Intragenomic variation in the second internal transcribed spacer of the ribosomal DNA of species of the genera *Culex* and *Lutzia* (Diptera: Culicidae)

Fabiana Tavares Vesgueiro[1], Bruna Demari-Silva[1], Rosely dos Santos Malafronte[2], Maria Anice Mureb Sallum[1], Mauro Toledo Marrelli[1]/+

[1]Departamento de Epidemiologia, Faculdade de Saúde Pública [2]Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, São Paulo, SP, Brasil

Culex *is the largest genus of Culicini and includes vectors of several arboviruses and filarial worms. Many species of* Culex *are morphologically similar, which makes their identification difficult, particularly when using female specimens. To aid evolutionary studies and species distinction, molecular techniques are often used. Sequences of the second internal transcribed spacer (ITS2) of ribosomal DNA (rDNA) from 16 species of the genus* Culex *and one of* Lutzia *were used to assess their genomic variability and to verify their applicability in the phylogenetic analysis of the group. The distance matrix (uncorrected p-distance) that was obtained revealed intragenomic and intraspecific variation. Because of the intragenomic variability, we selected ITS2 copies for use in distance analyses based on their secondary structures. Neighbour-joining topology was obtained with an uncorrected p-distance. Despite the heterogeneity observed, individuals of the same species were grouped together and correlated with the current, morphology-based classification, thereby showing that ITS2 is an appropriate marker to be used in the taxonomy of* Culex.

Key words: Diptera - *Culex* - ITS2 - rDNA - phylogeny - concerted evolution

Culex Linnaeus is the largest genus of the Culicine with 768 species subdivided into 26 subgenera (Harbach 2011). The *Culex* genus includes species suspected of being potential vectors for filarial worms and several arboviruses, including the Venezuelan equine encephalitis virus (Weaver et al. 2004) and the West Nile virus (Turell et al. 2001). In the Americas, members of this extensive genus are subdivided into 14 subgenera. The subgenera *Melanoconion* Theobald and *Culex* Linnaeus are those of greatest epidemiological importance in the region and include most of the neotropical species. In Brazil, the Saint Louis encephalitis virus has been isolated from *Culex* (*Culex*) *coronator* Dyar and Knab and *Culex* (*Cux.*) *declarator* Dyar and Knab (Vasconcelos et al. 1991). *Culex* (*Cux.*) *quinquefasciatus* Say is the main vector for *Wuchereria bancrofti*, with active transmission in areas in the Northeast of Brazil (Fontes et al. 1998, 2005). Despite the medical importance of some species of the genus *Culex* and the great discomfort they cause, little progress has been made in the taxonomy and systematics of the genus. Most *Culex* mosquito research focuses on the epidemiology of arboviruses and potential vectors (Turell et al. 2001, Weaver et al. 2004) and on the control of species that transmit pathogens to humans and other vertebrates (Regis et al. 1995, Melo et al. 2009).

The taxonomic history of *Culex* demonstrates the complexity of the group and the difficulty in differentiating its species using traditional morphological criteria alone. The morphological identification of these mosquitoes is difficult because many species of *Culex* are morphologically similar (Bram 1967, Williams & Savage 2009). Furthermore, some *Culex* species belong to species complexes (Smith & Fonseca 2004), there are hybrid forms (Humeres et al. 1998, Smith & Fonseca 2004) and intraspecific variations in established diagnostic characteristics are frequently reported (Rey et al. 2006). Lastly, field-collected adult specimens often lack ornamentation characteristics, making their identification difficult based on morphology alone (Knight & Nayar 2004). There are few taxonomic studies for groups of *Culex*. In Bram's (1967) revision of neotropical species of *Culex* and the subgenus *Culex*, a description of a new species, a re-description of several species and identification keys for the male genitalia and fourth instar larval characteristics are included. In another study, Tanaka (2003) elevated the subgenus *Lutzia* Theobald to the status of genus of the Culicine tribe.

Accurate species identification is fundamental for ecological, biological and epidemiological studies. Diagnosis based on traditional methods, including the external structures of the male genitalia, allows for initial identification that can be further tested using molecular techniques. Thus, many species previously considered unique and homogeneous turn out to consist of other isomorphic forms (Forattini 2002). Ribosomal DNA (rDNA) sequences have been used widely in mosquito taxonomy and phylogenetic analyses (Walton et al. 1999, Sallum et al. 2002, Marrelli et al. 2005). Due

Financial support: FAPESP (05/50225-2 for MTM and 05/53973-0), CNPq (BPP 300351/2008-9 to MAMS)
FTV was a Master's student supported by CAPES.
+ Corresponding author: mmarelli@usp.br
Received 23 April 2010
Accepted 4 November 2010

to the concerted evolution mechanism, multiple copies of rDNA tend to homogenize, maintaining a low level of intraspecific and intragenomic variation. Thus, rDNA transcribed spaces (ITS) sequences prove useful for solving evolutionary relationships at different taxonomic levels, including recently diverged taxa. It is also helpful for solving problems associated with the identification of morphologically similar species by polymerase chain reaction (PCR) (Collins & Paskewitz 1996, Djadid et al. 2007). The first molecular approach for investigating the phylogenetic relationships between 14 species of four subgenera of the *Culex* genus was carried out by Miller et al. (1996), using both ITS1 and ITS2 rDNA. The authors found sequence heterogeneity for both ITSs between species and, in some cases, between individual mosquitoes. Importantly, among the species included in Miller et al. (1996), two were analyzed in our study. Furthermore, a phylogenetic analysis of the Vomerifer and Pedroi groups of the Spissipes Section of *Culex* (*Melanoconion*) also employed ITS2 sequences (Navarro & Weaver 2004).

To gain a better understanding of group relationships, we investigated the intragenomic, intraspecific and interspecific variation among 16 mosquito species of the *Culex* genus, including those belonging to the subgenera, *Culex*, *Microculex* Theobald, *Melanoconion*, *Phenacomyia* Harbach and Peyton and one taxon of the genus *Lutzia*. The main objective of this study was to establish the taxonomic relationships within the *Culex* genus in the neotropics, in order to test the phylogenetic placement of the subgenera *Phenacomyia* and *Lutzia* relative to *Culex*.

MATERIALS AND METHODS

Mosquitoes - The species sampled for this study, specimen abbreviations, details about field collections and GenBank/EMBL clone accessions are shown in Supplementary data. The mosquitoes were collected from 2006-2008 in 14 localities in the four following Brazilian states: São Paulo (SP), Espírito Santo (ES), Rio Grande do Sul and Paraná. Most species were collected in their immature larval or pupal stage, though some adults were captured in the field. The immature specimens were reared in the laboratory to the adult stage. Male genitalia, larval and/or pupal exuviae, or all three, were mounted on Canada balsam on microscope slides and deposited for record purposes in the Entomological Collection of the School of Public Health of the University of São Paulo, Brazil. ITS2 sequences were characterized from adult males.

DNA extraction and amplification - Genomic DNA was extracted from specimens that were initially preserved in 100% ethanol at room temperature in the field and subsequently frozen at -80ºC. DNA was extracted from the specimens following the tissue DNA extraction protocol provided with the DNeasy Blood & Tissue Kit (QIAGEN, Crawley, UK). The DNA extracted from all samples was preserved at -80ºC for future use. The PCR amplification of the ITS2 region was carried out using the primers 5.8SF (5'-ATCACTCGGCTCGTGGATCG-3') and 28SR (5'-ATGCTTAAATTTAGGGGGTAGTC-3') (Djadid et al. 2007). PCR products were amplified in a 25 µL reaction mix containing 3-10 µL of genomic DNA (1st elution), 2.5 µL of 10X PCR buffer (Fermentas), 1.25 mM $MgCl_2$, 0.12 mM of each dNTP (Fermentas), 10 picomols of each primer, 8% dimethyl sulfoxide, 1.25 U of *Taq* DNA polymerase (Fermentas) and [DNase/RNase-free distilled water (Invitrogen)]. PCRs were performed at 94ºC for 2 min, 34 cycles at 94ºC, 55ºC and 72ºC for 30 s, followed by a final extension step at 72ºC for 20 min. PCR products were visualized by ethidium bromide staining after electrophoresis in a 1.5% agarose gel. The products were purified using isopropanol and electrophoresed again to quantify DNA.

DNA cloning and sequencing - Amplicons containing the ITS2 region and some of the flanking 5.8S and 28S genes were cloned using the InsTAclone™ PCR Cloning Kit (Fermentas) and *Escherichia coli* strain DH5α (Invitrogen) for transformation. The cloned sequences were extracted by PCR colony using the same protocol as ITS2 PCR, but using vector plasmid primers M13/pUC F (5'-GTAAAACGACGGCCAGT-3') and M13/pUC R (5'-CAGGAAACAGCTATGAC-3'). The clones were sequenced in both directions, using the same vector plasmid primers with a Big Dye® Terminator version 3.1 Cycle Sequencing Kit (PE Applied Biosystems, Warrington, England) in an ABI PRISM® 3100 automated sequencer (Genetic Analyzer/HITACH, Applied Biosystems, Foster City, CA, USA).

Sequence analysis - All ITS2 sequences obtained, including parts of the 5.8S and 28S genes, were analyzed and edited using Chromas Lite version 2.01 software (Technelysium Pty Ltd 2007). The ITS2 sequences obtained were compared with sequences available in the GenBank/EMBL database, using the Basic Local Alignment Search Tool (Zhang et al. 2000). The ITS2 sequences were annotated using the ITS2 annotation tool (Keller et al. 2009). Nucleotide sequences were aligned using the multiple alignment program CLUSTALX version 2.0 (Larkin et al. 2007) with a gap opening penalty of 10 and a gap extension penalty of 5. ITS2 sequences of three species available in GenBank were used as an out-group, including *Psorophora (Janthinosoma) ferox* (Von Humboldt) (M95129), *Aedes (Aedimorphus) vexans* Meigen (EF539857) and *Aedes (Stegomyia) aegypti* Linnaeus (AY512670). Because of the large interspecific variability, we edited the alignment using the program MacClade version 4.3 (Maddison & Maddison 2000). We used the proposed ITS secondary structure to minimize ambiguities in the sequence alignments.

Selection of clones by secondary structure - At least two sequences of each individual were used to obtain the secondary structures with the Model tool available in the ITS2 database (Schultz et al. 2006, Selig et al. 2008). Given the interspecific variability in the spatial pattern from structures, especially between different subgenera, we clustered one sequence of each species with known structures with three sequences of each

species with unknown structures. We then checked each cluster for the presence of sequences with assigned structures. Thus, the assigned structure that resulted in the best alignment with the template sequences was selected as a model for transferring the structure to the homologous sequences within all 144 clones. To ensure high prediction performance, the structure was transferred only when more than 75% of all base pairs (bp) of each helix could be used. Only clones with a high-quality model (> 75%) were selected.

Sequence alignment and distance analysis - Selected sequences were aligned in CLUSTALX version 2.0 (Larkin et al. 2007). The alignment of the ITS2 sequences, excluding 5.8S and 28S, was edited manually in MacClade version 4.0 (Maddison & Maddison 2000). The divergences between the ITS2 sequences were accessed by distance matrices, produced in Molecular Evolutionary Genetics Analysis version 4.0 (Tamura et al. 2007). A neighbour-joining (NJ) (Saitou & Nei 1987) topology was generated with an uncorrected p-distance in Phylogenetic Analysis Using Parsimony* (PAUP*) version 4.0 (Swofford 2003). Statistical support for the branches was estimated by bootstrap analysis with 1,000 replicates.

RESULTS

We sequenced ITS2 PCR products from 31 individuals belonging to the subgenera *Microculex*, *Melanoconion*, *Culex* and *Phenocomyia* and one of the genus *Lutzia* (Supplementary data). The number of clones sequenced per individual ranged from 3-7, with 144 sequences obtained in total. An alignment of 93 different sequences is shown in Supplementary data. The length of ITS2 fragments, including 41 bp of the final portion of the ribosomal 5.8S gene and 41 bp of the initial portion of the 28S of rDNA, ranged from 281-421 bp in *Culex* (*Microculex*) *imitator* Theobald (*imit 1*) and *Cux*. (*Cux*.) *quinquefasciatus* (*quin 1*), respectively. The GenBank/EMBL accessions are shown in Supplementary data.

Intragenomic variation - Of the 31 specimens analyzed only nine (*moll 2*, *bidens*, *nigri 2*, *decl 1*, *dyius*, *ybarmis*, *caud 1*, *bigoti 1* and *bigoti 2*) showed no variation in the length of cloned fragments within each individual. However, of these, only clones derived from *Culex* (*Cux*.) *mollis* Dyar and Knab (*moll 2*) showed no intragenomic variation. Differences in sequence length between clones were mainly due to insertions or deletions (indels) in ITS2 microsatellite repeat regions. There were no indels in either 5.8S or 28S. However, in the ITS2, clones from *Culex* (*Cux*.) *dolosus* Lynch Arribalzaga (*dolo 2*) showed differences of up to 16 bp. Details regarding the number of clones sequenced, the variants from each individual selected using the secondary structure, the length of ITS2 only (excluding the 5.8S and 28S flanking regions) and the mean uncorrected p-distance between clones are shown in Table I.

Intraspecific variation - Details of intraspecific variation, the number of specimens per species, the length of the ITS2 region (excluding the flanking regions 5.8S and 28S), the number of clones selected using the secondary structure, the mean uncorrected p-distance and the number of variants of each species are shown in Table II. *Lutzia* (*Lut*.) *bigoti* had the lowest average divergence of all selected sequences (0.3% for all clones, 0.4% for variants) and *Cux*. (*Mcx*.) *imitator* had the highest average divergence (3.8% for all of the selected clones, 5% for variants). In the distance matrix, with uncorrected p-distance made by selecting and grouping only variations per individual, there was greater divergence (7.1%) between the clones of specimens of *Cux*. (*Mcx*.) *imitator* from SP (*imit 2*) and ES (*imit 1*). *Culex* (*Mel*.) *caudelli* (*caud 1* and *caud 2*) showed a distance of 1.4% and the other distances between individuals of the same species were below 0.7% (distance matrix not shown).

Interspecific variation - Considering only the ITS2 region, the species of subgenus *Microculex* showed the shortest length sequences (199-213 bp), while species of subgenus *Culex* presented the largest fragments (277-339 bp). The length ranged from 205-213 bp in *Melanoconion*, from 268-276 bp in *Phenacomyia* and from 276-278 bp in *Lut. bigoti*. The range of guanine-cytosine contents of ITS2 variants selected by secondary structure was as follows: subgenus *Microculex* (50.2-50.3%), *Melanoconion* (45.4-50.9%), *Culex* (52.8-57.5%), *Phenacomyia* (50.6-53%) and *Lutzia* (55.4-55.8%). According to the data from the distance matrix with uncorrected p-distance between variants of each species (matrix not shown), the shortest distance (0.5%) in different species was observed between *Cux*. (*Cux*.) *declarator* Dyar and Knab and *Culex* (*Cux*.) *bidens* Dyar and between *Cux*. (*Cux*.) *coronator* and *Culex* (*Cux*.) *usquatus*. In the subgenus *Melanoconion*, the shortest distance was between *Culex* (*Mel*.) *ybarmis* and *Culex* (*Mel*.) *aliciae* (0.9%).

NJ topology - The identification of homologous sites and manual editing of the sequence alignment was difficult because there were many indels and nucleotide substitutions in the sequences. It was therefore not possible to produce a phylogenetic tree by the Maximum Parsimony method, using PAUP* version 4.0. Consequently, only the NJ topology was generated to examine the variability between cloned sequences.

In NJ topology, within the subgenus *Culex*, *Culex nigripalpus* + *Culex chidesteri* Dyar, *Cux. declarator* + *Cux. bidens* and *Cux. mollis* + *Cux. dolosus* formed well-supported groups by bootstrap values, which were 100% for the first two groups and 97% for the latter. The cluster of complex *coronator* species (*Cux. usquatus* and *Cux. coronator*) was also supported by a high bootstrap value (100%). Only *Cux. quinquefasciatus* was not grouped with all other taxa of the subgenus, occupying a position as a neighbouring group to the others. *Lutzia* and the subgenus, *Phenacomyia,* were placed within the *Culex* subgenus. Subgenera *Melanoconion* and *Microculex* formed two distinct but closely related groups.

TABLE I

Intragenomic variability between the internal transcribed sequences (ITS2) sequences of all clones and variants, both selected by the secondary structure in the same individual

		All clones selected		Variants selected	
Specimens	ITS2 length (bp)	Clones (n)	Mean uncorrected p-distance	Variants (n)	Mean uncorrected p-distance
coro 1	304-302	6	0.019	5	0.023
coro 2	298-304	3	0.002	2	0.003
coro 3	300-306	5	0.022	5	0.024
usqua 1	294-297	5	0.007	5	0.007
usqua 2	294-298	5	0.007	2	0.009
moll 1	281-285	5	0.009	4	0.007
moll 2	292	5	0	1	0
bidens 1	277	4	0.004	3	0.005
chid 1	286-288	5	0.009	4	0.009
dolo 1	283-285	5	0.011	2	0.018
dolo 2	268-284	6	0.011	2	0.019
nigri 1	277-280	5	0.014	5	0.014
nigr 2	280	5	0.002	2	0.004
quin 1	336-339	5	0.007	4	0.009
quin 2	324-335	4	0.011	3	0.015
quin 3	332-338	6	0.009	4	0.013
quin 4	333-336	5	0.009	2	0.015
decl 1	283-287	3	0.014	2	0.021
imit 1	199	2	0	1	0
imit 2	211-213	5	0	2	0
zete 1	206-207	5	0.002	3	0.003
aliciae 1	208-212	3	0.016	3	0.016
dyius 1	205	5	0.006	2	0.010
ybarmis 1	218	3	0.009	2	0.014
caud 1	205	5	0.029	4	0.032
caud 2	205	4	0.020	2	0.040
corni 1	279	4	0.002	2	0.004
corni 2	268-276	3	0.007	2	0.011
bigoti 1	276	4	0.002	2	0.004
bigot 2	278	3	0.002	2	0.004

bp: base pairs.

DISCUSSION

According to the current classification of the Culicine, this tribe includes four genera, *Culex*, *Deinocerites*, *Galindomyia* and *Lutzia*. The latter was previously considered a subgenus of the genus *Culex* (Bram 1967) and was elevated to genus by Tanaka (2003). It is interesting to note that in the NJ topology, *Lut. bigoti* was recovered within the genus *Culex* (Figure), which thus conflicts with Tanaka's (2003) classification proposal. Navarro and Liria (2000) inferred the phylogenetic relationships between seven subgenera of *Culex* and *Deinocerites* by parsimony analysis of the characteristics of the fourth instar larval mouthparts. Parsimony topologies showed

TABLE II

Intragenomic variability between the internal transcribed sequences (ITS2) sequences of all clones and variants, both selected by the secondary structure in the same species

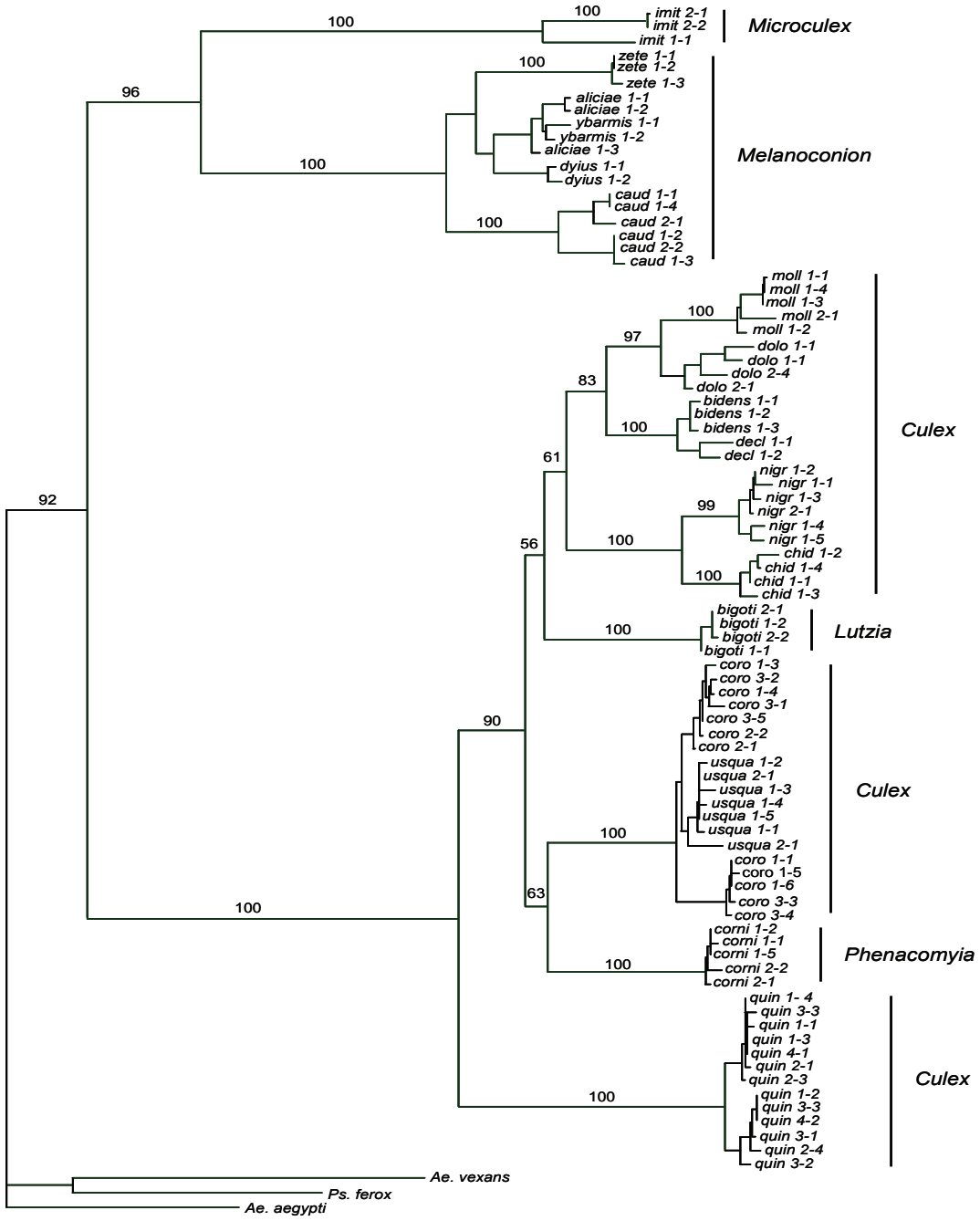
Species (number of specimens)	ITS2 length (bp)	All clones selected		Variants selected	
		Clones (n)	Mean uncorrected p-distance	Variants (n)	Mean uncorrected p-distance
Culex (*Cux.*) *coronator* (3)	298-306	14	0.019	12	0.019
Culex (*Cux.*) *usquatus* (2)	294-298	10	0.007	7	0.010
Culex (*Cux.*) *mollis* (2)	281-285	10	0.012	5	0.011
Culex (*Cux.*) *bidens* (1)	277	4	0.004	3	0.005
Culex (*Cux.*) *chidesteri* (1)	286-288	5	0.009	4	0.009
Culex (*Cux.*) *dolosus* (2)	268-285	11	0.017	4	0.020
Culex (*Cux.*) *nigripalpus* (2)	277-280	10	0.009	7	0.012
Culex (*Cux.*) *quinquefasciatus* (4)	324-339	20	0.009	13	0.010
Culex (*Cux.*) *declarator* (1)	283-287	3	0.014	2	0.021
Culex (*Mcx.*) *imitator* (2)	199-213	7	0.038	3	0.050
Culex (*Mel.*) *zeteki* (1)	206-207	5	0.002	3	0.003
Culex (*Mel.*) *aliciae* (1)	208-212	3	0.016	3	0.016
Culex (*Mel.*) *dyius* (1)	205	5	0.006	2	0.010
Culex (*Mel.*) *ybarmis* (1)	218	3	0.009	2	0.014
Culex (*Mel.*) *caudelli* (2)	205	9	0.027	6	0.030
Culex (*Phc.*) *corniger* (2)	268-276	7	0.006	4	0.008
Lutzia (*Lut.*) *bigoti* (2)	276-278	7	0.003	4	0.00

bp: base pairs.

Lutzia in a basal position sharing a sister-group relationship with the clade that includes species of *Culex*. This result confirmed the classification of Belkin (1962), which treated *Lutzia* as a specialized lineage that shares a similarity and therefore ancestry with members of the subgenus *Culex*. Belkin also corroborated the topology obtained by Miller et al. (1996) from their analysis of rDNA sequences. Nevertheless, the conflicting position of *Lutzia* presented in our study was supported by topologies generated using sequences of the mitochondrial cytochrome oxidase (COI) gene (Demari-Silva et al. 2011). The results of our molecular analysis employing the ITS2 and the COI sequence data do not support a genus position for *Lutzia*. The NJ topology (Figure) recovered the subgenus *Phenacomyia* embedded within a group that includes members of the subgenus *Culex* (90% bootstrap support).

As with *Lutzia*, our results do not support the current status of *Phenacomyia* as a subgenus of *Culex*, as was proposed by Harbach and Peyton (1992). The placement of *Phenacomyia* within the subgenus *Culex* also disagrees with the topology of Navarro and Liria (2000), in which *Phenacomyia* is placed in a basal position relative to the clade that includes species of the subgenus *Culex*. Again, analyses of the ITS2 sequence data were corroborated by analysis of the COI mtDNA (Demari-Silva et al. 2011). Furthermore, the results of the ITS2 sequence data showed that in the current classification, the subgenus *Culex* is paraphyletic because it excludes *Phenacomyia*.

Species of the subgenera *Microculex* and *Melanoconion* formed a monophyletic clade (96% bootstrap support) and these subgenera are clustered in two distinct groups, each supported by a 100% bootstrap analysis value (Figure). The results of the NJ analysis are in agreement with the current classification of *Culex*, with individuals of the same species and species complex grouped together. Considering the interspecific variation in ITS2 sequences, species belonging to the *Cux. coronator* (*Cux. usquatus* and *Cux. coronator*) complex formed a clade (Figure) and species with similar morphology (*Cux. bidens* + *Cux. declarator* and *Cux. nigripalpus* + *Cux. chidesteri*) also clustered. The positioning of *Cux.* (*Cux.*) *quinquefasciatus* as a sister group of the clade, which includes the others species of *Culex*, is similar to the topology obtained by Navarro and Liria (2000) based on the morphological characteristics of the larval maxilla and mandibula. The authors analyzed the phy-



Distance tree generated with the variants selected by internal transcribed sequences secondary structure (specimen codes-clone numbers). Only the model structures with high-quality (> 75%) in the transfer helices, using "Homology Modeling" tool were selected. The topology was constructed by neighbor-joining method, uncorrected p-distance, bootstrap confidence levels (%) from 1,000 replications and rooted with *Aedes vexans* (EF539857), *Psorophora ferox* (M95129) and *Aedes aegypti* (AY512670) sequences of GenBank/EMBL.

logenetic relationships of 18 species from eight genera of the tribe Culicine, including *Cux. quinquefasciatus*, *Cux. dolosus*, *Cux. nigripalpus* and *Cux. coronator* for subgenus *Culex*. In the clade formed by species of these subgenera, *Cux. quinquefasciatus* is a sister of a clade that includes *Cux. dolosus*, *Cux. nigripalpus* and *Cux. coronator*, which is consistent with the ITS2 NJ topology analysis. *Cux. ybarmis* and *Cux. aliciae* of the subgenus *Melanoconion* showed the lowest mean p-distance between their clones (0.9%). Due to the great morphological similarity and consequent difficulty in differentiating *Cux. aliciae* from *Culex dyius*, we expected to find that these two species were related at the molecular level. In this subgenus, dissection and examination of the lateral plate of the aedeagus from a lateral perspective is essential for species identification (Rozeboom

1950). In *Cux. dyius*, the lateral plate of the aedeagus is slender with a long, broad, blunt point at an angle in the middle, with a few small teeth at its apex (Lane 1953). In *Cux. aliciae*, this structure is very similar (Duret 1953). In *Cux. ybarmis* this structure is different, presenting the apical, dorsal and sterna processes, with its dorsal process curved (Lane 1953).

Cux. imitator, the only species of *Microculex* analyzed in this study, proved to have the greatest intraspecific divergence (7.1%) among specimens from SP (*imit 2*) and ES (*imit 1*) (data not shown). In addition, morphological variations were observed in the pupal exuviae of specimens from SP and ES, suggesting that the two individuals may belong to different species. The pupa of an individual of *Cux. imitator*, from the same location as the specimen from ES that was analyzed molecularly, showed typical characteristics of *Cux. imitator*, including a dark spot on segments II, III and IV and the arrangement pattern of setae 2, 3, 4 and 5 of segment II. The specimen from SP did not have the spot and had another arrangement pattern of its setae (morphological data not shown). Analysis of COI sequences also indicated high intraspecific variation for these specimens (Demari-Silva et al. 2011), thus corroborating the morphological hypothesis that these individuals may belong to different taxa of a species complex. In a phylogenetic analysis of species of the Pedroi and Vomerifer groups of *Melanoconion*, Navarro and Weaver (2004) used ITS sequence analysis and observed an intraspecific variation of 0-3.3% between individuals from the same location (*Culex pedroi* in Venezuela or *Cux. pedroi*-Peru form in Peru) and 0-4.5% between those from different locations (*Culex vomerifer*: Colombia vs. Peru).

The intragenomic and intraspecific variability of clones contradicted the theory of concerted evolution and prevented phylogenetic analysis because the alignment that is a fundamental step for reliability of any evolutionary analysis was compromised. There is a possibility of PCR-induced mutations, which might interfere in the assessment of intragenomic and intraspecific variability because the lack of 3'-5' proofreading ability of the *Taq* DNA polymerase used in our experiments results in an error rate (mutations per nucleotide per cycle) of approximately 1 in 10,000 bases (Eckert & Kunkel 1991, Barnes 1994). However, considering that PCR errors are also proportionally related to the length of the PCR products and that approximately 50,000 bases were sequenced in the present study, the interference of PCR-induced mutations in our final conclusions was not significant.

Some aspects of the phenomenon of intragenomic variability remains unclear, but the high intragenomic heterogeneity observed seems to be the result of a comparison between sequences of different clusters undergoing different levels of evolution. Similar results were observed by Miller et al. (1996), who showed heterogeneity between sequences of the same individual in different populations of the *Culex pipiens* complex, thus invalidating the use of ITS markers for phylogenetic analysis of taxa that exhibit intragenomic variation. However, there was general agreement between the groupings obtained in the topologies for species of the genus *Culex* and the recognition of taxa by morphology. Therefore, in both this study and the previously mentioned work, the trees obtained generally corroborated the traditional taxonomy of taxa, indicating that despite the presence of intragenomic variation, the ITS2 marker seems to be appropriate for solving taxonomic doubts in the group.

REFERENCES

Barnes WM 1994. PCR amplification of up to 35-kb DNA with high fidelity and high yield from lambda bacteriophage templates. *Proc Natl Acad Sci USA 91*: 2216-2220.

Belkin JN 1962. *The mosquitoes of the South Pacific (Diptera, Culicidae)*, vol. I, University of California Press, Berkeley and Los Angeles, 545 pp.

Bram RA 1967. Classification of *Culex* subgenus *Culex* in the New World (Diptera, Culicidae). *Proc US Nat Hist Mus 120*: 1-122.

Collins FH, Paskewitz SM 1996. A review of the use of ribosomal DNA (rDNA) to differentiate among cryptic *Anopheles* species. *Insect Mol Biol 5*: 1-9.

Demari-Silva B, Vesgueiro FT, Sallum MA, Marrelli MT 2011. Taxonomic and phylogenetic relationships between species of the genus *Culex* (Diptera: Culicidae) from Brazil inferred from the Cytochrome C Oxidase I mitochondrial gene. *J Med Entomol, in press.*

Djadid ND, Gholizadeh S, Tafsiri E, Romi R, Gordeev M, Zakeri S 2007. Molecular identification of Paleartic members of *Anopheles maculipennis* in northern Iran. *Malar J 6*: 6.

Duret JP 1953. Las especies argentinas de *Culex* (*Melanoconion*) (Diptera: Culicidae). *Rev Soc Entomol Argent 16*: 67-76.

Eckert KA, Kunkel TA 1991. DNA polymerase fidelity and the polymerase chain reaction. *PCR Methods Appl 1*: 17-24.

Fontes G, Braun RF, Fraiha Neto H, Vieira JB, Padilha SS, Rocha RC, da Rocha EM 2005. Filariose linfática em Belém, estado do Pará, Norte do Brasil e a perspectiva de eliminação. *Rev Soc Bras Med Trop 38*: 131-136.

Fontes G, Rocha EM, Brito AC, Antunes CM 1998. Lymphatic filariasis in Brazilian urban area (Maceió, Alagoas). *Mem Inst Oswaldo Cruz 93*: 705-710.

Forattini OP 2002. *Culicidologia médica: identificação, biologia e epidemiologia*, vol. II, EDUSP, São Paulo, 864 pp.

Harbach RE 2011. Mosquito taxonomic inventory. Culicidae classification. Natural History Museum, London, UK [cited 2009 Nov 11]. Available from: http://mosquito-taxonomic-inventory.info/em/taxonomy/term/6165.

Harbach RE, Peyton EL 1992. A new subgenus of *Culex* in the Neotropical region (Diptera: Culicidae). *Mosq Syst 24*: 242-252.

Humeres SG, Almirón WR, Sabattini MS, Gardenal CN 1998. Estimation of genetic divergence and gene flow between *Culex pipiens* and *Culex quinquefasciatus* (Diptera: Culicidae) in Argentina. *Mem Inst Oswaldo Cruz 93*: 57-62.

Keller A, Schleicher T, Schultz J, Müller T, Dandekar T, Wolf M 2009. 5.8S-28S rRNA interaction and HMM-based ITS2 annotation. *Gene 430*: 50-57.

Knight JW, Nayar JK 2004. Identification of four common *Culex* (*Culex*) (Diptera: Culicidae) species from Florida with isoenzyme analysis. *Fla Entomol 87*: 1-5.

Lane J 1953. *Neotropical* Culicidae, vol. I, EDUSP, São Paulo, 558 pp.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan

PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG 2007. Clustal W and CLUSTALX version 2.0. *Bioinformatics 23*: 2947-2948.

Maddison DR, Maddison WP 2000. *MacClade 4: analysis of phylogeny and character evolution*, Sinauer, Sunderland, 398 pp.

Marrelli MT, Floeter-Winter LM, Malafronte RS, Tadei WP, Lourenço-de-Oliveira R, Flores-Mendoza C, Marinotti O 2005. Amazonian malaria vector anopheline relationships interpreted from ITS2 rDNA sequences. *Med Vet Entomol 19*: 208-218.

Melo AL, Soccol CR, Thomaz-Soccol V, Nogueira M Jr 2009. Evaluation of *Bacillus sphaericus* bioinsecticide produced with white soybean meal as culture medium for the control of *Culex* (*Culex*) *quinquefasciatus*. *Cad Saude Publica 25*: 563-569.

Miller BR, Crabtree MB, Savage HM 1996. Phylogeny of fourteen *Culex* mosquito species, including the *Culex pipiens* complex, inferred from the internal transcribed spacers of ribosomal DNA. *Insect Mol Biol 5*: 93-107.

Navarro JC, Liria J 2000. Phylogenetic relationships among eighteen neotropical Culicini species. *J Am Mosq Control Assoc 16*: 75-85.

Navarro JC, Weaver SC 2004. Molecular phylogeny of the Vomerifer and Pedroi groups in the Spissipes section of the subgenus *Culex* (*Melanoconion*). *J Med Entomol 41*: 575-581.

Regis L, Silva-Filha MHNL, de Oliveira CMF, Rios EM, da Silva SB, Furtado AF 1995. Integrated control measures against *Culex quinquefasciatus*, the vector of filariasis in Recife. *Mem Inst Oswaldo Cruz 90*: 115-119.

Rey JR, O'Meara GF, O'Connell SM, Darsie RF 2006. Variation in the number and position of siphonal setae in *Culex quinquefasciatus* from Key West and Vero Beach, Florida, USA. *J Am Mosq Control Assoc 22*: 355-357.

Rozeboom LE 1950. A review of the species of *Culex* of the subgenus *Melanoconion* (Diptera: Culicidae). *Ann Entomol Soc Am 43*: 75-114.

Saitou N, Nei M 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol 4*: 406-425.

Sallum MAM, Schultz TR, Foster PG, Aronstein K, Wirtz RA, Wilkerson RC 2002. Phylogeny of Anophelinae (Diptera: Culicidae) based on nuclear ribosomal and mitochondrial DNA sequences. *Syst Entomol 27*: 361-382.

Schultz J, Müller T, Achtziger M, Seibel PN, Dandekar T, Wolf M 2006. The internal transcribed spacer 2 database: a web server for (not only) low level phylogenetic analyses. *Nucleic Acids Res 34*: 704-707.

Selig C, Wolf M, Müller T, Dandekar T, Schultz J 2008. The ITS2 Database II: homology modelling RNA structure for molecular systematics. *Nucleic Acids Res 36*: 377-380.

Smith JL, Fonseca DM 2004. Rapid assays for identification of members of the *Culex* (*Culex*) *pipiens* complex, their hybrids, and other sibling species (Diptera: Culicidae). *Am J Trop Med Hyg 70*: 339-345.

Swofford DL 2003. PAUP*. Phylogenetic analysis using parsimony (*and other methods), version 4.01b, Sinauer Associates, Sunderland, Massachusets.

Tamura K, Dudley J, Nei M, Kumar S 2007. MEGA 4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol 24*: 1596-1599.

Tanaka K 2003. Studies on the pupal mosquitoes of Japan. Genus *Lutzia*, with establishment of two new subgenera, *Metalutzia* and *Insulalutzia* (Diptera, Culicidae). *Syst Entomol 9*: 159-169.

Technelysium Pty Ltd 2007. [cited 2009 Oct 10]. Chromas lite version 2.01. Available from: http://www.technelysium.com.au/chromas_lite.html.

Turell MJ, O'Guinn ML, Dohm DJ, Jones JW 2001. Vector competence of North American mosquitoes (Diptera: Culicidae) for *West Nile Virus*. *J Med Entomol 38*: 130-134.

Vasconcelos PFC, Travassos-da-Rosa JFS, Travassos-da-Rosa APA, Dégallier N, Pinheiro FP, Sá-Filho GC 1991. Epidemiologia das encefalites por arbovírus na Amazônia brasileira. *Rev Inst Med Trop Sao Paulo 33*: 465-476.

Walton C, Sharpe RG, Pritchard SJ, Thelwell NJ, Butlin RK 1999. Molecular identification of mosquito species. *Biol J Linn Soc 68*: 241-256.

Weaver SC, Ferro C, Barrera R, Boshell J, Navarro JC 2004. Venezuelan equine encephalitis. *Annu Rev Entomol 49*: 141-174.

Williams MR, Savage HM 2009. Identification of *Culex* (*Melanoconion*) species of the United States using female cibarial armature (Diptera: Culicidae). *J Med Entomol 46*: 745-752.

Zhang Z, Schwartz S, Wagner L, Miller W 2000. A greedy algorithm for aligning DNA sequences. *J Comput Biol 7*: 203-214.

TABLE

Species studied, specimen codes, localities, geographical coordinates and GenBank/EMBL accessions of clones

Species	Specimens	Sex	Localities	Coordinates	GenBank accessions
Culex (Cux.) coronator Dyar and Knab	*coro 1*	♂	Campos do Jordão (SP)	22º45'50''S 45º30'87''W	GU299736-GU299740
	coro 2	♂	Linhares (ES)	19º20.917'S 40º07.103'W	GU299741-GU299742
	coro 3	♂	Aparecida do Norte (SP)	22º50'34.4"S 45º14'45"W	GU299743-GU299747
Culex (Cux.) usquatus Dyar	*usqua 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299731-GU299735
	usqua 2	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299729-GU299730
Culex (Cux.) mollis Dyar and Knab	*moll 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299706-GU299709
	moll 2	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299705
Culex (Cux.) chidesteri Dyar	*chid 1*	♂	Pindamonhangaba (SP)	22º57'35.1"S 45º27'08.6"W	GU299725-GU299728
Culex (Cux.) dolosus Lynch Arribalzaga	*dolo 1*	♂	Pindamonhangaba (SP)	22º57'35.1"S 45º27'08.6"W	GU299710-GU299711
	dolo 2	♂	Pindamonhangaba (SP)	22º57'35.1"S 45º27'08.6"W	GU299712-GU299713
Culex (Cux.) nigripalpus Theobald	*nigri 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299719-GU299723
	nigri 2	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299724
Culex (Cux.) quinquefasciatus Say	*quin 1*	♀	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299748-GU299751
	quin 2	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299752-GU299754
	quin 3	♂	Bento Gonçalves (RS)	29º09'28.90"S 51º30'43.55"W	GU299755-GU299758
	quin 4	♂	Bento Gonçalves (RS)	29º09'28.90"S 51º30'43.55"W	GU299759-GU299760
Culex (Cux.) declarator Dyar and Knab	*decl 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299717-GU299718
Culex (Cux.) bidens Dyar	*bidens 1*	♂	Aparecida do Norte (SP)	22º50'34.4"S 45º14'45"W	GU299714-GU299716
Culex (Mcx.) imitator Theobald	*imit 1*	♂	Santa Teresa (ES)	19º55'54.46"S 40º35'41.61"W	GU299677
	imit 2	♂	Pindamonhangaba (SP)	22º57'35.1"S 45º27'08.6"W	GU299678-GU299679
Culex (Mel.) zeteki Dyar	*zetk 1*	♀	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299680-GU299682
Culex (Mel.) aliciae Duret	*aliciae 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299683-GU299685
Culex (Mel.) dyius Root	*dyius 1*	♂	Linhares (ES)	19º20.917'S 40º07.103'W	GU299686-GU299687
Culex (Mel.) ybarmis Dyar	*ybarmis 1*	♂	Linhares (ES)	19º20.917'S 40º07.103'W	GU299688-GU299689
Culex (Mel.) caudelli (Dyar & Knab)	*caud 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299690-GU299693
	caud 2	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299694-GU299695
Culex (Phc.) corniger Theobald	*corni 1*	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299700-GU299702
	corni 2	♂	Pariquera-Açu (SP)	24º43.975'S 47º49.502'W	GU299703-GU299704
Lutzia (Lut.) bigoti (Bellardi)	*bigoti 1*	♀	São Paulo (SP)	23º26'12.44"S 46º38'05.09"W	GU299696-GU299697
	bigoti 2	♀	S. José dos Pinhais (PR)	25º33'14.48"S 49º14'08.69"W	GU299698-GU299699

ES: Espírito Santo; PR: Paraná; RS: Rio Grande do Sul; SP: São Paulo.