

Phylogenetic Analysis of Mitochondrial DNA Supports the Monophyly of Dacini Fruit Flies (Diptera: Tephritidae)

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ABSTRACT The tribe Dacini (Diptera: Tephritidae: Trypetinae) currently consists of ≈ 40 genera and 1,000 described species. Among others, it includes the genera *Bactrocera*, *Ceratitidis*, and *Dacus*, all of which are pests of agricultural importance because they oviposit in ripening fruit. Although previous studies, based on morphological and molecular data, suggested that the members of Dacini are monophyletic, no extensive molecular phylogenetic studies have been published on *Bactrocera*, *Ceratitidis*, or *Dacus*. Therefore, we undertook a phylogenetic analysis of 34 tephritid taxa, including 16 species of the tribe Dacini, utilizing 1,391 bp from the mitochondrial 16S rRNA, 12S rRNA, and cytochrome oxidase II + tRNA_{Lys} + tRNA_{Asp} genes. Combined maximum parsimony analysis suggested the following with strong quantitative support: (1) the subfamilies Tephritinae and Trypetinae are monophyletic, (2) Dacini is monophyletic, and (3) members of the subtribe Dacina are monophyletic. In addition, Ceratitidina + Gastrozonina are basal to Dacina. Within Dacina, *Dacus* and *Bactrocera* were each monophyletic. The results obtained here are generally congruent with those based on morphology.

KEY WORDS Dacini, Tephritidae, fruit flies, phylogenetics, mitochondrial DNA

THE DIPTERAN FAMILY Tephritidae, commonly known as fruit flies, includes some of the most biologically interesting and agriculturally important species of flies. Several tephritids have been the subject of seminal studies on behavior, ecology, sexual selection, speciation, and evolutionary biology (Bush 1975; Futuyma and Mayer 1980; Prokopy 1980; Fletcher 1987; Feder et al. 1988; McPheron et al. 1988; Headrick and Goeden 1998, 2000; Berlocher 2000; Díaz-Fleischer and Aluja 2000; Han and McPheron 2000). There are >450 genera and $\approx 4,300$ described species within Tephritidae worldwide, making it one of the largest families within Diptera (Norrbom et al. 1998). About half of the species within the family are frugivorous; however, the larvae of many species develop in flowers of Asteraceae, and others are associated with flowers of various plant families or their larvae are miners, borers, or gallmakers of various plant organs.

From an agricultural standpoint, the genera *Anastrepha*, *Bactrocera*, *Ceratitidis*, *Dacus*, and *Rhagoletis* are the most important economically because many species in these genera are serious pests of fruit (White and Elson-Harris 1992). *Bactrocera*, *Ceratitidis*, and *Dacus* are members of the tribe Dacini (Trypetinae), which includes ≈ 40 genera and 1,000 described spe-

cies (Norrbom et al. 1998). Three subtribes are currently recognized within Dacini: Ceratitidina (198 species), Dacina (723 species), and Gastrozonina (112 species).

The monophyly of Dacini is supported by morphological and nucleotide data (Hancock 1986, Foote et al. 1993, Han and McPheron 1997). From a morphological standpoint, the monophyly of Dacini is suggested on basis of the shape of the lobe of cell *bcu* of the wing, number of spermathecae (reduced to two), and surstyli shape (discussed in Norrbom et al. 1998). Nucleotide data from the mitochondrial 16S rRNA gene (Han and McPheron 1997) also supported the monophyly of Dacini; however, only four species of Dacini were included in the study. De Meyer (2000) examined the phylogenetic relationships of *Ceratitidis* using morphology; and Drew and Hancock (2000) and White (2000) proposed phylogenies for *Bactrocera*, and to a lesser extent *Dacus*, also based on morphological criteria. Although isozyme and nucleotide data have been used to examine the evolution of *Anastrepha* and *Rhagoletis* (Steck 1991, Smith and Bush 1997, McPheron and Han 1997, McPheron et al. 2000), currently no extensive molecular phylogenetic studies have been published on *Bactrocera*, *Ceratitidis*, or *Dacus*.

The primary aim of this study was to investigate the monophyly of Dacini using DNA sequence data from portions of the mitochondrial 16S rRNA, 12S rRNA, and cytochrome oxidase II + tRNA_{Lys} + tRNA_{Asp} (CO2KD) genes and parsimony analysis. Specifically,

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Table 1. List of taxa examined with geographic origin and GenBank accession numbers

Species	Origin	16S rRNA	12S rRNA	CO2KD
<i>Anastrepha grandis</i> Macquart	Brazil	AF152068*	AY037515	AY037527
<i>Bactrocera (Apodacus) viscenda</i> (Hardy)	Australia: Bamaga: Queensland	AY037366	AY037323	AY037409
<i>Bactrocera (Asiadaeus) apicalis</i> Meijere	Thailand: Kanchanaburi	AY037361	AY037318	AY037404
<i>Bactrocera (Austrodacus) cucumis</i> Perkins	Australia	AY037349	AY037306	AY037392
<i>Bactrocera (Bactrocera) carambolae</i> Drew & Hancock	Malaysia: Ipoh	AY037367	AY037324	AY037410
<i>Bactrocera (Bactrocera) caryae</i> Kapoor	India: Karnataka: Mudikere	AY037384	AY037341	AY037427
<i>Bactrocera (Bactrocera) dorsalis</i> Hendel	USA: Hawaii & Tahiti	AY037372	AY037329	AY037415
<i>Bactrocera (Daculus) oleae</i> Rossi	Italy & USA: California: Ventura Co.	AY037380	AY037337	AY037423
<i>Bactrocera (Hemigymnodacus) diversa</i> (Coquillett)	Sri Lanka: Kandy	AY037379	AY037336	AY037422
<i>Bactrocera (Paradacus) longicaudata</i> (Sabah)	Malaysia: Selangor	AY037364	AY037321	AY037407
<i>Bactrocera (Zeugodacus) cucurbitae</i> Coquillett	Brunei Darussalam: Bandar Seri Bagawan	AY037350	AY037307	AY037393
<i>Bactrocera (Zeugodacus) tau</i> Walker	India: Karnataka: Mudikere	AY037391	AY037348	AY037434
<i>Ceratitis capitata</i> (Wiedemann)	Venezuela: Granja Santa Rosa	U01231*	AJ242872*	AJ242872*
<i>Dacus (Callantra) pedunculatus</i> (Bezzi)	Philippines: Los Banos: Mt. Makiling	AY037390	AY037347	AY037433
<i>Dacus (Dacus) demmerezi</i> (Bezzi)	Reunion: La Bretagne	AF388192	AY037500	AY037530
<i>Dacus (Didacus) vertebratus</i> Bezzi	Malawi: Chiroambo Bay	U39384*	AY037501	AY037532
<i>Euaresta aequalis</i> Loew	USA: Illinois	AY037525	AY037511	AY037551
<i>Euaresta festiva</i> Loew	USA: Pennsylvania	AY037524	AY037512	AY037533
<i>Euleia fratris</i> (Loew)	USA: Pennsylvania: Centre Co.	AF177136*	AY037490	AY037534
<i>Paragastrozona japonica</i> (Miyake)	Japan: Hokkaido: Sapporo, Jozankei	U39385*	AY037504	AY037535
<i>Goniglossum</i> sp.	Israel: Alma	U39388*	AF388191	AY037550
<i>Gymnocarena mexicana</i> (Aczél)	Mexico: Michoacan	U39378*	AY037508	AF360541
<i>Hexachaeta fallax</i> Lima	unavailable	AF152089*	AY037481	AY037536
<i>Jamesomyia geminata</i> Loew	USA: Michigan	AY037522	AY037506	AY037548
<i>Myoleja lucida</i> (Fallén)	Switzerland: Schaffhausen	AF177129*	AY037495	AY037537
<i>Neotephritis</i> sp.	unavailable	AY037523	AF388190	AY037538
<i>Paramyiolia rhino</i> (Steyskal)	USA: Florida: Polk Co.	AF177130*	AY037497	AY037540
<i>Parastenopa limata</i> (Coquillett)	USA: Pennsylvania: Centre Co.	AF177128*	AY037488	AY037549
<i>Rhagoletis pomonella</i> (Walsh)	USA: Pennsylvania: Centre Co.	U39437*	AY037485	AY037544
<i>Rhagoletis suavis</i> (Loew)	USA: Pennsylvania: Centre Co.	U39419*	AY037492	AY037543
<i>Rhyncencina spilogaster</i> Steyskal	Mexico	AY037521	AY037478	AY037541
<i>Toxotrypana curvicauda</i> Gerstaecker	USA: Florida	U39381*	AF388189	AY037545
<i>Trypeta sigma</i> (Phillips)	USA: Illinois: Vermilion Co.	AF177137*	AY037496	AY037546
<i>Zonosemata electa</i> (Say)	USA: Texas: Nacogdoches	U39415*	AY037505	AY037547
Outgroup				
<i>Delphinia picta</i> (F.) (Diptera: Otitidae)	USA; Kansas: Manhattan	AF177121*	AY037484	AY037531
<i>Drosophila melanogaster</i> Meigen	Sequences from Genbank	NC001709	NC001709	NC001709

* Previously published sequence obtained from GenBank

we explore the utility of the mitochondrial gene fragments for phylogenetic inference of Dacini exemplars within a broad sampling of tephritid taxa.

Materials and Methods

Specimens. A list of analyzed taxa including origin and associated GenBank accession number is presented in Table 1. The specimens were either field collected (see Han and McPherson 1997 for details) or provided by colleagues in the form of genomic DNA or as dried or ethanol-preserved adults or larvae. *Delphinia picta* (F.) (Diptera: Tephritoidea: Otitidae) and *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) were used as outgroups. The choice of outgroup was partially based on the results of Korneyev (2000), who showed that *D. picta* is closely allied and basal to Tephritidae. DNA sequences of *D. melanogaster* were included as a representative non-tephritid fly.

DNA Extraction, Polymerase Chain Reaction (PCR) Amplification, and Sequencing. Genomic DNA was obtained using the procedures outlined by

Kambhampati and Smith (1995) and Han and McPherson (1997). For some flies, DNA was extracted using the DNAeasy Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Three different DNA fragments comprising portions of the 16S rRNA, 12S rRNA, cytochrome oxidase II, tRNA_{Lysine} and tRNA_{Aspartic Acid} mitochondrial genes were amplified and sequenced using the oligonucleotide primers listed in Table 2.

PCR amplification of the three mitochondrial gene fragments was performed in 50- μ l volume using annealing temperatures ranging from 40 to 50°C as previously described (Kambhampati et al. 1992, Smith et al. 1999). Amplified products (45–50 μ l) were purified on a QiaQuick PCR column. DNA sequencing was performed using the ABI d-Rhodamine Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA) in 5- μ l volume. Sequencing reactions were submitted to the University of Florida's DNA Sequencing Core Facility for sequencing of both strands on an ABI 377 DNA sequencer. Sequence electropherograms were read and edited using ABI's Sequence Navigator software. Multiple sequence

Table 2. Oligonucleotide primers used for polymerase chain reaction (PCR) amplifications

Name	Sequence
(16S-F) LR-J-13756	5'-TAGTTTTTTTTAGAAATAAATTTAATTTA-3'
(16S-R) LR-N-13308	5'-GCCITCAATTTAAAAGACTAA-3'
(CO2KD-F) C2-J-3549	5'-CAAATTCGAATTTTACTAGACAGC-3'
(CO2KD-R) TD-N-3884	5'-TTAGTTTGACAWACTAATGTATTAT-3'
(12S-F) SR-J-14199 ^a	5'-TACTATGTTACGACTTAT-3'
(12S-R) SR-N-14594 ^a	5'-AAACTAGGATTAGATACCC-3'

Nomenclature based on Simon et al. (1994); 3' nucleotide position based on sequence of *Drosophila yakuba* (Clary and Wolstenholme (1985)
^a From Kambhampati and Smith (1995).

alignment was initially carried out using CLUSTAL X (Thompson et al. 1997), and the resulting alignment was manually optimized. All sequences have been deposited in GenBank under the accession numbers listed in Table 1. Sequence alignments are available upon request from PTS in NEXUS format.

Phylogenetic Analysis. Permutation tail probability (PTP) (Archie 1989, Faith 1991, Faith and Cranston 1991) as implemented in PAUP* 4.0b6 (Swofford 1998) and relative apparent synapomorphy analysis (RASA) (Lyons-Weiler et al. 1996) were used to evaluate the extent to which the data were hierarchically structured. RASA, a tree-independent regression-based method, was carried out using RASA, ver. 3.0T (Lyons-Weiler 2001). To explore the conflict between data sets, the incongruence length difference (ILD) test (Mickelich and Farris 1981; Farris et al. 1994, 1995; Cunningham 1997 a, 1997b) was applied to the combined (pairwise) data matrices (invariant characters removed; 100 randomizations) using the partition homogeneity test option in PAUP*.

Phylogenetic analyses were conducted using maximum parsimony (MP) and neighbor-joining (NJ) methods in PAUP*. Neighbor-joining analysis (Saitou and Nei 1987) was conducted using the Tajima-Nei distance to account for the nucleotide bias in animal mtDNA (Tajima and Nei 1984). Unweighted and weighted MP analyses were carried out using the multiple equally parsimonious heuristic search option with tree bisection-reconnection and 100 random addition sequence replicates. For weighted parsimony, we used successive approximation (Farris 1969) based on the rescaled consistency index (Farris 1989). MacClade 4.0 (Maddison and Maddison 2000) was used to explore character state changes on our MP trees.

Support for specific nodes on the MP and NJ trees was estimated by bootstrap analysis (10,000 replications; "fast" stepwise addition search) (Felsenstein 1985). Bootstrap values >70% likely reflect true clades 95% of the time (Hillis and Bull 1993). Although high or unequal rates of evolutionary change may reduce this probability, the conservative nature of the bootstrap is robust across a range of rates of change typically encountered in molecular phylogenetic studies (Hillis and Bull 1993). Decay indices (Bremer 1994) were determined using AUTODECAY, ver. 4.0 (Erikson 1998) and extracted onto our MP tree using TREEVIEW (Page 1996).

Results

Characterization of the Nucleotide Data. A total of 1,391 aligned bases (including gaps) of DNA sequence were obtained from portions of the mitochondrial 16S rRNA (501 bp), 12S rRNA (442 bp), and CO2KD (448) genes. Of the 1,391 characters, 666 (48%) were variable, and 445 (32%) were parsimony informative. The base composition of the three mitochondrial gene fragments was biased toward adenine and thymine, which together constituted an estimated 76% of the total. A χ^2 test of homogeneity of base frequencies across taxa reported a *P* value of 0.63 ($\chi^2 = 99.51$, *df* = 105). Of the 1,391 characters, \approx 30% exhibited multiple changes (two or more) on our MP tree (data not shown). Summary statistics for the different substitutional changes are shown in Fig. 1. The estimated transition (ti)/transversion (tv) ratio was 0.69.

Phylogenetic Analyses. Phylogenetic signal in the data was explored using PTP and RASA. All PTP tests were statistically significant ($P \leq 0.01$). Rooted RASA analyses were conducted with constant and ambiguous nucleotide sites excluded. All *t*-RASA values were statistically significant (Table 3). These results suggested the presence of phylogenetic signal in the distribution of character states among the taxa.

Pairwise incongruence among the three nucleotide data sets was assessed using the incongruence length difference (ILD) test. The ILD test indicated that the data sets were not significantly heterogeneous ($P \geq 0.01$). Therefore, the data were combined and analyzed in all possible combinations ($n = 4$). A summary of character statistics and results of unweighted parsimony analyses is presented in Table 3.

Among the individual data sets, unweighted parsimony analysis identified 19 equally parsimonious trees of 1,107 steps for the 16S rRNA sequences; 3 equally parsimonious trees of 521 steps for the 12S rRNA sequences; and 20 equally parsimonious trees of 934 steps for the CO2KD sequences. The strict consensus trees for each individual data set indicated that Dacini was monophyletic, but this relationship was not strongly supported (bootstrap value [BV] < 50%). Combining the data resulted in increased resolution and support for the monophyly of the Dacini. Dacini was supported in 58, 70, and 58% of 10,000 bootstrap replicates for the 12S rRNA + CO2KD, 16S rRNA + 12S rRNA, and 16S rRNA + CO2KD data sets, respectively.

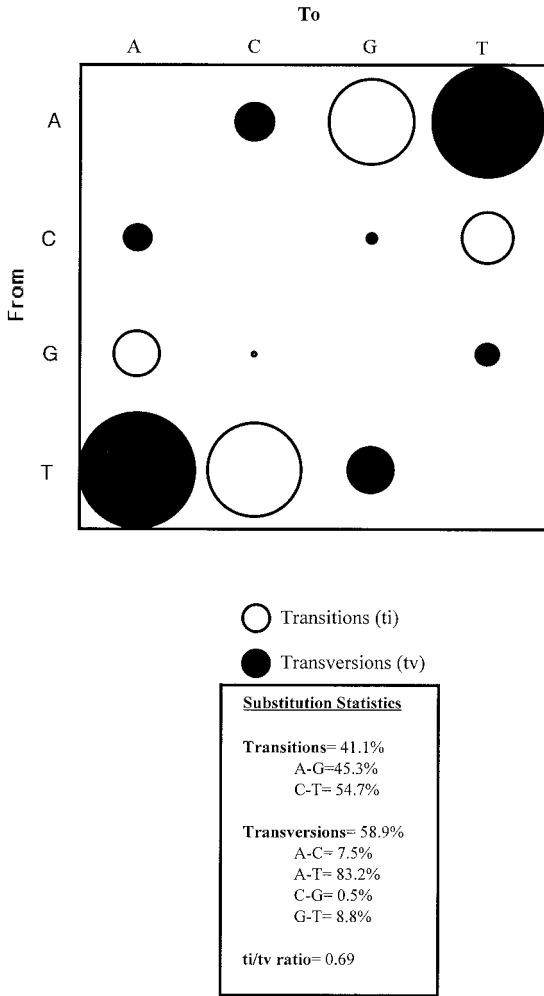


Fig. 1. Summary statistics for the different substitution classes.

When all molecular data were combined, the un-weighted MP analysis recovered a single most parsimonious tree of 2,627 steps (Fig. 2). Combining all the

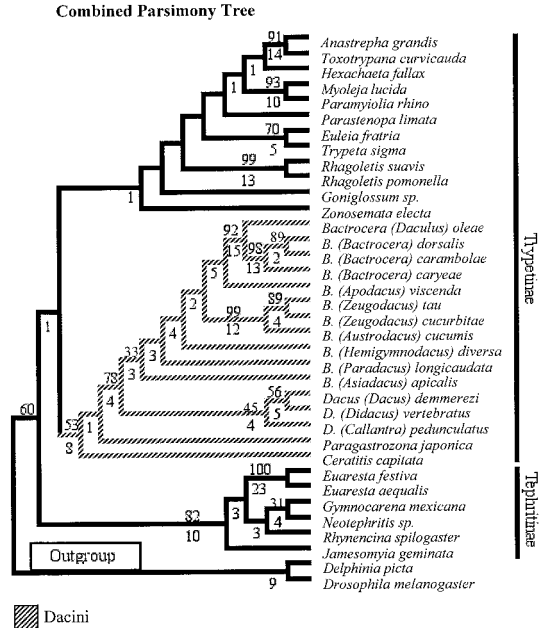


Fig. 2. Single most parsimonious tree for 34 tephritid taxa based on combined DNA sequence portions (1,391 bp including gaps) of the mitochondrial 16S rRNA, 12S rRNA, cytochrome oxidase II, tRNA_{Lys}, and tRNA_{Asp} genes and equal weighting of characters. Gaps were treated as the fifth base. Tree length: 2,627. Numbers above branches are bootstrap values (%) and those below branches are decay indices. Bootstrap values <30% and decay indices of 0 are not shown.

data resulted in a phylogenetic tree with increased resolution and higher support in the form of bootstrap values and decay indices (DI). The phylogenetic tree indicated the following relationships: (1) Tephritidae is monophyletic (BV = 60%). (2) The subfamily Tephritinae is monophyletic (BV = 82, DI = 10) and basal to Trypetinae, which is also monophyletic, but with low support (DI = 1). (3) Dacini is monophyletic (BV = 53, DI = 8). (4) Within Dacini, the following relationships were indicated (*Ceratitits* + (*Paragastrozona* + (*Dacus* + *Bactrocera*))) with varying levels of

Table 3. Summary of character statistics and results of parsimony analysis

Data partition	Characters	PICs ^a	TL ^b	EPTs ^c	CI ^d	RI ^e	ILD ^f	PTP ^g	tRASA ^h	Dacini monophyletic ?
All	1,391	445	2,627	1	0.38	0.46	NA	P = 0.01	4.47	Yes
16S + 12S	943	308	1,662	1	0.40	0.46	P = 0.19	P = 0.01	4.56	Yes
16S + CO2KD	949	333	2,085	13	0.38	0.45	P = 0.01	P = 0.01	3.19	Yes
12S + CO2KD	890	249	1,483	13	0.39	0.48	P = 0.18	P = 0.01	8.60	Yes
16S	501	196	1,107	19	0.39	0.45	NA	P = 0.01	2.26	Yes (except <i>C. capitata</i>)
12S	442	112	521	3	0.43	0.54	NA	P = 0.01	15.09	Yes
CO2KD	448	137	934	20	0.38	0.48	NA	P = 0.01	6.50	Yes

^a PICs, number of parsimony informative characters.

^b TL, most parsimonious tree length

^c EPTs, equally parsimonious trees

^d CI, consistency index

^e RI, retention index

^f ILD, incongruence length difference; only applicable for pairwise partition comparisons

^g PTP, permutation tail probability

^h tRASA, relative apparent synapomorphy analysis

Combined Parsimony Tree-Weighted

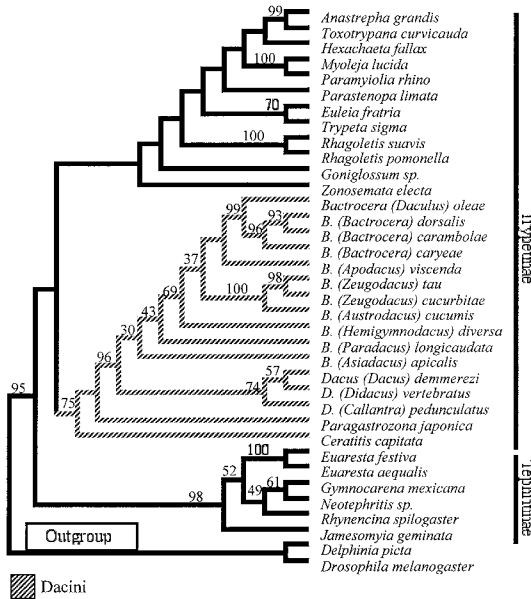


Fig. 3. Single most parsimonious tree for 34 tephritid taxa based on combined DNA sequence portions (1,391 bp including gaps) of the mitochondrial 16S rRNA, 12S rRNA, cytochrome oxidase II, tRNA_{Lys}, and tRNA_{Asp} genes and differential weighting of characters (see text for details). Gaps were treated as the fifth base. Tree length: 528. Numbers above branches are bootstrap values (%). Bootstrap values <30% are not shown.

branch support (Fig. 2). Successive weighting of the characters resulted in a single tree that was identical to the tree based on unweighted parsimony, but with higher branch support (Fig. 3). In terms of topology and branch support, the tree based on NJ analysis was congruent with those based on MP analysis (Fig. 4).

Discussion

In this article, we presented a phylogenetic analysis of relationships among 34 tephritid taxa, including 16 species of Dacini, based on the DNA sequence of portions of the mitochondrial 16S rRNA, 12S rRNA, and CO2KD genes. Congruence testing indicated that the data sets were not significantly heterogeneous. Thus, we refer to the trees that resulted from the combined analysis of all gene fragments. Combining data continues to be subject of much debate (Bull et al. 1993, De Quieroz 1993, De Quieroz et al. 1995, Huelsenbeck et al. 1996, Nixon and Carpenter 1996). However, all gene fragments used here were mitochondrial and thus, presumably, share the same evolutionary history.

Our results indicated that the 16 members of Dacini represent a monophyletic lineage within the subfamily Tephritinae. The monophyly of Dacini had strong quantitative support as measured by bootstrap (53–75%) and decay index (8 steps) values. Furthermore, the monophyly of Dacini was upheld in a tree derived

Neighbor-Joining Tree based on Tajima-Nei Distance

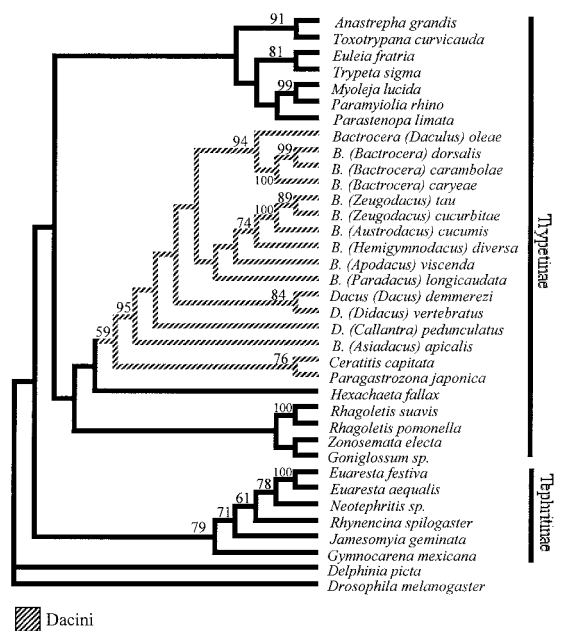


Fig. 4. Neighbor-joining tree based on Tajima-Nei distance and combined DNA sequence of portions (1,391 bp including gaps) of the mitochondrial 16S rRNA, 12S rRNA, cytochrome oxidase II, tRNA_{Lys}, and tRNA_{Asp} genes. Numbers above branches are bootstrap values (%).

from NJ analysis based on the Tajima-Nei distance. NJ analysis is only an approximate solution resulting in a single tree, ignoring other, equally likely, solutions. Nonetheless, the NJ approximation was highly congruent to the trees inferred using MP analysis.

Within Dacini, the following relationships were indicated. (Ceratitis + (Gastrozonina + (Dacina))), a relationship also hypothesized on the basis of morphological criteria (Munro 1984, Hancock 1986, Drew and Hancock 2000, reviewed in Norrbom et al. 1998). The close relationship of Ceratitis and Gastrozonina is apparently indicated by an often large, convex scutellum, wing pattern, and chaetotaxy. The monophyly of Dacina (*Bactrocera* + *Dacus* + *Monacrostichus*) is suggested by a long lobe of cell *bcu* of the wing, and the convoluted shape of the spermathecae (Norrbom et al. 1998). The sister group relationship of *Bactrocera* + *Dacus* is supported by the following synapomorphies: radial veins of the wings crowded anteriorly, medial wing cells broad, female tergite 6 separate from preceding tergites, and tergite 5 of males and females with ceromae (Munro 1984; Hancock 1986; Norrbom et al. 1998). The monophyly of *Dacus* (with respect to *Bactrocera*) is supported by the fusion of abdominal terga 2–5 (Drew and Hancock 2000).

The phylogenetic utility of the mitochondrial gene fragments used in this study was first indicated by the presence of phylogenetic signal in the data as suggested by significant PTP and RASA tests. The recov-

ery of Tephritidae, Tephritinae, and Trypetinae as monophyletic lineages strongly demonstrated the phylogenetic utility of the molecular characters used in this study. Our results strongly support the relationships of Dacini as suggested by morphology, indicating that the molecular data will be useful for further molecular phylogenetic study of Dacini.

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