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SHORT COMMUNICATION

Reassessment of the 16S rRNA Nucleotide Sequence from Members of the Parasitic Hymenoptera

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Recently we examined the phylogenetic utility of the large ribosomal subunit of mitochondrial DNA from the insect order Hymenoptera (Derr *et al.* 1992). That study included nucleotide sequence information for members of six superfamilies from the two hymenopteran suborders (Symphyta and Apocrita). After submitting the manuscript for publication we discovered an error regarding some of the sequences in our original report. By the time the problem was resolved the manuscript was in press and we could not prevent publication. The incorrect sequences were all from the terebrant ("parasitic Hymenoptera") group and consisted of sequences from three members of the superfamily Ichneumonoidea (*Xanthopimpla stemmator, Digonogastra kimballi,* and *Allorhogas pyralophagus*) and sequences from two representatives of the superfamily Chalcidoidea (*Aphytis yanonensis* and *A. lingnanensis*).

In this report we provide the correct nucleotide sequence for four members of the parasitic group along with a reanalysis of this gene region. In addition, we offer suggestions on how to prevent reporting spurious nucleotide sequence when limited amounts of comparative data are available.

We suspect that samples used in our original analyses were contaminated with small amounts of vertebrate DNA through the aqueous phase layered above the phenol used in the phenol–chloroform DNA extraction. We therefore eliminated this step when extracting new genomic DNAs by using a commercially available glass bead DNA purification kit (Schleicher & Schuell). This procedure eliminated the organic extraction/ethanol precipitation step and provided excellent template DNA for PCR amplification.

In our original studies template DNAs from all parasitic Hymenoptera failed to amplify due to a nucleotide substitution corresponding to the 3′ end of one "conserved" PCR primer. Therefore, we designed two new pairs of PCR primers that border this region of the 16S rRNA gene. The first primer pair, 16A1 and 16B1, are identical to the original primers 16SA and 16SB (Derr *et al.,* 1992) except a single base was deleted at each of the 3′ ends (Fig. 1). Although these primers amplified the appropriate size fragment from the new DNA samples, we could not be sure that this product was not an additional contaminant. In order to ensure that PCR amplifications were limited to insect templates, we constructed an additional primer pair that ended on 3′ positions that consistently differed between insect and vertebrate 16S rRNA sequences. Details of the placement of the A primers are provided in Fig. 1. The sequences of these "taxonomically limited" primers are as follows: 16A2—AGATTTTAAAAGTCGAACAGAC[CT]TAA and 16B2—CGCCTGTTTATCAAAAACATGT. PCR, DNA sequencing, sequence alignment, and the phylogenetic analysis were as reported earlier (Derr *et al.,* 1992).

With the use of the primers 16A2 and 16B2, nucleotide sequences were determined from both DNA strands from four members of the superfamily Ichneumonoidea. These included one individual from the family Ichneumonidae (*Xanthopimpla stemmator*) and three from the family Braconidae (*Digonogastra kim-*

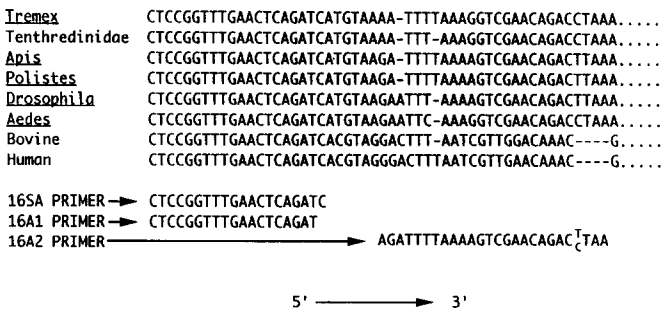

FIG. 1. Comparison of each of the A primers with aligned 16S rRNA sequence from four hymenopterans: *Tremex* and Tenthredinidae (both in the suborder Symphyta), *Apis* (Vlasak *et al.,* 1987) and *Polistes* (both in the suborder Apocrita); two dipterans: *Drosophila* (Clary and Wolstenholme, 1985) and *Aedes* (Hsu-Chen *et al.,* 1984); and two vertebrates: bovine (Anderson *et al.,* 1982) and human (Anderson *et al.,* 1981).

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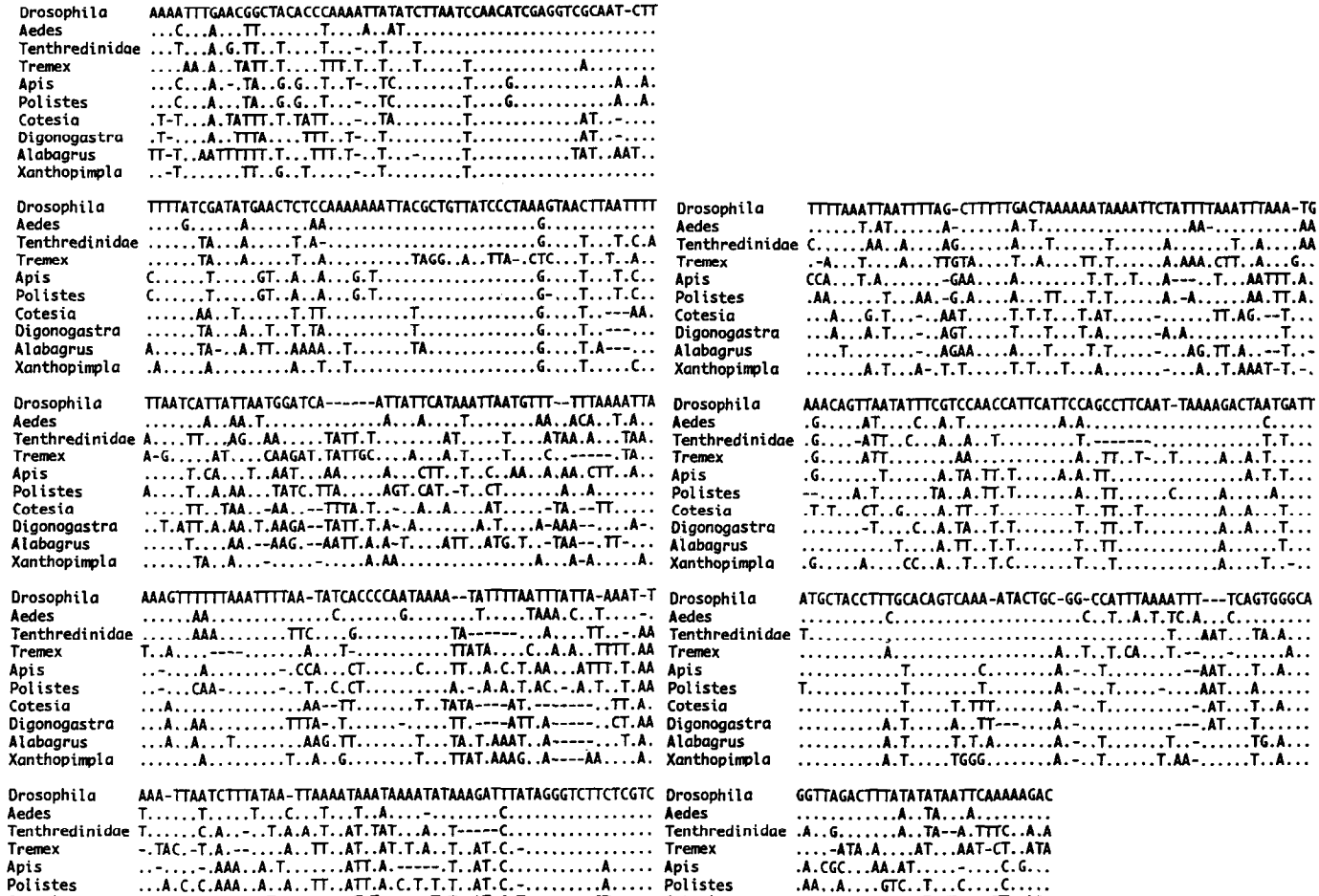

FIG. 2. Aligned nucleotide sequence used for the phylogenetic analyses.

balli, *Alabagrus stigma*, and *Cotesia flavipes*). PCR amplification of this region from the two representatives of the superfamily Chalcidoidea was successful, but complete nucleotide sequences are not available. Multiple alignment (Higgins and Sharp, 1988) of these sequences to the previously reported 16S rRNA sequences from the aculeates, the two symphytans, and the two outgroup dipterans resulted in a matrix consisting of 510 total positions (Fig. 2). Based on this alignment, 189 positions (37.0%) were monomorphic, and 104 positions (20.4%) were autapomorphic for a total of 293 positions (57.4%) that were uninformative. This left 217 polymorphic positions with the potential to provide phylogenetic information. Consistent with the previously reported Hymenoptera 16S rRNA sequences (Derr *et al.*, 1992), sequences from members of the superfamily Ichneumonoidea all displayed a bias for A and T bases; *Xanthopimpla* (0.795%), *Alabagrus* (0.862%), *Cotesia* (0.850%), and *Digonogastra* (0.839%). Evolutionary distance estimates based on these nucleotide sequences are provided in Table 1.

Using the two dipterans as outgroups (as in Derr *et al.*, 1992) parsimony analysis resulted in the production of a single tree with both members of the suborder Symphyta represented as a sister group to all representatives from the suborder Apocrita (Fig. 3A). Within the Apocrita, the sister-group relationship of the aculeates (*Apis* and *Polistes*) was retained as was the monophyly of Ichneumonoidea. Within this superfamily, the single Ichneumonidae (*Xanthopimpla*) displays a sister group relationship to a monophyletic Braconidae (Fig. 3A). A bootstrap analysis produced the same topology for the terminal taxa but resolution of the two suborders was not obtained in over 50% of the 100 bootstrap iterations (Fig. 3B).

As in our original analysis, we find support in these data for monophyly of Hymenoptera (but now obtain this result with two outgroup taxa), monophyly of Aculeata, and monophyly of Ichneumonoidea. Because we could not obtain sufficient sequence from the chalcidoid taxa, we could not further examine relationships within Apocrita. In view of the fact that the suborder

TABLE 1

Estimation of Evolutionary Distance from Nucleotide Sequence Data Using K (Kimura, 1980; Eq. (10))

	1	2	3	4	5	6	7	8	9
1. *Drosophila*									
2. *Aedes*	0.174								
3. Tenthredinidae	0.253	0.259							
4. *Tremex*	0.368	0.395	0.326						
5. *Apis*	0.335	0.373	0.323	0.453					
6. *Polistes*	0.325	0.369	0.339	0.461	0.196				
7. *Cotesia*	0.320	0.372	0.314	0.416	0.388	0.372			
8. *Digonogastra*	0.298	0.302	0.268	0.350	0.350	0.330	0.227		
9. *Alabagrus*	0.354	0.359	0.325	0.412	0.351	0.371	0.263	0.261	
10. *Xanthopimpla*	0.258	0.308	0.267	0.468	0.381	0.383	0.276	0.243	0.331

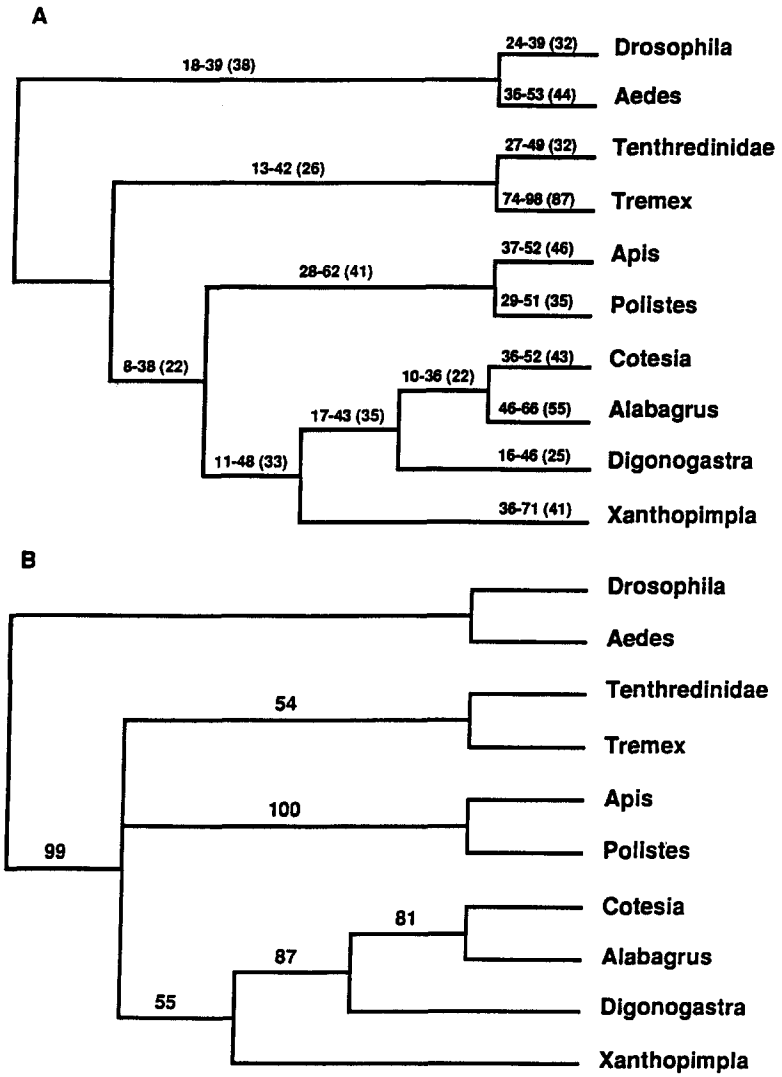

FIG. 3. (A) Parsimony tree of Hymenoptera based on 510 bp of nucleotide sequence from the 16S rRNA gene using two dipterans *Drosophila* and *Aedes* as outgroups. The minimum, maximum, and assigned number of character state transformations are provided along each branch. This tree is 657 steps in length and has a CI of 0.54. (B) Bootstrap majority-rule consensus tree based on the same data matrix as the parsimony tree above. Numbers at each node represent the percentage of bootstrap trees supporting each branch.

Symphyta is now widely regarded as paraphyletic (Gauld and Bolton, 1988), our result of a monophyletic Symphyta relative to Apocrita (Fig. 3A) is surprising. However, this sister group relationship was unambiguously supported in only 54% of the bootstrap iterations. Overall, the phylogenetic relationships are very similar to those presented in our original analysis due, at least in part, to the fact that the contaminating vertebrate DNAs and the real ichneumonoid sequences both represent distinct monophyletic groups.

We are now in a position to offer suggestions to other researchers regarding PCR contamination in phylogenetic studies. Although others have pointed out that extreme care must be taken throughout DNA isolation and PCR amplification this cannot be overstated. Contamination can occur through shared chemicals, pipettes, and quite possibly through aerosols. Nevertheless, contamination is generally not a serious problem with strong template DNA/PCR primer complementarity. However, conserved PCR primers may result in mismatch pairing when used with distantly related taxa and any DNA contaminants with sequences that better match the primers will preferentially amplify at the expense of the target DNAs. In such cases, we advise that primer design include safeguards that limit the taxonomic diversity of the amplified products. For example, the 16A2/16B2 primer pair will not amplify the 16S rRNA region from vertebrate mitochondrial DNAs. In addition, nucleotide sequences should be exhaustively compared to sequences found in data banks such as GenBank and EMBL, even though this will require substantial computer time. While data bank scans will not provide definitive answers in cases where sequences from a particular taxonomic group are not available, they can provide an important check for PCR-based contamination problems.

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