Molecular Phylogeny and Evolutionary Relationships Among Mosquitoes (Diptera: Culicidae) from the Northeastern United States Based on Small Subunit Ribosomal DNA (18S rDNA) Sequences

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ABSTRACT The phylogenetic relationships of Culicidae native to the northeastern United States were investigated by analyzing small subunit ribosomal DNA (18S rDNA) sequences obtained from 39 species representing nine genera. Molecular phylogenies were consistent with traditional classifications based on morphological characters except for the placements of *Psorophora* Robineau-Desvoidy and *Uranotaenia* Lynch Arribalzaga. In our analyses, 1) *Anopheles* Meigen was strongly supported as the sister taxon to the remaining Culicidae; 2) *Toxorhynchites* Theobald was represented as a distinct monophyletic sister group to the Culicinae; 3) *Psorophora* formed a basal clade to *Culiseta* Felt, *Coquillettidia* Dyar, and *Culex* L. but also was shown as a sister taxon to *Aedes* Meigen and *Ochlerotatus* Lynch Arribalzaga; 4) *Coquillettidia perturbans* (Walker) seems to be a sister group to *Culiseta*; 5) placement of *Uranotaenia* was inconclusive and seemed to be either a sister group to the *Aedes* and *Ochlerotatus* or a basal taxon to all other culicines; and 6) *Aedes* and *Ochlerotatus* formed two separate and distinct clades, providing phylogenetic data consistent with the recent elevation of *Ochlerotatus* to the generic level as proposed by Reinert (2000). The utility of 18S rDNA for evaluating phylogenetic and evolutionary relationships among mosquito taxa was demonstrated at the genus and species levels. To our knowledge, this study represents the first molecular-based phylogenetic study of mosquito species occurring within this geographic region of North America and contains the largest number of species that have been examined among the genera *Aedes* and *Ochlerotatus*.

KEY WORDS mosquito, *Ochlerotatus*, molecular phylogeny, evolutionary relationships, taxonomy

Traditional mosquito (Diptera: Culicidae) taxonomy has been based largely on comparisons of morphological features and life histories (Edwards 1932). Although the use of easily recognized morphological characters has greatly facilitated identification at the genus and species level, these physical characters may not be informative enough to resolve evolutionary relationships. The most commonly recognized taxonomic grouping system for Culicidae was published >70 yr ago by Edwards (1932) and has been amended only slightly since then. Further analysis of morphological character sets (Harbach and Kitching 1998, Reinert et al. 2004) and examination of thoracic setae in species of *Aedes* Meigen and *Ochlerotatus* Lynch Arribalzaga (=*Aedes*, see Reinert 2000; Lunt and Nielsen 1971a,b) resulted in phylogenies that were inconsistent with the classification of Edwards (1932). A high degree of morphological variation was observed among species within the tribe Aedini, which was proposed to be a polyphyletic taxon, leading the authors to call for further examination of the phylogenetic relationships within *Aedes* and *Ochlerotatus* (Harbach and Kitching 1998). The morphology of larval mouthparts also has been used to determine evolutionary relationships within Neotropical *Culex* L. species (Navarro and Liria 2000). However, this study was limited to mosquito taxa in the tribe Culicini.

Molecular taxonomic characters based on isozyme analyses were identified for a limited number of North American "snow pool" species of *Ochlerotatus* (Eldridge et al. 1986, Brust and Munstermann 1992, Gimnig and Eldridge 1999), but these studies did not focus on their phylogenetic relationships. Schultz et al. (1986) analyzed allozyme data to develop a phylogeny of saltmarsh *Ochlerotatus* and recognized the need to include additional genera and species in future studies.

Gene sequences obtained from the small subunit ribosomal DNA (18S rDNA) have been successfully used to examine the evolutionary relationships among species, genera, and higher taxonomic groups of carabid beetles (Maddison et al. 1999), Nemotocera (Miller et al. 1997), sandflies (Aransay et al. 2000), and species within the *Anopheles punctulatus* Doenitz group (Beebe et al. 2000a,b). The gene contains con-

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served regions that are comparatively stable and other portions that typically show a few nucleotide changes among closely related species (Hillis and Dixon 1991). In addition to the small subunit, the 5.8S portion, the large subunit, and internal transcribed spacer regions (ITS-1 and ITS-2) of ribosomal DNA also have been examined (Hillis and Dixon 1991, Paskewitz and Collins 1997).

There are currently 67 recognized species of mosquitoes representing 11 genera in the northeastern United States (Connecticut, Massachusetts, Maine, New Hampshire, New Jersey, New York, Rhode Island, and Vermont) (Andreadis et al. 2005, Darsie and Ward 2005). The largest generic group inhabiting the region is *Ochlerotatus*, which has recently been elevated from its former subgeneric status within the genus *Aedes* based on primary characters of the female and male genitalia (Reinert 2000). This reclassification has generated controversy among mosquito biologists and systematists (Black 2004, Savage and Strickman 2004, Savage 2005), leading several scientific journals (Higgs 2005) to take the position that "more research (including molecular evidence) and interpretation are needed to develop a consensus on the reclassification" (Anonymous 2005). The phylogenetic and evolutionary relationships among species within these two genera have not been extensively explored at the molecular level but have now clearly come into question.

The objective of this study was to develop a molecular phylogeny of the Culicidae based on 18S rDNA sequences obtained from species native to the northeastern United States, in an attempt to gain new insight into their evolutionary relationships and current systematics. In this article, we compare molecular-based cladistic groupings with traditional taxonomic groupings within the Culicidae, paying particular attention to *Aedes* and *Ochlerotatus.*

Materials and Methods

Mosquitoes were collected between 3 March 1999 and 3 October 2001 (Table 1). Adult female collections were made with dry ice-baited CDC miniature light traps with aluminum pans (model 512, J. W. Hock Co., Gainesville, FL) as part of statewide trapping program for arbovirus surveillance (Andreadis et al. 2004). Hand-held aspirators (Hausherr's Machine Works, Tom's River, NJ) were used to collect host-seeking females of certain species that were not readily obtained in light traps. Larval specimens were collected from vernal pools, freshwater swamps, bogs, and natural and artificial containers. Identification of mosquito species was determined using the keys of Carpenter and LaCasse (1955), Darsie and Ward (1981), and Means (1979, 1987). Specimens were placed into 2.0-ml cyrovials (Nalgene, Rochester, NY) and stored at −20°C until DNA extraction. Nucleotide primers for amplification and sequencing of mosquito 18S rDNA (Table 2) were designed by aligning available 18S rDNA sequences of arthropods from GenBank. Primers were synthesized at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT).

Genomic DNA was isolated from one adult or one to five larval specimens using the DNeasy tissue kit (QIAGEN, Valencia CA) according to the manufacturer's protocol. Polymerase chain reaction (PCR) amplification of nuclear 18S rDNA was performed using the *Taq*PCR core kit (QIAGEN) according to the manufacturer's protocol with 0.6 μM primers 28 F and 16SendR (Table 2). PCR reactions were performed in a thermal cycler (PTC-200 DNA Engine, MJ Research, Watertown, MA) under the following conditions: 94°C for 3 min followed by 35 cycles of 94°C for 45 s, 45°C for 30 s, and 72°C for 1 min 30 s, followed by a final extension at 72°C for 3 min. The PCR product was ≈1,860 nucleotides and was confirmed by standard 1% agarose gel electrophoresis. The amplified PCR product was purified using QIAquick PCR purification kit (QIAGEN) according to the manufacturer's protocol. The purified PCR product was submitted for direct nucleotide sequencing. For sequencing reactions, ≈100 ng of purified 18S rDNA PCR product was combined with 0.6 μM sequencing primer (Table 2) and sterile water. Sequencing reactions were performed at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). Additional 18S rDNA sequences for mosquito species and outgroup taxa were obtained from GenBank (see Table 1 for accession numbers). *Dixella cornuta* Johannsen was selected for inclusion in the data set as the closest nonmosquito outgroup taxon.

Three independent methods of alignment were used for analysis of the sequence data. First, nucleotide sequences (primary structure) were aligned using ClustalX 1.64b software (Thompson et al. 1994). The Clustal alignment was examined for errors by using line-editing software, and no obvious misaligned sequences were found. The resulting alignment of 43 taxa consisted of 2,075 characters, including gaps. A second alignment of 1,823 characters was created by removing the highly variable regions, which contained large gaps. A third alignment based on the secondary structure of 18S ribosomal RNA was produced (SequentiX–Digital DNA Processing, Germany). Full alignment of the primary structure was submitted to GenBank. The three alignments were analyzed using the Maximum Likelihood, Maximum Parsimony, and Neighbor-Joining options of PAUP* version 4.0b software (Swofford 1998). Maximum Likelihood analyses were performed using heuristic search settings, the HKY85 model of sequence evolution, and no molecular clock. Bootstrap analyses based on the same Maximum Likelihood criteria were conducted using 200 replicates for the primary structure alignment and 100 replicates for the secondary structure alignment. Additional Maximum Likelihood analysis was performed using ModelTest 3.7 (Posada and Crandall 1998) for determination of the optimal model of sequence evolution for the secondary structure alignment. The models selected by Hierarchical Likelihood Ratio Tests (hLRTs) and Akaine Information Criteria (AIC) in ModelTest were both TrN+I+G.

Table 1. Origin, date of collection, and GenBank accession numbers for 18S rDNA sequences for taxa included in the phylogenetic study

Taxa	Description	Origin and date	GenBank no.
Genus *Aedes* Meigen			
Subgenus *Aedes* Meigen			
Aedes cinereus Meigen	Adult	Barkhamsted, CT; 6/10/99	AY988441
Subgenus *Aedimorphus* Theobald			
Aedes vexans (Meigen)	Adult	Winsted, CT; 7/6/99	AY988439
Subgenus *Stegomyia* Theobald			
Aedes aegypti L.	Adult	Lab Colony, CAES (Rock strain; date unknown)	AY988440
Aedes albopictus Skuse		Baldridge and Fallon (1991)	X57172
Genus *Anopheles* Meigen			
Subgenus *Anopheles* Meigen			
Anopheles pseudopunctipennis Theobald		Miller et al. (1997)	U49735
Anopheles punctipennis (Say)	Adult	Winsted, CT; 7/6/99	AY988422
Anopheles quadrimaculatus Say	Adult	Lab Colony, USDA (Tallahassee, FL; ≈1995)	AY988423
Anopheles walkeri Theobald	Adult	East Haven, CT; 8/22/00	AY988424
Genus *Coquillettidia* Dyar			
Subgenus *Coquillettidia* Dyar			
Coquillettidia perturbans (Walker)	Larva	Cornwall, CT; 6/10/99	AY988454
Genus *Culex* L.			
Subgenus *Culex* L.			
Culex pipiens L.	Larvae	New Haven, CT; 6/20/00	AY988445
Culex pipiens form *molestus* Forskal	Adult	New York, NY; 10/3/01	AY988446
Culex restuans Theobald	Adult	New Haven, CT; 10/3/00	AY988448
Culex salinarius Coquillett	Adult	Stratford, CT; 9/2/01	AY988449
Culex quinquefasciatus Coquillett	Adult	Lab Colony, USDA (Gainesville, FL; ≈1995)	AY988447
Culex tritaeniorhynchus Giles		Miller et al. (1997)	U48385
Subgenus *Neoculex* Dyar			
Culex territans Walker	Adult	Stamford, CT; 6/12/00	AY988450
Genus *Culiseta* Felt			
Subgenus *Climacura* Howard, Dyar, and Knab			
Culiseta melanura (Coquillett)	Larvae	Lab Colony, CAES (Farmington, CT; 8/27/67)	AY988453
Subgenus *Culicella* Felt			
Culiseta minnesotae Barr	Adult	East Haven, CT; 7/26/01	AY988452
Culiseta morsitans (Theobald)	Adult	East Haven, CT; 8/22/00	AY988451
Genus *Ochlerotatus* Lynch Arribalzaga			
Subgenus *Finlaya* Theobald			
Ochlerotatus japonicus japonicus (Theobald)	Adult	Kent, CT; 5/15/00	AY988431
Subgenus *Ochlerotatus* Lynch Arribalzaga			
Ochlerotatus abserratus (Felt and Young)	Adult	Chester, CT; 4/23/99	AY988426
Ochlerotatus atropalpus (Coquillett)	Larvae	North Haven, CT; 10/22/99	AY988438
Ochlerotatus aurifer (Coquillett)	Adult	Stonington, CT; 7/2/01	AY988427
Ochlerotatus canadensis canadensis (Theobald)	Adult	Barkhamsted, CT; 4/22/99	AY988433
Ochlerotatus cantator (Coquillett)	Adult	East Haven, CT; 8/22/00	AY988428
Ochlerotatus communis (De Geer)	Larva	Barkhamsted, CT; 4/8/99	AY988425
Ochlerotatus excrucians (Walker)	Adult	Winsted, CT; 7/6/99	AY988430
Ochlerotatus punctor (Kirby)		Miller et al. 1997	U48378
Ochlerotatus sollicitans (Walker)	Adult	Old Lyme, CT; 6/9/00	AY988435
Ochlerotatus sticticus (Meigen)	Adult	Litchfield, CT; 8/30/00	AY988437
Ochlerotatus stimulans (Walker)	Larva	Hamden, CT; 3/3/99	AY988429
Ochlerotatus taeniorhynchus (Wiedemann)	Adult	Darien, CT; 6/28/00	AY988436
Ochlerotatus trivittatus (Coquillett)	Adult	Milford, CT; 6/15/00	AY988434
Subgenus *Protomacleaya* Theobald			
Ochlerotatus triseriatus (Say)	Adult	Laboratory colony, CAES (Waterford, CT; 7/95)	AY988432
Genus *Psorophora* Robineau-Desvoidy			
Subgenus *Janthinosoma* Lynch Arribalzaga			
Psorophora ferox (von Humboldt)	Adult	East Haven, CT; 8/22/00	AY988442
Subgenus *Psorophora* Robineau-Desvoidy			
Psorophora ciliata (F.)	Adult	Old Lyme, CT; 8/21/00	AY988443
Genus *Toxorhynchites* Theobald			
Subgenus *Lynchiella* Lahille			
Toxorhynchites rutilus septentrionalis (Dyar and Knab)	Adult	Stonington, CT; 9/5/00	AY988455
Subgenus *Toxorhynchites* Theobald			
Toxorhynchites ambionensis (Doleschall)		Miller et al. (1997)	U48377
Genus *Uranotaenia* Lynch Arribalzaga			
Subgenus *Uranotaenia* Lynch Arribalzaga			AY988444
Uranotaenia sapphirina (Osten Sacken)	Adult	East Haven, CT; 8/22/00	
Outgroup Taxa			
Dixella cornuta Johannsen		Miller et al. (1997)	U48381
Lutzomyia shannoni (Dyar)		Miller et al. (1997)	U48382
Nephrotoma altissima Osten Sacken		Miller et al. (1997)	U48379
Tipula L. species		Unpublished	X89496

CAES, The Connecticut Agricultural Experiment Station, New Haven, CT; USDA, United States Department of Agriculture, Gainesville, FL.

Table 2. Primers used for PCR and sequencing reactions

Primer name	Sequence (5′-3′)	Used for
ARA28F	CTGGTTGATCCTGCCAGTAG	Amplification, sequencing
INS326F	CTTTCAAATATCTGCCCTATC	Sequencing
ARA400R	GGGTCGATTCCGGAGAGG	Sequencing
INS647F	TTGTTGCGGTTAAAAMGYTCG	Sequencing
MOS788R	GAGCACTCTAATTTGTTCAAGG	Sequencing
ARA1026F	GGTTCGAAGGCGATCAGATAC	Sequencing
ARA1160R	GGAATTGACGGAAGGGCACCAC	Sequencing
MOS1441F	GACCGATAACAGGTCCGTGATGC	Sequencing
ARA1479R	ACGCGCGCTACACTGAAGGAA	Sequencing
ARA16SENDR	CGTAGGTGAACCTGCGGAAG	Amplification, sequencing

M, A/C; Y, C/T.

Parameters for base frequencies, substitution model, and among-site variation as determined by ModelTest were executed in Maximum Likelihood analysis in PAUP*. Neighbor-Joining analyses were calculated using heuristic search settings, with DNA distances calculated using the unrooted ("p") option, and among-site variation was assumed to be zero. Bootstrap analyses were performed using 1,000 replicates based on Neighbor-Joining settings for the primary and secondary structure alignments. For Maximum Parsimony analyses, all characters were unordered, assigned equal weight, and gaps were treated as missing data.

Results

Phylogenetic analysis based on primary (with and without gaps) and secondary structure alignments for 18S rDNA of 39 mosquito species and four additional dipteran outgroup taxa, demonstrated a number of consistent relationships. In all analyses, 1) *Aedes* and *Ochlerotatus* formed two distinct clades; 2) *Ochlerotatus abserratus* (Felt & Young) (=*Aedes abserratus;* see Reinert 2000), *Ochlerotatus cantator* (Coquillett) (=*Aedes cantator;* see Reinert 2000), *Ochlerotatus communis* (De Geer) (=*Aedes communis;* see Reinert 2000), and *Ochlerotatus punctor* (Kirby) (=*Aedes punctor;* see Reinert 2000) were consistently grouped in the same arrangement; 3) *Ochlerotatus sollicitans* (Walker) (=*Aedes sollicitans;* see Reinert 2000) and *Ochlerotatus taeniorhynchus* (Wiedemann) (=*Aedes taeniorhynchus;* see Reinert 2000) were consistently grouped together, as were the pairings of *Ochlerotatus sticticus* (Meigen) (=*Aedes sticticus;* see Reinert 2000) with *Ochlerotatus trivittatus* (Coquillett) (=*Aedes trivittatus;* see Reinert 2000) and *Ochlerotatus canadensis* (Theobald) (=*Aedes canadensis;* see Reinert 2000) with *Ochlerotatus triseriatus* (Say) (=*Aedes triseriatus;* see Reinert 2000); 4) *Anopheles* Meigen was a sister taxon to all of the included Culicidae; 5) *Toxorhynchites* Theobald was the sister taxon to the Culicinae; and 6) *Culex territans* Walker was a sister taxon to the other *Culex* species. The phylogenetic relationships among mosquito taxa, using the alignment based on the secondary structure of 18S rRNA, were best demonstrated by bootstrap analysis based on Neighbor-Joining criteria (Fig. 1), bootstrap analysis based on Maximum Likelihood criteria (Fig. 2), and a single Maximum Likelihood analysis with sequence evolution parameters optimized by ModelTest (Fig. 3.). The discrepancies among phylogenetic analyses were exemplified by the inconsistent placements of *Psorophora* Robineau-Desvoidy, *Uranotaenia* Lynch Arribalzaga, and *Coquillettidia* Dyar. Maximum Likelihood analysis of the aligned sequences based on secondary structure did not show phylogenetic relationships that differed at the generic or subgeneric level from those determined by sequence evolution model HKY85 (figure not shown) and with parameters based on the TrN+I+G model as calculated by ModelTest 3.7 (Fig. 3). The major exceptions were the relationships shown among *Culex* species (other than *Cx. territans*). The analysis produced using ModelTest criteria (Fig. 3) showed members of the *Culex pipiens* L. group as basal taxa relative to the subgenus *Culex* L. This relationship was in disagreement with both bootstrap analyses (Figs. 1 and 2).

A trichotomy consisting of *Psorophora*, *Culiseta* Felt (with *Coquillettidia*) and *Culex*, as shown in the bootstrap tree based on Neighbor-Joining (Fig. 1), also was demonstrated by Maximum Parsimony analysis of the primary sequence structure alignment (figure not shown). Based on Maximum Likelihood analysis with optimized sequence evolution parameters (Fig. 3) *Psorophora* was shown as a sister taxon to the *Culiseta*/*Culex* clade. Bootstrap analysis using Maximum Likelihood criteria (Fig. 2) based on the secondary structure alignment, placed *Psorophora* in a polychotomous relationship to the *Aedes*/*Ochlerotatus* clade, the *Culiseta*/*Culex* clade and *Uranotaenia sapphirina* (Osten Sacken).

In all of our phylogenetic analyses, the position of *Uranotaenia* was poorly resolved. The genus was shown in both bootstrap analyses (Figs. 1 and 2) as a trichotomy with clades consisting of *Aedes*/*Ochlerotatus* and *Psorophora*/*Culiseta* Felt (+ *Coquillettidia*)/*Culex*. *Ur. sapphirina* was shown as the sister group to the *Aedes*/*Ochlerotatus* clade in Maximum Likelihood bootstrap analysis based primary sequence alignment (figure not shown), Maximum Parsimony analyses (figure not shown), and in Maximum Likelihood analysis (Fig. 3). *Uranotaenia* also was shown to be the sister group to all genera except *Anopheles* and *Toxorhynchites* by Neighbor-Joining analysis based on the

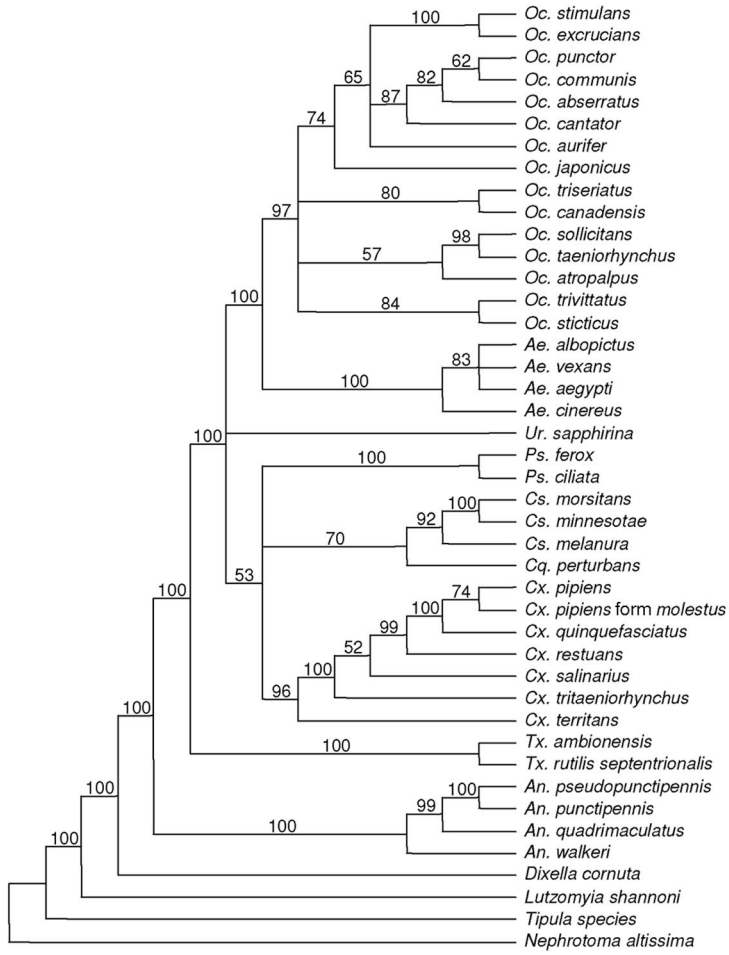

Fig. 1. Bootstrap analysis based on 1,000 replicates using the Neighbor-Joining method of PAUP* 4.0b (Swofford 1998). Tree produced for 39 mosquito species with *D. cornuta*, *L. shannoni*, *Tipula* sp., and *N. altissima* as outgroup taxa. Analysis based on alignment of sequences corresponding to secondary structure of 18S rRNA.

primary sequence structure alignment (figure not shown).

Coquillettidia perturbans Dyar was shown as a sister taxa to *Culiseta* (Figs. 1–3) except for Maximum Parsimony analysis and Maximum Likelihood analysis (using HKY85 model) based on the secondary structure alignment (figures not shown). In the former analysis, *Cq. perturbans* was shown in an unresolved position relative to *Culex*, *Culiseta*, *Psorophora*, and a clade comprised of *Uranotaenia*, *Aedes*, and *Ochlerotatus.* In the latter tree, *Cq. perturbans* was shown as a sister group to *Culex* and *Culiseta.*

Aedes and *Ochlerotatus* species formed two monophyletic groups in all of the analyses. The species retained in the genus *Aedes* and the species reclassified in the genus *Ochlerotatus* by Reinert (2000) were phylogenetically distinct groups. The separation of *Ochlerotatus* from *Aedes* was supported with high bootstrap values (Figs. 1 and 2). The relationships among the *Aedes* included in our analyses were not well resolved at the species level. The topologies of the bootstrap analyses based on Neighbor-Joining (Fig. 1) and Maximum Likelihood (Fig. 2) were similar, with *Ae. cinereus* Meigen forming a sister group to *Aedes albopictus* (Skuse), *Aedes aegypti* (L.), and *Aedes vexans* (Meigen). Maximum Parsimony analysis could not differentiate among the four species (figure not shown). Maximum Likelihood analysis based on ModelTest criteria (Fig. 3) placed *Ae. albopictus* as the ancestral species followed by *Ae. vexans*, *Ae. aegypti*, and *Ae. cinereus.*

Discussion

This study demonstrates the usefulness and limitations of 18S rDNA sequence analysis for evaluating phylogenetic relationships among mosquitoes. Our results reveal a monophyletic placement of *Ochlerotatus* consistent with its elevation to generic rank as proposed by Reinert (2000). Other notable findings presented here are 1) the placement of *Psorophora* species as a sister group to *Culex* and *Culiseta* or as sister

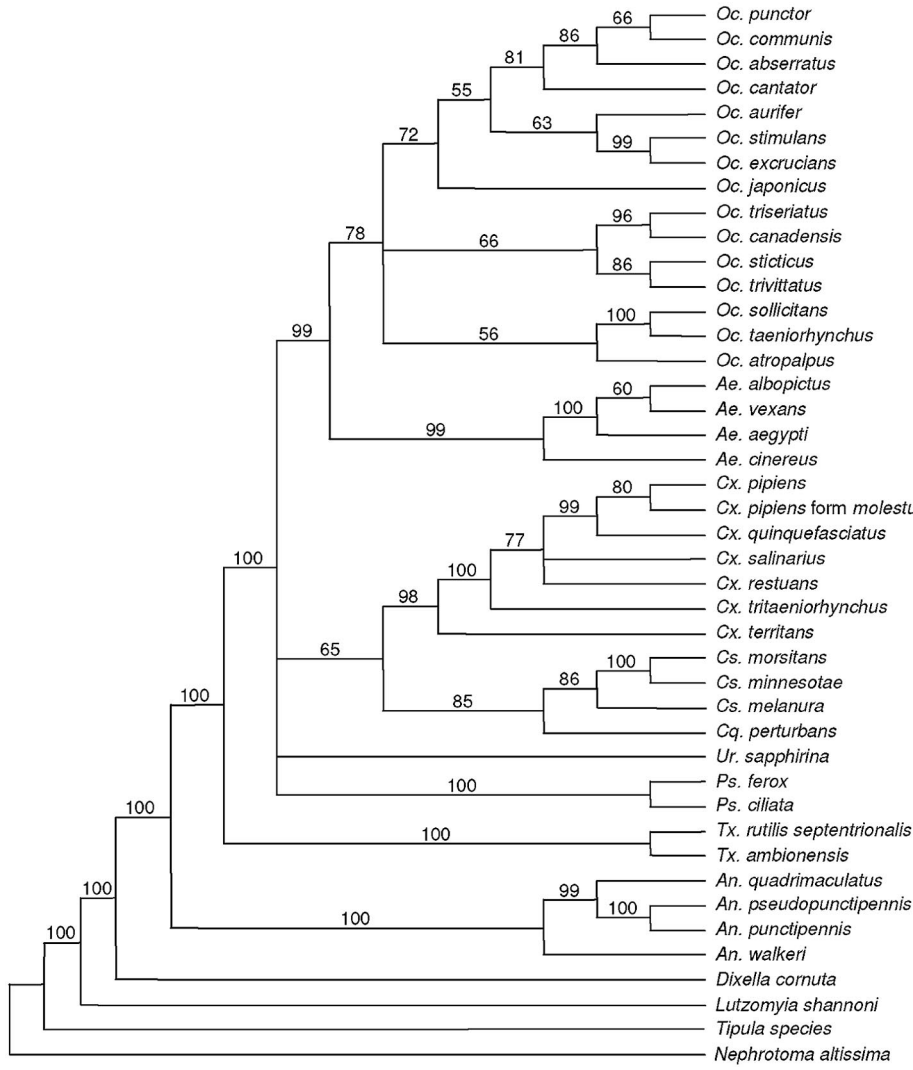

Fig. 2. Bootstrap analysis based on 100 replicates using the Maximum Likelihood method of PAUP* 4.0b (Swofford 1998). Tree produced for 39 mosquito species with *D. cornuta*, *L. shannoni*, *Tipula* sp., and *N. altissima* as outgroup taxa. Analysis based on alignment of sequences corresponding to secondary structure of 18S rRNA.

group to the *Aedes* and *Ochlerotatus* species; 2) the possibility that *Uranotaenia* is either the sister group to the *Aedes* and *Ochlerotatus* species, or the ancestor to all species included in the study except the *Anopheles* and *Toxorhynchites* species; and 3) the placement of *Cq. perturbans* with members of *Culiseta.* The phylogenetic relationships of each genus are addressed below.

Anopheles. Our data show that the genus *Anopheles* is the sister taxon to the remaining Culicidae. This is supported by previous phylogenetic analyses based on morphology (Harbach and Kitching 1998), single copy genes (Besansky and Fahey 1997, Krzywinski et al. 2001), and ribosomal RNA genes (Miller et al. 1997). The analysis of the 18S rDNA was effective in delineating the relationships among the major *Anopheles* species found in the region. Furthermore, *Anopheles walkeri* Theobald, which overwinters in the egg stage rather than as a diapausing adult (Means 1987), was the most distantly related of the four species.

Toxorhynchites. The two species representing the genus *Toxorhynchites* formed a monophyletic sister group to the other Culicinae in this study. This position is in agreement with classical systematics and consistent with phylogenetic examination of the white-gene (Besansky and Fahey 1997), 18S and 5.8S rDNA (Miller et al. 1997), and vitellogenin genes (Isoe 2000). Based on our findings, *Toxorhynchites* could be included in the subfamily Culicinae as proposed by Harbach and Kitching (1998) or considered as a separate subfamily as proposed by Mitchell et al. (2002).

Psorophora. The *Psorophora* species were shown as sister taxa to a clade consisting of *Culex*, *Culiseta*, and *Coquillettidia* in multiple analyses. Although the spe-

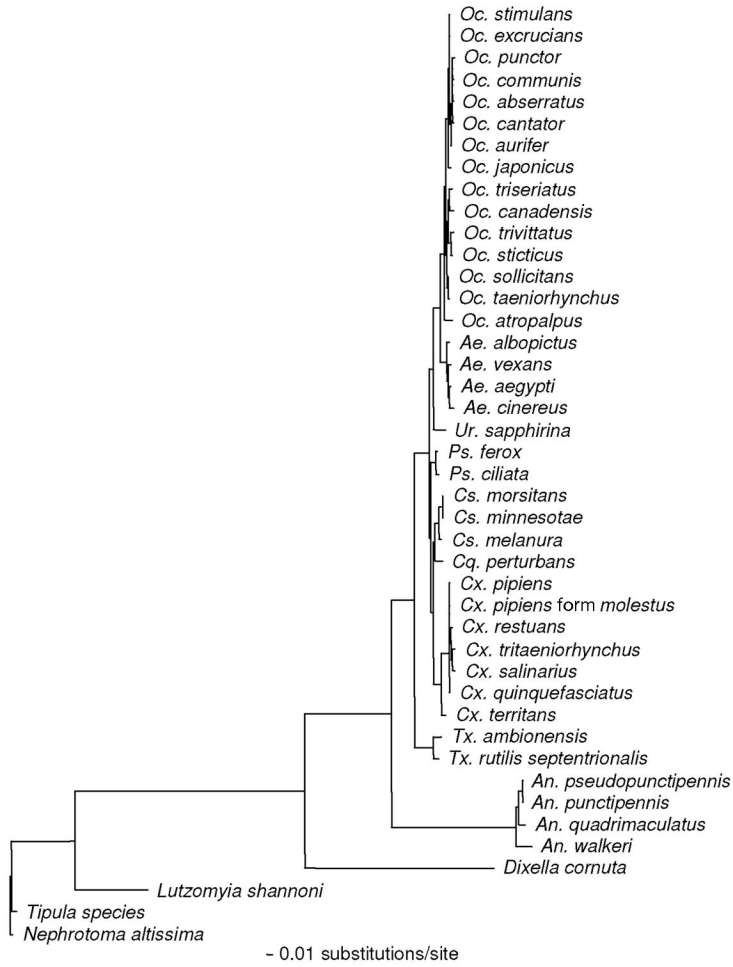

Fig. 3. Maximum Likelihood analysis with sequence evolution model TrN+I+G as calculated by ModelTest 3.7 (Posada and Crandall 1998) and executed using PAUP* 4.0b (Swofford 1998). Tree produced for 39 mosquito species with *D. cornuta*, *L. shannoni*, *Tipula* sp., and *N. altissima* as outgroup taxa. Analysis based on alignment of sequences corresponding to secondary structure of 18S rRNA.

cific placements of the genus with the *Culex* and *Culiseta* clades were in disagreement (Neighbor-Joining, *Culex* basal [figure not shown]; Maximum Likelihood, *Psorophora* basal; Fig. 3), these analyses of the 18S rDNA data differed radically from the traditional taxonomic and phylogenetic placement of *Psorophora* with *Aedes* and *Ochlerotatus.* Bootstrap analyses based on Neighbor-Joining (Fig. 1) and Maximum Likelihood trees (Fig. 2) show *Psorophora* in unresolved relationships with *Aedes*/*Ochlerotatus* and *Culex*/*Culiseta* clades.

The grouping of *Psorophora* with *Aedes*/*Ochlerotatus* has been previously supported based on morphology and biology (e.g., eggs laid singly, larval characters, and male genitalia) (Edwards 1932, Clements 1992, Savage and Strickman 2004). Additionally, phylogenetic studies based on morphological characters (Harbach and Kitching 1998, Reinert et al. 2004) sequences of the vitellogenin gene (Isoe 2000), and numerical taxonomy (Hendrickson and Sokal 1968) have supported the traditional classification (Edwards 1932). Wesson et al. (1992) placed *Psorophora ferox* (von Humboldt) as a sister taxon to *Oc. triseriatus* based on sequence and secondary structure data of ITS2 rDNA, but their study only included seven species from four genera.

Traditional morphological features show that adult female *Psorophora* have a characteristically pointed abdomen similar to *Aedes* and *Ochlerotatus.* In contrast, *Culex*, *Culiseta*, and *Coquillettidia* all possess bluntly rounded abdomens (Edwards 1932, Carpenter and LaCasse 1955, Darsie and Ward 1981). Adult *Psorophora* and *Culiseta* species possess prespiracular setae, a well established diagnostic character for both genera (Reinert et al. 2004). These setae are absent in *Coquillettidia* and *Culex* (Reinert et al. 2004). Harbach and Kitching (1998) observed two shared characters between *Psorophora* and *Coquillettidia* + *Mansonia* Blanchard species. Members of these genera possess 1) a complete saddle on larval abdominal segment X,

and 2) a constricted interocular space in females. Of the two characters, the complete saddle provides stronger support for their phylogenetic grouping (Harbach and Kitching 1998). Reinert el al. (2004) demonstrated a phylogenetic grouping of *Psorophora* species with *Mansonia titillans* (Walker) based on adult and immature morphological characters. In both of these analyses (Harbach and Kitching 1998, Reinert et al. 2004), *Culiseta* and *Culex* were shown in an ancestral position, and *Aedes* was shown in a derived position relative to *Psorophora.* Because there is little morphological evidence to support the placement of *Psorophora* as a sister group to *Culex* and *Culiseta* as observed in our analyses, additional genes should be sequenced for more *Psorophora* species.

Culiseta* and *Coquillettidia. Our phylogenetic analyses show *Cq. perturbans* and *Culiseta* species as sister taxa and follow the subgeneric groupings of the *Culiseta* proposed by Knight and Stone (1977): *Culiseta melanura* (Coquillett)=*Climacura* Howard, Dyar & Knab; *Culiseta minnesotae* Barr and *Culiseta morsitans* (Theobald)=*Culicella* Felt. *Cq. perturbans* is the sole representative of the tribe Mansoniini occurring in North America. The tribe Mansoniini is noted for its uniquely adapted respiratory siphon, which is used to pierce the stems of emergent vegetation (Carpenter and LaCasse 1955, Means 1987, Andreadis et al. 2005). The *Culiseta* species included in our study share few life history similarities with *Cq. perturbans* except the manner in which eggs are laid. *Cq. perturbans*, *Cs. melanura,* and *Cs. minnesotae* lay eggs in the form of rafts on the surface of the water, whereas *Cs. morsitans* lay single eggs on damp soil (Means 1987). *Cq. perturbans* and *Cs. melanura* also overwinter as larvae in this region, whereas *Cs. morsitans* and *Cs. minnesotae* overwinter as mated females (Means 1987). Additional species of *Culiseta* and *Mansonia* should be sequenced to clarify the relationship between *Cq. perturbans* and *Culiseta.*

Culex. The *Culex* species included in this study formed a monophyletic group that agreed with the subgeneric classifications (Edwards 1932); *Cx. territans,* a member of subgenus *Neoculex* Dyar, was the sister taxon to the remaining *Culex* in this study. These findings agree with previous sequence analysis of the ITS region (Miller et al. 1996).

Culex pipiens and *Culex quinquefasciatus* (Coquillett) differ in their behavior and geographical distribution. *Cx. pipiens* is widely distributed throughout northern latitudes (Darsie and Ward 2005) and is anautogenous (requires a bloodmeal for egg development), eurygamous (breeds in open areas), and diapausing (Clements 1992, Vinogradova 2000). Females occasionally bite mammals, including humans, but they are largely ornithophilic (Magnarelli 1977; Spielman 2001; Apperson et al. 2002, 2004). *Cx. quinquefasciatus* is widespread in tropical and subtropical regions (Vinogradova 2000, Darsie and Ward 2005). It is also anautogenous but exhibits more stenogamous (breeds in confined areas) behavior and does not diapause (Vinogradova 2000). Females are also ornithophilic but seem to feed much more readily on mammalian hosts, including humans than do *Cx. pipiens* (Edman 1974, Tempelis 1975, Vinogradova 2000). Hybrids have been reported in areas where the habits or ranges overlap, exhibiting morphological or behavioral differences from parental forms (Barr 1957, Miller et al. 1996, Spielman 2001, Fonseca et al. 2004). Four nucleotide differences were observed between *Cx. pipiens* and *Cx. quinquefasciatus.* This was of interest because these two species are very similar morphologically. The only reliable characters for separating these two species, in regions where they overlap, are structures of the male genitalia (Barr 1957).

No differences were found in the 18S rDNA sequences obtained from *Cx. pipiens* and *Cx. pipiens* form *molestus* Forskal collected from an underground habitat in New York City. These two forms have morphologies that are virtually identical but exhibit well documented behavioral and physiological traits (Spielman 1964, 1971, 2001; Byrne and Nichols 1999; Vinogradova 2000). *Cx. pipiens* form *molestus*, which has adapted to inhabiting artificial subterranean structures, such as subway tunnels and sewers, is autogenous and stenogamous (Clements 1992). Unlike aboveground populations of *Cx. pipiens*, it breeds throughout the year and will readily bite humans. Populations of these two forms will interbreed at certain times of the year (Spielman 1964, 1971, 2001) and can be differentiated using highly polymorphic DNA microsatellite loci (Fonseca et al. 2004).

Uranotaenia. The classification of the genus *Uranotaenia* is not well defined and has not been extensively studied (Peyton 1972). Our findings are inconclusive, indicating that *Ur. sapphirina* is either the sister group to 1) *Aedes* and *Ochlerotatus* as observed in Maximum Parsimony analysis and Maximum Likelihood analyses based on primary sequence alignments (figures not shown), or 2) both the *Culex*/*Culiseta* and *Aedes*/*Ochlerotatus* clades (Figs. 1 and 2). This close relationship with *Aedes* and *Ochlerotatus* is surprising because adult *Uranotaenia* differ greatly in morphology. Harbach and Kitching (1998) described shared morphology between *Uranotaenia* and *Aedeomyia* Theobald. Additionally, morphological similarities of male genitalia were shared with members of the Anophleinae and Aedini, and the shape of the larval head capsule was similar to Anopheleinae and *Toxorhynchites.* However, the authors were unable to fully resolve the phylogenetic relationships of *Uranotaenia* with other taxa.

Aedes. Our phylogenetic analyses show *Aedes* and *Ochlerotatus* to be two distinct, monophyletic sister taxa. This supports the elevation of *Ochlerotatus* to the genus level as proposed by Reinert (2000), or at least indicates that they are not polyphyletic taxa. Neither we, using our 18S rDNA data set, nor Rey et al. (2001), using cytochrome oxidase subunit I (COI) sequence, could obtain any conclusive results concerning the relationships among the *Aedes* species. The four *Aedes* species used in this study seem to be too closely related for differentiation by 18S rDNA analysis. They have >99% sequence similarity on a pairwise basis, and there are only four parsimony informative characters

among the four *Aedes* species. We acknowledge that our data only includes *Aedes* that are currently distributed in the New World and that additional Old World species will need to be analyzed to more fully resolve this important issue.

Recently, Reinert et al. (2004) conducted phylogenetic analyses on species classified in the tribe Aedini based on the morphologies of immature and adult specimens and elevated the subgenus *Stegomyia* Theobald to generic ranking. This included *Ae. aegypti* and *Ae. albopictus*, which would now become *Stegomyia aegypti* (L.) and *Stegomyia albopicta* Skuse. In their analyses (Reinert et al. 2004), the subgenus *Aedes*, including the type species *Ae. cinereus*, was shown as a monophyletic group. Additionally, the subgenus *Aedimorphus* was designated as a polyphyletic group, and as a result, the authors were reluctant to elevate the subgenus to the genus level, thus retaining both *Ae. cinereus* and *Ae. vexans* in the genus *Aedes* (Reinert et al. 2004). Our data are not informative enough to allow us to address these issues.

Ochlerotatus. The phylogenetic relationships among the *Ochlerotatus* species included in our study largely follow traditional groupings founded on morphology. *Oc. atropalpus* seems to be the most divergent taxon based upon parsimony analysis (figure not shown) of the 18S rDNA sequence. Reinert et al. (2004) similarly placed *Oc. atropalpus* in a basal group among the *Ochlerotatus.* This species exhibits a preference for oviposition in rock holes located along river and stream beds (Means 1979, Andreadis et al. 2005), and unlike most other members of the genus, females are autogenous for the first gonotrophic cycle (Carpenter and LaCasse 1955, Means 1979, Bowen et al. 1994).

The next phylogenetic grouping included two multivoltine species, *Oc. sollicitans* and *Oc. taeniorhynchus*, that develop in costal saltmarshes and have immature stages that tolerate high salinities (Means 1979, Crans and McNelly 1997). These species are also widely distributed throughout temperate eastern North America, with *Oc. sollicitans* having a broader range, developing in brackish waters located in the midwestern United States as well (Darsie and Ward 1981). Based on shared morphological characters of the male genitalia, bands of light scales on the tarsi, and common breeding habitats, taxonomists have long considered that these two species form a separate group within the subgenus *Ochlerotatus* (Edwards 1932). Isozyme analysis (Schultz et al. 1986) placed *Oc. sollicitans* and *Oc. taeniorhynchus* as a basal group to other brackish water *Ochlerotatus* species such as *Oc. cantator*, and freshwater species such as *Oc. canadensis*, a relationship that cannot be completely supported by our findings.

The next two species that formed a phylogenetic group were *Oc. trivittatus* and *Oc. sticticus.* These two early season "flood-water" species share similar life histories (Means 1979). However, *Oc. trivittatus* is multivoltine (Andreadis et al. 2005), and it is not entirely clear whether the second generation of *Oc. sticticus* represents delayed or "installment" egg hatch (Crans and McNelly 1997). Edwards (1932) included *Oc. trivittatus* in the *scapularis* group along with species thought to arise from South America, a hypothesis also accepted by Ross (1964). In the northeastern United States, the only species representing the *scapularus* group is *Oc. trivittatus*, perhaps demonstrating an evolutionary adaptation to cold temperatures. *Oc. sticticus* was placed within the *communis* group (Edwards 1932), a group consisting of dark-legged species inhabiting North America and Europe. One of the characters used by Edwards to separate the *scapularis* and *communis* groups was the presence or absence of lower mesepimeral setae. Lunt and Nielsen (1971a,b) observed that although both species lack these structures, there was not sufficient variation to remove *Oc. sticticus* from the *communis* group. Rey et al. (2001) included *Oc. sticticus* in a phylogeny of European *Ochlerotatus* species, by using COI sequence data, and showed *Oc. sticticus* as an ancestral species within the *communis* group. Based on our 18S rDNA analysis, *Oc. sticticus* seems likely to have shared a common ancestor with *Oc. trivittatus* as well as other North American *Ochlerotatus* species. This relationship is in contradiction to the taxonomic groupings that have been traditionally accepted (Edwards 1932).

The grouping of *Oc. canadensis* with *Oc. triseriatus* represents a clear deviation from their subgeneric placements in *Ochlerotatus* and *Protomacleaya* Theobald, respectively (Zavortnik 1972). The two species differ greatly in their biology and morphology. *Oc. triseriatus* typically develops in natural and artificial container habitats, whereas *Oc. canadensis* develops in vernal woodland pools. Morphologically, the two seem vastly different in larval and adult forms (Carpenter and LaCasse 1955, Means 1979, Andreadis et al. 2005). However, both species are considered to have evolved in the New World (Ross 1964), and our analyses suggest they share a common ancestor.

Oc. triseriatus was previously grouped within the subgenus *Finlaya* Theobald until reclassified within the subgenus *Protomacleaya* by Zavortnik (1972), a subgenus consisting entirely of New World species. The reclassification of *Oc. triseriatus* into the subgenus *Protomacleaya* from *Finlaya* by Zavortnik (1972) is supported in our analyses of 18S rDNA.

The last phylogenetic group consists of *Oc. aberratus*, *Ochlerotatus aurifer* (Coquillett), *Oc. cantator*, *Oc. communis*, *Ochlerotatus excrucians* (Walker), *Oc. japonicus*, *Oc. punctor*, and *Ochlerotatus stimulans* (Walker), species that are widely distributed in the Holarctic realm. Ross (1964) proposed that these species (except *Oc. japonicus*) evolved from a common ancestor and dispersed throughout what is now North America and Europe. All of these species are well adapted to cold climates, and larvae are commonly found as early instars in water that is partially or completely covered with ice (Means 1979). With the exception of *Oc. cantator* and *Oc. japonicus*, they are all univoltine (Andreadis et al. 2005).

Oc. excrucians and *Oc. stimulans* have nearly identical biologies and life histories, are morphologically similar as adults, but differ in the larval form. Isozyme

analysis (Eldridge et al. 1986) indicated a close but undefined relationship between *Oc. excrucians* and *Oc. stimulans.* Our 18S rDNA sequences contain three nucleotide differences between the two. Both species have been traditionally classified in the *annulipes* group (Edwards 1932) of the subgenus *Ochlerotatus*, a group of "snow pool" species, possessing tarsi with bands of light-colored scales. Our analyses place *Oc. excrucians* and *Oc. stimulans* as a sister group to the other snow pool *Ochlerotatus.*

Oc. cantator is another species traditionally classified in the *annulipes* group (Edwards 1932). However, the 18S rDNA sequence data place it with species of the *communis* group. Unlike the other species in this phylogenetic group, *Oc. cantator* is multivoltine, and larvae develop in coastal saltmarshes, similar to *Oc. sollicitans* and *Oc. taeniorhynchus*, as well as in inland pools with moderate salinity (Means 1979, Crans and McNelly 1997). Additionally, *Oc. cantator* is distributed only in North America and is not found in Europe (Knight and Stone 1977). Schultz et al. (1986) placed *Oc. cantator* as a sister group to *Oc. canadensis* and other western *Ochlerotatus* species, a relationship that is not supported in the current study. Based on the phylogenetic analyses of 18S rDNA, *Oc. cantator* seems likely to have evolved with other cold climate species, as a descendent of *Oc. sollicitans* and *Oc. taeniorhynchus*, and retained its tolerance for high salinities.

The remaining members of this clade have evolved to maximize the short summer experienced in colder regions of North America, Europe and northern Asia (Knight and Stone 1977). The northeastern region is near the southern end of the distribution for *Oc. abserratus*, *Oc. aurifer*, *Oc. communis*, and *Oc. punctor* in North America (Darsie and Ward 1981). *Oc. abserratus*, *Oc. aurifer*, *Oc. communis*, and *Oc. punctor* all develop in vernal pools formed by melted snow in woodland habitats, produce a single generation each year, and are predominantly mammalian feeders (Means 1979, Andreadis et al. 2005).

Conclusion. The utility of 18S rDNA for evaluating phylogenetic and evolutionary relationships among mosquito taxa has been demonstrated and seems to produce reliable results at the generic and subgeneric levels. The phylogenies presented here agree with the substantiated classifications based on morphological (Edwards 1932) and molecular data (Besansky and Fahey 1997, Miller et al. 1997, Harbach and Kitching 1998, Isoe 2000). We feel that our data add significantly to understanding the evolutionary relationships among the Culicidae. *Psorophora* has traditionally been grouped as a sister taxon to *Aedes* and *Ochlerotatus*, but we conclude from our analysis that this genus may be more closely related to *Culiseta, Coquillettidia*, and *Culex. Uranotaenia* seems to be a sister taxon to the *Aedes* and *Ochlerotatus* clade or a basal taxon to all other culicines. We have described a consistent relationship among four species of *Ochlerotatus* (*Oc. punctor, Oc. communis, Oc. abserratus*, and *Oc cantator*). Although the exact relationships among a number of *Ochlerotatus* species could not be resolved, we saw three groups of very closely related species (*Oc triseriatus*/*Oc. canadensis*, *Oc. sollicitans*/*Oc. taeniorhynchus*, and *Oc. trivittatus*/*Oc. sticticus*). The phylogenetic relationships determined by our analysis of 18S rDNA show that *Ochlerotatus* and *Aedes* are two separate monophyletic groups. The morphology of female (insula) and male (proctiger) genitalia as described Reinert (2000), provide an apomorphic character set, which was likely to have arisen once, separating adult *Ochlerotatus* from *Aedes.* In conclusion, our phylogenetic analyses provide data consistent with the elevation of *Ochlerotatus* to the generic level as proposed by Reinert (2000), however, additional *Aedes* species, from other regions, should be analyzed to completely resolve this change in nomenclature.

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