the loci are shown in Table 2. Only the 5.8S rDNA data are best explained by a single substitution rate, a result perhaps due to small length of the locus. Most loci are not adequately represented by the simplest model and require different substitution rates (Table 2). Note that in our analysis we assumed that substitution rates are stable among evolutionary lineages (but see Yang and Yoder, 1999).

Mutation rates and saturation rates across loci. The graphs depicting the proportion of observed versus estimated substitutions for each locus (with the ML parameters estimated from Modeltest) are shown in Fig. 2. In this dataset certain loci become saturated more quickly than others, as shown by the divergence ranges in Table 3. The most rapidly saturated loci are the mitochondrial loci (mtDNA; see Fig. 2C), which also show the highest divergences across the eight sampled taxa (the percentages for the most divergent pair of sequences are 44 and 54% for COI and Cytb, respectively; see Table 3). In contrast, the nuclear loci (Figs. 2B and 2D–2F) saturate more slowly and show a range of rates of divergence, with the rDNA array being the slowest (8.65%) and the LW *Rh* the fastest (29.87%; see Table 3).

Gene and Species Phylogenies

The consensus topologies obtained from ML reconstruction of 100 bootstrap replicates are shown in Fig. 3. Comparison of the topologies generated by the different loci and combined datasets shows that the generated trees are substantially different. The only clade supported by all datasets, and in agreement with morphology, is that uniting the two *Andricus* species. Most datasets also support the oak gallwasp lineage (*Plagiotrochus* and the two *Andricus* species) (Figs. 1 and 3). However, the molecular datasets offer no support for monophyly of morphologically well-established clades (according to Liljeblad and Ronquist (1998)) such as the woody rosid gallers (*Diplolepis* and oak gallwasps), the inquilines (*Periclistus* and *Synergus*), or the *Panteliella–Periclistus–Synergus* lineage (Figs. 1 and 3).

Statistical significance of differences among our (molecule-generated) topologies and the morphological phylogeny of Liljeblad and Ronquist (1998) was tested with the SOWH test (Goldman *et al.*, 2000). Due to computational limitations, comparisons were made only between the morphological phylogeny and the three following datasets: mtDNA (all the mitochondrial loci), nuclear loci (all the nuclear loci), and all loci (all the datasets combined). Given the all loci dataset, the morphological phylogeny and the all loci ML topology gave significantly different likelihood scores ($\Delta = 28.9312$, $P < 0.01$), with the morphological topology providing a significantly worse fit for this dataset. Significant differences were also found when the morphological topology was compared with ML topologies generated under the mtDNA and the nuclear loci datasets ($\Delta = 14.1253$, $P < 0.01$, and $\Delta = 18.6775$, $P < 0.01$, respectively).

Which Gene for What Taxonomic Level?

The percentage sequence divergences within Cynipidae and within various morphologically well-established clades within the family (oak gallwasps, woody rosid gallers, inquilines) are shown in Table 3.

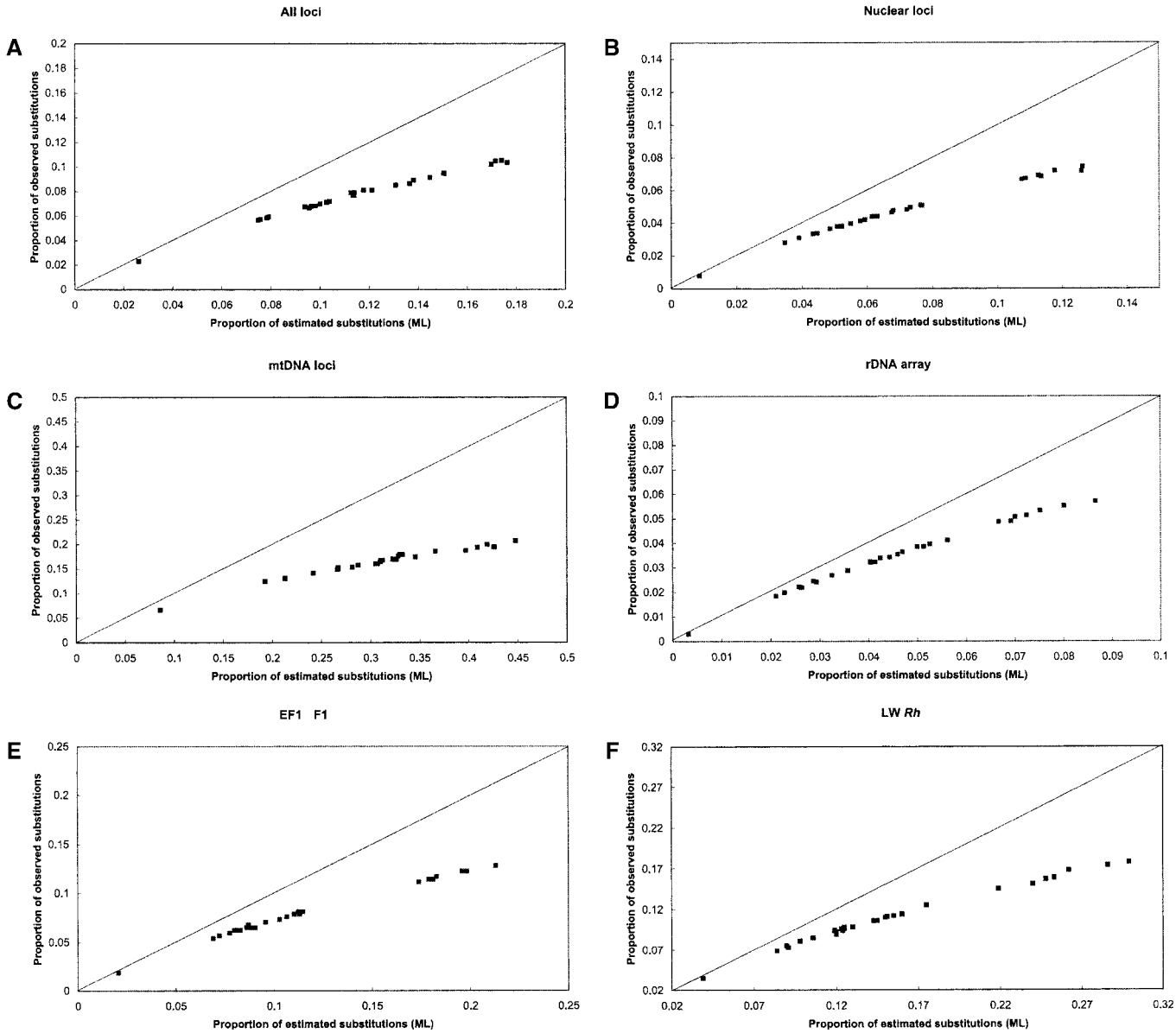

FIG. 2. Saturation plots of the proportion of uncorrected (observed) sequence divergence on the y axis versus the proportion of the estimated sequence divergence on the x axis, for (A) all loci, (B) nuclear loci, (C) mtDNA, (D) rDNA array, (E) EF1α F1, and (F) LW *Rh*. Mitochondrial loci (COI and Cytb) are shown combined since the plots for each locus look very similar; for the same reason, saturation levels of the rDNA array loci are also shown in a single plot. Estimated sequence divergence values were calculated with the phylogenetic package PAUP* (Swofford, 2000), with the ML model suggested by Modeltest (Posada and Crandall, 1998).

DISCUSSION

Aspects of Molecular Evolution for the Eight Loci

Rate heterogeneity. Apart from the 5.8S locus, in which all sites are evolving at the same rate (very probably because of its small length), best-fit models for all other loci require a parameter allowing rate heterogeneity (Table 2). Rate heterogeneity in most loci is adequately explained by the gamma-distributed-sites model. In the few cases in which the invariable-sites model is used, it is in addition to the gamma-distributed-sites model (Table 2). This result is intuitive since, although both models are designed to explain rate heterogeneity, the gamma-distributed-rates model is more parameter rich and so allows a better fit to the data than the invariable-sites model alone. However, this comes at the cost of increased computational effort. The shape parameter α of the gamma-distributed-rates model is small in most cases, denoting strong rate heterogeneity (Table 2). This is concordant with results from other studies (Yang *et al.*, 1994; for a review see Yang, 1996). We note, however,

TABLE 3

Percentage Sequence Divergence within Cynipidae

Lineage (→) Locus (↓)	Cynipidae (observed/estimated)	Oak gallwasps (observed/estimated)	Woody rosid gallers (observed/estimated)	Inquilines (observed/estimated)
18S rDNA	0.06–1.17/0.06–1.73	0.06–0.17/0.06–0.18	0.06–0.7/0.06–0.9	0.8/1.1
28S rDNA	0.28–8.89/0.29–22.99	0.28–4.18/0.29–7.13	0.28–8.89/0.29–22.99	4.83/7.97
5.8S rDNA	0–3.39/0–3.52	0/0	0–1.69/0–1.75	2.46/2.53
ITS	1.33–16.69/1.36–26.94	1.33–7.22/1.36–8.74	1.33–16.88/1.36–25.52	14.46/22.47
Ef1α F1	1.91–12.81/2.07–21.28	1.91–6.54/2.07–8.87	1.91–11.72/2.07–18.28	8.17/11.23
LW *Rh*	3.53–17.82/3.89–29.87	3.53–10.6/3.89–14.26	3.53–17.41/3.89–28.6	9.77/12.77
COI	6.68–20.15/8.68–43.89	6.68–13.11/8.68–21.94	6.68–18.89/8.68–39.23	19.77/42.87
Cytb	6.7–22.97/8.31–53.90	6.7–13.16/8.31–19.72	6.7–21.36/8.31–46.07	20.55/39.87
rDNA array	0.31–5.72/0.32–8.65	0.31–2.43/0.32–2.93	0.31–5.51/0.32–8.00	4.12/5.61
mtDNA loci	6.69–20.77/8.55–44.76	6.69–13.12/8.55–21.28	6.69–19.38/8.55–40.86	20.0/41.83
Nuclear loci	0.80–7.45/0.85–12.63	0.80–3.68/0.85–4.85	0.80–7.45/0.85–12.63	5.09/7.68
All loci	2.32–10.54/2.6–17.62	2.32–5.94/2.6–7.89	2.32–10.54/2.6–17.39	8.94/13.8

Note. Estimation of sequence divergences was performed with ML with the parameter values specified in Table 2.

that our conclusion should be taken with some caution, given some recent results suggesting that in certain cases estimates of rate heterogeneity might be sensitive to taxon sampling (Sullivan *et al.*, 1999).

Variation in base composition across loci. Variation in base composition is important in the modeling of sequence evolution because of its effect in the reduction of the number of character states for a given site. For example, the extreme A-T bias observed in insect mitochondrial genomes reduces many sites from four-state (A, C, G, or T) to two-state characters (A or T), the consequence being that these sites become saturated more quickly than others. Variation such as this observed in the base composition of mtDNA genes among Hymenoptera seriously reduces the utility of mtDNA genes (e.g., Whitfield and Cameron, 1998; Dowton and Austin, 1999), at least for higher-level phylogenetics, due to fast mutational saturation (see below). In contrast, the nuclear loci show smaller deviations from equal base frequencies (even those that show variation in base composition) and are less affected by the problem of character state reduction.

Rates of different substitution types. It is interesting to note that certain widely used substitution models such as Kimura's (1980), which assumes one rate for all types of transitions and one rate for all types of transversions, are not utilized frequently by the loci analyzed here (only the 28S rDNA dataset fits the assumptions of Kimura's model). From Table 2 it is evident that genes are idiosyncratic in terms of substitution types and that there is considerable variation within transitions or transversions. For example, the rDNA array is best explained by a ML model with one transversion rate and two transition rates, in contrast to the mtDNA loci which are best explained by a model with one transition rate and four transversion rates (see Table 2 for more details).

Mutation rates and saturation rates across loci. The plotting of observed versus estimated values of sequence divergence can be used as a way to select genes that may give the correct topology with most methods, by simply selecting those loci that exhibit low saturation levels at the level of divergence exhibited by the sampled taxa. However, saturation plots cannot be taken as *prima facie* predictors of data quality. First, there must be adequate sequence divergence for the phylogeny to be resolved. Second, at the other end of the mutation rate continuum, the phylogenetic performance of apparently saturated loci, or partitions of a locus (e.g., codon positions), is dependent on taxonomic sampling (cf. Hillis, 1996; Björklund, 1999). Nevertheless, in a study like this (i.e., with limited taxonomic sampling), this approach gives valuable information on the relative evolutionary rates for the compared loci. For example, it is clear that mitochondrial loci become saturated most rapidly—perhaps partly due to their strong A-T bias—and hence they might not be the best first candidate loci for higher-level phylogenetics or for nuclear loci that are less saturated.

Gene and Species Phylogenies

The significant differences in likelihood scores observed between the morphological topology and the three molecular topologies (all loci, nuclear loci, and mtDNA loci) indicate a conflict between morphology and molecules. However, given that most of the consensus trees for the loci (Fig. 3) agree (partially or completely) with the morphological phylogeny and that our molecular analyses have used relatively few species (especially compared with the morphological analysis), we do not want to emphasize this conflict, until more data are generated.

An interesting feature of the consensus trees of most loci presented in Fig. 3 is their poor resolution, which may be explained in two ways. The first is that the

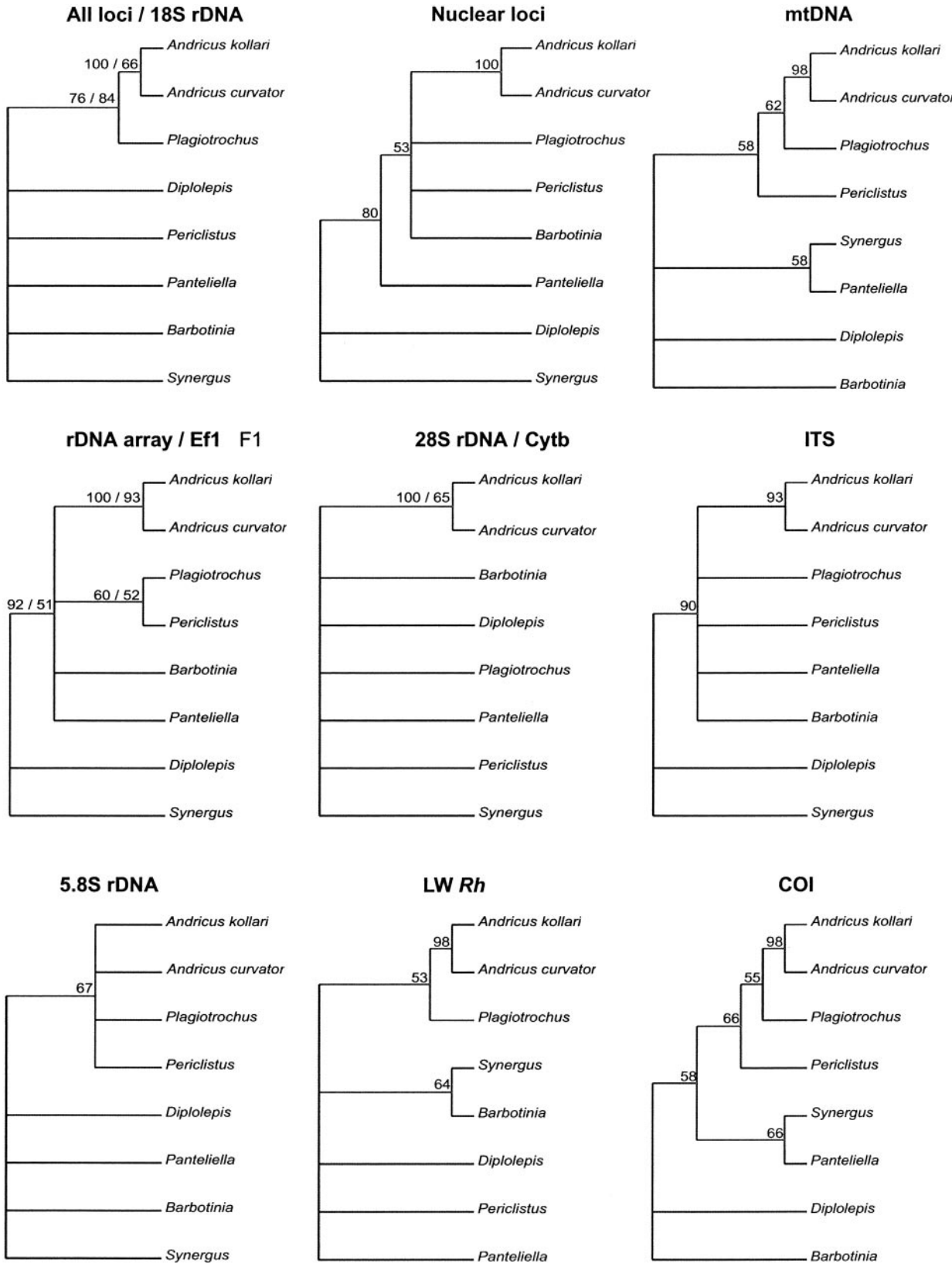

FIG. 3. The 50% majority rule consensus unrooted trees for all datasets. Numbers above branches denote bootstrap support (100 pseudoreplicates with ML and a branch and bound algorithm).

topologies might represent what has really happened, i.e., an adaptive radiation. If the rate of speciation for a given time window is relatively high (emergence of many new species in a small amount of time), interspecific differentiation will be low. This has as a consequence a poorly resolved phylogeny, which, however, is an accurate depiction of the historical associations between the taxa analyzed. Liljeblad and Ronquist (1998) alluded to this scenario for gallwasps, after having difficulty in resolving basal cynipid relationships. A rapid early radiation of gallwasps represents an attractive scenario, since the radiation might have been correlated with the transition to a new adaptive zone (the evolution of the gall-induction mechanism being the key adaptation). Such a scenario would predict not only poorly resolved topologies, but also similar sequence divergence estimates between the major cynipid clades. Data shown in Table 3 suggest that distances for woody rosid gallers and inquilines are similar (although major exceptions are LW *Rh* and 28S) to each other and to estimates for the whole family of Cynipidae (these comparisons are valid under the assumption that the assemblages of the inquilines, the woody rosid gallers, and the Cynipidae are monophyletic). Although more data are needed, these preliminary results are intriguing. A point that should also be considered is that when we are attempting to resolve the phylogenetic history of an adaptive radiation, where divergence times between taxa in a well-sampled data matrix range between millions of years, it is likely that a single locus will not be effective at resolving all the nodes. A combined approach, employing several loci which diverge at various rates, is probably the only way to obtain well-supported phylogenetic hypotheses (Hillis *et al.*, 1996a).

A second explanation for lack of resolution might be the absence of data (be it characters or taxa). Certain clades are resolved in most cases (e.g., the oak gallwasps), although this is not true for the inquiline lineage or for higher clades (Fig. 3), and the addition of more taxa and/or characters might resolve the currently observed polytomies.

Which Gene for What Taxonomic Level?

Selection of a gene for phylogenetic analysis requires matching of the level of sequence variation to the desired taxonomic level of study (i.e., adequate sequence diversity to resolve taxonomic affinities, but minimal artifacts due to saturation). Because of the arbitrariness of taxonomic categories (there is no guarantee that a genus of beetles is the same age as a genus of aphids and even less that it is the same age as a genus of fish), generalizations about the taxonomic rank at which particular genes might be useful should be made with caution. Families across insect orders probably show a wide range of genetic divergences (although to our knowledge there is no review available on this topic) as shown in other groups (Johns and Avise, 1998; Avise and Johns, 1999). Reasons for this diversity at a given taxonomic level include variation among lineages in age, in rate of evolution (molecular or morphological), or simply in the alpha taxonomist's philosophy (the "splitters versus lumpers" debate) (Johns and Avise, 1998). Furthermore, a given protein gene may vary in rate from one taxon to the next (Gillespie, 1986; Crozier *et al.*, 1989; Jermiin and Crozier, 1994). Nevertheless, generalizations can be made about the comparative evolutionary rate of specific genes. Divergence time, if it is known (e.g., from fossils), is another predictor of degree of genetic divergence (usually better than taxonomic rank) (Simon *et al.*, 1994) and will also be briefly discussed. What follows is a preliminary comparison (the subject is too large to be fully discussed here) of published findings that have employed the same markers for other hymenopterans and, more generally, insect taxa.

rDNA array. 18S is the standard marker for insect phylogenetics, especially for higher-level categories (Caterino *et al.*, 2000). Levels of divergence of 18S rDNA within Cynipidae are very small (Table 3), and the largest part of the molecule is resistant to substitutions, as indicated by the fact that the best-fit model of evolution for this locus estimates that 91.95% of the sites are invariable (Table 2). However, it is one of the least saturated genes (Fig. 2D) and the generated topology, although not very well resolved, is concordant with morphological phylogeny (Fig. 1). 18S will probably be more useful as a marker for interfamily and interorder insect phylogenies (i.e., for divergences >85 mya, under the consideration that gallwasps originated at least 85 mya). Wiegmann *et al.* (2000) recently suggested that 18S will be useful for resolving insect phylogenetic splits of Mesozoic age (~65–250 mya). Our result suggests that, at least in Cynipidae, 65 mya may be too recent for good resolution.

There is an extensive literature on the use of 28S rDNA in insect phylogenetics, and compared with 18S, 28S is more frequently used in hymenopteran systematics (for a compilation of published work see Caterino *et al.*, 2000). Levels of divergence observed within Cynipidae (0.28–8.89%, uncorrected; Table 3) generally agree with levels observed within Microgastrinae (Hymenoptera: Braconidae) (0.7–12%, uncorrected) (Mardulyn and Whitfield, 1999) and within therevid flies (Diptera: Therevidae, with divergence percentages ranging from 0.26 to 6.90%) (Yang *et al.*, 2000).

The ITS regions might be poor markers for higher-level phylogenetics since a large amount of data is ambiguous due to alignment problems. Table 4 shows the variation in nucleotide length in the ITS regions observed for each of the eight taxa in this study. However, alignment ambiguities might be dependent on density of taxon sampling. Notwithstanding alignment

TABLE 4

Variation in Nucleotide Length in the ITS Region (ITS1 and ITS2) for the Eight Taxa Used in This Study

Species (tribe)	Nucleotide length (unaligned)
Panteliella bicolor (Aylacini)	1560
Synergus gallaepomiformis (Synergini)	1330
Periclistus brandti (Synergini)	1516
Barbotinia oraniensis (Aylacini)	1636
Diplolepis rosae (Diplolepidini)	1758
Plagiotrochus quercusilicis (Cynipini)	1657
Andricus kollari (Cynipini)	1447
Andricus curvator (Cynipini)	1335

problems, the usefulness of ITS regions in the resolution of intrageneric and intraspecific relationships has already been demonstrated (e.g., Beebe *et al.,* 1999; Rokas *et al.,* 2001). 5.8S rDNA has properties similar to those of 18S and 28S but its small length is a disadvantage due to the small number of variable positions.

Long-wavelength opsin. This is the first study after Mardulyn and Cameron (1999) to use LW *Rh* in insect, and more specifically hymenopteran, phylogenetics. Levels of genetic distance are similar in both studies (within the bee family Apidae the uncorrected pairwise divergence ranges between 1.93 and 19.83%, whereas within Cynipidae the values are between 3.53 and 17.82%). Direct comparison shows similar average base frequencies for the two groups with a slight A-T bias in both data sets (bees: A-25.52%, C-21.86%, G-22.42%, and T-30.2%; gallwasps: A-26.08%, C-20.45%, G-22.32%, and T-31.15%). We agree with Mardulyn and Cameron (1999) that LW *Rh* represents a promising candidate gene for insect phylogenetics and stress the importance of studies both within and outside Hymenoptera. Preliminary data within the gallwasp genus *Andricus* indicate that LW *Rh* might be useful also for the resolution of intrageneric relationships (A. Rokas, unpublished data).

Ef1α F1. Ef1α is a marker that has proved very useful in resolving within-family relationships (Cho *et al.,* 1995; Belshaw and Quicke, 1997; Mitchell *et al.,* 2000). Current evidence suggests that Hymenoptera possess two copies of Ef1α, F1 and F2 (Danforth and Ji, 1998). The Ef1α primers used in this study (see Appendix) seem to amplify the F1 copy preferentially (the one analyzed here). However, in one case we did amplify the F2 copy (in *Diplolepis;* data not shown) but the high sequence divergence allowed easy discrimination between the paralogous and the orthologous copy by comparison with the *Apis* (bee) F1 and F2 copies. This suggests that—for lower-level phylogenetics at least—the discrimination between paralogy and orthology in the Ef1α is not a serious obstacle. The uncorrected distances reported for gallwasps (1.91–12.81%) roughly agree with observed distances within the butterfly superfamily Noctuoidea (1–10.7%) (Mitchell *et al.,* 2000), whereas the upper limit is higher within the dipteran family Therevidae (2–17%) (Yang *et al.,* 2000). The Ef1α data presented here agree with published findings about the usefulness of the Ef1α marker for lower-level phylogenetics.

mtDNA loci. The two mitochondrial loci in this study (COI and Cytb), and their combined dataset (mtDNA loci), are best explained by the same ML model (Table 2). Mitochondrial loci essentially have a single history (since they do not recombine) and, although studies using many mtDNA markers (e.g., Crespi *et al.,* 1998; Naylor and Brown, 1998; Koulianos and Schmid-Hempel, 2000) may avoid problems associated with low amounts of character data (given the large size of a typical eukaryote mitochondrial genome) (Cummings *et al.,* 1995), their conclusions are based on data from what is essentially a single locus. Mitochondrial markers are also more susceptible to loss of variation due to small population sizes than nuclear loci (e.g., Rokas *et al.,* 2001).

Cytochrome oxidase subunits and cytochrome *b* are the most conserved insect mtDNA genes (Simon *et al.,* 1994). They are nonetheless the fastest evolving (with the exception of the ITS region) among the set of loci used in this study, showing high levels of divergence (in the range of 40–50%). This has been found for other hymenopterans, thus explaining their success in recovering inter- and intrageneric phylogenies within the Hymenoptera (e.g., Stone and Cook, 1998; Nyman *et al.,* 2000) and even intraspecific phylogenies (Rokas *et al.,* 2001; Stone *et al.,* 2001). They have been less useful for recovering higher-level phylogenies both within Hymenoptera (i.e., lineages that have diverged more than 50 mya) (Belshaw and Quicke, 1997; this study) and within other lineages of insects (Howland and Hewitt, 1995). Additionally, Crozier and Crozier (1993) noted a rate acceleration in the mtDNA of *Apis* (Hymenoptera) resulting in *Drosophila* proteins being more similar in amino acid composition to those of *Locusta* (Orthoptera) than to those of *Apis,* despite Hymenoptera being phylogenetically closer to Diptera than to Orthoptera (the phenomenon of long branch attraction). This rate acceleration (if widespread in Hymenoptera) may represent a serious constraint for the use of mitochondrial data for higher-level phylogenetics.

CONCLUSION

These results suggest a rough match between particular loci and certain taxonomic ranks. mtDNA loci and ITS regions appear promising for genus-level or even within-species-level phylogenies and indeed the first results are encouraging (Stone and Cook, 1998;

Rokas *et al.*, 2001; Stone *et al.*, 2001). However, their fast substitution rate and other peculiarities (high A-T bias for mtDNA, gaps and unalignable parts for ITS regions) make them less suitable for higher-level phylogenetics. In contrast to the mtDNA and ITS regions, the rest of the loci analyzed here show lower substitution rates. At one extreme, 18S rDNA is evolving too slowly to be of use for cynipid phylogenetics or even for within-family insect phylogenetics in general. However, 28S rDNA, EF1α F1, and LW *Rh* are very promising candidates for the resolution of cynipid relationships, with low saturation levels (Fig. 2) and a useful range of divergence. At least in gallwasps, LW *Rh* is faster evolving than 28S and EF1α F1, making it a more appropriate marker for within-tribe phylogenetics.

The analysis presented here has enabled us to identify a number of useful loci for various taxonomic ranks within gallwasps (and possibly within insects in general). More extensive sampling of species will be essential in our attempt to resolve phylogenetic relationships among insects and test evolutionary hypotheses.

APPENDIX

18S rDNA. Primers used: 18e from Palumbi (1996) and the reverse of ITS5 from White *et al.* (1990). For the direct sequencing, two internal primers were designed from gallwasp sequences. Their sequences are 18SF2: 5′-CTA CCA CAT CCA AGG AAG GCA G-3′ (22 nucleotides) and 18SR2: 5′-AGA GTC TCG TTC GTT ATC GGA-3′ (21 nucleotides). Sequencing was performed directly from the PCR product.

Concentration of PCR ingredients: PCRs were performed in 25-μl volumes and they consisted of 1 μl of DNA sample, 2.5 μl of 10× PARR Buffer (HYBAID), 1.5 μl of $MgCl_2$ (25 mM), 0.5 μl of dNTPs (10 mM), 0.35 μl of each primer (20 mM), 0.25 μl of *Taq* (Promega), and 18.55 μl of distilled, deionized H_2O.

PCR program: one step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 60 s, 72°C for 2 min, and a final extension step at 72°C for 10 min.

GenBank Accession Nos.: AF395142–AF395149. TreeBASE Matrix Accession No.: M1002.

28S rDNA. Primers used: 28SF and 28Sbout from the Wheeler/DeSalle lab at the American Museum of Natural history, New York.

Their sequences are 28Sbout 5′-CCC ACA GCG CCA GTT CTG CTT ACC-3′ (24 nucleotides) and 28SF 5′-AGT CGT GTT GCT TTG ATA GTG CAG-3′ (24 nucleotides). These primers were also used for the direct sequencing, together with two internal primers designed from gallwasp sequences. Their sequences are 28SFAf: 5′-GGT ACT TTC AGG ACC CGT CTT-3′ (21 nucleotides) and 28Sin1: 5′-ACC TTC ACT TTC ATT AYG CCT TTA-3′ (26 nucleotides). Sequencing was performed directly from the PCR product.

Concentration of PCR ingredients: PCRs were performed with premixed, predispensed reactions (Ready-to-Go PCR Beads; Amersham Pharmacia Biotech, Cat. No. 27-9553-01). Each reaction contained 5 μl of DNA sample, 4 μl of each primer (final concentration 1.6 μM), 1.5 units of *Taq* DNA polymerase, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 200 μM dNTPs and stabilizers, including bovine serum albumin (BSA). Sterile distilled water was added to give a final reaction volume of 25 μl.

PCR program: one step at 94°C for 5 min followed by 38 cycles of 94°C for 60 s, 60°C for 60 s, 72°C for 60 s, and a final extension step at 72°C for 5 min.

GenBank Accession Nos.: AF395150–AF395157. TreeBASE Matrix Accession No.: M1003.

ITS1–5.8S rDNA–ITS2. Primers used: ITS5 and ITS4 from White *et al.* (1990). For the direct sequencing, two internal primers were designed from gallwasp sequences. Their sequences are ITS5.8F: 5′-GTC CAC GGA TAC AAT TCC CGG ACC A-3′ (25 nucleotides) and its reverse complement ITS5.8R: 5′-TGG TCC GGG AAT TGT ATC CGT GGA C-3′ (25 nucleotides). Sequencing was performed from clones.

Concentration of PCR ingredients: PCRs were performed in 25-μl volumes and they consisted of 1 μl of DNA sample, 2.5 μl of 10× PARR Buffer (HYBAID), 1.5 μl of $MgCl_2$ (25 mM), 0.5 μl of dNTPs (10 mM), 0.35 μl of each primer (20 mM), 0.25 μl of *Taq* (Promega), and 18.55 μl of distilled, deionized H_2O.

PCR program: one cycle of 94°C for 2 min, 55°C for 60 s, and 72°C for 2 min, followed by 35 cycles of 94°C for 30 s, 55°C for 60 s, 72°C for 2 min, and a final extension step at 72°C for 10 min.

GenBank Accession Nos.: AF395158–AF395165. TreeBASE Matrix Accession Nos.: M1004 (for 5.8S rDNA) and M1008 (for ITS1 and ITS2).

Elongation factor 1α F1. There are two paralogs of EF1α in hymenopterans (Danforth and Ji, 1998). In this analysis we use only EF1α F1. Primers used: M44-1 and rc51-1 from Cho *et al.* (1995) (these primers can amplify both F1 and F2 paralogs). Sequencing was performed from cloned fragments.

Concentration of PCR ingredients: PCRs were performed in 25-μl volumes and they consisted of 1 μl of DNA sample, 2.5 μl of 10× PARR Buffer (HYBAID), 1.5 μl of $MgCl_2$ (25 mM), 0.5 μl of dNTPs (10 mM), 0.35 μl of each primer (20 mM), 0.25 μl of *Taq* (Promega), and 18.55 μl of distilled, deionized H_2O.

PCR program: one step at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 55°C for 60 s, 72°C for 2 min, and a final extension step at 72°C for 10 min.

GenBank Accession Nos.: AF395166–AF395173. TreeBASE Matrix Accession No.: M1007.

Long-wavelength opsin. Primers used: LWRhF and LWRhR from Mardulyn and Cameron (1999). Sequencing was performed from cloned fragments.

Concentration of PCR ingredients: PCRs were performed with premixed, predispensed reactions (Ready-to-Go PCR Beads; Amersham Pharmacia Biotech, Cat. No. 27-9553-01). Each reaction contained 8 μl of DNA sample, 3 μl of each primer (final concentration 1.2 μM), 1.5 units of *Taq* DNA polymerase, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 200 μM dNTPs and stabilizers, including BSA. Sterile distilled water was added to give a final reaction volume of 25 μl.

PCR program: one step at 94°C for 5 min followed by 36 cycles of 94°C for 60 s, 59°C for 60 s, 72°C for 60 s, and a final extension step at 72°C for 5 min.

GenBank Accession Nos.: AF395182–AF395189. TreeBASE Matrix Accession No.: M1009.

Cytochrome b. Primers used: CB1 and CB2 (Jermiin and Crozier, 1994; Stone and Cook, 1998). The PCR protocol for cytochrome *b* used in this study has been described in detail elsewhere (Stone and Cook, 1998).

GenBank Accession Nos.: AF395136–AF395141. TreeBASE Matrix Accession No.: M1006.

Cytochrome Oxidase I. Primers used: lco and hcoexternb from the Wheeler/DeSalle lab at the American Museum of Natural History, New York. The amplified fragment corresponds to positions 1835 to 2911 in the *Apis mellifera* mtDNA sequence (GenBank Accession No.: L06178, Crozier and Crozier, 1993). Their sequences are lco 5′-TCW ACM AAT CAT AAA RAT ATT GG-3′ (23 nucleotides) and hcoexternb 5′-CCT ATT GAW ARA ACA TAR TGA AAA TG-3′ (26 nucleotides).

Sequencing was performed from cloned fragments.

Concentration of PCR ingredients: PCRs were performed with premixed, predispensed reactions (Ready-to-Go PCR Beads; Amersham Pharmacia Biotech, Cat. No. 27-9553-01). Each reaction contained 8 μl of DNA sample, 4 μl of each primer (final concentration 1.6 μM), 1.5 units of *Taq* DNA polymerase, 10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl, and 200 μM each dNTP and stabilizers, including BSA. Sterile distilled water was added to give a final reaction volume of 25 μl.

PCR program: one step at 94°C for 5 min followed by 38 cycles of 94°C for 30 s, 46°C for 75 s, 72°C for 75 s, and a final extension step at 72°C for 5 min.

GenBank Accession Nos.: AF395174–AF395181. TreeBASE Matrix Accession No.: M1005.

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