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The second internal transcribed spacer of nuclear ribosomal DNA as a tool for Latin American anopheline taxonomy - A critical review

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Among the molecular markers commonly used for mosquito taxonomy, the internal transcribed spacer 2 (ITS2) of the ribosomal DNA is useful for distinguishing among closely-related species. Here we review 178 GenBank accession numbers matching ITS2 sequences of Latin American anophelines. Among those, we found 105 unique sequences corresponding to 35 species. Overall the ITS2 sequences distinguish anopheline species, however, information on intraspecific and geographic variations is scarce. Intraspecific variations ranged from 0.2% to 19% and our analysis indicates that misidentification and/or sequencing errors could be responsible for some of the high values of divergence. Research in Latin American malaria vector taxonomy profited from molecular data provided by single or few field capture mosquitoes. However we propose that caution should be taken and minimum requirements considered in the design of additional studies. Future studies in this field should consider that: (1) voucher specimens, assigned to the DNA sequences, need to be deposited in collections, (2) intraspecific variations should be thoroughly evaluated, (3) ITS2 and other molecular markers, considered as a group, will provide more reliable information, (4) biological data about vector populations are missing and should be prioritized, (5) the molecular markers are most powerful when coupled with traditional taxonomic tools.

Key words: *Anopheles* - Neotropical - anophelines - malaria vectors - internal transcibed spacer 2 - ITS2 - taxonomy - systematics

ANOPHELES SIBLING SPECIES

Many anopheline mosquitoes belong to species complexes that are difficult to differentiate using morphological criteria alone. These complexes may include both malaria vectors and nonvector species, which either may occur sympatrically, or have distinct geographical distributions (Collins & Paskewitz 1996, Krzywinski & Besansky 2003, Black & Munstermann 2005). The *Anopheles gambiae* complex, as an example of an extensively studied taxonomic group, is composed of seven species, of which five are human malaria vectors. *A. gambiae s.s.* and *A. arabiensis* are both major malaria vectors in Africa, however the former feeds mostly on humans while the latter feeds on both humans and cattle. Differently, *A. quadriannulatus* species A and B, members of the same complex, are mainly cattle feeders and do not transmit human malaria (Coetzee 2006). In Asia a similar scenario occurs in which *A. mininus s.s.* and *A. varuna*, both members of the *A. minimus* complex, have distinct feeding behaviors. *A. minimus s.s.* feeds both on humans and animals, and is a major malaria vector, while *A. varuna* is essentially zoophilic and does not play a role in *Plasmodium vivax* and *P. falciparum* transmission (Kittayapong 2006). Therefore, the proper identification of malaria vectors and their sibling species, together with the mapping of their geographical distribution, is of fundamental importance for malaria control programs that depend on this information to set up their actions and to establish protocols that will result in useful epidemiological data. Actions focused on areas where malaria vector species occur, in contrast with a widespread intervention, are beneficial since the burden of malaria is primarily situated in developing countries, which have limited economic resources to invest in public health programs (Van Bortel et al. 2001, McMichael et al. 2005).

Evidences for anopheline sibling species were first discovered during the 1920s and 1930s when mosquitoes of the *A. maculipennis* complex were shown to belong to distinct species by comparative studies of their eggshell coloring patterns, feeding behaviors and mating incompatibility (Black & Munstermann 2005). Subsequently, different chromosomal forms were observed in specimens of *A. maculipennis* and these forms assigned to distinct species within the complex. While the cytogenetic approach has proven to be powerful for mosquito taxonomy [i.e. *A. maculipennis* and *A. gambiae* complexes (Frizzi 1947, 1953, Coluzzi 1966, Coetzee et al. 2000)], its application is time consuming and requires specialized skills.

Since then, researchers from around the world have pursued ways to recognize species within the various

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complexes of anophelines. As a result, ecological, behavioral, biochemical, and molecular studies have provided substantial data resulting in the improvement of mosquito taxonomy, and thus, *A. albitarsis* (Li & Wilkerson 2005), *A. crucians* (Wilkerson et al. 2004), *A. culicifacies* (Subbarao & Sharma 1997), *A. dirus* (Huong et al. 2001), *A. farauti* (Beebe et al. 2000), *A. gambiae* (Powell et al. 1999), *A. maculipennis* (Romi et al. 2000), *A. minimus* (Jaichapor et al. 2005), and *A. quadrimaculatus* (Levine et al. 2004), are now recognized as species complexes. In addition, several other anopheline mosquito taxonomic groups are suspected to be complexes of cryptic species (Lounibos et al. 1998, Tadei & Dutary-Thatcher 2000).

THE INTERNAL TRANSCRIBED SPACER 2 (ITS2) AS A MOLECULAR MARKER

Among the molecular markers used for mosquito taxonomy, the ITS2 of the ribosomal DNA genes is useful for distinguishing among specimens belonging to closely-related species. For example, the *A. maculipennis* complex (Porter & Collins 1991), *A. quadrimaculatus* complex (Cornel et al. 1996) and *A. culicifacies* complex (Goswami et al. 2005) have been analyzed using this marker. In other complexes, composed of recently-diverged members, such as the *A. gambiae,* the ITS2 interspecies differences are minor, from 0.4 to 1.6% (Paskewitz et al. 1993), not allowing the use of this marker alone for taxonomic purposes. The sequences of the ITS2 of several Neotropical anophelines have been determined during the last decade and deposited in the GenBank public database. While these sequences have been analyzed and compared within each taxonomic group as contributions of individual researchers and laboratories, little or no efforts have been made to look at the more global pattern that this comprehensive data collection is generating. Therefore the organization and comparison of all the ITS2 sequences of Latin American anopheline mosquitoes, and an analysis of their relationships is the purpose of this review.

LATIN AMERICAN ANOPHELINES

Of the more than 500 species of *Anopheles* in the world approximately 100 occur in the Neotropical Region (Papavero & Guimarães 2000). Of those found in Latin America, 29 have been indicated as confirmed or potential human malaria vectors (Table I). The principal vectors in Mexico and Central America are *A. pseudopunctipennis* and *A. albimanus* (Rodriguez 2006). In the Amazon area, where 95% of all Latin America malaria cases are reported, the major vector is *A. darlingi* (Deane 1986, Tadei & Dutary-Thatcher 2000), an endophilic and antropophilic species highly susceptible to infection with *Plasmodium* parasites (Klein et al. 1991a,b). However, as mentioned above, these are not the only malaria vectors and research based on radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) for the detection of *Plasmodium* antigens in mosquitoes extracts, and mosquito dissection for the direct observation of parasites (oocysts and/or sporozoites), has identified other anopheline species playing potentially important roles in malaria transmission in specific locations in Latin America (de Arruda et al. 1986, Klein et al. 1991a,b, Zimmermann 1992, Branquinho et al. 1993,1996, Calderon et al. 1995, Marrelli et al. 1998, Tadei & Dutary-Thatcher 2000). For example, Conn et al. (2002) conducted entomological surveys in malaria afflicted areas of Macapá (state of Amapá, Brazil) and found that *A. marajoara* was the most abundant anopheline, replacing *A. darlingi* as the primary vector. Most likely the observed change in mosquito population was a result of human activity (deforestation and newly created ground pools for agricultural use) that provided adequate breeding sites for *A. marajoara*. These data emphasize the challenges that malaria control agencies face in identifying specific target vectors in a dynamic scenario driven by constant human intervention in the environment. The possibility that the mosquitoes inhabiting these areas are distinct members of complexes of anopheline species, with varying degrees of vectorial capacity, further complicates the overall picture and makes it more difficult to define effective vector control interventions. A fundamental step towards the recognition of the target species is the accurate identification of field captured specimens. For most of the anopheline mosquitoes from Latin America, taxonomy based only on morphological characters has not presented much problem, however, molecular data have indicated that for some taxonomic groups the apparent simplicity of species identification may be deceptive.

ITS2 OF LATIN AMERICAN ANOPHELINES

The Neotropical anopheline species are classified into five subgenera: *Nyssorhynchus*, *Kerteszia*, *Anopheles, Stethomyia*, and *Lophopodomyia* (Faran & Linthicum 1981, Sallum et al. 1999, Wilkerson & Sallum 1999), but malaria vectors are only found within the first three subgenera. This fact dictated the interest of researchers and most of the Latin American anopheline biology information generated so far is concentrated on the *Nyssorhynchus*, *Kerteszia,* and *Anopheles* subgenera. Accordingly, our search of the GenBank data base for Latin American anopheline ITS2 sequences resulted in more than 150 entries that include data for most species registered as vectors of human malaria (Table I) (Tadei & Dutary-Tatcher 2000, Deane 1986, Consoli & Lourenço-de-Oliveira 1994, Marrelli et al. 2005). *A. calderoni*, *A. punctimacula*, *A. neomaculipalpus, A. vestitipennis,* and *A. neivai* have been indicated as human malaria vectors but have no ITS2 sequences available in the database. The acquisition of ITS2 sequences for these species should therefore be considered as the objective of near future studies. No ITS2 sequence data was found for mosquito species of the *Stethomyia* and *Lophopodomyia* subgenera.

One hundred seventy-eight ITS2 sequences were downloaded from the GenBank (data available on January 2006), corresponding to 35 different anopheline species. For the majority of species, more than one sequence is available. In these cases, the sequences were derived

TABLE I
Latin American malaria vectors [a]

Genus	Subgenus	Species	ITS2 sequence	Reference
Anopheles	*Anopheles*	*calderoni*	No	Wilkerson (1991)
Anopheles	*Anopheles*	*mattogrossensis*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Anopheles*	*mediopunctatus*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Anopheles*	*near fluminensis*	Yes	Hayes et al. (1987)
Anopheles	*Anopheles*	*neomaculipalpus*	No	Moreno et al. (2005)
Anopheles	*Anopheles*	*peryassui*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Anopheles*	*pseudopunctipennis*	Yes	Rodriguez et al. (2000)
Anopheles	*Anopheles*	*punctimacula*	No	Rubio-Palis & Zimmernan (1997)
Anopheles	*Anopheles*	*vestitipennis*	No	Grieco et al. (2005)
Anopheles	*Anopheles*	*quadrimaculatus*	Yes	Robert et al. (2005)
Anopheles	*Kerteszia*	*bellator*	Yes	Forattini et al. (1999)
Anopheles	*Kerteszia*	*cruzii*	Yes	Carvalho-Pinto & Lourenco de Oliveira (2004)
Anopheles	*Kerteszia*	*homunculus*	Yes	Rubio-Palis & Zimmernan (1997)
Anopheles	*Kerteszia*	*neivai*	No	Carvajal et al. (1989)
Anopheles	*Nyssorhynchus*	*albimanus*	Yes	Rodriguez et al. (2000)
Anopheles	*Nyssorhynchus*	*albitarsis*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Nyssorhynchus*	*aquasalis*	Yes	Zimmermann (1992)
Anopheles	*Nyssorhynchus*	*benarrochi*	Yes	Flores-Mendonza et al. (2004)
Anopheles	*Nyssorhynchus*	*braziliensis*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Nyssorhynchus*	*darlingi*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Nyssorhynchus*	*deanorum*	Yes	Rubio-Palis & Zimmernan (1997)
Anopheles	*Nyssorhynchus*	*galvaoi*	Yes	Tadei et al. (1998)
Anopheles	*Nyssorhynchus*	*marajoara*	Yes	Moreno et al. (2005)
Anopheles	*Nyssorhynchus*	*nuneztovari*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Nyssorhynchus*	*oswaldoi*	Yes	Rosa-Freitas et al. (1998)
Anopheles	*Nyssorhynchus*	*rangeli*	Yes	Povoa et al. (2001)
Anopheles	*Nyssorhynchus*	*strodei*	Yes	de Oliveira-Ferreira et al. (1990)
Anopheles	*Nyssorhynchus*	*triannulatus*	Yes	Tadei & Dutary-Thatcher (2000)
Anopheles	*Nyssorhynchus*	*trinkae*	Yes	Hayes et al. (1987)

a: the species included in this table are those in which *Plasmodium* parasites have been detected by direct microscopic observation, immunological and molecular approaches. This comprehensive compilation allows the possibility of including false-positive data for mosquitoes that have just or recently fed on human blood infected with malaria. Also some mosquitoes may develop oocysts that do not complete their development into salivary gland sporozoites. As a result, one should be cautious about assuming that all these species are human malaria vectors. Supplemental biological and epidemiological data are a requirement to conclude that a species in fact transmits malaria to humans.

mostly from specimens collected in distinct localities and by different laboratories. Some species had several identical sequences deposited in the GenBank, corresponding to data obtained from different specimens. Therefore, sequences were first grouped, aligned and compared within each species. When individuals of a single species had identical sequences, we opted to consider only the first identical entry (Table II) in order to perform further analyses. For instance, among the 51 *A. nuneztovari* ITS2 entries, five different sequences were found (3% of intraspecific nucleotide divergence) with the sequence abbreviated as NUN5 representing 90% of the total (46 of 51 entries) (Tables II, III). Despite the dogma that concerted evolution makes the individual representative of the entire species (Liao 1999), extensive intraspecific variation has been reported for anophelines. Among the species included in this study (according to data from GenBank), ITS2 intraspecific variations ranged from 0.2 to 19.0%. While ITS2 divergence does occur, we argue that where extensive ITS2 intraspecific variation is found, it should be taken as a signal that further investigation is necessary. As discussed below, misidentification and mislabeling of specimens and/or sequencing errors may account for some of the reported variability. Conversely, intraspecific heterogeneity of ITS sequences has been reported in other mosquito species, blackflies, and several other organisms (Tang et al. 1996). It is possible that in some cases low level of interbreeding occurs between different, closely related, sibling species concealling the effets of concerted evolution. Supporting this hypothesis, limited genetic introgression has been observed among members of the *An. gambiae* complex (Besansky et al. 1994, Kamau et al. 1998)

After all the sequences were organized and all identical sequences removed from the pool, the remaining 105 unique entries were used to construct a similarity tree (Fig. 1, nucleotide distances estimated

TABLE II

Nucleotide sequences of the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA of Latin American *Anopheles* mosquitoes available in the GenBank (April 2006) and included in this study

Species	Subgenus	Seq. abbr.	Accession number	References
A. bradleyi	*Anopheles*	BRAD1	AY386967.1	Wilkerson et al. (2004)
A. crucians	*Anopheles*	CRUC1	AY386966.1	Wilkerson et al. (2004)
		CRUC2	AY386965.1	
		CRUC3	AY386964.1	
		CRUC4	AY386963.1	
		CRUC5	AY245553.1	
A. eiseni	*Anopheles*	EIS1	AF462380.1	Marrelli et al. (2005)
A. fluminensis	*Anopheles*	FLU1	DQ328638	Brelsfo et al. (unpublished)[a]
A. freeborni	*Anopheles*	FREE1	M64484.1	Porter & Collins (1991)
A. hermsi	*Anopheles*	HERM1	M64483.1	Porter & Collins (1991)
A. mattogrossensis	*Anopheles*	MATTO1	AF461754.1	Marrelli et al. (2005)
A. mediopunctatus	*Anopheles*	MED1	AF462379.1	Marrelli et al. (2005)
A. occidentalis	*Anopheles*	OCCI1	M64482.1	Porter & Collins (1991)
A. peryassui	*Anopheles*	PER1	AF461755.1	Marrelli et al. (2005)
A. pseudopunctipennis	*Anopheles*	PSEU1	U49735.1	Miller et al. (1997)
A. quadrimaculatus	*Anopheles*	QUADRI1	U32506.1	Cornel et al. (1996)
		QUADRI2	U32505.1	
		QUADRI3	U32504.1	
		QUADRI4	U32503.1	
A. bellator	*Kerteszia*	BELLA1	DQ364652	Malafonte at al. (unpublished)
A. cruzii	*Kerteszia*	CRUZ1	AF027165.1	Malafronte et al. (unpublished)
		CRUZ2	AF035227.1	
		CRUZ3	DQ364653	
		CRUZ4	DQ364654	
A. homunculus	*Kerteszia*	HOMUN1	DQ364656	Malafonte at al (unpublished)
A. laneanus	*Kerteszia*	LANEA1	DQ364655	Malafonte at al (unpublished)
A. albimanus	*Nyssorhynchus*	ALBIM1	U92323.1	Danoff-Burg & Conn (unpublished),
		ALBIM2	L78065.1	Collins (unpublished)
A. albitarsis	*Nyssorhynchus*	ALBIT1	AF462387.1	Marrelli et al. (2005)
		ALBIT2	AF462386.1	
		ALBIT3	AF462385.1	
A. aquasalis	*Nyssorhynchus*	AQUA1	DQ020135.1	Fairley et al (2005)
		AQUA2	DQ020136.1	Marrelli et al. (2005)
		AQUA3	DQ020134.1	
		AQUA4	DQ020133.1	
		AQUA5	DQ020132.1	
		AQUA6	DQ020131.1	
		AQUA7	DQ020130.1	
		AQUA8	DQ020129.1	
		AQUA9	DQ020128.1	
			DQ020127.1,	
		AQUA10	DQ020125.1	
		AQUA11	DQ020126.1	
		AQUA12	DQ020124.1	
		AQUA13	DQ020123.1	
		AQUA14	AF462376.1	
		AQUA15	DQ020137.1	
A. argyritarsis	*Nyssorhynchus*	ARGY1	AY849554.1	Wilkerson et al. (2005),
		ARGY2	AY849553.1	Danoff-Burg & Conn (unpublished)
		ARGY3	U92356.1	
		ARGY4	U92347.1	

↳

Species	Subgenus	Seq. abbr.	Accession number	References
A. benarrochi	*Nyssorhynchus*	BEN1	U92325.1	Marrelli et al. (2005),
		BEN2	AY684976.1,	Ruiz et al. (2005),
			AY684977.1,	Danoff-Burg & Conn (unpublished)
			AY684978.1,	
			AY684979.1,	
			AY684980.1,	
			AY684981.1,	
			AY684982.1,	
			AY684983.1,	
			AY684984.1	
		BEN3	AF462384	
		BEN4	AF463483	
A. braziliensis	*Nyssorhynchus*	BRAZ1	AF461753.1,	Marrelli et al. (2005),
			U92336.1	Danoff-Burg & Conn (unpublished)
A. darlingi	*Nyssorhynchus*	DAR1	AF032133.1	Malafronte et al. (1999),
			U36780.1,	Marrelli et al. (2005),
		DAR2	U92337.1	Danoff-Burg & Conn (unpublished)
		DAR3	AF462388.1	
		DAR4	AF462389.1	
A. deaneorum	*Nyssorhynchus*	DEAN1	U92335.1	Li & Wilkerson (2005),
		DEAN2	AF461751.1	Marrelli et al. (2005),
		DEAN3	AF461752.1	Danoff-Burg & Conn (unpublished)
		DEAN4	AY828343.1	
A. dunhami	*Nyssorhynchus*	DUN1	U92326.1	Danoff-Burg & Conn
		DUN2	AF462378.1	(unpublished), Marrelli et al. (2005)
A. evansae	*Nyssorhynchus*	EVAN1	U92327.1	Marrelli et al. (2005),
		EVAN2	Y09240.1	Fritz (1998),
		EVAN3	AF461750.1	Danoff-Burg & Conn (unpublished)
A. galvaoi	*Nyssorhynchus*	GALV1	U92328.1	Danoff-Burg & Conn (unpublished)
A. konderi	*Nyssorhynchus*	KON1	U92342.1	Danoff-Burg & Conn (unpublished)
		KON2	U92348.1	
		KON3	U92349.1	
A. marajoara	*Nyssorhynchus*	MARA1	AY028127.1,	Li & Wilkerson (2005),
			U92334.1,	Linton et al (unpublished),
			AY828353.1,	Danoff-Burg & Conn (unpublished)
			AY828345.1,	
			AY828349.1,	
			AY828340.1,	
			Y828347.1,	
			AY828328.1	
		MARA2	AY828354.1,	
			AY828352.1,	
			AY828350.1,	
			AY828339.1	
		MARA3	AY828344.1	
			AY828351.1,	
		MARA4	AY828348.1	
		MARA5	AY828346.1	
		MARA6	AY828329.1	
		MARA7	DQ077808.1	
A. nuneztovari	*Nyssorhynchus*	NUN1	U92351.1	Fritz et al. (1994),
		NUN2	AY028126.1,	Marrelli et al. (2005),
			AY028125.1	Linton et al. (unpublished),
		NUN3	U92350.1	Danoff-Burg & Conn (unpublished)
		NUN4	U92343.1	
		NUN5	AY028128.1,	
			AY028103.1,	
			AY028100.1,	
			AY028099.1,	
			AY028098.1,	

↳

Species	Subgenus	Seq. abbr.	Accession number	References
			AY028097.1, AY028096.1, AY028095.1, AY028094.1, AY028093.1, AY028092.1, AY028091.1, AY028090.1, AY028089.1, AY028087.1, AY028088.1, AY028083.1, AY028086.1, AY028084.1, AY028081.1, AY028124.1, AY028123.1, AY028122.1, AY028121.1, AY028120.1, AY028119.1, AY028118.1, AY028117.1, AY028116.1, AY028115.1, AY028114.1, AY028113.1, AY028112.1, AY028111.1, AY028110.1, AY028109.1, AY028108.1, AY028107.1, AY028106.1, AY028105.1, AY028104.1, AF461749.1, AY028082.1, L22462.1, AY028102.1, AY028101.1	
A. oswaldoi	*Nyssorhynchus*	OSW1	AF055070.1, AF056318.1	Marrelli et al. (1999), Ruiz et al. (2005),
		OSW2	AF055069.1	Danoff-Burg & Conn (unpublished)
		OSW3	AY679154.1, AY679150.1, AY679149.1, AY679152.1, AY679155.1, AY679153.1, AY679151.1	
		OSW4	AF055072.1	
		OSW5	AF055071.1	
		OSW6	AF055068.1	
		OSW7	U92352.1, U92344.1	
		OSW8	AF056317.1	
		OSW9	U92353.1	
A. rangeli	*Nyssorhynchus*	RAN1	U92329.1, Y09239.1	Fritz (1998), Marrelli et al. (2005),
		RAN2	AF462381.1	Danoff-Burg & Conn (unpublished)
		RAN3	AF462382.1	

↳

Species	Subgenus	Seq. abbr.	Accession number	References
A. rondoni	*Nyssorhynchus*	RON1	U92330.1	Danoff-Burg & Conn (unpublished)
A. strodei	*Nyssorhynchus*	STRO1	Y09241.1	Fritz (1998),
		STRO2	U92354.1	Danoff-Burg & Conn (unpublished)
		STRO3	U92345.1	
A. triannulatus	*Nyssorhynchus*	TRIA1	U92331.1	Marrelli et al. (2005),
		TRIA2	AF462377.1	Danoff-Burg & Conn (unpublished)
A. trinkae	*Nyssorhynchus*	TRIN1	Y09075.1	Fritz (1998),
		TRIN2	U92355.1	Danoff-Burg & Conn (unpublished)
		TRIN3	U92346.1	

a: sequences indicated as (unpublished) are available in the GenBank but have not been published as part of a scientific article or report.

TABLE III

Intraspecific variations of the second internal transcribed spacer (ITS2) of nuclear ribosomal DNA of Latin American *Anopheles* mosquitoes, according to published data

Species	Number of different sequences	Intraspecific divergence (%)
Anopheles crucians	5	14.0
Anopheles quadrimaculatus	4	2.4
Anopheles cruzii	4	0.3
Anopheles albimanus	2	0.4
Anopheles albitarsis	3	0.2
Anopheles aquasalis	15	1.9
Anopheles argyritarsis	4	3.0
Anopheles benarrochi	4	19.0
Anopheles darlingi	4	5.7
Anopheles deaneorum	3	2.5
Anopheles dunhami	2	8.8
Anopheles evansae	3	13.0
Anopheles konderi	3	0.3
Anopheles rangeli	3	0.3
Anopheles strodei	3	8.0
Anopheles triannulatus	2	0.2
Anopheles trinkae	2	0.3
Anopheles marajoara	7	15.0
Anopheles oswaldoi	8	18.0
Anopheles nuneztovari	5	0.3

by the Neighbour Joining algorithm and number of differences) using the MEGA software (Molecular Evolutionary Genetics Analysis, version 3.01) (Kumar et al. 2003). The sequences were aligned using the CLUSTAL W software (1.60) (Thompson et al. 1994). To obtain a better alignment, both pairwise and multiple alignments parameters were changed from the default set: DNA substitution matrix from the Clustal program, decreased the open gap penalty to 10, and also decreased the transition/transversion rate to 0.25. The ITS2 sequences were clustered into three major clades that represent the three subgenera, *Nyssorhynchus*, *Kerteszia,* and *Anopheles*. Although *Anopheles* and *Kerteszia* subgenera have fewer species and ITS2 sequences represented in this study, as compared to the *Nyssorhynchus* subgenus, the three groups formed are compatible with the morphological hypothesis of classification of the genus *Anopheles*. Based on their ITS2 sequences, *Nyssorhynchus* and *Kerteszia* mosquitoes grouped together in a major phylogenic branch (Fig. 1-a) supporting the *Nyssorhynchus* and *Kerteszia* monophyletic lineage previously proposed (Sallum et al. 2000, Krzywinski et al. 2001a, b).

SUBGENUS *KERTESZIA*

Kerteszia is a small Neotropical subgenus of *Anopheles*, composed of only 12 species (Zavortink 1973, Collucci & Sallum 2003), with a geographic distribution extending from South of Mexico to the state of Rio Grande do Sul in Brazil (Aragão 1964). *A. cruzii* and *A. bellator* were the primary vectors of the once malaria endemic area in Southeastern and Southern Brazil (Rachou 1958). Up to now, however, these species have been involved in the transmission of malaria to human populations that live in close association with the Atlantic Forest (Deane 1988, Branquinho et al. 1997, Machado et al. 2003).

Of the four *Kerteszia* species having available ITS2 sequences, the NJ tree shows that the ITS2 sequences of *A. cruzii* cluster in a single clade (Fig. 1-b). Four different ITS2 sequences assigned to organisms identified as *A. cruzii* exist in the data base. These four sequences could either represent intraspecific polymorphism or the existence of a complex of sibling species yet to be described. Specimens of *A. cruzii* also have been analyzed on the basis of their polytene chromosome patterns (Ramirez & Dessen 2000 a,b) providing evidence for genetically distinct populations, and suggesting an ongoing process of speciation within this group of mosquitoes. Future studies should verify if there is a correlation between the chromosomal forms and the distinct ITS2 sequences. The placement of *A. laneanus* next to *A. cruzii* (Fig. 1-c) is consistent with the interpretation that these two species belong to a group that includes *A. cruzii*, *A. laneanus* and possibly other unnamed new species. *A. laneanus* is an endemic species of the Serra da Mantigueira, Southern Brazil, and is

morphologically more similar in all life stages to *A. cruzii* than to any other *Kerteszia* species. Characters that separate these two species are mainly those of the male genitalia. The *Kerteszia* subgenus and the presumed *A. cruzii* complex deserve further investigation, since they include human malaria vectors. The present situation in Southern Brazil, where malaria parasites and vectors coexist alongside developed and highly populated areas, demands constant surveillance from public health authorities to prevent epidemic episodes.

SUBGENUS *ANOPHELES*

The ITS2 sequences of mosquitoes of the subgenus *Anopheles* (11 species) cluster together (Fig. 1-d). As mentioned above, several malaria vectors belonging to this group do not have ITS2 sequences available. All ITS2 sequences generated from *A. quadrimaculatus* grouped together (Fig. 1-e). *A. quadrimaculatus* belongs to the Quadrimaculatus Subgroup and the samples are clustered with three species of the Freeborni Subgroup (*A. freeborni*, *A. hermsi,* and *A. occidentalis*) (Fig. 1-f). Both subgroups belong to the Maculipennis Group. The Crucians Complex of the Punctipennis Subgroup includes *A. crucians*, *A. bradleyi*, *A. georgianus,* and four informally designated species. Except for the sequence CRUC3 that was recovered within the clade leading to species of the Maculipennis Group, the remaining four *A. crucians* plus BRAD1 sequences clustered together, reflecting their relative positions determined by classical taxonomy (Fig. 1-g). Wilkerson et al. (2004) subdivided the North American specimens of the taxon *A. crucian*s into *A. crucians* species A, B, C, D, and E (here designated CRUC1-5) based on rDNA ITS2 sequence data. Further investigation is needed to determine if *A. crucians* from Latin America belongs to those previously described species. The placement of *A. bradleyi* (BRAD1) within the *A. crucians* Group could indicate that gene flow occurs among these two closely-related species. However, this hypothesis has already been tested and discarded by the analysis of the progeny of *A. bradleyi* and *A. crucians* intraspecific crosses (Kreutzer & Kitzmiller 1971).

A. mediopunctatus and *A. fluminensis*, both of the Arribalzagia Series (Wilkerson & Peyton 1990) are grouped together, however, *A. peryassui* and *A. mattogrossensis* of the same series were placed in different branchs of the tree. *A. eiseni* and *A. pseudopunctipennis*, both of the Pseudopunctipennis Group were placed in distinct clades (Fig. 1-h). These molecular data do not support the classic systematic placement of the group, however, it is important to consider that only one ITS2 sequence is available for each one of these five species. While the sequences may have some taxonomic value, evolutionary inferences could not be derived from such a small sample.

SUBGENUS *NYSSORHYNCHUS*

The subgenus *Nyssorhynchus* comprises 33 species (Harbach 2004) that are largely known to be problematic for identification using only female morphological characters, mainly due to their intraspecifc variation and interspecific similarity (Hribar 1995, 1997). It is worth noting that several of the ITS2 sequences presently available in the GenBank were generated from DNA extracted from field captured female specimens and this could lead to misidentification as discussed in the following paragraphs. Male genitalia traits are generally less variable (Faran 1980, Linthicum 1988), however, sometimes they lack elements for species identification or may vary geographically (Hribar 1994). Consequently other life stages need to be examined as well. The external morphology of the eggs also has been investigated in order to provide species-diagnostic characters (Lounibos et al. 1998). Forattini et al. (1998) showed that this character is polymorphic among populations of a single species and Deane and Causey (1943) provided evidence that the eggshell morphology could vary according to environmental conditions. Therefore, the external morphology of the egg also should be used cautiously when distinguishing between closely-related species.

Identification difficulties are not evident for all the *Nyssorhynchus* species. Some species of the *Nyssorhynchus* subgenus are easily distinguishable by morphological characters and ITS2 sequences. Hence, all 15 sequences from *A. aquasalis* were recovered in a single clade, and so were four from *A. darlingi*, four from *A. argyritarsis*, three from *A. trinkae*, two from *A. triannulatus*, and three from *A. rangeli* specimens (Fig. 1-i, j, k, l, m, n). In contrast, other species are not easily discernible from each other and their ITS2 sequences were placed in more than one group of the NJ tree.

The *A. albitarsis* complex includes *A. albitarsis s. s.*, *A. marajoara*, *A. deaneorum,* and an unnamed species designated *A. albitarsis* B (Wilkerson et al. 1995). Lehr et al. (2005), using data derived from the sequences of the cytochrome c oxidase subunit I (COI) gene, hypothesized a fifth species in this complex, designated species E, which occurs in Boa Vista municipality, state of Roraima, Brazil. Species included in the *A. albitarsis* complex are notoriously difficult to identify. Their external morphology is similar in all life stages with interspecific and intraspecific variation. *A. deaneorum* can be distinguished from other species only by the larval seta 3-C, which is branched in *A. deaneorum*, whereas it is single in the remaining species. In the adult stage, *A. deaneorum* can be distinguished by the absence of posterolateral scales on abdominal segment III. Both traits are difficult to evaluate and need well-preserved samples. Morphology traits for separating *A. marajoara*, *A. albitarsis,* and *A. albitarsis* B are more problematic and may overlap among the species. Wilkerson et al. (1995) noted that the extent of dark pigmentation on the hindtarsus 2 could be used to distinguish *A. albitarsis* and *A. albitarsis* B. The former species has more than 0.5 basal dark, while *A. albitarsis* B has less than 0.5. However, if this is correct *A. albitarsis* overlaps *A. marajoara* in which the basal dark varies from 0.6-0.9 length of segment. Members of the *A. albitarsis* complex are apparently largely allopatric. According to Li and Wilkerson (2005), *A. albitarsis* occurs in Southern Brazil, Northern Argentina, and Paraguay, *A. deaneorum*

in Northern Argentina and Western Brazil, *A. albitarsis* B in South, Central, and Eastern Brazil, *A. marajoara* in Brazil, Venezuela, Colombia, Southern Central America, and *A. albitarsis* E is restrict to Northern Amazonas River. Even with that information, geographical distribution of members of the *A. albitarsis* complex is poorly described, especially along the Brazilian coast and savannah where few captures have been performed, and there are no specimens from those areas available in collections for study. In conclusion, discriminating among species of the *A. albitarsis* complex based on either morphology or molecular markers is still problematic (Wilkerson et al. 1995, Lehr et al. 2005). Accordingly, we found the ITS2 sequences of *A. marajoara*, *A. deaneorum,* and *A. albitarsis* distributed in more than one group of the tree (Fig. 1-o). This pattern could indicate large intraspecific variations of the ITS2 sequences, a recent process of speciation within this group, or could represent the misidentification of the specimens that had their DNA extracted and sequenced. Recent advances reported by Li and Wilkerson (2005) indicate that the polymerase chain reaction technique with a set of species-specific oligonucleotide primers enables the separation of four species of the *A. albitarsis* complex, except species E. Nevertheless, further efforts to clarify the taxonomic status of these mosquitoes are being made and the morphology of *A. albitarsis* species E is being evaluated by Motoki et al. (unpublished results).

Marrelli et al. (1999) analyzing the ITS2 from specimens identified as *A. oswaldoi* found four distinct sequences and hypothesized that at least one of these could be from *A. konderi*. Morphological distinction between *A. konderi* and *A. oswaldoi* is based on the shape

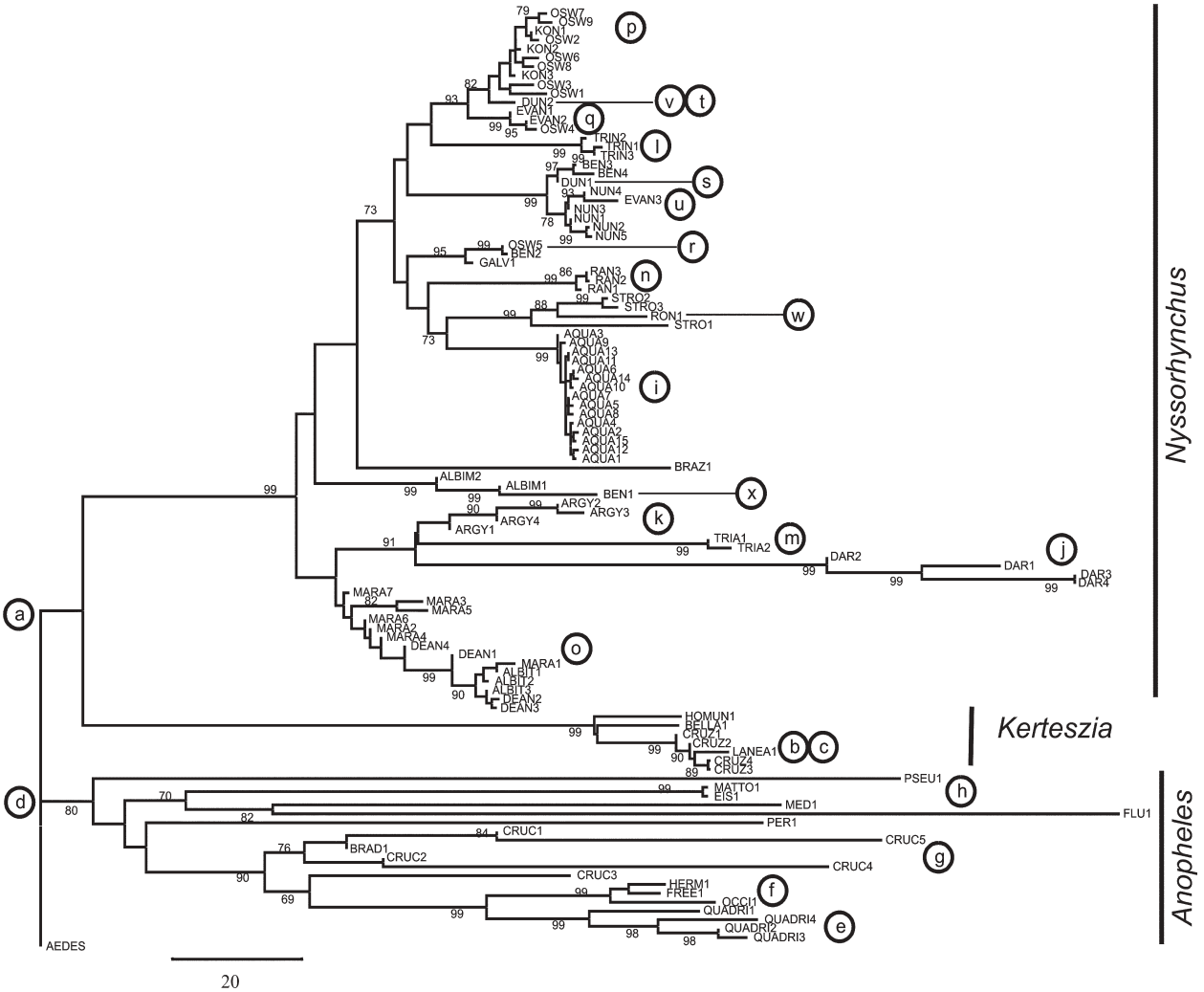

Fig. 1: Neighbor-Joining bootstrap tree based on the second internal transcribed spacer (ITS2) of the nuclear ribosomal DNA genes from Latin American *Anopheles* mosquitoes. The tree was rooted with *Aedes aegypti* ITS2 sequence (GenBank/EMBL: M95126). Numbers above the branches show bootstrap confidence levels (from 1000 replications) for the split. Bootstrap values lower than 70 were omitted in the figure. Species names abbreviated as in Table I. The grouping of the species belonging to each subgenus is indicated in the right of the figure. Letters (a-x) indicate the positions of the sequences discussed in the text. Aligned sequences (Clustal W) were examined with the MEGA software (Molecular Evolutionary Genetics Analysis, version 3.01). Sequences alignment and an alternative tree by Maximum Parsimony method are available upon request to the authors.

of the apical area of the aedeagus of the male genitalia (Causey et al. 1946). Because the type locality of the former species is Coari (Rio Solimões, state of Amazonas, Brazil) it is reasonable to consider that the specimen that Marrelli et al. (1999) collected in the state of Amazonas is in fact *A. konderi*. Except for OSW4 and OSW5, most sequences of specimens identified as *A. oswaldoi* and *A. konderi* clustered in a major clade (Fig. 1-p). Further studies need to be performed to determine if there is a true overlap of ITS2 sequences of *A. oswaldoi* and *A. konderi* or the present data is due to misidentification of specimens. Interestingly, the OSW4 sequence generated from a specimen captured in Vale do Rio Doce, Espírito Santo, Brazil, which was identified as *An. oswaldoi* by Marrelli et al. (1999) grouped with EVAN1 and EVAN2 (Fig. 1-q), whereas OSW5 isolated from specimen collected in Peru appeared in a clade formed by sequences of *A. galvaoi* and *A. benarrochi* (BEN2) (Fig. 1-r). Position of an *A. oswaldoi* sequence in a cluster with two *A. evansae* sequences suggests that the specimen identified as *A. oswaldoi* (OSW4) was misidentified. Morphological separation of adult females of *A. evansae* and *A. oswaldoi* is based mainly on the basal dark band of the hindtarsal segment 2 which is usually < 0.25 (0.12 - 0.25) the length of segment in *A. oswaldoi* and *A. konderi* or ≥ 0.25 the length of segment in *A. evansae* (0.2 - 0.4). In addition to the hindtarsus 2, Faran (1980) pointed out the ratio between the humeral light spot and basal dark spot of wing vein C that in *A. oswaldoi* varies from 1.10 to 3.80, while in *A. evansae* is 1.75 - 4.5. In summary, these characters are polymorphic and overlap between the two species. Therefore, the proper identification of these mosquitoes requires a wide-ranging study of how characters vary in distinct population of each species and the observation of male-associated characters. Male genitalia aedeagus clearly distinguish *A. evansae* and *A. oswaldoi* indicating that the analyses of male progeny of captured females is necessary to solve these impasses.

Morphological separation between *A. oswaldoi*, *A. galvaoi,* and *A. benarrochi* may be problematic when using only adult female traits (Sallum et al. 2002), and thus we believe that some of the specimens that generated the cluster containing BEN2, GALV1, and OSW5 sequences (Fig. 1-r) could have been misidentified. Quiñones et al. (2001), Calle et al. (2002), and Estrada et al. (2003) noted that some individuals of *A. benarrochi* from Southern Colombia can be misidentified as either *A. evansae* or *A. oswaldoi* due to overlapping characters when using female morphology. *A. evansae*, *A. oswaldoi,* and *A. benarrochi* can be easily distinguished based on male genitalia traits. The occurrence of *A. benarrochi* in the state of São Paulo, Brazil (Sallum et al. 1997) is suggestive that this population differs from that of the type locality, which is in La Ceiba, Trujillo Department, Venezuela. ITS2, as well as other molecular markers could be informative about similar and/or differential characters among *A. benarrochi* populations from distinct geographic origins. Populations of *A. benarrochi* of São Paulo are currently been investigated, and there are morphological evidences that distinguish São Paulo form from that illustrated by Faran (1980) (Sallum et al. unpublished results).

The ITS2 sequences of *A. dunhami* specimens also are problematic. One sequence, DUN1 groups with *A. benarrochi* (BEN3 and BEN4, both from specimens collected in the state of Rondonia, Brazil), whereas DUN2 (specimen collected in the state of Acre) clusters within the *A. oswaldoi* group (Fig. 1-s,t). The geographical distribution of *A. dunhami* is poorly known. Currently, this species was registered in Tabatinga and Tefé (type-locality) both in Amazonas, Brazil. The fact that DUN1 clusters with BEN3 and BEN4 raises a question about the presence of *An. dunhami* in Rondonia. However, this hypothesis might be tested by generating sequences from specimens correctly identified. The adult female of *A. dunhami* is morphologically more similar to *A. nuneztovari* than to any other *Nyssorhynchus* species, however both species can be misidentified as either *A. oswaldoi* or *A. evansae* when using only female traits. Characters of the hindtarsomere 2 and the ratio of humeral light spot and basal dark spot, although distinct among those species, vary and may overlap among distinct populations and consequently makes species separation more difficult. For example, the placement of *A. evansae* among sequences generated from specimens identified as *A. nuneztovari* (Fig. 1-u) and *A. oswaldoi* (Fig. 1-q), and that of *A. dunhami* within the *A. oswaldoi* group (Fig. 1-v) makes it reasonable to conclude that a specimen of *A. nuneztovari* has been misidentified as *A. evansae* (EVAN3), and DUN2 belongs, in fact, to a specimen of a taxa of the *A. oswaldoi* Group. Distinction between *A. evansae* and *A. nuneztovari* is based mainly on ratio of lengths of the vein C humeral light spot and basal dark spot, which is < 2.0 in *A. nuneztovari* but ≥ 2.0 in *A. evansae*. However, Hribar (1995) showed that variation occur within and among isofemale progeny lines of *A. nuneztovari* from distinct geographic localities, and thus they could be misidentified as other species, i.e. *A. evansae* when that value is > 2. Consequently, the presence of *A. dunhami* in Acre state needs to be confirmed.

Wilkerson et al. (1990) have determined that *A. rondoni* can be easily distinguished from other species based on the dark spot of the prescutellar area, which is well developed in *A. rondoni*, however a character frequently used for identification of *A. rondoni* is a dark basal band on hindtarsome 3. It is important to note that this dark band is absent in some individuals of this species and thus *A. rondoni* can be misidentified if the dark spot in the prescutellar area is not taken into consideration. The position of the RON1 sequence within the *A. strodei* group (Fig. 1-w) suggests that either the sample used for generating the ITS2 sequence was misidentified as *A. rondoni* or that *A. strodei* may be a species complex. Moreover, Sallum et al. (unpublished results) found strong morphological evidences that there are two distinct forms of *A. strodei* in São Paulo. This is going to be tested by sequencing specimens of both forms.

The placement of BEN1 among sequences of specimens of *A. albimanus* (Fig. 1-x) is probably due to misidentification or mislabeling of that specimen (BEN1) because the species can be easily separate using female external morphology (see Faran 1980, for morphological details).

CONCLUDING REMARKS

We have reviewed the available ITS2 sequences from Latin American anophelines and antecipate that it will further stimulate needed research in malaria vector taxonomy. The analyses indicate that ITS2 provides valuable molecular markers that could be applied for the identification of some species and also could be used to further investigate population genetics and the occurrence of sibling species of Latin America anophelines. Although morphological characters are available to identify most of the studied species, the distinction of closely-related species may be problematic, and additional differential traits must be utilized. DNA-based methods are advantageous in these cases because they can be applied to specimens and situations unsuitable for morphological taxonomy. For example, DNA may be obtained and analyzed from specimens in all developmental stages, of both sexes, fresh, preserved in alcohol, dried or frozen.

One possibility that has emerged from this comprehensive analysis is that because of the difficulties in precise identification of field captured female specimens based only on their morphological characters, a number of them could have been misidentified and therefore their corresponding ITS2 sequences were deposited into GenBank with incorrect organism source assignment. Unfortunately, most of the publications regarding the ITS2 sequences from Neotropical anophelines do not provide indications that siblings of the analyzed specimen are preserved and available for further morphological and/or molecular verification. Therefore, those questionable sequences can not be reevaluated and validated, or have their records corrected. In conclusion, while research in malaria vector taxonomy has profited from data provided by single or few field captured mosquitoes, the problems pointed out in the preceding paragraphs indicate that caution should be taken and a minimum of requirements should be considered by researchers working with Latin American anophelines in the design of future studies. Following we outline basic characteristics of taxonomic studies in order to continue producing reliable and high-quality results that will provide basis for effective surveillance and control strategies in the countries where malaria occurs. We propose that:

1) *Voucher specimens need to be deposited in collections (Museums)* - The progeny of field captured female mosquitoes can usually be raised in the laboratory. DNA could then be extracted from some of the siblings of the family while other specimens should be preserved to allow future verification. DNA sequences should be of high quality and attached to a voucher specimen whose origin and current status are registered. The placement of samples in repository institutions will serve as a platform to ensure preservation of specimens and communication between repositories and researchers (Hebert & Gregory 2005, Corthals & Desalle 2005)

2) *Intraspecific variation should be considered* - Species identification either needs to be based on invariant characters, or the variability of the characters determined among the individuals of the species. Larger samples composed of several specimens from each locality, and from distinct localities covering the known geographic distribution of the species, should be analyzed. Furthermore, intragenomic heterogeneity may occur, and in those circunstances direct sequencing of PCR products may be misleading. Intragenomic polymorphism occurring, cloning of PCR products and sequencing of individual clones from each specimen is required. Unfortunately, due to the cost and time-consuming mosquito captures, researchers are sometimes reluctant to perform these large scale studies, incurring the risk of proposing or defining species complexes without an adequate evaluation of intraspecific variation (van Bortel & Coosemans, 2003). As an example, Fairley et al. (2005) detected 15 different *A. aquasalis* ITS2 sequences (72 clones examined) from mosquito samples from two geographic locations in Brazil, two in Venezuela, and one in Suriname. The observed intraspecific divergence could suggest a complex of species, nevertheless that accurate study led to the conclusion that the nucleotide variations were not informative in distinguishing populations, supporting the status of *A. aquasalis* as a single species.

3) *Other molecular markers should be used in addition to ITS2* - Molecular biological techniques are becoming readily available in most scientific institutions and the cost of DNA sequencing is no longer prohibitive. ITS2 is only one of the molecular markers, therefore, once the DNA has been extracted from mosquito specimens, a wealth of information could be generated, including the sequences of other marker genes such as those of the nuclear and mitochondrial ribosomal loci (ITS1 and IGS) and mitochondrial genomes (COI, COII). Random amplified polymorphic DNA (RAPD) and restriction fragment length polymorphic (RFLP) markers as well as microsatellite DNAs could also be utilized to distinguish between anopheline species (Norris 2002).

4) *Additional biological data are necessary* - Laboratory autonomous colonies of most of the Neotropical anophelines, including some of the major malaria vectors such as *A. darlingi*, are still unavailable. Efforts should be made to colonize these mosquitoes and this would facilitate the acquisition of biological data that nowadays are only possible through time-consuming and costly field captures. These colonies also would be beneficial in providing the opportunity of performing mating compatibility studies between specimens of potential sibling species (Lima et al. 2004 a,b). Cytogenetics has proven immensely useful for differentiating among sympatric anopheline taxa, however,

with a few exceptions (Pérez & Conn 1992, Ramirez & Dessen 2000 a, b), this technique has not been applied for neotropical anophelines. Ecological (Guimarães et al. 2004), behavioral (da Silva Vasconcelos et al. 2002) and biochemical (Phillips et al. 1988, dos Santos et al. 2003) studies also provide important information for mosquito taxonomy and should be encouraged.

5) *A mosquito integrative taxonomy is valuable and desirable* - Molecular markers are to support and not replace traditional taxonomy. The value of molecular markers is most powerful in disclosing hidden diversity when coupled with traditional taxonomic tools. The continuous generation of quality data and the application of an integrative taxonomy, which uses all available characters including DNA sequences and other types of data, to delimit, discover, and identify meaningful, natural species and taxa (Faran 1979, Rubio-Palis & Zimmerman 1997, Lounibos & Conn 2000, Will et al. 2005) will contribute to a more detailed understanding of the anophelines species and complexes in Latin America and serve as a basis for effective malaria vector control programs in this part of the world.

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