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Molecular Systematics of the Tephritoidea (Insecta: Diptera): Phylogenetic Signal in 16S and 28S rDNAs for Inferring Relationships Among Families

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Phylogenetic signal present in the mitochondrial 16S ribosomal RNA gene (16S rDNA) and the nuclear large subunit ribosomal RNA gene (28S rDNA) was explored to assess their utility in resolving family level relationships of the superfamily Tephritoidea. These two genes were chosen because they appear to evolve at different rates, and might contribute to resolve both shallow and deeper phylogenetic branches within a highly diversified group. For the 16S rDNA data set, the number of aligned sites was 1,258 bp, but 1,204 bp were used for analysis after excluding sites of ambiguous alignment. Among these 1,204 sites, 662 sites were variable and 450 sites were informative for parsimony analysis. For the 28S rDNA data set, the number of aligned sites was 1,102 bp, but 1,000 bp were used for analysis after excluding sites of ambiguous alignment. Among these 1000 sites, 235 sites were variable and 95 sites were informative for parsimony analysis. Our analyses suggest that: (1) while 16S rDNA is useful for resolving more recent phylogenetic divergences, 28S rDNA can be used to define much deeper phylogenetic branches; (2) the combined analysis of the 16S and 28S rDNAs enhances the overall resolution without losing phylogenetic signal from either single gene analysis; and (3) additional genes that evolve at intermediate rates between the 16S and 28S rDNAs are needed to further resolve relationships among the tephritoid families.

The superfamily Tephritoidea currently includes the families Lonchaeidae, Ulidiidae (=Otitidae, =Pterocallidae), Platystomatidae, Tephritidae (including Tachiniscidae), Pyrgotidae, Richardiidae, Pallopteridae, and Piophilidae (Korneyev, 1999). Among them, Tephritidae (fruit flies) is the most important group of agricultural pests of all fly families, and much research attention has been focused on this family. While the tephritids are almost exclusively phytophagous, the pyrgotids are internal parasites of scarabaeoid beetles and the other families are mostly saprophagous.

Relationships among the tephritoid families have been the subject of morphological investigations by several dipterists, including Hennig (1973), Griffiths (1972), McAlpine (1989), and Korneyev (1999). Their results, however, are discordant due to the conflict between selected character sets, how the characters were interpreted and the huge number of taxa involved. Furthermore, their analyses were limited by the small number of available morphological characters in the family-level comparison. Recently, nucleotide sequences have become an increasingly important source of data to resolve many controversial or unresolved phylogenetic questions, and it has become clear that molecular characters are needed to further resolve the phylogenetic relationships of Tephritoidea (Korneyev, 1999). There have been a few molecular sequence analyses of family level relationships in Diptera (e.g., Vossbrink and Friedman, 1989; Pawlowski et al., 1996; Friedrich and Tautz, 1997a, 1997b; Skevington and Yeates, 2000; Wiegmann et al., 2000), but no attempt has yet been made to infer the relationships among the tephritoid families.

In this study, we explored the phylogenetic signal present in the mitochondrial 16S ribosomal RNA gene (16S rDNA), and the nuclear large subunit ribosomal RNA gene (28S rDNA) to assess their utility in resolving family level relationships within the superfamily Tephritoidea. These two genes were chosen because they appear to evolve at different rates, and might contribute to the resolution of both shallow and deeper relationships within this highly diversified group. The fast evolving 16S rDNA has been used in many insect phylogenetic studies, including recent attempts to

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resolve relationships within the family Tephritidae (Han and McPheron, 1997, 1999; McPheron and Han 1997; McPheron et al., 1999; Han, 2000; Han and Ro, 2002). The more slowly evolving 28S rDNA appears highly informative in resolving relationships at the family level within the infraorder Culicomorpha (Diptera: Nematocera) (Pawlowski et al., 1996). Because of the preliminary nature of this study, we selected taxa not to resolve many pending phylogenetic questions but to show the degree of resolution at wider taxonomic levels.

Materials and Methods

For sequence analysis, we selected 20 species representing four families of Tephritoidea and nine other fly familes. Multiple species were included for Tephritidae and Uliidae to look at the resolution in the shallower phylogenetic branches, but non-tephritoid taxa including the ones thought to be remotely related to Tephritoidea were also chosen to elucidate phylogenetic signal in the deeper branches. We sequenced all the taxa except for *Drosophila melanogaster*, whose sequences were available from GenBank (Table 1). For Lonchaeidae, we used *Silba adipata* for the 16S rDNA and *Lonchaea* sp. for 28S rDNA because of technical difficulty in PCR and sequencing.

Nucleic acid extractions from freshly frozen flies followed the standard protocol optimized for individual flies (Sheppard et al., 1992). Pinned or alcohol-preserved specimens were extracted by the modified protocol described in Han and McPheron (1997). Some samples were also prepared using a DNeasy tissue kit (Quagen Inc.). Either a whole body or a single leg was used for each DNA extraction. In both cases, voucher specimens (wings or remaining body parts) are deposited in the Department of Life Science, Yonsei University, Korea (Table 1).

The regions to be analyzed were amplified using standard PCR approaches (e.g., Kocher et al., 1989). Double-stranded amplification product (40 amplification cycles of 93℃ (1 min.), 45-55℃ (1 min.), 72℃(2 min.)) was purified by isolating the desired band using 2% agarose gel electrophoresis in 1× TAE buffer. Preparation of gel-purified template followed the freeze and thaw method of Palumbi et al. (1991). This product was re-amplified asymmetrically using one of the PCR primers or an internal primer as a limiting primer (1:25-100 ratio). Single-stranded DNA was purified and concentrated using the isopropanol precipitation method (Palumbi et al. 1991). Single-stranded DNA was sequenced by the dideoxy, chain-termination method (Sanger et al., 1977) using Sequenase version 2.0 (Amersham Co.).

The following primers were used to conduct PCR and sequencing reactions for the nearly entire 16S rDNA: LR-N-13398 (5'-CGCCTGTTTATCAAAAACAT-3') and LR-J-12883 (5'-CTCCGGTTTGAACTCAGATC-3') (primers A and B, respectively, of Xiong and Kocher (1991)); LR-J-13021 (5'-ACGCTGTTATCCCTAAAGTA-3'); LR-N-13182 (5'-TTAAAAGACGAGAAGACCCTA-3'); LR-J-13323 (5'-ACTAATGATTATGCTACCTT-3'); LR-J-

Table 1. Collection and voucher information of the flies sequenced

TABANIDAE Chrysops carbonarius Walker. USA: Pennsylvania: Centre Co., 500m E. of Spring Creek Hatchery, 16.VI.1994, H.-Y. Han (1 female from the same collecting lot; 16S-AY123341; 28S-AY123353).
ASILIDAE Laphria thoracica Fabricius. USA: Pennsylvania: Centre Co., E. of Spring Creek Hatchery, 16.VI.1992, H-Y. Han (the sequenced specimen without one leg; 16S-AY123342; 28S-AY123354).
DOLICHOPODIDAE Dolichopus sp. USA: Pennsylvania: Centre Co., 1.5mi W of Port Matila, Malaise trap, VII.1992, B. A. McPheron (1 male and 2 females from the same collecting lot; 16S-AY123343; 28S-AY123355).
SYRPHIDAE Eristalis tenax (Linnaeus). USA: Pennsylvania: Centre Co., W. of Spring Creek Hatchry, 28.IX.1992, H.-Y. Han (3 individuals from the same collecting lot; 16S-AY123344; 28S-AY123356).
MUSCIDAE Musca domestica Linnaeus. USA: Pennsylvania: Pennsylvania State University, Department of Entomology, from lab colony, VII.1992 (no voucher specimen; 16S-AY123346; 28S-AY122258).
TACHINIDAE Archytas nivalis Curran. USA: Pennsylvania: Centre Co., W. of Spring Creek Hatchry, 28.IX.1992, H.-Y. Han (1 male and 1 female from the same collecting lot; 16S-AY123345; 28S-AY123357).
DROSOPHILIDAE Drosophila melanogaster Meigen. (sequences obtained from GenBank; 16S-M21017, M29800; 28S-NC_001709).
CONOPIDAE Physocephala sagittaria (Say). USA: Pennsylvania: Cerntre Co., Spring Creek Hatchery, 30.VI.1992, H.-Y. Han (1 female from the same collecting lot; 16S-AY123349; 28S-AY123361).
MICROPEZIDAE Rainieria antennaepes (Say). USA: Pennsylvania: Centre Co., 1.5mi W of Port Matila, Malaise trap, 19~21.IX.1993, B.-A. McPheron (1 male and 2 females from the same collecting lot; 16S-AY123347; 28S-AY123359).
DIOPSIDAE Cyrtodiopsis dalmanni (Wiedemann). MALAYSIA: Ex. Maryland Culture 27. Feb. 1992 G. Wilkinson (1 male from the same culture; 16S-AY123348; 28S-AY123360).
LONCHAEIDAE Lonchaea sp. USA: Pennsylvania: Centre Co., 1.5mi W of Port Matila, Malaise trap, 19~21.IX.1993, B. A. McPheron (1 male and 1 female from the same collecting lot; 28S-AY123362).
LONCHAEIDAE Silba adipata McAlpine. Israel: Golan Heights, Baniyas Spring, 29.V.2000, H.-Y. Han (over 100 individuals from same collecting lot; 16S-AY123350).
ULIDIIDAE Delphinia picta (Fabricius). USA: Pennsylvania: Centre Co., 1.5 mile west of Port Matilda, Malaise trap, VII.1992, B. A. McPheron (both wings of the sequenced specimen glued on card point; 16S-AF177121; 28S-AY123364).
ULIDIIDAE Tritoxa flexa (Wiedemann). USA: Pennsylvania: Centre Co., Scotia Barren, 14.VII.1992 (2 males and 2 females from the same collecting lot; 16S-AY123351; 28S-AY123363).
PYRGOTIDAE Pyrgota undata Wiedemann. USA: Pennsylvania: Centre Co., along Spring Creek near Hatchery, 30.V.90, H.-Y. Han (right wing of the sequenced specimen mounted on a rectangular paper; 16S-AY123352; 28S-AY123365).
TEPHRITIDAE Anastrepha serpentina (Wiedemann). VENEZUELA: Maracay, 1988, G. Steck (1 male & 1 female from the same collecting lot; 16S-AF177125; 28S-AY123366).
TEPHRITIDAE Ceratitis capitata (Wiedemann). VENEZUELA: Granja Santa Rosa, Dtto Mara, Estado Zulia, 18.V.1992, K. Katiyuar & A. Romero (1 male & 1 female from the same collecting lot; 16S-AF177123; 28S-AY123367).
TEPHRITIDAE Chetostoma curvinerve Rondani. ISRAEL: Mt. Meron 1,200 m, 16.IV.1992, A. Freidberg (both wings of the sequenced specimen glued on card point; 16S-AF177131; 28S-AY123368).
TEPHRITIDAE Euleia fratria (Loew). USA: Pennsylvania: Centre Co., along Spring Creek, west of fish hatchery, reared from leaf mine of Angelica atropurpurea, 22.VIII.1989, em. 1-12.IX.1989, H.-Y. Han (1 male & 1 female from the same collecting lot; 16S-AF177136; 28S -AY123370).
TEPHRITIDAE Itosigo bellus Ito. KOREA: Gangwon-do, Inje-gun, Mt. Jeombong, 18-VIII-1996, H.-Y. Han & H.-W. Byun (both wings of the sequenced specimen glued on card point; 16S-AF177133; 28S-AY123369).
TEPHRITIDAE Trypeta tortilis Coquillett. USA: Illinois: Vermilion Co., Forest Glen Preserve, Malaise trap, 1.VI.1986, B. A. McPheron (both wings of the sequenced specimen glued on card point; 16S-AF177137; 28S-AY123371).

Status of the voucher specimens and GenBank accession numbers are indicated in parentheses. Both 16S and 28S rDNA sequences for each species were obtained from a single individual except for Lonchaeidae. Vouchers are either remaining body parts of the sequenced specimens or additional individuals from same collecting lots.

13677 (5'-AGCTTATCCCATAAAATATT-3'); LR-N-13770 (5'-AGAAATGAAATGTTATTCGT-3'); and TV-N-14112 (5'-AGCATTTCATTTACATTGAA-3'). For each relatively fresh specimen (i.e., less than 2 year old pinned or alcohol specimen kept in ambient temperature), two gel-purified templates were made using the primers LR-J-12883/LR-N-13398 and the primers LR-J-13323/ TV-N-14112, respectively. For specimens with high DNA degradation, shorter segments were amplified for gel-purification. The DNA fragment used in this study represents the portion bracketed by the primers LR-J-12883 and TV-N-14112. The 16S rDNA primers were designed based on the alignment between *Drosophila yakuba* and *Anopheles gambiae*.

For the 28S rDNA, the fragment starting at position 4339 (1053th from 5' end of 28S rDNA) and ending at position 5475 of *Drosophila melanogaster* (Tautz et al., 1988) was amplified and sequenced. It contains expansion segments D4, D5, D6, D7a and D7b (Hancock et al., 1988). The PCR and sequencing primers used in this study are as follows: S28C (5'-GTGCAAATCGATTGTCAGAA-3') (Primer 10i of Pawlowski et al. (1996)); S28E (5'-AGCAGGACGGTGGACATGGA-3'); A28D (5'-ACTTAAGCGCCATCCATTTT-3'); S28G (5'-GAAGTGGAGAAGGGTTTCGT-3'); A28F (5'-TGGAACCGTATTCCCTTTCG-3'); A28HL (5'-CTTACCTACATTATTCTATCGACT-3'). The DNA fragment used in this study represents the portion bracketed by the primers S28C and A28HL; the rest are internal primers. All the 28S rDNA primers were designed based on the alignment between *Drosophila melanogaster* and *Aedes albopictus*.

Alignment of the sequences was conducted using CLUSTAL X software (Thompson et al., 1997; version 1.81, 2000). No additional manual adjustment was made, but ambiguous portions of the alignment were identified using GBLOCKS software (version 0.91b; Castresana, 2002), and eliminated from the data set. Neighbor-joining (NJ) analysis was performed using MEGA software (version 2.1; Kumar et al., 2001). The Kimura two-parameter model of nucleotide substitution (Kimura, 1980) was selected for the analysis based on Nei's (1991) guideline for choosing the most appropriate distance measure. The reliability of clustering pattern in trees was tested by the standard error test for the internal branches of NJ trees (Rzhetsky and Nei, 1992) and bootstrapping (Felsenstein, 1985) (2,000 replications). Maximum parsimony (MP) and maximum likelihood (ML) analyses were performed with PAUP software (version 4.0b10; Swofford, 2002), using the heuristic search procedure, with the tree bisection-reconnection (TBR) branch swapping method and the start tree derived by stepwise addition. Bootstrapping of the MP analysis (2,000 replications) and ML analysis (100 replications) was also conducted under the heuristic search procedure, with a maxtree setting of 200 trees. All characters were treated as unordered

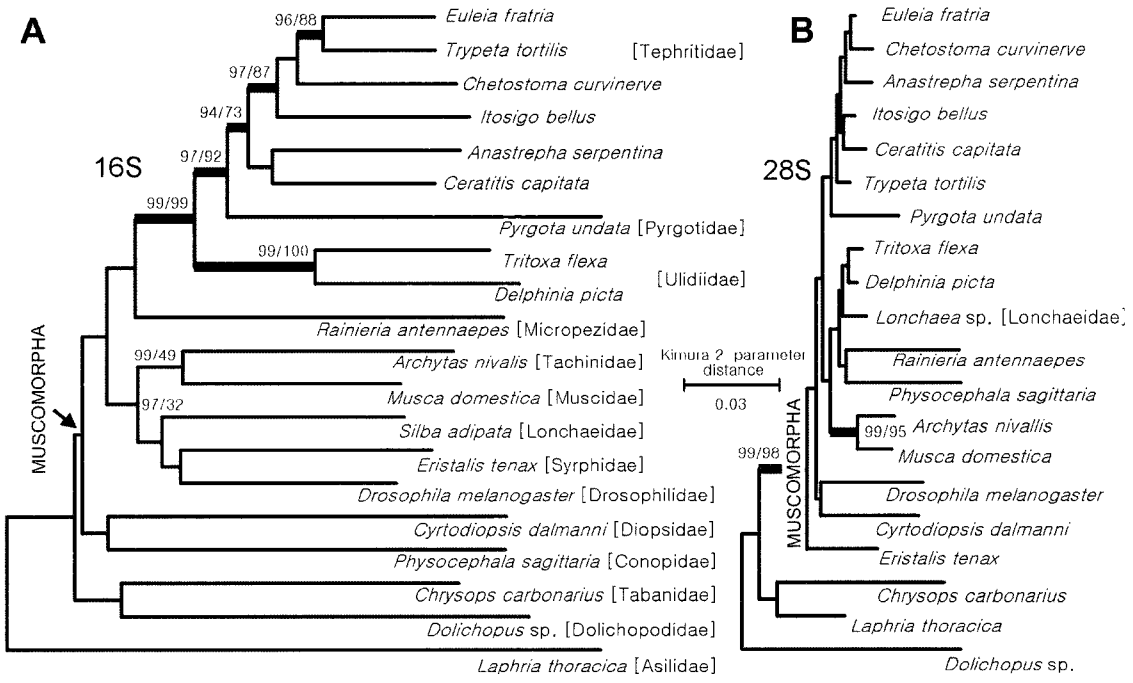

Fig. 1. Unrooted neighbor-joining trees based on Kimura two parameter distances with pairwise deletion of gaps and missing data, using 16S rDNA sequences (A, 1,204 bp after excluding sites of ambiguous alignment) and 28S rDNA sequences (B, 1,000 bp after excluding sites of ambiguous alignment). The first number on each branch is the Pc value from the standard error test, and the second number is the Pb from the bootstrap test (2,000 replications). Only values same or greater than either 95% of the standard error test or 70% of bootstrap test are presented. In both cases, ambiguously aligned sites were eliminated using Gblocks (parameters: default except for minimum length of block = 5, allowed gap = with half).

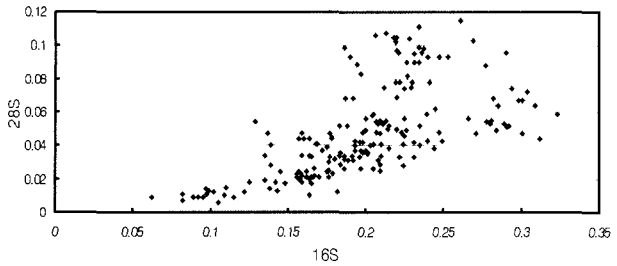

Fig. 2. Kimura two parameter distances for pairwise comparisons between 16S rDNA sequences plotted against corresponding distances between 28S rDNA sequences.

and equally weighted for MP.

Sequence data newly generated in this study have been deposited in GenBank (Accession Nos. AY123341-123371). The alignment is reproducible using the methods described above, but is also available as a Nexus file from the authors upon request.

Results

For the 16S rDNA data set, the number of aligned sites was 1,258 bp, but 1,204 bp were used for analysis after excluding sites of ambiguous alignment identified using the Gblocks analysis (default except for minimum length of block = 5, allowed gap = with half). Among these 1,204 sites, 662 sites were variable and 450 sites were informative for parsimony analysis. The average proportion of A:T:C:G was 37.9:44.0:5.9:12.2.

For the 28S rDNA data set, the number of aligned sites was 1,102 bp, but 1,000 bp were used for analysis after excluding sites of ambiguous alignment using the Gblocks analysis with the same parameters as the 16S rDNA data. Among these 1,000 sites, 235 sites were variable and 95 sites were informative for parsimony analysis. The average proportion of A:T:C:G was 30.6:29.6:16.6:23.2.

For the combined analysis of the 16S and 28S rDNA data sets, we simply combined the above alignments, but we also generated an additional data set using more stringent parameters for the Gblocks analysis (default except for minimum length of block = 5, sites with gaps and missing data eliminated altogether). For the latter data set, 304 additional sites were eliminated because of containing gaps or missing data.

When the 16S and 28S rDNA trees based on NJ analysis were compared under a same distance scale, the 16S rDNA appeared to evolve much faster (Fig. 1). This was also evident when the Kimura two parameter distances for the pairwise comparisons between the 16S rDNA sequences were plotted against the corresponding distances between the 28S rDNA sequences (Fig. 2).

When the 16S and 28S rDNA data sets were combined, the results (Figs. 3, 4) appeared to have much more phylogenetic signal than either of the

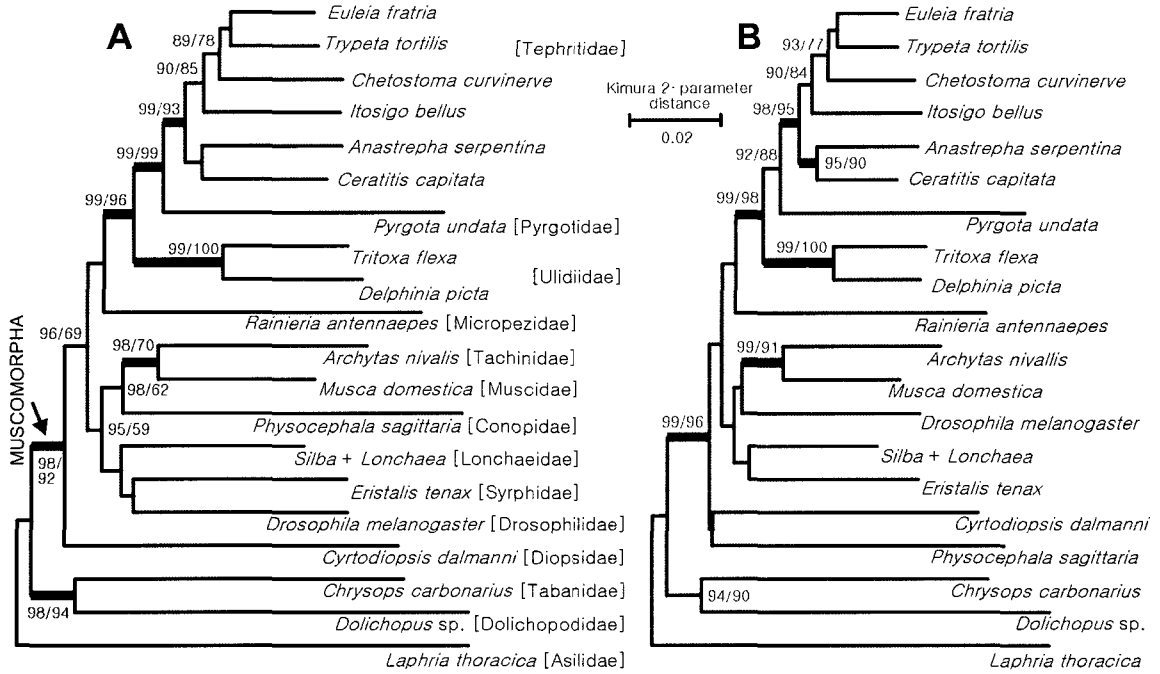

Fig. 3. Unrooted neighbor-joining trees based on Kimura two parameter distances with pairwise deletion of gaps and missing data, using combined sequences of 16S (A) and 28S rDNAs (B). The first number on each branch is the Pc value from the standard error test, and the second number is the Pb from the bootstrap test (2,000 replications). Only values same or greater than either 95% of the standard error test or 70% of bootstrap test are presented. The Gblocks parameters used to eliminate ambiguously aligned sites are as follows. A, Default except for minimum length of block = 5, allowed gap = with half (2,204 bp resulted). B, Default except for minimum length of block = 5, allowed gap = None (1,900 bp resulted).

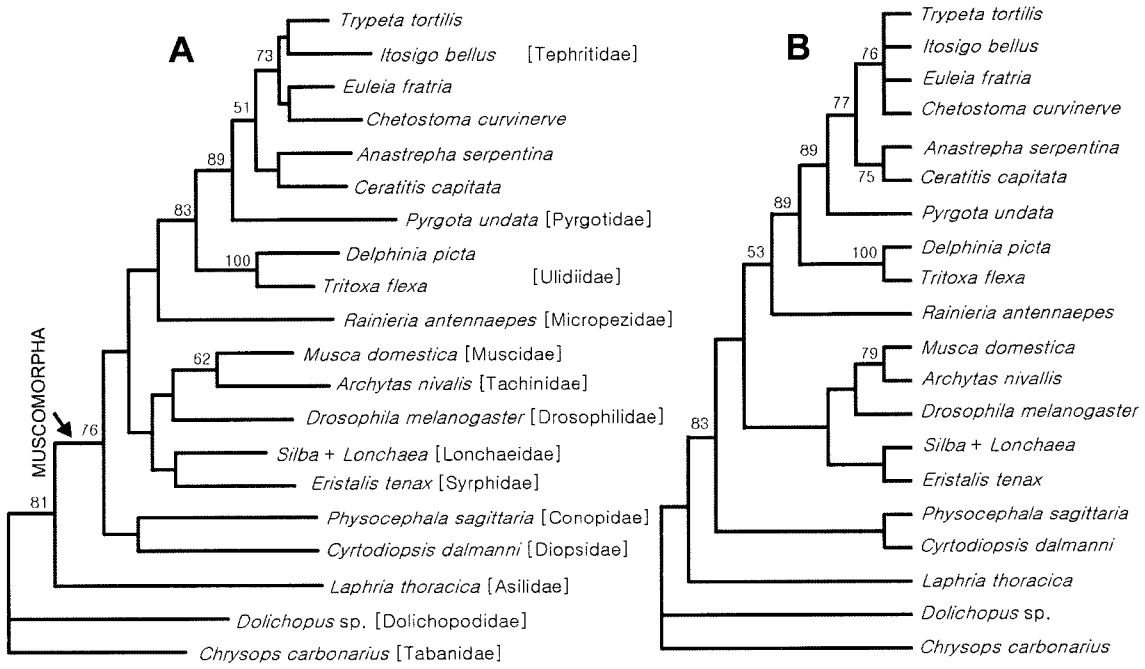

Fig. 4. Relationships inferred from unrooted maximum likelihood tree based on HKY85 model (A), and unrooted strict consensus tree of five most parsimonious trees using combined sequences of 16S rDNA and 28S rDNAs (B). Statistics for each most parsimonious tree: length = 2,530, CI = 0.4854, HI = 0.5146, RI = 0.3770, RC = 0.1830. Bootstrap support is indicated on the nodes (only values greater than 50% are presented). Ambiguously aligned sites were eliminated using Gblocks (parameters: default except for minimum length of block = 5, allowed gap = with half; 2,204 bp resulted). Alignment gaps were treated as missing data.

separate analyses in terms of both statistical support of the interior branches and compatibility with the previously established classification. The inferred phylogenetic trees based on NJ (Figs. 3A, 3B), ML (Fig. 4A) and MP (Fig. 4B) are quite similar, especially for the branches with higher statistical support, and our discussion is mostly based on the NJ trees (Figs. 3A, 3B), for which more rigorous statistical testing of the interior branches was possible. Based on our previous NJ analyses with several 16S rDNA data sets, we used the following empirical guidelines to evaluate the inferred phylogeny (Han and McPheron, 1997, 1999; Han, 2000; Han and Ro, 2002): Pc values (P values from standard error test) of at least 95% - statistically significant; Pb values (P values from bootstrap test) of at least 70% - generally informative; branches supported both by at least 95% of Pc and 70% of Pb - highly likely to represent true phylogeny.

Discussion

When the 16S and 28S rDNA trees are compared (Figs. 1A vs. 1B), the fast evolving 16S rDNA tree appears far better resolved. However, this is simply because the nine closely related species (Tephritidae-Pyrgotidae-Ulidiidae clade) were included in the analysis. This result is compatible with the conventional classification, but, for the rest of the taxa, there is no phylogenetic resolution other than topological support for the close relationship between Tachinidae and Muscidae. On the other hand, the 28S rDNA topology, even though seemingly uninformative in resolving overall relationships, clearly supports two deeper branches (infraorder Muscomorpha and Tachinidae/Muscidae clade) not well supported by the 16S rDNA topology (Fig. 1B).

The poor overall phylogenetic resolution within the infraorder Muscomorpha based on the partial 28S rDNA (Fig. 1A) strongly contrasts with the high information content of the tree for the infraorder Culicomorpha based on the nearly identical portion of 28S rDNA (Pawlowski et al., 1996). It may be because the infraorder Muscomorpha is much younger than the Culicomorpha, and a large number of Muscomorpha families might have diverged in a relatively short period of geological time, resulting in numerous short basal phylogenetic branches. Such short branches are often very difficult to detect by any phylogenetic analysis due to the limited number of associated character changes. That the Muscomorpha explosively radiated is suggested by the huge diversity of this group: among the 10 infraoders of Diptera (sensu McAlpine, 1989), Muscomorpha contains more than 80 families (over 60% of fly families).

In the combined analysis of the 16S and 28S rDNAs (Fig. 3A), there is much more resolution without the loss of phylogenetic signal from either single tree. Even when more stringent Gblocks parameters are

used for the alignment, the topology and statistical support remain similar (Fig. 3B). In these trees, the Dolichopodidae and Tabanidae clade has much higher statistical support (98/94 in Fig. 3A and 94/90 in Fig. 3B) than in the separate analyses (Fig. 2). This result, however, should be viewed with caution because these trees are unrooted. If Tabanidae is considered an outgroup (as according to conventional classification), the basal branches should be rearranged (with Dolichopodidae as the basalmost branch next to the outgroup and Asilidae as the sister group of Muscomorpha - arrangement similar to Figs. 4A, 4B). Under this assumption, the sister group relationship between Asilidae and Muscomorpha is supported by the same interior branch test values. These basal relationship is also topologically recognized in the ML or MP trees (Figs. 4A, 4B).

Neither separate nor combined analysis supports the monophyly of the superfamily Tephritoidea. Furthermore, no clear phylogenetic signal elucidates the relationships of the included tephritoid families with other Muscomorpha families. This problem may be alleviated if we explore additional genes which evolve at intermediate rates between the 16S and 28S rDNAs.

In conclusion, the combined analysis of the 16S and 28S rDNAs provides relatively high resolution in both shallow and deep phylogenetic branches, and, thus, is a useful tool for phylogenetic analysis of the highly diverged fly families. Additional genes and more extensive taxon sampling, however, would certainly enhance our ability to resolve family-level relationships of the higher Diptera in the future.

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