Universidade de São Paulo
Faculdade de Saúde Pública

Estudos sistemáticos sobre espécies da Seção Myzorhynchella do subgênero *Nyssorhynchus* (Diptera: Culicidae)

Sandra Sayuri Nagaki

Dissertação apresentada ao Programa de Pós-Graduação em Saúde Pública para obtenção do título de Mestre em Saúde Pública.

Área de Concentração: Epidemiologia

Orientadora: Profa. Dra. Maria Anice Mureb Sallum

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RESUMO

Nagaki, SS. Estudos sistemáticos sobre espécies da Seção Myzorhynchella do Subgênero *Nyssorhynchus* (Diptera: Culicidae) [Dissertação de Mestrado]. São Paulo: FSP- USP; 2009.

Introdução – Anopheles (*Nsssorhynchus*) constitui o grupo de anofelinos que encerra o maior número de vetores de plasmódios que causam a malária humana na Região Neotropical. Em vista disso, são as espécies que têm sido mais frequentemente estudadas. O subgênero possui 33 espécies e está dividido em três seções, Myzorhynchella, Albimanus e Argyritarsis. A última revisão da seção Myzorhynchella é a de Galvão (1941) e são raros os estudos com a seção que é formada pelas espécies *An. lutzii*, *An. parvus*, *An. nigritarsis* e *An. antunesi*. Embora estas espécies sejam consideradas zoofílicas, estudos taxonômicos são necessários para estabelecer a identificação morfológica e para diferenciar estas espécies de outros Anophelinae, fornecendo assim condições adequadas para avaliar as espécies que estão envolvidas na transmissão da malária. *Objetivos* – Caracterizar morfologicamente e molecularmente as espécies da seção Myzorhynchella e estabelecer caracteres morfológicos que permitam a separação entre as mesmas. *Métodos* – Foram realizadas coletas de mosquitos em diferentes localidades da Mata Atlântica, além da análise de caracteres morfológicos de larva, pupa, adultos macho e fêmea e ovos de espécimes disponíveis na coleção entomológica da Faculdade de Saúde Pública – FSP/USP, do Museu de Zoologia – MZUSP e do Instituto Oswaldo Cruz – IOC. Foram realizadas análises moleculares utilizando sequências de bases nucleotídicas da região do Espaçador Interno Transcrito 2 - ITS2 do DNA ribossômico e do gene mitocondrial Citocromo Oxidase Subunidade I - COI. *Resultados* - Foram caracterizados os adultos, machos e fêmeas, as formas imaturas e os ovos de *An. antunesi* e de *An. lutzii*. *Anopheles guarani* e *An. niger* foram retiradas da sinonímia de *An. lutzii.* Foi descrita uma espécie nova que é encontrada em simpatria com *An. antunesi* na Serra da

Mantiqueira. Os resultados das análises filogenéticas corroboraram a existência de pelo menos cinco espécies dentro da seção Myzorhynchella e indicam que *An. parvus* e *An. antunesi* podem representar complexos de espécies. Acresce considerar que *An. lutzii* foi redescrita com o emprego de espécimes do Vale do Ribeira. No entanto, a falta de espécimes de *An. lutzii* da localidade tipo com as formas adultas e imaturos associados, impediram a caracterização adequada da espécie. *Conclusão* – Foram caracterizadas quatro espécies da seção Myzorhynchella, foi descrita uma espécie nova que ocorre na Serra da Mantiqueira e demonstrou-se que *An. parvus* e *An. antunesi* podem ser complexos de espécies. Há a necessidade de continuar os estudos da Seção Myzorhynchella e obter topotipos de *An. lutzii*.

Descritores: *Nyssorhynchus* - Myzorhynchella – nova espécie – sinonímia – análise molecular

ABSTRACT

Nagaki, SS. Estudos sistemáticos sobre espécies da Seção Myzorhynchella do Subgênero *Nyssorhynchus* (Diptera: Culicidae). / Systematic studies on species of Myzorhynchella Section of the subgenus *Nyssorhynchus* (Diptera: Culicidae) [Dissertation]. São Paulo (BR): FSP-USP; 2009.

Introduction – *Anopheles* (*Nsssorhynchus*) is the group of anophelines that has the largest number of vectors of plasmodium that causes human malaria in the Neotropics. Because of this, species of this subgenus have been most frequently studied. The subgenus has 33 nominal species, subdivided into three sections, Myzorhynchella, Albimanus and Argyritarsis. The last revision on species of the Myzorhynchella section is that by Galvão (1941) and taxonomic studies are rare in the section that is formed by *An. lutzii*, *An. parvus*, *An. nigritarsis* and *An. antunesi*. Although these species are considered to be zoophilic, taxonomic studies are necessary to fix the morphological identification, and to differentiate these species from other Anophelinae, thus providing appropriate conditions to evaluate which species are involved in the transmission of malaria. *Objetives* – To fix the morphological and molecular identification of species of the Myzorhynchella section and to define morphological characters to separate the species. *Methods* – The mosquitoes were collected in different localities in the Mata Atlântica. These specimens were employed in analyses of morphological characters of eggs, larva, pupa, adult male and female, and were compared to specimens deposited in the Entomological Collection of Faculdade de Saúde Pública – FSP/USP, Museu de Zoologia – MZUSP and Instituto Oswaldo Cruz – IOC. Molecular analysis were performed using sequences of of the internal transcribed spacer 2 – ITS2 of ribosomal DNA and the mitochondrial cytochrome oxidase subunit I gene – COI. *Results* – We characterized the adults, male and female, immature forms and the eggs of *An. antunesi* and *An. lutzii*. *Anopheles guarani* and *An. niger* were removed from the synomym of *An. lutzii*. A new species that was found in sympatry with *An. antunesi* in Serra da Mantiqueira was described. Results of

phylogenetic analysis corroborate the existence of at least five species within Myzorhynchella Section and indicate that *An. parvus* and *An. antunesi* may represent complexes of species. Besides *An. lutzii* was redescribed using specimens of Ribeira Valley. However, the lack of specimens of *An. lutzii* from the type locality with associated adults and immature forms prevented the proper characterization of the species. *Conclusion* – We characterized four species of Myzorhynchella section, a new species that occurs in Serra da Mantiqueira was described, and it showed that *An. parvus* and *An. antunesi* may be species complex. There is a need to continue the studies of the section and get topotypes of *An. lutzii*.

Descriptors: *Nyssorhynchus* – Myzorhynchella – new species – synonymy – molecular analysis.

ÍNDICE

1. INTRODUÇÃO

1.1. GÊNERO ANOPHELES

O gênero *Anopheles* Meigen inclui todos os vetores conhecidos da malária humana. A malária é uma doença tropical que ameaça mais de 40% da população mundial em mais de 90 países e territórios (SALLUM et al., 2000). Reside nesse fato o maior interesse que esses mosquitos despertam sob o ponto de vista epidemiológico (FORATTINI, 2002).

De acordo com HARBACH (2007), a maioria das espécies de anofelíneos pertence ao gênero *Anopheles*, que inclui 455 espécies e está subdividido em sete subgêneros: *Anopheles* (cosmopolita), *Baimaia* (Oriental), *Cellia* (Velho Mundo), *Kerteszia* (Neotropical), *Lophopodomyia* (Neotropical), *Nyssorhynchus* (Neotropical) e *Stethomyia* (Neotropical).

No Brasil, foram registradas 54 espécies que estão incluídas em cinco subgêneros (*Nyssorhynhus, Kerteszia, Stethomyia, Lophopodomyia*, *Anopheles*) (ROSA-FREITAS et al., 1998).

Embora o gênero *Anopheles* seja o mais estudado e mais conhecido grupo de todos os culicídeos, não se pode afirmar que a última classificação do gênero é a correta. Há muitas questões para serem respondidas e muitas espécies a serem descobertas.

1.2. SUBGÊNERO NYSSORHYNCHUS

O subgênero *Nsssorhynchus* Blanchard constitui o grupo de anofelíneos que encerra o maior número de espécies que atuam como vetores, principais ou auxiliares, de plasmódios que infectam o ser humano na Região Neotropical. Em todo o mundo, ao redor de três bilhões de pessoas vivem sob o risco de serem infectadas por plasmódios. A malária

mata mais de um milhão de pessoas todos os anos – a maior parte crianças que vivem na África subsaariana. Em 2002, o Brasil registrou aproximadamente 40% do total dos casos de malária das Américas (OMS, 2005). Mais de 99% dos casos registrados no Brasil ocorrem na Amazônia na qual a transmissão se deve a espécies de *Nyssorhynchus* (ROSA-FREITAS et al., 1998). Em vista disso, são as espécies de *Anopheles* que têm sido mais frequentemente focalizadas em estudos epidemiológicos dessa infecção (FORATTINI, 2002).

O subgênero *Nyssorhynchus* possui 33 espécies e está dividido em três seções, Myzorhynchella, Albimanus e Argyritarsis (HARBACH, 2004). FARAN (1980) dividiu o subgênero *Nyssorhynchus* em duas seções, a Albimanus e a Argyritarsis, distinguindo-as através de caracteres morfológicos. Paralelamente, o autor reconheceu o Grupo Myzorhynchella, mas considerou como incerto o status taxonômico do mesmo. LINTHICUM (1988) revisou a seção Argyritarsis. Em contraste, a última revisão da seção Myzorhynchella é a de Galvão (1941).

1.3. SEÇÃO MYZORHYNCHELLA

1.3.1. Histórico

A Seção Myzorhynchella foi descrita como gênero de Culicidae por THEOBALD (1907). Baseado nos caracteres da terminália do macho, CHRISTOPHERS (1924) considerou cinco subgêneros no gênero *Anopheles*: *Anopheles, Bironella, Chagasia, Myzomyia* e *Nyssorhynchus.* Dessa maneira, as espécies do gênero *Myzorhynchella* foram transferidas para o subgênero *Nyssorhynchus*. Posteriormente, ROOT (1926) dividiu as espécies de *Nyssorhynchus* em três grupos, Nyssorhynchus, Myzorhynchella e Kerteszia. COSTA LIMA (1929) elevou *Nyssorhynchus* a gênero de Culicidae e os três grupos para subgênero. EDWARDS (1932)

adotou a classificação de ROOT (1926), considerando o grupo Myzorhynchella junto com os grupos Nyssorhynchus e Kerteszia no subgênero *Nyssorhynchus*. LANE (1939) seguiu a proposta de EDWARDS (1932) e ROOT (1926) e também considerou Myzorhynchella como um grupo de *Nyssorhynchus*. Na revisão de Myzorhynchella, GALVÃO (1941) considerou que as espécies da seção formavam um agrupamento zoologicamente homogêneo dentro do gênero *Anopheles* e, por isso, representariam um subgênero. Nesse sentido, o grupo Myzorhynchella foi elevado à categoria de subgênero de *Anopheles*. Finalmente, PEYTON et al. (1992) definiram Myzorhynchella como seção do subgênero *Nyssorhynchus*, por considerarem que ambos reuniam espécies derivadas de ancestral comum baseado em semelhança morfológica.

1.3.2. Espécies da Seção

A Seção Myzorhynchella inclui as espécies:

- *Anopheles lutzii* CRUZ, 1901;
- *Anopheles parvus* (CHAGAS, 1907);
- *Anopheles nigritarsis* (CHAGAS, 1907);
- *Anopheles antunesi* GALVÃO & AMARAL, 1940.

Todas foram descritas a partir de espécimes coletados no Brasil (BELKIN et al., 1971). No entanto, não foram feitas associações entre os diversos estádios de desenvolvimento do inseto que foram descritos por outros autores, com animais coletados fora das localidades tipo. Acresce considerar que o adulto macho e os estádios imaturos de *Anopheles nigritarsis* são desconhecidos (FORATTINI et al., 1997).

Quanto às informações sobre a fonte e localização do material tipo das espécies de acordo com BELKIN et al. (1971):

Anopheles (Nys.) parvus (Chagas, 1907). Tipo: Macho(s) e fêmea(s), fêmea (tubo 994) e lâmina de asa (no. 2112). Localidade-tipo: Oliveira

(Minas Gerais). Depositado na coleção IOC (Instituto Oswaldo Cruz, Rio de Janeiro).

Anopheles (Nys.) nigritarsis (Chagas, 1907). Tipo: Fêmea (s). Localidade-tipo: Oliveira (Minas Gerais). Desconhecido o local depositado.

Anopheles (Nys.) antunesi Galvão & Amaral, 1940. Tipo: Holótipo fêmea (no. 370) com lâmina de exúvia de larva (no. 235). Localidade-tipo: Vila Emílio Ribas, Campos do Jordão (São Paulo). Originalmente depositado na coleção FMSP (Departamento de Parasitologia, Faculdade de Medicina, São Paulo, SP). No entanto, o material da mencionada coleção foi transferido para a Faculdade de Saúde Pública, sem os tipos das espécies descritas por Galvão. O tipo não foi encontrado. Existe um parátipo fêma (nº 409) depositado na Coleção da Faculdade de Saúde Pública da Universidade de São Paulo.

Anopheles (Nys.) lutzii Cruz, 1901. Tipo: 3 síntipos fêmeas (no. 1965) em tubo 993 e lâmina de asa no. 2111. Localidade-tipo: Lagoa Rodrigo de Freitas, Guanabara, (Rio de Janeiro). Depositados na coleção IOC (Instituto Oswaldo Cruz, Rio de Janeiro).

Sinônimos de *An. lutzii*:

Anopheles (Nys.) niger (Theobald, 1907). Tipo: Lectótipo fêmea. Localidade-tipo: Cantareira (São Paulo). Depositado na coleção NHM (Natural History Museum, Londres).

Anopheles (Nys.) guarani Shannon, 1928. Tipo: Holótipo fêmea. Localidade-tipo: Iguaçu (Paraná). Depositado na coleção USNM (U.S. National Museum of Natural History).

As principais características morfológicas que identificam espécies da seção Myzorhynchella são a ausência completa de escamas nos oito segmentos abdominais dos adultos, havendo escamas apenas nos segmentos genitais, na genitália masculina, presença de folíolos no edeago e claspete dorsal com dois folíolos apicais e um implantado subapicalmente, e nas larvas, cerda 6 dos segmentos abdominais 4, 5 e 6, longas e ramificadas (GALVÃO, 1941).

Pouco se conhece sobre a biologia das espécies da seção Myzorhynchella. De acordo com a literatura, são mosquitos essencialmente zoófilos e silvestres. As formas imaturas têm sido encontradas em pequenas coleções de água, como poças de chuva, pequenos charcos, riachos e buracos em rochas. Os criadouros estão geralmente situados em ambientes florestais, sombreados e contendo água limpa e com vegetação escassa (FORATTINI, 1962).

As fêmeas parecem ter acentuada preferência por sangue de animais não humanos e, seu encontro intradomiciliar reveste-se de certo caráter de raridade. Contudo, em algumas regiões do Brasil, *Anopheles parvus* e *Anopheles lutzii* foram observadas sugando o homem fora das habitações e mesmo, a segunda espécie, no intradomicílio (ANTUNES e LANE, 1933). Por outro lado, GALVÃO (1941) assinalou que somente com dificuldade, conseguiu alimentar espécimes de *Anopheles parvus* com sangue humano. GALVÃO e AMARAL (1940) observaram que os alados de *Anopheles antunesi* não picavam as pessoas que permaneciam ao lado da isca animal e DEANE et al. (1948) capturaram exemplares de *Anopheles parvus* com esse tipo de isca, fora das habitações.

NEIVA (1909) observou espécies da seção Myzorhynchella predominando no Piau, localidade situada próxima de Juiz de Fora, Minas Gerais. As espécies mais frequentes foram *Anopheles lutzii* e *An*. *parvus*. Por serem abundantes, NEIVA (1909) julgou-as suspeitas de envolvimento na transmissão de malária.

Apesar das evidências mencionadas, há quem atribua certo papel vetor a algumas das espécies da seção Myzorhynchella. Todavia, não existem até o momento evidências epidemiológicas que permitam confirmar essa hipótese (FORATTINI, 1962).

Os trabalhos mais recentes com espécies da Seção Myzorhynchella são os de FORATTINI et al. (1997) com a descrição dos ovos de *An. antunesi,* onde os autores compararam com outras espécies de *Nyssorhynchus* e encontram caracteres distintos de todos os outros. Os ovos foram examinados e fotografados em microscópio eletrônico de

varredura que permite a análise detalhada da estrutura externa. FORATTINI et al. (1998) descreveram os ovos de *An. parvus.* Comparando os ovos de *An. parvus* com o de outras espécies do subgênero *Nyssorhynchus*, nota-se que eles são parecidos com aqueles de *An. darlingi* Root, *An. rangeli* Gabaldón, Cova García & López and *An. dunhami* Causey, porém diferem por características da micrópila e dos flutuadores. No mesmo trabalho, os autores descreveram os ovos de *An. lutzii* a partir de espécimes coletados em duas localidades do Estado de São Paulo, Dourado e Pariquera-Açu. Vale assinalar que os ovos das duas populações estudadas diferem em características morfológicas. A presença de diferenças morfológicas nos ovos sugere que se trata de espécies distintas.

Nesse sentido vale assinalar o estudo de LOUNIBOS et al. (1997) que demonstrou que o *Anopheles trinkae* Faran é espécie válida, retirando-a da sinonímia de *Anopheles dunhami*. Semelhantemente, SALLUM et al. (2004) observaram que *Anopheles mediopunctatus* (Lutz) e *Anopheles costai* Fonseca & Ramos podem ser diferenciadas por aspectos da morfologia externa do exocório dos ovos.

1.4. SISTEMÁTICA

A ciência da Culicidologia atravessa atualmente período crítico, em virtude da carência de especialistas que se dediquem à sistemática. Esta situação é preocupante para a ciência da culicidologia, pois a maioria dos mosquitos é pouco conhecida e os estudos filogenéticos restringem-se a grupos de maior importância médica (ZAVORTINK, 1994).

A tendência atual é desenvolver métodos moleculares para a identificação de espécies e o reconhecimento de complexos de espécies. Idealmente, o treinamento de taxonomistas deveria incluir tanto técnicas moleculares como morfologia. Nesse sentido, vale lembrar que os mosquitos da subfamília Anophelinae necessitam ser mais bem conhecidos

morfológicamente para que possamos entender as diferenças moleculares observadas e definir a presença de complexos de espécies.

No momento, confiar somente em dados depositados no banco de dados genéticos de domínio público (GenBank) para fazer comparações e identificações de organismos pode induzir a erros. Nesse contexto, vale lembrar o estudo de MARRELLI et al. (1999) que levantou a hipótese de que o *An. oswaldoi* compreende grupo formado por pelo menos quatro espécies crípticas. No entanto, comparando-se a sequência de DNA da amostra de *An. oswaldoi* proveniente da localidade tipo (Vale do Rio Doce, Espírito Santo), observou-se que se trata de um exemplar de *An. evansae* que foi confundido com *An. oswaldoi* (MARRELLI et al. 2006). Além disso, sequências são submetidas ao banco genético sem a preocupação de depósito de espécimes testemunhas em coleções, sem detalhes das coletas, ou associações das sequências com determinados indivíduos, ou outros dados, como o nome da pessoa que identificou o espécime (HARBACH, 2004).

1.5. MARCADORES MOLECULARES

Tanto a detecção como a interpretação da estrutura populacional dependerá da classe de marcador e da localização do locus no genoma. Resultados obtidos a partir da mesma espécie utilizando diferentes tipos de marcadores ou diferentes conjuntos gênicos não irão necessariamente concordar, particularmente se baseados em estatísticas que abrangem loci que se afastam da tendência evolutiva do genoma (KRZYWINSKY e BESANSKY, 2003).

Dois marcadores foram empregados no presente estudo, o gene Citocromo Oxidase subunidade I (COI) do genoma mitocondrial e o segundo espaçador interno transcrito (ITS2) do genoma nuclear. Ambos já se mostraram bons marcadores para caracterizar molecularmente espécies de *Anopheles.* Por esta razão eles têm sido utilizados como ferramentas para a

delimitação de complexos de espécies de *Anopheles* (*Nyssorhynchus*). Como exemplos, vale citar o trabalho de SALLUM et al. (2008) que, empregando sequências do ITS2, apresentou evidências de que o *An. benarrochi*, *An. oswaldoi* e *An. konderi* de Acrelândia, Acre, Brasil, representam complexos de espécies. Paralelamente, os resultados das análises de COI e ITS2 de diversas populações de *An. nuneztovari* e *An. goeldii* do Brasil, Venezuela a Colombia demonstraram que são espécies distintas (CALADO et al., 2008). Os dois marcadores também tem sido amplamente utilizados para definir complexos de espécies de outros subgêneros de *Anopheles.* Espécies do complexo *An. maculipennis* foram definidas por LINTON et al. (2003) com emprego do ITS2 e COI. Sequências do gene COI foram empregadas em estudos sobre relações filogenéticas de Anophelinae (SALLUM et al., 2002) e ITS2 em complexo *An*. *maculipennis* (MARINUCCI et al., 1999). Vale asssinalar o estudo de MOTOKI et al. (2007) que caracterizou molecularmente *An. oswaldoi,* o que possibilitará a definição das outras espécies do complexo *An. oswaldoi*. Dessa maneira, SALLUM et al. (2008) definiram a população de *An. oswaldoi* de Acrelândia como espécie distinta do complexo *An. oswaldoi*.

O DNA ribossômico (rDNA) tem sido utilizado como ferramenta para responder perguntas sobre taxonomia e relações filogenéticas em ampla variedade de organismos. As regiões funcionais que produzem os ribossomos são altamente conservadas, ao mesmo tempo, existem regiões espaçadoras transcritas e não-transcritas que possuem alta variabilidade interespecífica e baixa variabilidade intraespecífica. Dessa maneira, sequências de nucleotídeos dessas regiões podem ser úteis para o estudo das relações de espécies próximas e para a identificação de complexos de espécies isomórficas (WILKERSON et al., 2004). Embora as regiões codificantes do rDNA tendam a ser altamente conservadas na evolução, as espaçadoras parecem relativamente livres para divergir, mesmo em organismos muito próximos (MARINUCCI et al.,1999).

Tradicionalmente, o DNA mitocondrial (mtDNA) tem sido uma escolha de marcador para estudar as variações genéticas nas espécies de insetos.

Sequências de gene mitocondrial têm sido utilizadas em estudos filogenéticos e de genética de população para construir a história evolutiva de espécies relacionadas (BEHURA, 2006). O DNA mitocondrial é utilizado para análises de marcador, em grande parte devido à sua herança materna, status haplóide, e alta taxa de evolução que na maioria dos organismos é de 5 a 10 vezes maior do que dos genes nucleares de cópias únicas (RAI, 1991; BEHURA, 2006)

HERBERT et al. (2003) demonstraram que a análise de regiões genômicas curtas e padronizadas (*DNA barcodes*) podem ser empregadas para discriminar espécies de animais. Em particular, os autores consideraram que o gene mitocondrial citocromo oxidase subunidade 1 (COI) pode servir como modelo de um gene-alvo para um sistema de bioidentificação. O estudo de CYWINSKA et al. (2006) forneceu o primeiro COI *barcodes* para mosquitos do Canadá e estabeleceu sua eficácia em discriminar espécies de mosquitos reconhecidos através de estudo taxonômico prévio.

2. OBJETIVOS

2.1. OBJETIVO GERAL

- Contribuir para a taxonomia de espécies da Seção Myzorhynchella do subgênero *Nyssorhynchus de Anopheles.*

2.2. OBJETIVOS ESPECÍFICOS

- Redescrever os adultos macho e fêmea, genitália masculina e formas imaturas de *Anopheles lutzii*;
- Avaliar o status específico de *Anopheles niger* e *Anopheles guarani*;
- Descrever os adultos macho e fêmea, genitália masculina, formas imaturas e ovos de *Anopheles guarani*;
- Caracterizar morfologicamente e molecularmente *Anopheles antunesi*;
- Estimar as relações filogenéticas entre espécies da seção Myzorhynchella.

3. MATERIAL E MÉTODOS

3.1. COLETAS

Durante as coletas, foram obtidos espécimes adultos com o uso de armadilha de Shannon (Figura 1A). As fêmeas eram atraídas pela luz e presença de humanos, pousando nas paredes externas e internas da armadilha. As coletas ocorreram aproximadamente entre 180hs 00mim e 20hs 30min, próximo a criadouros e possíveis áreas de repouso. Os espécimes foram coletados individualmente em tubos de vidro denominado “tubo Correa” e armazenados vivos em caixa de isopor. Paralelamente foram coletadas formas imaturas em criadouros naturais (Figura 1B).

Figura 1. (A) Coleta de adultos em armadilha de Shannon. (B) Coleta de formas imaturas

Os espécimes de *Anopheles* (*Nyssorhycnhus*) da Seção Myzorhynchella foram coletados nas localidades listadas na tabela 1.

Tabela 1. Espécimes de *Anopheles* da seção Myzorynchella coletados no Brasil, conforme UF, município, localidade, data, coordenadas e altitude.

UF	Município	Localidade	Data	Coordenadas	Altitude
RJ	Itatiaia	Parque Nacional do Itatiaia	III-2008	22º24’58,7”S 44º37’19,7”W	1100 m
PR	Foz do Iguaçu	Próximo ao Rio Almada	IV-2008	25º28’50”S 54º35’12”W	185 m
SP	Pindamonhangaba	Pico do Itapeva, Fazenda Saint Clair	I-2009	22º45’30,5”S 45º30’55,0”W	1781 m
SP	Pariquera-Açu	Vale do Ribeira, Sítio Galiléia	IV-2006	24º44.975'S 47º56.944'W	-

Entre os locais de coleta, o Pico do Itapeva que está entre Campos do Jordão e Pindamonhangaba fica próximo da localidade tipo de *An. Antunesi.* O município de Foz do Iguaçu é a localidade tipo de *An. guarani*, que esá na sinonímia de *An. lutzii*. Os espécimes foram identificados utilizando as chaves de FORATTINI (2002) até a categoria taxonômica de espécie, no Laboratório da Coleção Entomológica da FSP-USP.

3.2. OBTENÇÃO DE GERAÇÃO PARENTAL EM LABORATÓRIO

As formas imaturas foram mantidas em laboratório para a obtenção de adultos. As fêmeas foram alimentadas com sangue e mantidas em recipiente com umidade ao redor de 80% por período de 48 a 60 horas. Após esse período os mosquitos foram anestesiados com acetato de etila, identificados e foi removida uma das asas para induzir a oviposição. Em seguida, a fêmea foi colocada em recipiente pequeno contendo água mineral ou destilada, e após alguns minutos ocorreu a oviposição (Figura 2).

Figura 2. Postura dos ovos após indução à oviposição.

Parte dos ovos foi utilizada para análise morfológica. O restante foi mantido até a eclosão. As larvas foram transferidas para recipientes de plástico com volume aproximado de 250ml de água e mantidas em condições adequadas até a emergência dos adultos. A manutenção incluiu a troca diária da água e alimentação dos imaturos com comida para peixes e pólen triturados. As pupas foram transferidas individualmente, para pequenos frascos de plástico contendo água e tampa, e foram mantidas até a emergência dos adultos.

Os adultos foram mortos com acetato de etila e parte destes foi imediatamente armazenada em etanol 100% PA em freezer -70ºC. O restante foi montado em alfinetes entomológicos (Figura 3B). As exúvias da larva de quarto estádio e da pupa de cada espécime foram montadas entre lâmina e lamínula, em bálsamo do Canadá (Figura 3A). As genitálias masculinas foram dissecadas e montadas em lâminas. Este material serviu tanto como testemunha dos respectivos adultos que foram empregados para a extração de DNA como para a identificação das espécies.

O material testemunha foi depositado na Coleção Entomológica da Faculdade de Saúde Pública da Universidade de São Paulo (FSP-USP).

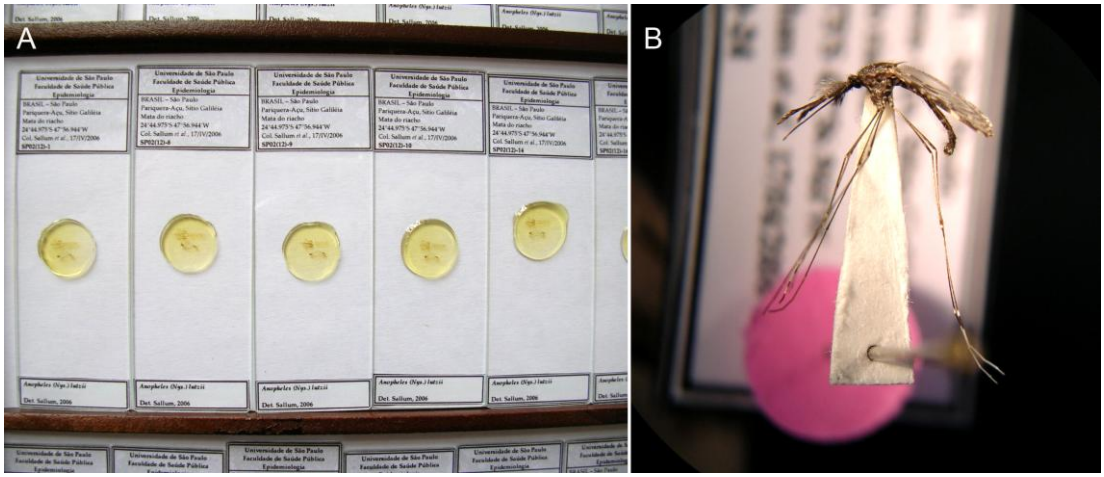

Figura 3. (A) Lâminas com as exúvias de larvas de 4º estádio e pupas; (B) Espécime adulto montado em alfinete entomológico.

3.3. MICROSCOPIA ELETRÔNICA DE VARREDURA

Os ovos foram mantidos na água por cerca de 36 horas para o embrionamento. Com o auxílio de papel filtro, eles foram cuidadosamente recolhidos e preservados em solução alcoólica de Bouin. A preparação e análise foram realizadas na Faculdade de Saúde Pública, onde os ovos foram quimicamente fixados, desidratados e a seguir, cobertos com carbono e ouro. Após a preparação, estes foram montados para exame em microscópio eletrônico de varredura (MEV) como descrito por FORATTINI e MARUCCI (1993). O exame das amostras foi realizado em microscópio JEOL JSM P-15 (JEOL Ltd., Akishima Tokyo, Japan). Foram analisados aproximadamente 30 ovos de duas fêmeas selvagens de Foz do Iguaçu – PR e cerca de 40 ovos de três fêmeas de Itatiaia – RJ. As amostras de Pariquera-Açu já foram estudadas por FORATTINI et al. (1998).

3.4. MATERIAL EXAMINADO

Além do material coletado para este projeto, foram utilizados adultos fêmeas e estágios imaturos da Seção Myzorhynchella que foram coletados em outras localidades do Brasil e depositados na Coleção da FSP-USP e do MZUSP. Foram analisados exemplares de *An. lutzii, An. antunesi* e *An. parvus*. Acresce considerar o exame do material tipo de *Anopheles lutzii;* três síntipos, fêmeas adultas e a asa de um dos exemplares montada entre lâmina e lamínula depositada na Coleção Entomológica do Instituto Oswaldo Cruz, Rio de Janeiro, Brasil (IOC - RJ). Foi examinado um parátipo de *An. antunesi* depositado na Coleção da Faculdade de Saúde Pública, Universidade de São Paulo, Brasil (FSP-USP).

3.5. ESTUDO MORFOLÓGICO

Dentre as inúmeras atividades relacionadas aos estudos morfológicos de Culicidae está a medição de caracteres dos adultos, imaturos e estruturas anatômicas das genitálias masculinas, além da quetotaxia de larvas de quarto estádio e pupas. Objetiva-se com isso o encontro de caracteres morfológicos que permitam diferenciar as espécies nos diferentes estádios de desenvolvimento.

No processo de quetotaxia das larvas e pupas, foram analisados 10 indivíduos da população de Foz do Iguaçu e 10 de Pariquera-Açu. Nesta etapa utilizou-se microscópio óptico modelo Diaplan, Leitz Wetzlar (Heidelberg, Germany) para medir as estruturas e descrever a quetotaxia.

Foram analisados adultos machos e fêmeas, associados aos imaturos, além das estruturas da genitália masculina.

As medidas dos adultos foram obtidas com ocular micrométrica digital acoplada a um estereomicroscópio que apresenta os valores com precisão de três casas decimais. Tanto o estereomicroscópio quanto a ocular

micrométrica são da marca Wild Heerbrugg (Heerbrugg, Switzerland). Para as medidas das larvas, pupas e genitálias masculinas utilizou-se ocular micrométrica da marca Leitz, adaptada ao microscópio óptico modelo Diaplan, Leitz.

Foram medidas as manchas de escamas claras e escuras da veia costa de cinco machos e cinco fêmeas, das populações de Foz do Iguaçu e Pariquera-Açu. As medidas foram obtidas de adultos de ambos os sexos para examinar possíveis diferenças.

Os caracteres morfológicos foram apresentados em forma de descrição dos adultos, macho e fêmea, genitália do macho, exúvias das larvas de 4º estádio e da pupa. Os caracteres analisados foram comparados entre espécies e indivíduos de populações distintas.

As nomenclaturas adotadas para os adultos seguem HARBACH e KNIGHT (1980), para as manchas das asas WILKERSON e PEYTON (1990), e para as veias, BELKIN (1962).

Tanto as coletas dos dados morfológicos como as análises foram realizadas no Laboratório de Sistemática Molecular do Departamento de Epidemiologia e na Coleção Entomológica da Faculdade de Saúde Pública da Universidade de São Paulo FSP-USP.

3.6. ESTUDO MOLECULAR

Todos os processos de extração, amplificação e sequenciamento de DNA foram realizadas no Laboratório de Sistemática Molecular do Departamento de Epidemiologia da FSP-USP.

3.6.1. Extração de DNA

O DNA foi extraído de espécimes que foram inicialmente preservados em etanol 100% em ultrafreezer à -70ºC. Foram obtidas amostras do DNA de 34 indivíduos de *Anopheles* da seção Myzorhynchella. A Tabela 1 lista as espécies e a origem das amostras que foram caracterizadas molecularmente.

As extrações dos espécimes foram feitas com o kit QIAGEN DNeasy® blood and tissue kit (QIAGEN, Crawley, United Kingdom), seguindo-se o protocolo fornecido pela companhia. Todos os reagentes foram fornecidos com o kit.

Dentre os espécimes analisados, dois exemplares eram mais antigos (nº E-11803 e E-12370), coletados em 1994 e 2001, respectivamente. Portanto, estes indivíduos tiveram o DNA extraído apenas do abdômen, para preservação de outros caracteres para estudos morfológicos. E devido ao tempo de deterioração do DNA, medidas especiais foram tomadas a fim de evitar contaminação, além de modificações no protocolo de extração.

Todo o processo de extração ocorreu dentro de uma Cabine de Segurança Biológica VLFS-12 (Grupo VECO, Campinas, São Paulo) que foi previamente esterilizada por luz ultravioleta. As extrações dos dois espécimes seguiram o protocolo fornecido pela QIAGEN DNeasy® blood and tissue kit até a etapa de eluição. Devido ao pouco material, foram adicionados apenas 50 μl de tampão de eluição. Na segunda eluição foram adicionados 20 μl do tampão. Os produtos das eluições foram armazenados separadamente, em microtubos de 1,5 ml em freezer -30°C.

Tabela1. Espécimes que tiveram o DNA extraído, código usado, sexo, localidade, espécie, coordenadas e data de coleta.

Código	Sexo	Localidade	Gênero (Subgênero) espécie	Coordenadas	Data coleta
PR29	♀	Foz do Iguaçu, PR	*Anopheles (Nys.) guarani*	25º28’50”S 54º35’12”W	28/IV/2008
PR29(8)	♀				
PR29(9)-6	♂				
SP02(9)-2	♂	Pariquera Açu, SP	*Anopheles (Nys.) lutzii*	24º44.975'S 47º56.944'W	17/IV/2006
SP02(10)-5	♂				
SP02(11)-9	♂				
SP02(12)-1	♂				
SP02(13)-3	♂				
SP02(14)-6	♂				
SP02(15)-5	♂				
PR28(5)-1	♂	Guaira, PR	*Anopheles (Nys.) parvus*	24º16'17,4"S 54º17'26,0"W	5/IV/2007
PR28(15)-1	♂				
PR28(65)-6	♂				
MG07(9)-20	♂	Frutal, MG	*Anopheles (Nys.) parvus*	20º01'31,0"S 49º04'35,4"W	21/XI/2006
RJ03(6)	♀	Itatiaia, RJ	*Anopheles (Nys.) antunesi*	22º24’58,7”S 44º37’19,7”W	26/III/2008
RJ03(11)	♀				
RJ03(12)	♀				
RJ03(13)	♀				

continua

VP11a	♀	Pindamonhangaba, SP	*Anopheles (Nys.) antunesi*	22º45'31,7"S 45º30'55,8"W	27/X/2006
VP11d	♀				
VP11c	♀				
VP11b	♂		*Anopheles (Nys.) antunesi* - Forma 1		
VP19-17	♂				
SP50a	♀	Pindamonhangaba, SP	*Anopheles (Nys.) antunesi* - Forma 1	22º45’30,5”S 45º30’55,0”W	14-26/I/2009
SP50b	♀				
SP51-100	♀				
SP55(2)	♀				
SP55(4)	♀				
SP53-100	♂				
SP53-101	♀				
SP53-4	Pupa				
SP53-5	Pupa				
E-11803	♀	Dourado, SP	*Anopheles (Nys.) guarani*	22º05’00”S 48º26’33”W	30/IV/1994
E-12370	♀	Campos do Jordão, SP	*Anopheles (Nys.) antunesi*	22º45’50”S 45º30’87”W	20/XI/2001

3.6.2. Amplificação de ITS2

As reações de polimerização em cadeia (PCR) da região ITS2 do DNA ribossomal empregaram os iniciadores (primers), desenhados para anelamento em regiões conservadas das subunidades 5.8S e 28S, recomendados por DJADID et al. (2007):

5.8S: 5' - ATC ACT CGG CTC GTG GAT CG - 3'

28S: 5' - ATG CTT AAA TTT AGG GGG TAG TC - 3'

Os produtos de PCR foram obtidos em reação com volume final de 25 µl contendo 1 µl de DNA da primeira eluição; 2,5 µl tampão 10x PCR (New England BioLabs® Inc); 200 µM de cada dNTPs; 0,5 µl de DMSO; 5 picomoles de cada primer; 2,5 U de Taq DNA Polimerase (New England BioLabs® Inc, Ipswich, MA). Adotou-se o seguinte perfil de amplificação para o marcador moleculaar utlizando o termociclador Mastercycler epgradient (Eppendorf, Hamburg, Germany): um ciclo de desnaturação inicial a 94ºC por 3 minutos, seguido por 35 ciclos (desnaturação a 94ºC por 30 segundos, anelamento a 60ºC por 30 segundos e extensão a 72ºC por 30 segundos), terminando com extensão de 10 minutos a 72ºC. Os produtos amplificados foram visualizados em gel de agarose 1,5% corado com GelRed™ (Biotium, Hayward, CA) após a eletroforese.

3.6.3. Amplificação de COI

O fragmento do gene COI do DNA mitocondrial com 658 pares de base foram amplificados por PCR usando os primers LCO1490 e HCO 2198 (FOLMER et al., 1994);

LCO1490: 5' - GGT CAA CAA ATC ATA AAG ATA TTG G - 3'

HCO2198: 5' - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3'

Os produtos de PCR foram obtidos em reação com volume final correspondente a 25 µl contendo 1 µl de DNA da segunda eluição; 2,5 µl

tampão 10x PCR (New England BioLabs® Inc); 200 µM de cada dNTPs; 5 picomoles de cada primer; 2,5 U de Taq DNA Polimerase (New England BioLabs® Inc).

O protocolo de amplificação por PCR consistiu em: desnaturação inicial – 1 ciclo a 95ºC por 2 minutos, seguido por 35 ciclos (desnaturação a 94ºC por 1 minuto, anelamento a 55ºC por 1 minuto e extensão a 72ºC por 1 minuto e meio), seguido por uma extensão final de 7 minutos a 72ºC. Os produtos amplificados foram visualizados em gel de agarose 1,5% corado com GelRed™ (Biotium) após a eletroforese.

3.6.4. Purificação e quantificação dos produtos de PCR

O excesso de iniciadores, dNTP e de sais presentes no DNA amplificado foram eliminados por precipitação com solução de PEG/NaCl (20% polietileno glicol 8000/2.5 M NaCl). Para isso, adicionou-se aos tubos contendo os produtos amplificados igual volume de solução de PEG/NaCl, permanecendo a mistura em incubação por 15 minutos à 37ºC. Ao final deste período o material foi centrifugado em 13200 rpm por 15 minutos à temperatura ambiente na microcentrífuga Eppendorf, modelo 5415R, o sobrenadante foi descartado e o precipitado lavado duas vezes por centrifugação em 13200 rpm por 10 minutos à 4ºC, com etanol 80%. O etanol residual foi eliminado por evaporação no concentrador à vácuo Eppendorf, modelo 5301.

O DNA foi resuspendido em 25µl de H_2O ultrapura, deionizada livre de nucleases e em seguida quantificado por análise eletroforética em gel de agarose 1,5%, corado com GelRed™ (Biotium). Utilizou-se o marcador “Low mass DNA™ Ladder” (Invitrogen, Carlsbad, CA).

3.6.5. Sequenciamento

As sequências de nucleotídeos do ITS2 e do gene COI foram determinadas diretamente dos produtos amplificados, após purificação com PEG/NaCl. As reações de sequenciamento foram conduzidas em ambas as direções utilizando os mesmos primers empregados nas PCRs e kit "ABI Prism® BigDye™ Terminator version 3.1 Cycle Sequencing Ready Reaction" (PE Applied Biosystems, Warrington, England) com algumas modificações. O volume final foi de 10 µl, contendo 0,5 µl da mistura de enzima-terminadores fluorescentes (*Terminator Ready Reaction Mix*); 2 µl de tampão de diluição 5X (*Sequence Dilution-buffer*) composto por 5 mM $MgCl_2$, 200 mM Tris-HCl, pH 9.0; 3,6 picomoles de cada primer e cerca de 10 ng de cada produto amplificado. As amostras foram colocadas no termociclador, seguindo o protocolo de 25 ciclos de desnaturação a 96ºC por 15 segundos, anelamento a 50ºC por 15 segundos e extensão a 60ºC por 4 minutos, finalizando em 4ºC por tempo indeterminado. Após o término da reação, as amostras foram armazenadas em geladeira ou imediatamente submetidas à etapa de purificação efetuada por cromatografia de gel filtração em colunas de Sephadex® G50 (GE-Healthcare-Pharmacia, Buckinghamshire, UK).

3.6.6. Cromatografia de gel filtração

As microcolunas com Sephadex® G50 (medium size, GE-Healthcare-Pharmacia, Buckinghamshire, UK) empregadas na purificação dos produtos pós-sequenciamento foram preparadas no laboratório. Para isto, 1g de Sephadex® G50 foi hidratada com 15 ml de H_2O ultrapura e mantida em repouso por 4 horas. Após este período o sobrenadante foi removido, adicionando-se novamente 15 ml de água deionizada, permanecendo a resina em repouso por, no mínimo 4 horas. Cerca de 800 µl de resina hidratada foram transferidos para as micro colunas apoiadas em tubos de

1,5 mL. A água intersticial foi eliminada por centrifugação por 2 minutos a 300 RPM.

A seguir, as reações de sequenciamento acrescidas de 10µl de H_2O deionizada foram aplicadas no topo das colunas que foram novamente centrifugadas a 300 RPM por 2 minutos. Os produtos eluídos foram secos em centrífuga à vácuo e armazenados a -20ºC ao abrigo de luz, até serem submetidos à análise eletroforética em um sequenciador modelo 3130xl da Applied Biosystems (Foster City, CA).

3.7. ALINHAMENTO E ANÁLISE DE ITS2

As sequências nucleotídicas do espaçador interno transcrito 2 (ITS2) assim como regiões dos genes 5.8S e 28S foram editadas utilizando o programa Sequencher^{TM} version 4.9 versão para Windows (Genes Codes Corporation, Ann Arbor, Michigan) e alinhadas utilizando o programa CLUSTAL X (THOMPSON et al., 1997). Os alinhamentos foram visualizados e editados manualmente com o programa MacClade 4.0b10 PPC (MADDISON e MADDISON, 2000).

A anotação da região ITS2 foi no sítio eletrônico 'The ITS2 database' (SCHUILTZ et al., 2006; SELIG et al., 2008) (http://its2.bioapps.biozentrum.uni-wuerzburg.de/cgi-bin/index.pl?about). Na opção 'Annotation' foi escolhido o modelo 'Diptera'.

A similaridade das sequências de ITS2 geradas neste estudo com as outras previamente disponíveis no GenBank foram acessadas usando o algorítmo FASTA (http://www.ncbi.nlm.nih.gov/BLAST/).

3.8. ALINHAMENTO E ANÁLISE DE COI

As sequências nucleotídicas do gene mitocondrial COI foram alinhadas usando o programa CLUSTAL 1.6 (THOMPSON et al., 1997). Para a análise foram excluídas as regiões dos primers.

A precisão dos alinhamentos das sequências de nucleotídeos de COI foi examinada usando sequências de aminoácidos. Os dados consistem de 658 pares de bases (pb) do gene COI.

3.9. ANÁLISES FILOGENÉTICAS

As análises filogenéticas foram realizadas apenas com sequências do gene mitocondrial COI. Como grupos externos foram utilizadas sequências de *An. nuneztovari* (AF368094) e *An. strodei* (SALLUM et al. dados não publicados). Foram conduzidas análises de máxima parcimônia, máxima verossimilhança e análise Bayesiana.

3.9.1. Máxima Parcimônia (MP)

Análises de Parcimônia foram conduzidas no programa PAUP 4.0 b10 (SWOFFORD, 2004) usando a opção de busca heurística com a opção TBR (Tree bisecton-reconection) branch swapping, que executa uma série de rearranjos da topologia originada a partir de uma análise simples da matriz, visando encontrar o cladograma mais curto. Para essas análises, o peso dos caracteres foi baseado no valor máximo do índice de consistência e continuaram até o peso dos caracteres se estabilizarem (FARRIS, 1969; CARPENTER, 1988). O teste de Boostrap (FELSENSTEIN, 1985) utilizou 1000 pseudo-réplicas. Os caracteres de parcimônia não informativos foram excluídos.

3.9.2. Máxima Verossimilhança (ML)

A análise de Máxima Verossimilhança foi realizada com o programa PAUP 4.0 (SWOFFORD, 2004). O modelo de evolução empregado nas análises foi escolhido com o programa ModelTest 3.0 (POSADA & CRANDALL, 1998), que compara quatorze modelos básicos. Todos os quatorze modelos foram avaliados com e sem taxa de heterogeneidade. A taxa de heterogeneidade foi ajustada de três maneiras: usando o modelo gama com seis categorias, usando um modelo de sítios invariáveis e usando um modelo gama com os sítios invariáveis (SWOFFORD et al., 1996). Usando um teste padrão de razão de verossimilhança, as pontuações de verossimilhança de cada topologia de parcimônia foram comparadas através de modelos aninhados com o programa Modeltest 3.0 (POSADA & CRANDALL, 1998). Os modelo foi escolhido usando o método denominado Akaike Information Criterion (AIC).

3.9.3. Bayesiana

A fim de encontrar o melhor modelo de evolução de nucleotídeos, a análise bayesiana foi conduzida com o programa MrBayes versão 3.1.2 (RONQUIST e HUELSENBECK, 2003). Nas análises foi empregado o modelo selecionado pelo programa Modeltest 3.0 (POSADA & CRANDALL, 1998) pelo método AIC. A análise MCMC (Markov Chain Monte Carlo) foi realizada com 6.000.000 gerações, salvando uma árvore a cada 100 gerações. Um “burn-in” de 1.000.000 das amostras foi usado.

4. RESULTADOS

Como resultados serão apresentados três manuscritos:

MANUSCRITO 1 – “Redescription of *Anopheles* (*Nyssorhynchus*) *antunesi* Galvão and Amaral and description of a new species of the Myzorhynchella Section (Diptera: Culicidae) from Serra da Mantiqueira, Brazil.”

(em preparação)

MANUSCRITO 2 – “Redescription of *Anopheles* (*Nyssorhynchus*) *lutzii*, and resurrection of *An. niger* and *An. guarani* (Diptera: Culicidae) from the synonymy“

(em preparação)

MANUSCRITO 3 – “Molecular phylogeny of species of the Myzorhynchella Section of the *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae)“

(em preparação)

4.1. MANUSCRITO 1

"Redescription of *Anopheles* (*Nyssorhynchus*) *antunesi* Galvão and Amaral and description of a new species of the Myzorhynchella Section (Diptera: Culicidae) from Serra da Mantiqueira, Brazil."

(em preparação)

Redescription of *Anopheles* (*Nyssorhynchus*) *antunesi* Galvão and Amaral **and description of a new species of the Myzorhynchella Section (Diptera: Culicidae) from Serra da Mantiqueira, Brazil.**

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Running title: Systematics of two species of Myzorhynchella Section

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Summary

Anopheles (Nyssorhynchus) antunesi Galvão and Amaral is characterized and a new species of the Myzorhynchella section of *Anopheles* (*Nyssorhynchus*) is described based on morphology, ITS2 sequences of the ribosomal DNA and a fragment of the COI gene of the mitochondrial genome. *Anopheles antunesi* and the new species are compared with morphologically similar species of the Myzorhynchella section. Morphological and molecular data suggest that the new species has been misidentified as both *An. antunesi* and *An. lutzii*. *Anopheles antunesi* and the new species are sympatric, occurring in high altitudes in Serra da Mantiqueira, southeastern Brazil.

Key words: ITS2 – COI – morphology – sympatry – mosquitoes - high altitudes

Introduction

The genus *Anopheles* Meigen includes 455 species and is divided into seven subgenera (Harbach, 2007). The subgenus *Nyssorhynchus* Blanchard includes some of the most important vectors of human malaria parasites in Central and South America (Sallum & Wilkerson, 1997). The *Nyssorhynchus* is divided into three sections, Albimanus, Argyritarsis and Myzorhynchella (Harbach, 2004). The Albimanus Section includes 19 species (Faran, 1980), whereas Argyritarsis is composed of 10 species (Linthicum, 1988), and Myzorhynchella has 4 nominal species, *An. lutzii* Cruz, *An. parvus* (Chagas), *An. nigritarsis* (Chagas) and *An. antunesi* Galvão & Amaral (Galvão, 1941; Harbach, 2004). These four taxa were described from specimens collected in Brazil (Belkin et al., 1971), and there is no

epidemiological evidence to support the involvement of any species of the Myzorhynchella as vector of pathogens.

Galvão & Amaral (1940) described *An. antunesi* based on morphological characters of all life stages, including those from eggs. Specimens employed in the description were from Vila Emilio Ribas, Campos do Jordão municipality, Serra da Mantiqueira, southeastern Brazil. Since the description of *An. antunesi*, few studies have been carried out for species of the Myzorhynchella section.

Galvão (1941) described and illustrated eggs and male genitalia of specimens of *An. antunesi* collected in Casa Grande, Salesópolis, State of São Paulo. By examining these specimens from Casa Grande deposited in the Entomological Collection of Faculdade de Saúde Pública (FSP-USP), Universidade de São Paulo, and by comparing both illustrations and description data of specimens from Casa Grande, it is evident that those specimens belong to *An. antunesi*. More recent, Forattini et al. (1997) described the eggs of specimens identified as *An. antunesi* collected in Campos do Jordão. The type specimen of *An. antunesi* was deposited by Galvão in Faculdade de Medicina, Universidade de São Paulo and posteriorly donated to Faculdade de Saúde Plública Collection. However, the type specimen was not found in the collection, except one adult female paratype that is deposited in FSP-USP. Similarly, Senise & Sallum (2007) could not find the type of *Anopheles lanei* Galvão and Amaral.

When examining specimens identified as *An. antunesi* from Serra da Mantiqueira, we observed that those individuals could be separated into two morphological forms on the basis of larval characteristics and male genitalia. One

form corresponds to *An. antunesi*, whereas the second form may represent an undescribed taxon.

The objective of this study is to characterize *Anopheles antunesi* and validate a new species of the Myzorhynchella section, employing morphological characters, ITS2 and COI DNA sequences.

Materials and Methods

Two adult female of *An. antunesi* were collected in Shannon trap, in the vicinities of the Parque Nacional do Itatiaia, State of Rio de Janeiro, Brazil. The females were blood fed and traumatized by removing one wing to induce oviposition. Eggs were fixed in alcoholic Bouin's solution 36 hours after oviposition. Eggs were prepared for scanning electron microscope (SEM) following the protocol described by Forattini & Marucci (1993) and examined in a JEOL JSM-P15 scanning electron microscope.

Nucleotide sequences of rDNA (ITS2) and mtDNA (COI) were generated for 19 specimens. Morphological characters of the adult male, female, male genitalia, pupal and larval exuviae were analyzed. Abbreviations for the life stages are: F, adult female; M, adult male; G, male genitalia; L, larva; P, pupa; Le, larval exuviae; Pe, pupal exuviae; E, eggs. Terminology follows that of Harbach & Knight (1980). All the specimens used in this study are deposited in the Entomological Collection of Faculdade de Saúde Pública da Universidade de São Paulo FSP-USP.

DNA Extraction

DNA was extracted from the specimens following the tissue DNA extraction protocol provided by the QIAgen Dneasy blood and tissue kit (QIAGEN, Crawley,

United Kingdom). All buffers were supplied in the kit. Because DNA often remains bound to the membrane after the first elution, the elution step was repeated and stored in a separate tube. For one specimen, only the abdomen was used to generate sequences. The extraction protocol for this specimen was the same used for fresh specimens except that the DNA was eluted in 50 µl of buffer AE. Since the chance of cross contamination is high, DNA was extracted in a separate room in a flow microbiological safety cabinet.

Amplification and Sequencing

ITS2 region products were amplified using 5.8SF, 5'-ATC ACT CGG CTC GTG GAT CG-3', and 28SR, 5'-ATG CTT AAA TTT AGG GGG TAG TC-3' primers (Djadid et al., 2007). Polymerase chain reactions (PCR) was carried out in a 25 µl reaction mix containing 1 µl of DNA of first elution, 2.5µl 10x PCR buffer (New England BioLabs® Inc), 0.5 µl of dimethyl sulfoxide, 5 pmol of each primer, 200 µM each dNTPs, and 2.5 U of *Taq* polymerase (New England Biolabs, Ispwich, MA). PCR amplification protocol consisted of a 3 min denaturation at 94ºC, 34 cycles at 94ºC, 60ºC and 72ºC for 30 s each, followed by a 10 min extension at 72ºC.

COI gene fragments were amplified using LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' primers (Folmer et al., 1994). PCR was carried out in a 25 µl reaction mix containing 1 µl of DNA of the second elution, 2.5µl 10x PCR buffer (New England BioLabs® Inc), 200 µM each dNTPs, 5 pmol of each primer; 2.5 U of *Taq* polymerase (New England BioLabs® Inc). PCR amplification protocol consisted of 2 min denaturation at 95ºC followed by 35 cycles at 94ºC for, 55ºC and 72ºC for 1

mim each, followed by a final extension at 72ºC for 7 min. PCR products were electrophoreses in 1.5% TAE agarose gels stained with GelRed™ (Biotium).

Sequencing reactions were carried out in both directions using the PCR primers and the Big Dye Terminator® kit, version 3.1 (PE Applied Biosystems, Warrington, England). Sequences were analyzed on a 3130 sequencer (Applied Biosystems).

Sequence Analysis

Sequences were edited using Sequencer version 4.9 for windows (Gene Code Corporation, Ann Arbor, USA), aligned in CLUSTAL X 1.8 (Thompson et al. 1997) and optimized manually in MacClade, version 4.3 (Maddison & Maddison, 2000). Sequence similarity of the ITS2 sequences generated in this study with those previously available in GenBank was assessed using FASTA search (http://www.ncbi.nlm.nih.gov/BLAST/).

Vouchers. Slides of larval and pupal exuviae, male genitalia, wings and legs of the specimens used in the extraction are deposited in FSP-USP. Template DNA from this study is retained dry at -70ºC in the FSP-USP for future reference.

Taxonomic Treatment

Anopheles (*Nyssorhynchus*) *antunesi* of Galvão & Amaral, 1940: 150. Type: holotype female (no. 370), alotype male (no. 371), deposited in Collection of Departamento de Parasitologia da Faculdade de Medicina, Brazil. Type-locality: Vila Emilio Ribas, Campos do Jordão, São Paulo, Brazil Galvão, 1941:552 (systematic, bionomics); Rodriguez & Varela, 1962:246 (first record in Uruguay); Gorham et al., 1967:42 (distribution, illustrated key); Forattini, 1962:432 (taxonomy); Belkin et al.,

1971:4 (type information, bionomics); Knight & Stone, 1977:61 (distribution); Forattini, 2002:213 (identification key).

Morphological characterization. *Anopheles antunesi* can be recognized by the following characteristics of the male genitalia: ventral claspette without spicules, apex straight (Fig.1A), ventral surface with distinct swollen lobes striated; dorsal claspette with three long setae, a dorsal seta subapically, narrow, two ventral setae at apex, large, contorted, flattened setae; aedeagus long, slender, strongly sclerotized laterally; aedeagal subapical leaflets present, well developed, straight, parallel to the longitudinal axis, strongly sclerotized, serrated along dorsal and lateral surfaces; apex of aedeagus somewhat rounded in shape (Fig.2A, C); proctiger membranous, without spicules (Fig.1C). Adult female can be recognized by possessing abdominal tergite II-VII without scales; R_{4+5} vein is variable, with pale scales upon dark scales along the vein, and small dark spots at proximal and distal ends; CuA vein with approximately 0.5 proximal pale and 0.5 distal dark; foretarsomeres 1-3 with small apical rings of pale scales, foretarsomeres 4.5 entirely dark, midtarsomere 1 with few pale scales at apex dorsally, midtarsomeres 2-5 dark, hindtarsomere1 with an apical ring of white scales; hindtarsomere 2 dark-scaled at approximatelly 0.5 and white-scaled apically; hindtarsomeres 3-5 white-scaled. Based on fourth instar larvae characteristics, *An. antunesi* can be recognized as follow: 2-C weakly aciculate, 4-C single, long aciculate, seta 14-P usually with 3 long thin branches (Fig.3C); 1-II-VII palmate, with a short pedicel, with pointed leaflets (Fig. 3A); 6-IV-VI long, with two branches (Fig. 3E). Pupae has a trumpet darkly pigmented at middle length, paddle obovate, unpigmented or very weakly pigmented, lighter than posterior abdominal

segments, outer edge of paddle distal to external buttress with spicules. Eggs with floats lateral in position, long, covering part of ventral surface, ribs weakly divided into lobes (Fig. 4A-4D); deck narrow, covered uniformly with fine tubercles, irregular in shape (Fig. 4C); ventral and lateral plastron have several somewhat circular pores (Fig. 4B). The eggs were examined and photographed in the scanning electron microscope that allows detailed analysis of the external structure.

Molecular characterization. The ITS2 region was sequenced for 6 individuals, two from Pindamonhangaba and four from Itatiaia. The amplicon length was consistent at 472-bp (without genes 5.8 and 28), and the 6 sequences revealed a single haplotype. The ITS2 haplotype comprised the following bases: 19% T, 19% A, 29% C and 33% G. A FASTA search using the algorithm 'Database: nucleotide collection - Optimize for: Somewhat Similar' revealed that the ITS2 sequences of *An. antunesi* shares a similarity of 77% and a Query coverage of 84% with *An. pictipennis* Phillip.

The fragment of gene COI was sequenced for the same individuals of ITS2. The amplicon length was consistent at 658-bp (without primers), and the sequences revealed two haplotypes, one different from the other five. The COI haplotype 1 comprised 39% T, 29% A, 16% C and 16% G bases and 11 bases varied. Haplotype 2 comprised 38% T, 29% A, 16% C and 17% G bases.

Distribution.

Distinction between *An. antunesi* and *An. antunesi* Form 1 and also with other species from Myzorhynchella section by adult female could have been largely misidentified through their distribution range. Consequently, it would be important to

collect and raise immatures to adult in order to have male and female with associated larval and pupal exuviae for accurate identification and to have a better understanding of the distribution of those species. Distribution data for *An. antunesi* in the present study are from literature records, and thus may be either underestimated or overestimated.

An. antunesi is known from Brazil, Argentina and Uruguay (Gorham et al., 1967). In Uruguay (Rodriguez & Varela, 1962). In Brazil it was found in the State of São Paulo, Campos do Jordão (Forattini et al., 1997); State of Maranhão, northeast (Rebêlo et al., 2007); State of Rio Grande do Sul (Cardoso et al., 2004), Guaíba municipality (Deane & Neto, 1969); State of Paraná (Rachou & Ricciardi, 1951), Foz do Iguaçu municipality (Consolim et al., 1993).

Medical importance.

Not known, and there is no epidemiological evidence to support the involvement of any species of the Myzorhynchella as vector of pathogens.

Bionomics.

Galvão & Amaral (1940) collected immature of *An. antunesi* in Vila Emilio Ribas, Campos do Jordão, São Paulo about 1570 meters above sea level, in Serra da Mantiqueira. The larvae were collected in ground pools, in small streams in Fonte Simão, and in rockholes along the edges of Capivari river. The larval habitat was shaded, the water was fresh, clean, cold, with little or none vegetation. On those habitats no other anophelines immatures were found. Adults were captured at night using animal (horse) bait. *Anopheles antunesi* was considered to be zoophilic by

Galvão and Amaral (1940), and during the field collections none of the collectors was bitten by specimens of this species. Forattini (1962) described a similar behavior for other Myzorhynchella species.

Immatures of *An. antunesi* were also collected in the vicinities of Parque Nacional do Itatiaia, located on Serra da Mantiqueira. Larvae were taken from stagnant, clean, fresh water from ground pools connected to a running stream (Fig. 5). The water was cold, well oxygenated, with pH approximately 5, with some decomposing leaves, with little vegetation around, on partial shady place. The altitude is about 1100 meters. Galvão (1941) collected larvae in Casa Grande, on the River Claro shores, situated on Serra do Mar, Salesópolis near Mogi das Cruzes municipality. The focus were in clean water in backwaters of small streams, inside the forest.

Fig.5. *An. antunesi* immature habitat in Itatiaia.

Brazil has its southern mountain ranges in the Atlantic massive, in the State of São Paulo there are two major branches which run parallel: Serra da Mantiqueira, located further inland and Serra do Mar which runs along the coast (Unti & Ramos, 1942). And it seems to be that *An. antunesi* is an endemic species of these mountain ranges.

Material examined – *Anopheles antunesi*

For morphology examination of *An. antunesi*, all the individuals were collected in Brazil. State of São Paulo, Vila Emilio Ribas, Campos do Jordão municipality, Galvão & Amaral coll., 1940, det. Galvão & Amaral, 1940, immatures collected: E-2034 [F,wing-paratype], E-2038 [MG], E-2042 [FLePe], E-2044 [MLePe], E-2047 [MG], E-2048 [Le], E-2049 [MG]. State of São Paulo, Campos do Jordão municipality, Sallum & Wilkerson coll., 20-XI-2001, det. Sallum, 2001, immatures collected: E-12438 [FLePe], E-12449 [MLePe], E-12461 [LePe], E-12462 [LePe], E-12463 [LePe], E-12464 [FLePe]. State of São Paulo, Pindamonhangaba municipality, Pico do Itapeva, Fazenda Saint Claire, Sallum *et al.* coll., 27-VII-2006, det. Sallum, 2006, immatures collected: VP09-17 [LePeG]. State of Rio de Janeiro, Itatiaia municipality, Parque Nacional do Itatiaia (22º24’58.7”S, 44º37’19.7”W), Motta coll., VII-2007, det. Motta, 2007, larva collected: RJ-0. Nagaki & Motta coll., 25-III-2008, det. Sallum & Nagaki, 2008, adults collected in Shannon trap: RJ03(11) [E], RJ03(12) [E], RJ03(13) [E]. State of São Paulo, Salesópolis municipality, Casa Grande, Galvão Coll., 1940, det. Galvão, 1940: E-2037 [G], E-2039 [Le], E-2041 [G], E-2050 [G].

For molecular examination of *An. antunesi*, all the individuals were collected in Brazil. State of São Paulo, Pindamonhangaba municipality, Pico do Itapeva, Fazenda Saint Claire Sallum *et al.*, coll., 27-VII-2006, det. Sallum, 2006, larva collected: VP09-17 [M], adult collected in Shannon trap: VP11b [F]. State of Rio de Janeiro, Itatiaia municipality, Parque Nacional do Itatiaia (22º24'58.7"S, 44º37'19.7"W), Nagaki & Motta, coll., 25-III-2008, det. Sallum & Nagaki, 2008, adults collected in Shannon trap: RJ03(6) [F], RJ03(11) [F], RJ03(12) [F], RJ03(13) [F].

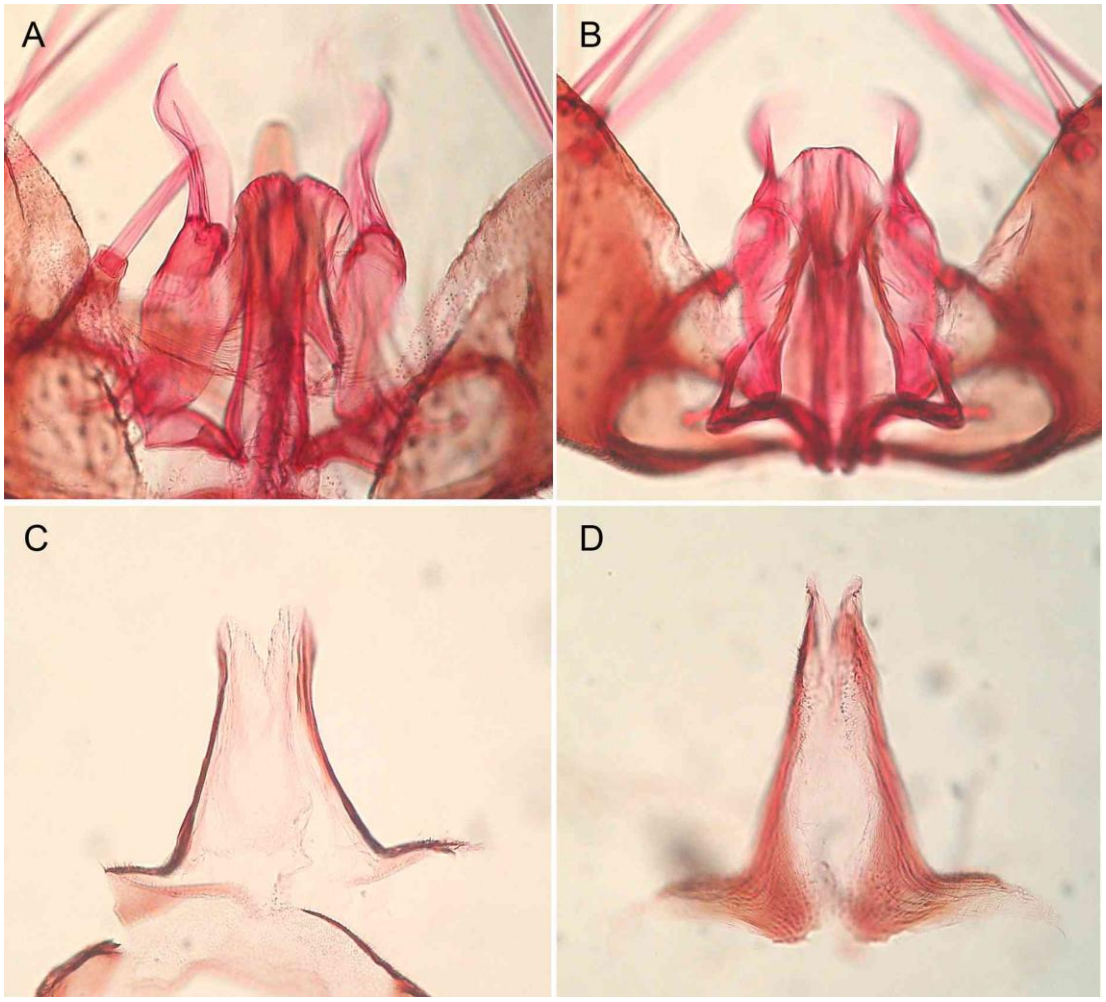

Fig.1. Male genitalia of *An. antunesi* and *An. antunesi* Form 1, both from Pico do Itapeva, State of São Paulo, Brazil. *An. antunesi*: ventral claspette (dorsal aspect) (A), proctiger bare (C). *An. antunesi* Form 1: ventral claspette (dorsal aspect) (B), proctiger with spicules (D).

Fig.2. Male genitalia of *An. antunesi* and *An. antunesi* Form 1, State of São Paulo, Brazil. *An. antunesi*: aedeagus from Pico do Itapeva, showing the position of leaflets (A) and dissected aedeagus from Casa Grande (slide from Galvão (1941), showing the position of ventromesal subtriangular projection (C). *An. antunesi* Form 1: aedeagus (B) and dissected aedeagus from Pico do Itapeva (D).

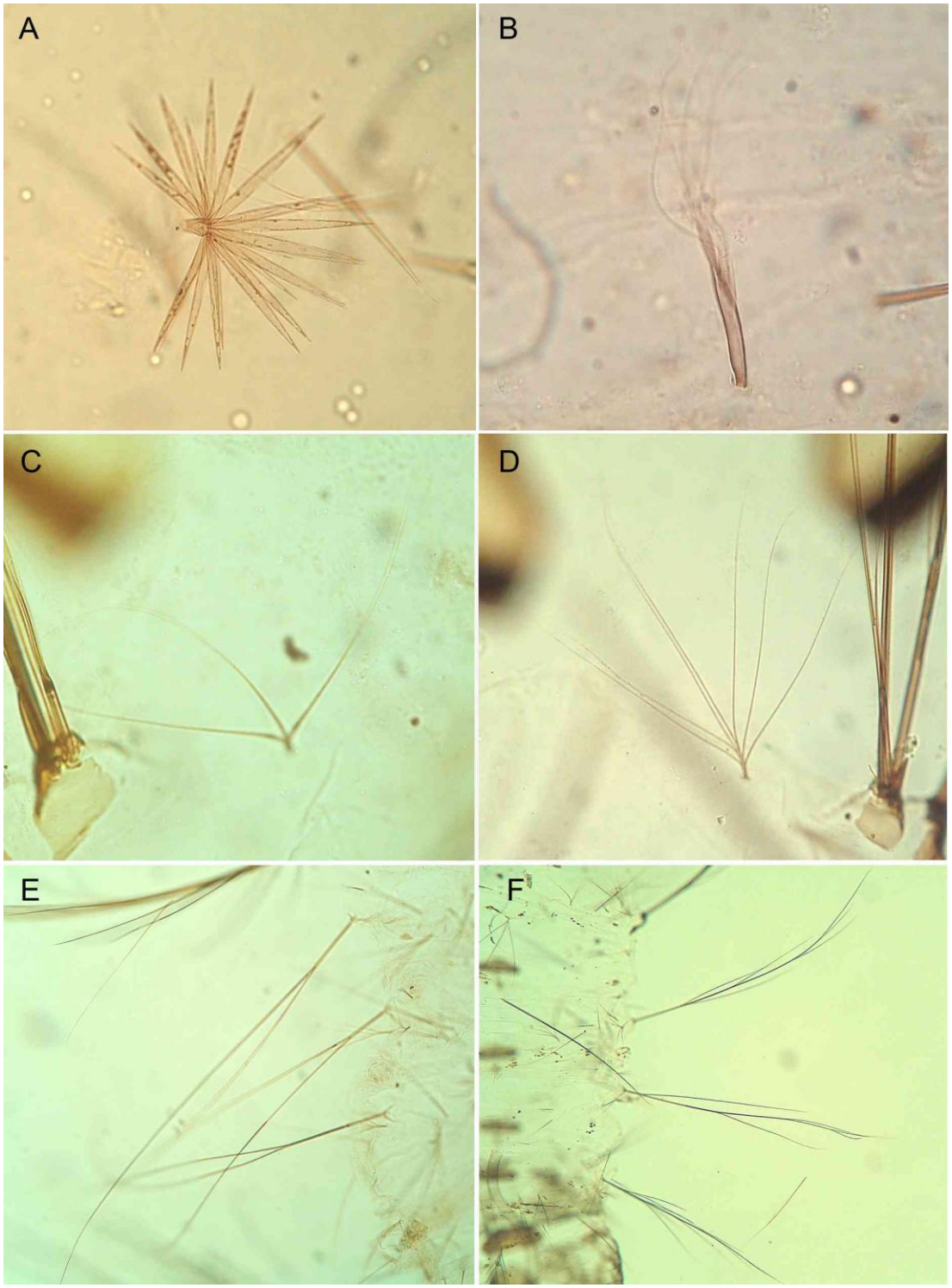

Fig.3. Larval exuvia of *An. antunesi* and *An. antunesi* Form 1, both from Pico do Itapeva, State of São Paulo, Brazil. *An. antunesi*: 1-II-VII palmate (A), 14-P (C) and 6-IV-VI (E). *An. antunesi* Form 1: 1-II-VII palmate (B), 14-P (D) and 6-IV-VI (F).

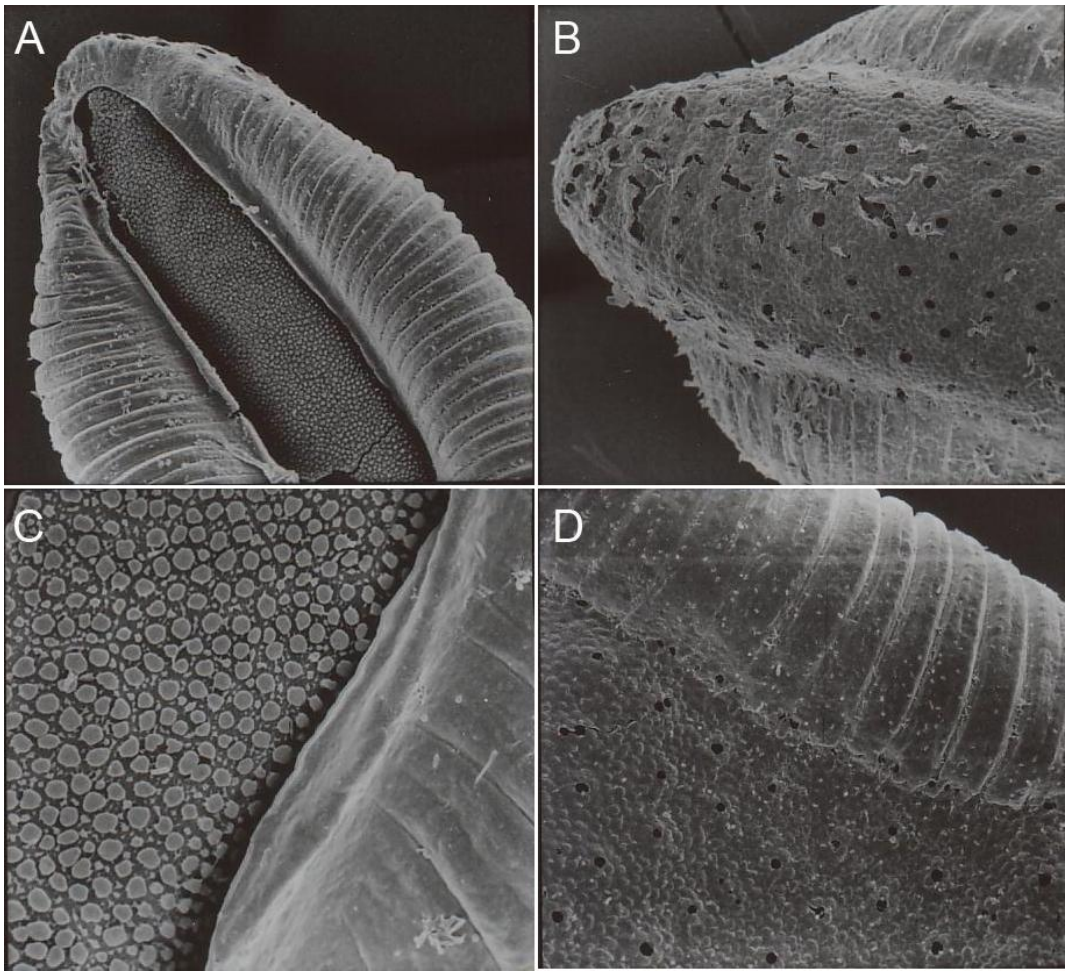

Fig.4. *Anopheles (Nys.) antunesi*. (A) Posterior end, ventral view. (B) Posterior end, dorsal view. (C) Deck tubercles and float. (D) lateral view.

Anopheles (*Nyssorhynchus*) a*ntunesi* Form 1 Nagaki & Sallum

Morphological characterization. *Anopheles antunesi* Form 1 can be recognized by the following characteristics of the male genitalia: ventral claspette without spicules, apex with a rounded shape (Fig. 1B), ventral surface with distinct lobes, median sulcus in a inverted bell shape; dorsal claspette with three long setae, a dorsal seta

subapically, narrow, two ventral setae at apex, large, contorted, flattened setae; aedeagus long, slender, strongly sclerotized laterally; aedeagal subapical leaflets present, forming an angle of 30º, well developed, strongly sclerotized, strongly serrated (Figs. 2B-2D); proctiger membranous mesally, well sclerotized at base, with minute spicules dorsally (Fig. 1D). Adult female can be recognized by possessing abdominal tergite II-VII without scales; R_{4+5} vein is mostly pale above dark scales, with small spots of dark scales at proximal and distal ends; foretarsomeres 1-3 with small apical rings of pale scales, foretarsomeres 4,5 entirely dark, midtarsomere 1-2 with few pale scales at apex, midtarsomeres 2-5 entirely dark, hindtarsomere 1 with an apical ring of white scales; hindtarsomere 2 dark-scaled at approximatelly 0.5 and white-scaled apically; hindtarsomeres 3-5 white-scaled. Based on fourth instar larvae characteristics, *An. antunesi* Form 1 can be recognized as follow: 2-C single, 4-C single aciculate; seta 14-P with a median of 7 long thin branches (Fig. 3D); seta 0-II-VII minute; 1-III-VII palmate, with a long pedicel and thin, hyaline branches (Fig. 3B); 6-IV-VI long, with a median of 5 branches (Fig. 3F). Pupae has a trumpet darkly pigmented at middle length, paddle obovate, unpigmented or very weakly pigmented, lighter than posterior abdominal segments, outer edge of paddle distal to external buttress with spicules.

Molecular characterization. The ITS2 region was sequenced for 12 individuals, all specimens from Campos do Jordão/Pindamonhangaba. The amplicon length was consistent at 470-bp (without genes 5.8 and 28), and the 12 sequences revealed a single haplotype. The ITS2 haplotype comprised the following bases: 20% T, 20% A, 27% C and 33% G. A FASTA search using the algorithm ‘Database: nucleotide

collection - Optimize for: Somewhat Similar' revealed that the ITS2 sequences of *An. antunesi* Form 1 shares a similarity of 81% and a Query coverage of 81% with *An. pictipennis*.

The fragment of gene COI was sequenced for 12 individuals, being one different of ITS2 specimens. The amplicon length was at approximately 658-bp (without primers), and the sequences revealed four haplotypes, but the percentage was similar. It comprised 38% T, 29% A, 16% C and 17% G bases.

Distribution.

Anopheles (Nys.) antunesi Form 1 could be misidentified through their distribution range. Consequently it has probably the same distribution of *An. antunesi.*

Medical importance

Not known, and there is no epidemiological evidence to support the involvement of any species of the Myzorhynchella as vector of pathogens so far.

Bionomics

The field collection carried in Pico do Itapeva, between Pindamonhangaba and Campos do Jordão municipalities, at approximately 1781 meters from the sea level, immatures of *An. antunesi* Form 1 were collected in small ground pool with grass vegetation around in a open field only with the vegetation shade (Fig.6).

An. antunesi Form 1 is probable an endemic species of high altitudes and as the specimens collected were from these places, probably because the climate and

environment, we could not raise the early larval instars neither the eggs in the laboratory.

Fig.6. Larval habitat of *An. antunesi* Form 1.

Material examined – *An. antunesi* Form 1

For morphology examination of *An. antunesi* Form 1, all the individuals were collected in Brazil. State of São Paulo, Campos do Jordão municipality, Sallum & Wilkerson., coll., 20-XI-2001, det. Sallum, 2001, immatures collected: E-12370 [LePe]. State of São Paulo, Pindamonhangaba/Campos do Jordão municipalities, Pico do Itapeva, Fazenda Saint Claire (22º45’30.5”S, 45º30’55.0”W), Nagaki *et al.*, coll., 14/26-I-2009, det. Nagaki & Sallum, 2009, immatures collected: SP51-100 [Pe], SP53-1 [LePe], SP53-3 [LePe], SP53-4 [Le], SP53-5 [Le], SP53-101 [Pe].

For molecular examination of *An. antunesi* Form 1, all the individuals were collected in Brazil. State of São Paulo, Campos do Jordão municipality, Sallum & Wilkerson.,

coll., 20-XI-2001, det. Sallum, 2001, immatures collected: E-12370 [F, abdomen]. State of São Paulo, Pindamonhangaba municipality, Sallum *et al.*, coll., 27-VII-2006, det. Sallum, 2006, adult collected in Shannon trap: VP11a [F], VP11c [F], VP11d [F]. State of São Paulo, Pindamonhangaba/Campos do Jordão municipalities, Pico do Itapeva, Fazenda Saint Claire (22º45'30.5"S, 45º30'55.0"W), Nagaki *et al.*, coll., 14/26-I-2009, det. Nagaki & Sallum, 2009, immatures collected: SP51-100 [F], SP53-100 [M], SP53-101 [F], SP53-4 [P], SP53-5 [P], adults collected in Shannon trap: SP50a [F], SP50b [F], SP55(2) [F], SP55(4) [F].

Discussion

During field collection carried out in the vicinities of Parque Nacional do Itatiaia, Serra da Mantiqueira, Rio de Janeiro, and in Pico do Itapeva, Pindamonhangaba, Serra da Mantiqueira, São Paulo, specimens of mosquitoes were collected and identified as *An. lutzii* and *An. antunesi*, respectively. Detailed morphological studies of specimens from Pico do Itapeva showed that individuals could be separated into two morphological groups based on wing spots, male genitalia and fourth-instar larva, suggesting that there were at least two species misidentified as *An. antunesi* and that specimens identified as *An. lutzii* from Itatiaia could belong to *An. antunesi*. Results of the ITS2 and COI sequences analyses corroborated the morphological hypotheses. Consequently, specimens from Pico do Itapeva were identified as *An. antunesi* and *An. antunesi* Form 1, and from Itatiaia were found to be conspecific with *An. antunesi*.

In the larval stage *An. antunesi* and *An. antunesi* Form 1 can be easily recognized as members of the Myzorhynchella section by possessing seta 6 of 4-6

abdominal segments long and branched; in the adult stage by the absence of scales in abdominal segments and hindtarsomeres 3-5 usually white scaled. In the male genitalia, both *An. antunesi* and *An. antunesi* Form 1 can be easily distinguished from species of the Albimanus and Argyritarsis sections in having dorsal claspette with two apical and 1 subapical setae, accessory setae ending in a sharply acute apex (Galvão, 1941).

Adults male and female of *An. antunesi* and *An. antunesi* Form 1 are larger when compared to those of *An. lutzii* and *An. parvus*. However, the size may be related to the altitude, where the climate is mild and water is usually cool. Based on morphological characters of the adults *An. antunesi* and *An. antunesi* Form 1 can be distinguished from *An. nigritarsis* by the absence of dark scales on hindtarsomeres 3, 4 and 5, which is a key characteristic to identify *An. nigritarsis*. Additionally, *An. antunesi* and *An. antunesi* Form 1 can be distinguished from *An. lutzii* and *An. parvus* in having R_{4+5} vein mostly pale, whereas R_{4+5} is mostly dark scaled in *An. lutzii.* In the male genitalia *An. antunesi* and *An. antunesi* Form 1 can be separated from *An. lutzii* and *An. parvus* by the shape of aedeagus, which has its leaflets forming distinct angles, and the position of the ventromesal subtriangular projection.

Further details of the wing dark and white spots are important to distinguish *An. antunesi* and *An. antunesi* Form 1 from *An. lutzii* and *An. parvus*. Details are as follow: vein R_{4+5} is mostly dark with one subdistal and one subproximal pale spots in *An. lutzii*. *An. parvus* R_{4+5} has three dark areas intermixed with two white spots well defined. In *An. antunesi*, it is mostly pale with dark spots at subproximal and subdistal ends according to Galvão & Amaral (1940). However, specimens collected in Itatiaia showed variation in the dark and white scale spots in $R_{4+5,}$ individuals

presented mostly dark or pale scales. Galvão (1941) calls the attention to the possibility of misidentification of *An. antunesi* and *An. lutzii* because adult female of both species could have melanism forms, which often leads to the presence of a bigger black portion of the R_{4+5} vein. In considering the polymorphism in R_{4+5}, we observed that CuA_2 of *An. antunesi* is white scaled in proximal 0.5 and dark in distal 0.5, whereas in *An. antunesi* Form 1 CuA_2 has a proximal pale spot, a dark spot, a pale spot and a distal dark spot. Consequently, the pattern of pale and dark spots in CuA_2 is an accurate character to distinguish *An. antunesi* from *An. antunesi* Form 1.

By male genitalia characteristics, *An. antunesi* Form 1 can be distinguished from *An. antunesi* by possessing the apex of the ventral claspette moderately rounded apically (Fig. 1A), the leaflets of the aedeagus are straight and parallel to its longitudinal axis (Fig. 2A), and the proctiger is spiculate dorsally (Fig. 1C), whereas in *An. antunesi* the apex of ventral claspette is somewhat straight (Fig. 1B), the aedeagal leaflets arise subapically forming an angle of 25º with the longitudinal axis (Fig. 2B), and the proctiger is entirely bare (Fig. 1D). Beyond that, the ventromesal subtriangular projection os both especies shows a different position according to its leaflets (Fig. 2CD).

Besides while examining characters of the fourth instar larva, it became evident that *An antunesi* has seta 1-II-VII palmate with a short pedicel (Fig. 3A), seta 6–IV-VI bifid (Fig. 3E), while in *An. antunesi* Form 1 seta 1-II-VII has a long pedicel, supporting thin hyaline leaflets (Fig. 3B), and seta 6–IV-VI is branched, with a central branch from which arise approximately five lateral branches (Fig. 3F).

Another evident character to distinguish both species were the studies of eggs, we could notice that the eggs described by Forattini et al. (1997) as *An. antunesi* could be *An. antunesi* Form 1. The character that most calls the attention is the outer chorion of ventral surface that in *An. antunesi* previously characterized, it is covered with circular pores (Fig. 4B) , while in the study of Forattini et al. (1997) it has several irregularly open areas. And it is clear that as both species are similar, they misidentified as being *An. antunesi*.

Finally, the morphological hypotheses for a new, sympatric species with *An. antunesi* in Pico do Itapeva, Serra da Mantiqueira was corroborated by molecular characters of rDNA (ITS2) and mtDNA (COI). It showed that the sequences from Itatiaia were the same of *An. antunesi* from Pico do Itapeva. Sequences of ITS2 showed two very distinct haplotypes, each group of one species (Fig. 7). The COI sequences were also separated into two groups respectively linking with the ITS2 sequences and confirming two distinct species.

```
SP55_2   CACATTCATC CACATATCGA ACTAGCGA-- -----CGCGG GGTCTCC--G GACCCGGCCG -----AGCTG CGTACTGATG  [ 80]
SP55_4   .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP53_101 .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP53_100 .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP53_5   .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP53_4   .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP51_100 .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP50b    .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
SP50a    .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
VP11d    .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
VP11a    .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
b12370   .......... .......... ........-- -----..... .......--. .......... -----..... ..........  [ 80]
RJ03_6   .......... .G........ ........AG CCTCG..... ....C..CG. .......... GGCCG..... ..........  [ 80]
RJ03_11  .......... .G........ ........AG CCTCG..... ....C..CG. .......... GGCCG..... ..........  [ 80]
RJ03_12  .......... .G........ ........AG CCTCG..... ....C..CG. .......... GGCCG..... ..........  [ 80]
RJ03_13  .......... .G........ ........AG CCTCG..... ....C..CG. .......... GGCCG..... ..........  [ 80]
VP09_17  .......... .G........ ........AG CCTCG..... ....C..CG. .......... GGCCG..... ..........  [ 80]
VP11b    .......... .G........ ........AG CCTCG..... ....C..CG. .......... GGCCG..... ..........  [ 80]

SP55_2   ATTTGATTGA CGC--CGGT- -AACACGCGC GTGTGGTCAA GCATTGAAGA TACTGTGGCG TTGAGTGTGG CACCGTGTCG  [160]
SP55_4   .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP53_101 .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP53_100 .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP53_5   .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP53_4   .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP51_100 .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP50b    .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
SP50a    .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
VP11d    .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
VP11a    .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
b12370   .......... ...--....- -......... .......... .......... .......... .......... ..........  [160]
RJ03_6   .......... ...GC.C.CC CGG.GG...T .......... .......... .......... .......... ..........  [160]
RJ03_11  .......... ...GC.C.CC CGG.GG...T .......... .......... .......... .......... ..........  [160]
RJ03_12  .......... ...GC.C.CC CGG.GG...T .......... .......... .......... .......... ..........  [160]
RJ03_13  .......... ...GC.C.CC CGG.GG...T .......... .......... .......... .......... ..........  [160]
VP09_17  .......... ...GC.C.CC CGG.GG...T .......... .......... .......... .......... ..........  [160]
VP11b    .......... ...GC.C.CC CGG.GG...T .......... .......... .......... .......... ..........  [160]

SP55_2   C-GGCTTAGC ACGACTCTCT CGTGCGCGCC TGAGCGGGCT ACCAGTCACG GAACCGGTTT ATCGTCGCCA AAGTAGGTGC  [240]
SP55_4   .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP53_101 .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP53_100 .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP53_5   .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP53_4   .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP51_100 .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP50b    .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
SP50a    .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
VP11d    .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
VP11a    .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
b12370   .-........ .......... .......... .......... .......... .......... .......... ..........  [240]
RJ03_6   .A...C.... .......... .......... .....A.... .......... .......... ....G..... ..........  [240]
RJ03_11  .A...C.... .......... .......... .....A.... .......... .......... ....G..... ..........  [240]
RJ03_12  .A...C.... .......... .......... .....A.... .......... .......... ....G..... ..........  [240]
RJ03_13  .A...C.... .......... .......... .....A.... .......... .......... ....G..... ..........  [240]
VP09_17  .A...C.... .......... .......... .....A.... .......... .......... ....G..... ..........  [240]
VP11b    .A...C.... .......... .......... .....A.... .......... .......... ....G..... ..........  [240]

SP55_2   GTCGCCACGG ATTTAGGTGA CAGGGTAGCT GGGATTTGCT TACTTGAAAT TTCAAATGGG TAGGCTCTTC CTTGCTCAGG  [320]
SP55_4   .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP53_101 .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP53_100 .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP53_5   .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP53_4   .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP51_100 .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP50b    .......... .......... .......... .......... .......... .......... .......... ..........  [320]
SP50a    .......... .......... .......... .......... .......... .......... .......... ..........  [320]
VP11d    .......... .......... .......... .......... .......... .......... .......... ..........  [320]
VP11a    .......... .......... .......... .......... .......... .......... .......... ..........  [320]
b12370   .......... .......... .......... .......... .......... .......... .......... ..........  [320]
RJ03_6   .......... .......... .......... .......... .......... .......... .........T ..G.......  [320]
RJ03_11  .......... .......... .......... .......... .......... .......... .........T ..G.......  [320]
RJ03_12  .......... .......... .......... .......... .......... .......... .........T ..G.......  [320]
RJ03_13  .......... .......... .......... .......... .......... .......... .........T ..G.......  [320]
VP09_17  .......... .......... .......... .......... .......... .......... .........T ..G.......  [320]
VP11b    .......... .......... .......... .......... .......... .......... .........T ..G.......  [320]
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Fig 7. Partial ITS2 sequence alignment of specimens of *An. antunesi* and *An. antunesi* Form1.

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4.2. MANUSCRITO 2

"Redescription of *Anopheles* (*Nyssorhynchus*) *lutzii*, and resurrection of *An. niger* and *An. guarani* (Diptera: Culicidae) from the synonymy"

(em preparação)

Redescription of *Anopheles* (*Nyssorhynchus*) *lutzii*, and resurrection of *An. niger* and *An. guarani* (Diptera: Culicidae) from the synonymy

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Running title: Species of the Myzorhynchella section

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Summary

Anopheles (*Nyssorhynchus*) *lutzii* is redescribed based on specimens collected in Pariquera–Açu, Vale do Ribeira, São Paulo state, Brazil. *Anopheles niger* and *An. guarani* are revived from synonymy with *An. lutzii*. Specimens from Vale do Ribeira are compared with the syntypes of *An. lutzii* and two females from Nova Friburgo, Rio de Janeiro state. Adult female of *An. guarani* is redescribed and the adult male, fourth-instar larvae and pupae are described for the first time. *Anopheles niger* is characterized and the male genitalia is compared with that of *An. lutzii* and *An. guarani*. Adults, male and female, male genitalia, fourth instar larvae and pupae of *An. lutzii* and *An. guarani* are described, and the male genitalia and larval characteristics are illustrated. Scanning electron micrographs of the eggs of *An. lutzii* and *An. guarani* are given. Male genitalia of *An. niger* is described and illustrated for the first time.

Key words: Myzorhynchella – Distribution - Mata Atlantica - Southern Brazil

Introduction

The subgenus *Nyssorhynchus* consists of three sections based on unique combinations of larval, pupal and adult characters (Peyton et al., 1992). The Albimanus Section is the largest with approximately 19 species, the Argyritarsis Section contains approximately 10 species, and the Myzorhynchella Section is the smallest with four valid species, *An. lutzii* Cruz, *An. parvus* (Chagas), *An. nigritarsis* (Chagas) and *An. antunesi* Galvão & Amaral (Galvão, 1941; Harbach, 2004). Additionally, a fifth species from Serra da Mantiqueira, southeastern Brazil, which is morphologically similar and sympatric to *An. antunesi*, will be described by Nagaki

et al. (unpublished). Additionally, *An. niger* Theobald and *An. guarani* Shannon were described, and later considered to be synonymous of *An. lutzii*.

The last revision on the Myzorhynchella section is that by Galvão (1941). Following, Lane (1953), Forattini (1962) and Gorham (1967) provided information for members of the Myzorhynchella section, including morphological identification keys. The geographical distribution range of each species is not well known, however, in Table 1, we list the type localities of taxa of the Myzorhynchella section, including *An. niger* and *An. guarani*. Species identification can be problematic if based only on the pattern of dark and pale scales of vein R_{4+5} of adult female because this character was found to be polymorphic (Galvão 1941, Nagaki et al. unpublished). On the other hand, characteristics of the external morphology of the eggs can be used for species distinction. For example, Forattini et al. (1998) described and compared the eggs of *An. lutzii* employing two populations, one from inland Mata Atlantica, and a second from southeast Mata Atlantica, both in the state of São Paulo. Interesting, Forattini et al. (1998) observed differences in the exocorion of eggs and hypothesized that they were suggestive of distinct species.

Table 1. Type localities of species of the Myzorhynchella section of Anopheles (Nyssorhynchus) according to Belkin (1971).

Species	Type locality (State)
Anopheles (Nys.) parvus	Oliveira (Minas Gerais)
Anopheles (Nys.) nigritarsis	Oliveira (Minas Gerais)
Anopheles (Nys.) antunesi	Vila Emilio Ribas, Campos do Jordão (São Paulo)
Anopheles (Nys.) lutzii	Lagoa Rodrigo de Freitas (Rio de Janeiro)
Anopheles (Nys.) niger	Cantareira (São Paulo)
Anopheles (Nys.) guarani	Iguaçu (Paraná)

Anopheles lutzii was described by Cruz (1901) based on morphology of the adult female, larvae, and eggs of specimens collected in Jardim Botânico and Sarapuhy, Rio de Janeiro. Peryassú (1908) redescribed the larvae and the eggs of *An. lutzii* from Rio Grande do Sul, and Ramos (1937) used specimens from Cotia and São Vicente municipalities, both in São Paulo state to describe the male genitalia. Following, Galvão (1938) showed a microphoto of the eggs of *An. lutzii* from the vicinities of São Paulo municipality, São Paulo state. By comparing the microphoto showed by Galvão (1938) with that of Peryassú (1908), we noticed that they are different, and thus may belong to distinct species. Later, Galvão (1941) redescribed all the stages and illustrated the male genitalia of *An. lutzii* employing specimens collected in Vila Ema, São Paulo municipality, São Paulo state. Lane (1953) redescribed the female, male, pupae, larvae and egg of *An. lutzii* mostly based on specimens of Galvão (1941). Later, Forattini (1962) considered Myzorhynchella a subgenus of *Anopheles*, and redescribed the adults male and female, male genitalia, larvae and eggs of *An. lutzii*. The male genitalia illustrated by Forattini (1962) is that of Galvão (1941).

Anopheles niger was named by Theobald (1907) from adults female collected in Cantareira, São Paulo, however, in 1907, Chagas synonymyzed *An. niger* with *An. lutzii*. The lectotype of *An. niger* was designated by Belkin (1968), and it is deposited in the Natural History Museum, London, UK.

Shannon (1928) validated and described *An. guarani* in Dyar (1928). The type locality is Iguaçú, Paraná state, and the type is deposited in United State National Museum (USNM). Later, in Lane (1953) *An. guarani* was considered a

synonymy of *An. lutzii*, however, there is no evidence that was Lane, who transferred the species to the synonymy.

When examining specimens deposited in the Entomological Collection of Faculdade de Saúde Pública, Universidade de São Paulo, Brazil, and comparing the records in the published literature on species of the Myzorhynchella Section, we noticed morphological differences that were indicative of distinct species under the name *An. lutzii*. Additionally, the hypothesis of Forattini et al. (1998) regarding to the SEM egg morphology was congruent with differences registered in the literature. Furthermore, results of a molecular study by Nagaki & Sallum (unpublished) employing nucleotide sequences of the ribosomal DNA internal transcribed spacer 2 (ITS2) and mitochondrial DNA citochrome oxidase subunit I (COI) corroborated the hypothesis of Forattini et al. (1998) that populations from Pariquera Açu and Dourado are not conspecific. As a consequence, the objectives of this study are: 1) to examine if *An. niger* is a valid species; 2) to examine the taxonomic status of *An. guarani*; 3) to redescribe the adults male and female, fourth instar larvae and pupae of *An. lutzii*.

Materials and methods

Ten specimens of *Anopheles lutzii* were collected in Shannon trap. Six individuals are from Sítio Galiléia (24º44.975'S, 47º56.944'W), Pariquera-Açu municipality, Vale do Ribeira, State of São Paulo, Brazil, and three from near Almada River (25º28’50”S, 54º35’12”W), Foz do Iguaçu municipality, state of Paraná, Brazil. Specimens from the latter locality represent topotypes of *An. guarani*, a synonymous of *An. lutzii*. The mosquitoes were blood–fed and induced to lay eggs

by removing one wing. Approximately 30 eggs of each female of *An. lutzii* from Foz de Iguaçu were fixed in alcoholic Bouin's solution 36 hours after oviposition, and then prepared for scanning electron microscopy (SEM) following the protocol described by Forattini & Marucci (1993). Eggs were examined in a JEOL JSM–P15 (JEOL Ltd., Akishima Tokyo, Japan).

The remaining eggs of each female were maintained in separate recipients with distilled water. Larvae were fed with a mixture of fish food and pollen. Fourth–instar larvae were transferred to individual plastic vials and kept until adults emerged. Both larval and pupal exuviae were preserved in 80% ethanol, prior to mount in microscope slides in Canada balsam. Male genitalia were dissected and mounted in microscope slides in Canada balsam.

Morphological characters of the adults male and female and the male genitalia were examined and measured. Pupal and larval chaetotaxy were examined, measured and counted for the description. Terminology for morphological characters follows Harbach & Knight (1980), except we used Belkin (1962) for wing veins and Wilkerson & Peyton (1990) for wing spots. Abbreviations for mosquito life stages are: F, adult female; M, adult male; G, male genitalia; L, larvae; Le, larval exuviae; P, pupae; Pe, pupal exuviae; E, egg.

Results

Taxonomic treatment

Anopheles (Nysshorhynchus) lutzii (Figs. 1 and 2)

Anopheles (Nys.) lutzii Cruz, 1901:423. Type: Syntypes 3 females (1965) in tube no. 993, one slide (no. 2111) with one wing of one female, Lagoa Rodrigo de Freitas, Rio de Janeiro (Guanabara) (IOC–FIOCRUZ), deposited at Costa Lima Collection. Belkin et al., 1971: 5 (type information); Marchon–Silva et al., 1996: 472 (type information); Forattini et al. 1998: 256 (egg description, in part specimens from Vale do Ribeira).

Description.

Female. Integument brown. *Head*: interocular space with frontal tuft of long, pale setae, and decumbent, pale, piliform scales; vertex bare along dorsal suture, with decumbent, pale spatulate scales and few long, browish setae anteriorly, lateral to bare dorsal area, remainder of vertex and occiput with semierect, grayish spatulate scales, posterolateral scales decumbent, dark spatulate; postgena with decumbent dark scales; clypeus bare. Pedicel of antenna brown, bare; flagellomere 1 with semierect pale scales on dorsal surface. Proboscis dark scaled, length 2.10–2.26 mm (mean = 2.16 ± 0.06) (n = 5), length of forefemur 1.66–2.11 mm (mean = 1.83 ± 0.17) (n = 5), and length of maxillary palpus 1.83–2.24 mm (mean = 2.05 ± 0.17) (n = 5). Maxillary palpomere 1 and 2 dark–scaled with few pale scales at apex of dorsal surface, scales erect or semierect; palpomere 3 dark–scaled with few pale scales at apex of dorsal surface; palpomere 4 dark–scaled with a conspicuous patch of white scales at apex of dorsal surface; palpomere 5 white–scaled with dark scales at base.

Thorax: integument with darker area between dorsocentral area and lateral margin, on anterior acrostichal area, on posterior edge of scutal fossa and posteriorly on prescutellar area extending posteriorly to median scutellar lobe; pale, narrow, decumbent scales on acrostichal area and anteriorly on prescutellar area; dorsocentral, supraalar and scutal fossa with pale, spatulate, decumbent scales; elongate, narrow and erect pale scales along lateral margin of antealar area extending posteriorly onto supraalar area; scutum bare anteriorly between acrostichal and dorsocentral areas, posteriorly to scutal fossa and posteriorly on prescutellar area; anterior promontory with erect piliform, pale scales. Scutellum with few, piliform pale scales and long and short brown setae on posterior margin. Mesopostnotum bare. Antepronotum with light brown setae and a patch of spatulate dark scales and few pale scales. Pleura with small patches of pale, spatulate scales on upper mesepimeron, upper mesokatepisternum, posterior border of middle mesokatepisternum, prespiracular area and prealar knob; dark brown setae on proepisternum and upper mesokatepisternum, light brown setae on prealar knob, minute pale setae on preespiracular area and pale setae on upper mesepimeron. *Wing*: length 3.24–3.54 mm (mean = 3.38 ± 0.11) (n = 10); wing spots measurements in Table 2; veins dark–scaled with spots of pale scales as follows: costa always with basal pale, prehumeral dark, humeral pale, humeral dark, presector pale, presector dark, setor pale, proximal setor dark, acessory sector pale, distal sector dark, subcostal pale, preapical dark, preapical pale and apical dark spots; presector pale absent in 20% of wings examined; remigium pale–scaled, vein R proximal pale; R_1 with acessory sector pale, subcostal pale, preapical pale spots; R_2 mostly dark–scaled with a small apical pale spot and a pale spot at furcation with vein R_3; R_3 with a pale

spot in the middle of the vein; Rs with few pale scales at base and a patch of pale scales at junction of R_{4+5}; R_{2+3} dark–scaled with few pale scales at apex and base; R_{4+5} variable, mostly pale–scaled, with small patches of pale scales at proximal and distal ends, dark scales at proximal 0.3, and pale scales along middle area on top of dark scales following the vein; vein M mostly pale scaled proximal to bifurcation level of CuA, mostly dark–scaled at distal 0.5, with patches of pale scales at middle length and at furcation of veins M_{1+2} and M_{3+4}; M_{1+2} with pale scales at base and apex, and a patch of pale scales at middle of the vein and two spots of dark scales bordering the pale patch; M_{3+4} dark–scaled with pale scales at proximal and distal ends; CuA with a patch of pale scales at proximal 0.5 and a patch of dark scales at distal 0.5; CuA_1 with four pale spots intermixed with three dark spots; CuA_2 mostly pale–scaled at proximal 0.5, with two dark spots; 1A mostly dark–scaled with pale scale patches at proximal and distal ends and one pale patch at middle of proximal 0.5; pale fringe spots at apices of veins R_2, R_{4+5}, M_{1+2}, M_{3+4}, Cua_1, Cua_2 and 1A. *Halter*: scabellum with pale integument; pedicel light brown; capitelum entirely dark–scaled.

Table 2. Wing spot measurements (in mm) for adult male (M) and female (F) of *Anopheles lutzii* collected in Pariquera Açu municipality, Ribeira Valley , State of São Paulo, Brazil (SP).

Wing spot	Range		Mean		SD (±)		n =	
	(F)	(M)	(F)	(M)	(F)	(M)	(F)	(M)
Basal pale	0.06–0.08	0.04–0.09	0.07	0.07	0.01	0.01	10	10
Prehumeral dark	0.23–0.29	0.22–0.29	0.27	0.26	0.02	0.02	10	10
Humeral pale	0.06–0.11	0.08–0.13	0.07	0.10	0.02	0.01	10	10
Humeral dark	0.15–0.19	0.11–0.16	0.17	0.14	0.02	0.02	8	10
Presector pale	0.07–0.10	0.04–0.13	0.08	0.08	0.01	0.02	8	10
Presector dark	0.31–0.34	0.27–0.41	0.32	0.35	0.01	0.04	3	10
Sector pale	0.04–0.08	0.07–0.14	0.06	0.10	0.02	0.02	5	8
Proximal sector dark	0.10–0.16	0.04–0.16	013	0.11	0.02	0.04	5	8
Accessory sector pale	0.09–0.17	0.15–0.18	0.12	0.17	0.02	0.01	10	8
Distal sector dark	0.74–0.88	0.65–0.76	0.80	0.69	0.04	0.03	10	10
Subcostal pale	0.22–0.30	0.27–0.36	0.25	0.33	0.02	0.03	10	10
Preapical dark	0.68–0.77	0.49–0.67	0.73	0.57	0.03	0.05	10	10
Preapical pale	0.15–0.24	0.18–0.25	0.20	0.22	0.03	0.02	10	10
Apical dark	0.08–0.14	0.08–0.18	0.11	0.12	0.01	0.03	10	10

Legs: anterior surface of forecoxa with few pale spatulate scales and long, dark setae on upper part; posterolateral surface with a patch of pale scales at apex; midcoxa with a patch of pale spatulate scales at apex of posterior surface, and at base and apex of anterior surface; posterior surface of hindcoxa with 2 long setae on upper part and a patch of pale spatulate scales. Fore–, mid– and hindtrochanters pale–scaled. Foretarsomeres 1–3 with apical, pale scales dorsally, tarsomere 1 with a small patch of pale scales at apex, tarsomere 2 with apical 0.7 pale and tarsomere 3 with apical 0.8 pale; foretarsomeres 4 and 5 entirely dark–scaled; midtarsomeres dark–scaled, tarsomere 1 with small patch of pale scales at apex; hindtarsomere 1 with an apical

ring of white scales, hindtarsomere 2 dark–scaled at basal 0.5, white scaled at approximatelly apical 0.5; hindtarsomeres 3–5 white–scaled. *Abdomen*: integument dark brown; terga I–VII without scales, covered with dark setae with golden reflections; sterna with basolateral pale spots; laterotergite I with spot of silver reflection; sternum VII with dark spatulate scales; cerci with dark scales.

Male. Similar to female except for sexual differences. Maxillary palpus dark–scaled; scales semierect on palpomeres 1 and 2, decumbent on palpomeres 3, 4 and 5; palpomere 2 with pale scales at apex, palpomere 4 with long, strong setae along border of ventral surface; palpomere 5 dark–scaled with withe scale along dorsal surface. Wing with with less quantity of scales in comparison to female; length of the wing similar to females; wing spots measurements in Table 2; vein R_{4+5} mostly pale–scaled. Abdominal segments dark–scaled and pale scales at apex of the last.

Male genitalia: Segment VIII: Tergum and sternum narrow, without scales and with long and short setae. *Segment IX*: Sternum rectangular, anteromedial and posteromedial border shallow emarginated without apodeme. *Proctiger*: membranous mesally, strongly sclerotized laterally, spiculose dorsally; apex narrow. *Gonocoxite*: length about 0.32–0.63 (mean = 0.34 ± 0.02) (n = 5); tergal surface laterally covered with large, obovate scales, with 4 long and 3 short tergomedial setae, 2 apicolateral setae and one apicomesal setae immediately based of gonostylus; parabasal seta slightly retrorsely hooked; dorsomedial rim long, strongly developed; accessory setae moderately long, broad, tapering to apex, ending in a narrow sharply pointed apex, dorsal seta slightly curved posteriorly, about 0.39–0.44 (mean = 0.42 ± 0.02) (n = 5) length of gonocoxite, ventral seta straight, about 0.70–0.93 (mean = 0.84 ± 0.07) (n = 5) length of dorsal setae; internal setae slender, about

0.84–0.99 (mean = 0.93 ± 0.05) (n = 5) length of ventral accessory seta, curved anteriorly. Sternal surface covered with white scales. *Gonostylus*: curved mesally along entire length, broad at base, narrow at middle part, broad at apical 0.3; dorsal margin with 9–12 minute setae; seta b slender, single, inserted basolateral to gonostylar claw; gonostylar claw short, moderately sclerotized, slender. *Dorsal Claspette*: paired, short, pedicel about 0.23–0.30 (mean = 0.26 ± 0.02) (n = 5) length of gonocoxite; pedicel moderately broad rounded at base, broad and sclerotized at apex. Apex with 2 ventral setae at apex and 1 dorsal seta arising subapically, setae curved mesally, dorsal seta without basomesal projection, the most lateral ventral seta with weakly developed basomesal projection, the mesal seta strongly developed, swollen at middle length, tapering to apex, without developed basomesal projection. *Ventral Claspette* (Fig. 1A): about 0.3 length of gonocoxite, somewhat trapezoidal in shape, without spicules, apex narrow, straight; dorsal surface mostly plain, with two small subapical lobes arising mesally; ventral surface with distinct, swollen lobes, projecting ventrally, basally developed as a pair of large basal lobules connected by a membrane, mesal border of each lobe close together bordering mesal cleft; mesal cleft long, extending ventrally from apex to base of ventral claspette, more sclerotized at posterior 0.5; median sulcus in shape of V. *Phallosome*: extending posteriorly beyond ventral claspette, composed of a central aedeagus, a pair of parameres, a pair of basal pieces; aedeagus long, narrow, strongly sclerotized laterally; aedeagal subapical leaflets present, well developed, slightly curved, forming an angle of 45º, strongly sclerotized, serrated along lateral surfaces; apex of aedeagus somewhat rounded in shape, moderately sclerotized, outer border of apex at level of insertion of leaflets hyaline; ventromesal subtriangular projection subapical

in position, mesally fused forming a collar positioned approximately at apex length of leaflets apex (Fig 1B).

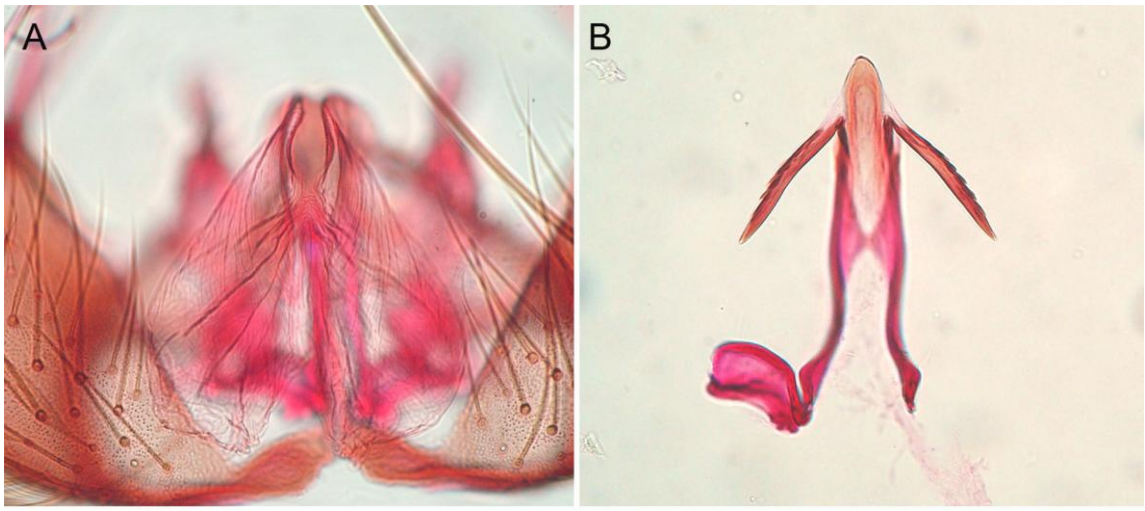

Fig. 1. *Anopheles lutzii*: (A) ventral claspette, ventral view; (B) aedeagus

Fourth–instar larva. Range and modes of branches in Table 3. Measurements from 10 specimens unless otherwise indicated. *Head*: length 0.68– 0.81 mm (mean = 0.76 ± 0.05) (n = 10); width 0.66–0.77 mm (mean = 0.74 ± 0.03) (n = 10); integument weakly sclerotized, somewhat pigmented yellowish with dark spots on posterior region of dorsal apotome; dorsomentum strongly sclerotized, blackish, median tooth broad, pointed, stronger than lateral teeth. Seta 2–C single, weakly aciculated on 0.5 distal**,** 0.95–1.33 (mean = 1.10 ± 0.08) (n = 17) length of 3–C; 0.04 mm (n = 10) distance between bases of 2–C; 3–C aciculate (brushlike) on 0.5 distal, 0.75–1.06 (mean = 0.90 ± 0.07) (n = 17) length of 2–C; clypeal index (distance between bases of 2–C and 3–C one side / distance between bases of 2–C) 1.10–1.63 (mean = 1.39 ± 0.11) (n = 18). Seta 4–C with 1–2 branches, short; seta 7–C shorter than 6–C, and 6–C shorter than 5–C (Fig. 2A). Collar dark brown, strongly pigmented. *Antenna*:

0.22–0.27 mm (mean = 0.23 ± 0.02) (n = 10), with spicules on mesal margin and ventral surface with longer spicules; 1–A with 2–4 short branches, inserted 0.04–0.05 mm (mean = 0.04 ± 0.00) (n = 10) distance from base. *Thorax*: granules under integument, seta 1,2–P not sharing a common tubercle, 1–P with 6–11 narrow, pointed leaflets, 2–P with 7–15 branches; 14–P with 2–4 short branches; 1–M strongly plumose, 17–27 branches; 3–T with 5–6 somewhat transparent leaflets; 10,11–T single aciculated. *Abdomen*: integument with minute spicules on ventral surface of segments II–VIII; segment I with two setae 12 on 20% of specimens observed; seta 0–II–VII minute, only few have been counted in segments II, III , IV; 1–I mostly single, 1–II–VII palmate, narrow, both pointed and lanceolate leaflets (Fig. 2C); 2–II with 3–5 large branches, 2–III with 2–5 large branches, 2–IV single or double; 5–I with 2–5 branches, inserted on lateral margin of abdomen, 13–IV with 3–5 large branches, 13–V with 2–5 branches larger than 13–IV. Seta 6–IV–VII with 2 branches (Fig. 2D). Spiracular apparatus (Fig. 2B), pecten plate with 8–10 long spines, 5–7 short spines. Segment X: covered with fine spicules dorsally, spicules longer ventrally; seta 1– X as long as saddle, inserted on ventral margin of saddle.

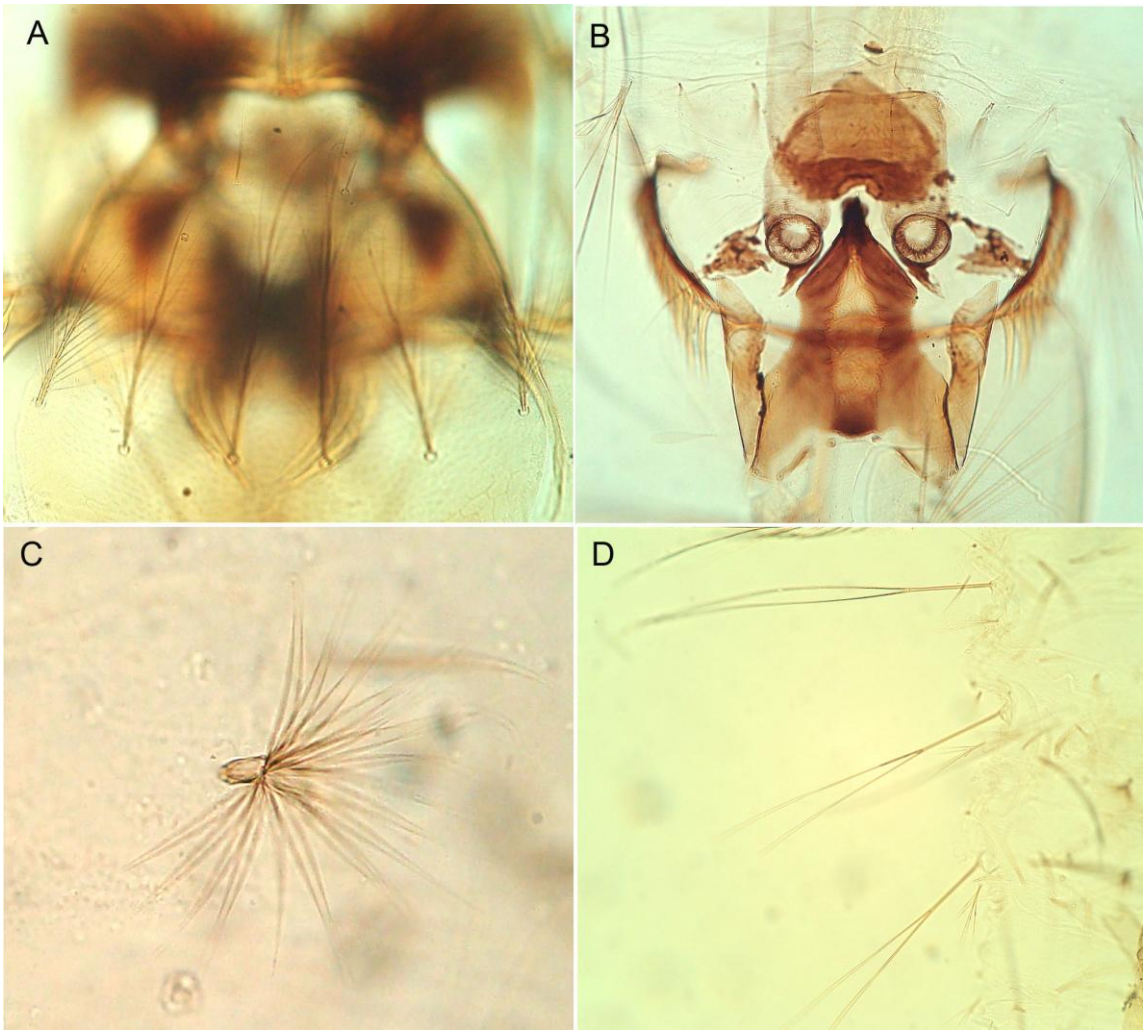

Fig. 2. *Anopheles lutzii*. (A) Showing setae 5,6,7C; (B) spiracular apparatus; (C) seta palmate from abdominal segments; (D) seta 6 from IV–VI abdominal segments.

Table 3. number and range (mode of setal branches of the fourth–instar larva of *Anopheles lutzii* (n = 10) (n.c. = not counted).

Seta	Head	Thorax		
No	C	P	M	T
0	0	1	–	–
1	1	6 – 11 (6)	17 – 27 (23)	1 – 2 (1)
2	1	7 – 15 (10)	1	1 – 2 (1)
3	1	1 – 2 (1)	1	5 – 6 (6)
4	1 – 2 (2)	15 – 20 (20)	2 – 4 (3)	2 – 3 (3)
5	13 – 21 (16)	20 – 28 (22)	1 – 2 (1)	22 – 45 (34)
6	13 – 17 (15)	1	2 – 5 (3)	2 – 4 (3)
7	12 – 18 (16)	19 – 28 (22)	2 – 4 (3)	27 – 42 (32)
8	1 – 3 (2)	24 – 32 (30)	11 – 19 (12)	33 – 40 (35)
9	2 – 4 (3)	1	1	1
10	1 – 2 (1)	1	1	1
11	0	2 – 5 (2)	1	1
12	2 – 5 (4)	4 – 7 (5)	1 – 2 (1)	1 – 3 (2)
13	2 – 5 (3)	3 – 7 (6)	3 – 5 (4)	2 – 5 (3)
14	0	2 – 4 (3)	2 – 5 (4)	–
15	1 – 3 (2)	–	–	–

continued.

Seta	Abdominal segments								
No	I	II	III	IV	V	VI	VII	VIII	X
0	–	1	1	1	0	0	0	0	–
1	1 – 3 (1)	9 – 14 (10)	11 – 15 (13)	13 – 17 (13)	11 – 17 (14)	13 – 16 (13)	nc	1	1
2	1 – 3 (2)	3 – 5 (4)	2 – 5 (3)	1 – 2 (1)	1 – 3 (3)	3 – 4 (3)	2 – 6 (4)	3 – 7 (5)	nc
3	1 – 2 (1)	1	1	2 – 3 (2)	1	1	2 – 4 (2)	4 – 8 (7)	3 – 5 (3)
4	4 – 7 (5)	3 – 5 (4)	2 – 3 (2)	1 – 3 (2)	1 – 3 (3)	1	1	1	7 – 9 (8)
5	2 – 5 (4)	3 – 5 (3)	2 – 3 (3)	1 – 3 (2)	3 – 5 (3)	2 – 6 (5)	3 – 7 (5)	4 – 7 (4)	–
6	32 – 41 (36)	25 – 42 (36)	26 – 33 (31)	2	1 – 2 (2)	1 – 2 (2)	2 – 5 (4)	1–S	3 – 8 (6)
7	33 – 36 (33)	31 – 36 (36)	2 – 4 (4)	2 – 4 (3)	2 – 4 (3)	2 – 3 (3)	2 – 5 (4)	2–S	2 – 3 (3)
8	–	2 – 3 (2)	1 – 3 (2)	2 – 3 (2)	2 – 3 (2)	1 – 3 (2)	2 – 3 (3)	6–S	1 – 2 (1)
9	5 – 6 (6)	4 – 7 (5)	2 – 5 (4)	3 – 4 (3)	2 – 5 (4)	3 – 5 (4)	2 – 5 (3)	7–S	1 – 2 (2)
10	1 – 3 (2)	1 – 3 (2)	1 – 2 (1)	1 – 2 (1)	1 – 3 (1)	1 – 4 (4)	2 – 4 (4)	8–S	1 – 3 (2)
11	3 – 6 (4)	1 – 2 (1)	1 – 3 (2)	1 – 3 (2)	2 – 3 (2)	2	1 – 2 (2)	9–S	1 – 2 (2)
12	2 – 4 (4)	2 – 4 (3)	1 – 2 (2)	2	1 – 2 (2)	1	1 – 2 (1)	–	–
13	2 – 4 (3)	2 – 4 (3)	2 – 4 (3)	2 – 5 (3)	2 – 5 (5)	2 – 4 (3)	2 – 4 (3)	–	–
14	–	–	–	1	0	0	0	0	–
15	–	–	–	–	–	–	–	–	–

Pupa. Range, number and mode of branches in Table 4. All measurements from 10 specimens, unless otherwise indicated. *Cephalothorax*: integument weakly pigmented, lightly yellowish with dark areas in legs cases and dorsal part, without a pattern of dark areas; trumpet length 0.30–0.35 mm (mean = 0.33 ± 0.02) (n = 10), pinna moderately to heavily pigmented, light to dark brown, 0.20–0.38 (mean = 0.29 ± 0.06) (n = 10) length of meatus, trumpet appearing truncate and flared apically in lateral aspect; seta 1–3–CT short, 10–CT usually single (1–3) aciculated, longer than 11–CT, 12–CT mostly single. *Abdomen*: integument weakly pigmented, pale yellow; abdomen length 2.67–3.10 mm (mean = 2.91 ± 0.12) (n = 10); 0–II–VIII minute; 1–II, III with median branches; 1–IV–VII mostly single and long; 2–I with 1–4 branches; 3–V normally double (1–3 branches); 5–IV–VII normally single and as long as seta 1 from the same segments; 6–I mostly single, rarely double or triple; 6–II single; 7–I with 1–3 branches, 7–III–V short, with 4 or fewer branches, generally with 2 or 3 branches, 7–VI, VII single and long; 14–IV–VIII minute. 9–II–IV small and unpigmented (mean = 0.02 ± 0.01) (n = 10), 9–IV 0.87–1.20 (mean = 1.0 ± 0.12) (n = 10) length of 9–III, 9–V strong, 7.83–13.04 (mean = 10.24 ± 1.45) (n = 10) length of 9–IV, 9– VI strong 1.03–1.24 (mean = 1.14 ± 0.08) (n = 10) length of 9–V, 9–VII strong, weakly curved, 0.92–1.13 (mean = 1.04 ± 0.08) (n = 10) length of 9–VI, 9–VIII 0.97–1.09 (mean = 1.05 ± 0.04) (n = 10) length of 9–VII. Paddle longer than wide, length 0.82– 0.92 mm (mean = 0.86 ± 0.04) (n = 10), width 0.51–0.60 mm (mean = 0.56 ± 0.03) (n = 10), presence of serration externally at midlateral margin; midrib distinct basally, indistinct distally; seta 1–P single, 2–P mostly single.

Table 4. Number and range (mode) of setal branches of the pupa of *Anopheles lutzii* (n = 10) (n.c. = not counted).

Seta	Cephalothorax		Abdominal segments								Paddle
No.	CT	I	II	III	IV	V	VI	VII	VIII	IX	P
0	–	–	1	1	1	1	1	1	1	–	–
1	1 – 2 (1)	n.c.	1 – 7 (3)	1 – 3 (2)	1 – 2 (1)	1 – 2 (1)	1 – 2 (1)	1 – 2 (1)	–	0	1
2	1 – 2 (1)	1 – 4 (2)	2 – 3 (2)	1 – 3 (2)	1 – 2 (2)	1 – 2 (1)	1 – 2 (1)	1 – 2 (1)	–	–	1 – 2 (1)
3	1 – 2 (1)	1 – 2 (1)	1	1 – 3 (1)	1 – 4 (3)	1 – 3 (2)	1 – 2 (1)	1 – 3 (1)	–	–	–
4	1 – 3 (1)	1 – 4 (4)	1 – 3 (1)	1 – 4 (3)	2 – 3 (2)	1 – 4 (3)	1 – 2 (1)	1 – 2 (2)	1 – 2 (2)	–	–
5	1 – 4 (3)	1 – 2 (1)	1 – 2 (1)	1 – 3 (2)	1 – 2 (1)	1 – 2 (1)	1 – 2 (1)	1 – 2 (1)	–	–	–
6	1 – 2 (1)	1 – 3 (1)	1	1 – 2 (1)	1	1	1	1	–	–	–
7	2	1 – 3 (2)	1 – 3 (1)	1 – 4 (3)	1 – 4 (2)	1 – 3 (2)	1	1	–	–	–
8	1	–	–	1 – 4 (3)	1 – 3 (3)	1 – 3 (2)	1 – 4 (2)	1 – 3 (2)	–	–	–
9	1 – 3 (2)	1	1	1	1	1	1	1	1	–	–
10	1 – 3 (1)	–	–	1 – 3 (2)	1 – 2 (1)	1 – 2 (1)	0	1 – 3 (1)	–	–	–
11	1 – 3 (2)	–	–	1 – 2 (1)	1 – 2 (1)	1	1 – 2 (1)	1 – 2 (2)	–	–	–
12	1 – 2 (1)	–	–	–	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–
14	–	–	–	–	1	1	1	1	1	–	–

Distribution

Considering that *An. guarani* and *An. niger* has been misidentified as *An. lutzii*, and that the species were described from specimens collected in localities inland Mata Atlantica, in this study, we opt to consider that the geographical range of *An. lutzii* is along the coastal Mata Atlantica. Unfortunately, based only on the register of the published literature, it was not possible to conclude which species the register refers to. However, assuming that the hypothesis of occurrence of *An. lutzii* along the coastal Mata Atlantica is correct, we can affirm that the species has been registered in several localities in Brazil. It was reported in Rio Grande do Sul state (Peryassú, 1908), Taquara (Pinto, 1932); Santa Catarina state, Ipuaçu and São Domingos (Marcondes et al., 2006), Joinvile (Deane, 1971); São Paulo state, Vale do

Ribeira, Pariquera–Açu (Forattini et al, 1998); Minas Gerais state, Juíz de Fora, Piau (Neiva, 1909); Espírito Santo state (Cerutti et al., 2007), Santa Leopoldina (Deane, 1971).

Bionomics

In this study, *An. lutzii* was collected in secondary forest in east Mata Atlantica bioma in the Vale do Ribeira. Adults were collected in Shannon trap using both light and human attraction, from 18 pm to 21 pm. The Shannon trap was located at the border of forest, near a permanent rocky stream situated inside the forest. The water was fresh, turbulent, fast running, much oxygenated. Along the margins of the stream, there were several rocky pools and ground pools connected to the stream. These ground pools were rich in debris, with muddy bottom and deeply shaded. Forattini et al. (1998) also collected one female in a Shannon trap in Pariquera-Açu.

According to Pinto (1932), *An. lutzii* was collected at –8ºC in the winter, in forest at 600 m from domicile, never invading human habitation in Gramado. In São Francisco de Paula, Rio Grande do Sul State at 900 meters of altitude, Pinto et al. (1940) observed that *An. lutzii* was bitten on man at 16°C in forest and enter human houses situated inside forest . Neiva (1909) observed that larvae of *An. lutzii* and *An. parvus* were frequent in small swamps, and the adults were captured on horses.

According to Howard et al. (1917) and Cruz (1901), *An. lutzii* has behavior similar to that of other *Anopheles* species. Adults peaked at dusk, rest in places without wind, were found in great number in the cattle stables, during the day rest in moist and dark places in human habitations, dark places under the curtains, tables and beds, attacking men only at night. When in laboratory, it takes too long to bite

and the bite is very painful. There are some contradictions among the authors, but little is know about the biology of this anopheline. In addition, identification of species from Myzorhynchella section by adult female can be largely misidentified.

Medical importance

Anopheles lutzii does not seem to be involved in either human or monkey malaria transmission. Neiva (1909) found species of Myzorhynchella predominating in the locality of Piau near Juiz de Fora, Minas Gerais, where *An. lutzii* and *An. parvus* were abundant. Because of the high frequency of these two species in Piau, the author hypothesized that these species could be involved in the dynamics of malaria transmission.

According to Peryassú (1940), a map of Amazon area put *An. lutzii* as possibly having epidemiological importance as a vector of malaria parasite. In contrast, results of other studies carried out in distinct localities in Brazil found *An. lutzii* and other species of the Myzorhynchella section to be zoophilic, sylvatic mosquitoes (Galvão, 1941; Forattini, 1962). Therefore it would be of great interest to verify if the specimens studied by Peryassú (1940) belong to *An. lutzii* or any other species of the Myzorhynchella section.

Material examined

*Type specimens***.** Brazil, State of Rio de Janeiro, Lagoa Rodrigo de Freitas. Syntypes: 3 females no. 1965 in glass vial 993, and a microscope slide with a wing no. 2111.

*Other material***.** Brazil, State of São Paulo, Pariquera–Açu municipality, Sítio Galiléia (24º44.975'S 47º56.944'W), Sallum *et al*., coll., 17–IV–2006, det. Sallum, 2006, progeny broods from females collected in Shannon trap: SP02(9)–3 [MLePe], SP02(9)–4 [G], SP02(9)–10 [F], SP02(9)–18 [FLePe], SP02(10)–3 [MLePe], SP02(10)–9 [G], SP02(11)–1 [G], SP02(11)–3 [MLePe], SP02(11)–8 [FLePe], SP02(12)–2 [MLePe], SP02(12)–3 [G], SP02(12)–5 [FLePe], SP02(12)–15 [F], SP02(13)–9 [FLePe], SP02(13)–10 [MLePe], SP02(14)–3 [FLePe], SP02(14)–5 [G]. Brazil, State of Rio de Janeiro, Nova Friburgo municipality, Wygodzinsky coll., I–1946, det Wygodzinsky: 5.922 [F], 5.923 [F]. All the specimens used in this study are deposited in the Entomological Collection of Faculdade de Saúde Pública, Universidade de São Paulo, Brazil.

Anopheles (Nyssorhynchus) guarani (Figs. 3 and 4)

Anopheles (Nys.) guarani Shannon, 1928: 444. Type: Holotype female, Iguaçu (Paraná), 5 Oct 1927, R.C. and E.M. Shannon (USNM). Belkin et al., 1971: 6 (type information); Forattini et al. 1998: 256 (egg description, in part specimens from Dourado). RESSURECTED FROM SYNONYMY WITH *AN. LUTZII*.

Description

*Female***.** Integument black. *Head*: interocular space with frontal tuft of long, white setae, and decumbent, pale, piliform scales; vertex bare along dorsal suture, with decumbent, pale spatulate scales and few long, browish setae anteriorly, remainder of vertex and occiput with decumbent, dark spatulate scales, postgena with decumbent

dark scales; clypeus bare. Pedicel of antenna bare; flagellomere 1 with semierect white scales at base of dorsolateral surface. Proboscis dark scaled, length 1.86–2.03 mm (mean = 1.98 ± 0.07) (n = 5), length of forefemur 1.45–1.68 mm (mean = 1.61 ± 0.09) (n = 5), and length of maxillary palpus 1.78–1.99 mm (mean = 1.93 ± 0.09) (n = 5). Maxillary palpomere 1 dark–scaled; palpomere 2 dark–scaled with few pale scales at apex of dorsal surface; palpomere 3 mostly dark–scaled with white scales at apex of dorsal surface; palpomere 4 dark–scaled with white scales at apex; palpomere 5 mostly white–scaled with dark scales at base. Scales erect or semierect on palpomeres 1 and 2, decumbent on 3–5. *Thorax*: integument black; pale, narrow, decumbent scales on acrostichal and dorsocentral areas and anteriorly on prescutellar area; supraalar, antealar and scutal fossa with pale, spatulate, decumbent scales; scutum bare anteriorly between acrostichal and dorsocentral areas with integument weakly darker; anterior promontory with erect piliform, white scales. Scutellum darker on central area, with few, spatulate, pale scales and long and short dark setae on posterior margin. Mesopostnotum bare. Antepronotum with dark setae and a patch of spatulate black scales. Pleura with small patches of pale, spatulate scales on upper mesokatepisternum, upper mesepimeron, posterior border of middle mesokatepisternum, prespiracular area and prealar knob; dark setae on proepisternum, upper mesokatepisternum, brown setae on prealar knob, pale setae on upper mesepimeron and minute pale setae on prespiracular area. *Wing*: length 3.05–3.24 mm (mean = 3.13 ± 0.06) (n = 10); wing spots measurements in Table 5 ; veins dark–scaled with spots of pale scales as follows: costa without basal pale, pre–humeral dark, humeral pale, humeral dark, sector pale, proximal sector dark, acessory sector pale, distal sector dark, subcostal pale, preapical dark, preapical pale

and apical dark spots; humeral dark, sector pale and proximal sector dark present in 30% of female wings examined; humeral pale and presector dark absent in 100% of wings examined; remigium pale–scaled, vein R proximal pale about 0.5 distance to sector pale spot; R_1 with acessory sector pale, subcostal pale, preapical pale spots; R_2 mostly dark–scaled with a pale spot at distal end and a patch of pale scales at furcation with vein R_3; Rs with a patch of pale scales at junction of R_{4+5}, and a few pale scales at base; R_{2+3} dark–scaled with pale scales at furcation of R_2 and R_3 and junction of R_{4+5}; R_{4+5} mostly dark–scaled with two pale spots at proximal 0.3 and distal end; vein M mostly dark–scaled, with a pale spot at region of Rs and at furcation of M_{1+2} and M_{3+4}; M_{1+2} with one pale spot in the middle of the vein and few pale scales at proximal and distal ends; M_{3+4} dark–scaled with few pale scales at proximal and distal end; CuA with a 0.4 proximal pale–scaled; CuA_1 with three patches of pale scales at 0.5 proximal and few pale scales at distal end; CuA_2 pale–scaled at 0.5 proximal; 1A mostly dark–scaled with pale scale patches at proximal and distal ends and one pale patch at proximal 0.5. Pale fringe spots at apices of veins R_2, R_3, R_{4+5}, M_{1+2}, CuA_1, CuA_2 and 1A. *Halter*: scabellum and pedicel with pale integument; capitelum entirely dark–scaled.

Table 5. Wing spot measurements (in mm) for adult male (M) and female (F) of *Anopheles guarani* collected in Foz do Iguaçu municipality, State of Paraná, Brazil (PR).

Wing spot	Range		Mean		SD(±)		n =	
	(F)	(M)	(F)	(M)	(F)	(M)	(F)	(M)
Basal pale	–	–	–	–	–	–	10	10
Prehumeral dark	0.26–0.32	0.25–0.29	0.29	0.27	0.02	0.01	10	10
Humeral pale	0.04–0.06	0.07–0.13	0.05	0.10	0.00	0.02	10	10
Humeral dark	0.22–0.24	–	0.23	–	0.01	–	3	10
Presector pale	–	–	–	–	–	–	10	10
Presector dark	–	–	–	–	–	–	10	10
Sector pale	0.05–0.07	–	0.06	–	0.01	–	3	10
Proximal sector dark	0.08–0.14	–	0.12	–	0.03	–	3	10
Accessory sector pale	0.11–018	0.12–0.19	0.14	0.15	0.02	0.02	10	10
Distal sector dark	0.81–0.90	0.61–0.72	0.85	0.66	0.03	0.04	9	10
Subcostal pale	0.10–0.16	0.13–0.21	0.13	0.16	0.02	0.02	9	10
Preapical dark	0.54–0.68	0.51–0.62	0.63	0.55	0.05	0.03	9	10
Preapical pale	0.09–0.18	0.16–0.19	0.13	0.17	0.03	0.01	10	10
Apical dark	0.14–0.25	0.09–0.16	0.19	0.12	0.03	0.02	10	10

Legs: anterior surface of forecoxa with long, dark setae, posterolateral surface with one patch of pale spatulate scales at apex; midcoxa with a patch of pale spatulate scales and few long setae at base and apex of anterior/lateral surface, and at apex of posterior surface; posterior surface of hindcoxa with two long, dark setae on upper part and few pale scales at apex. Fore–, mid– and hindtrochanters pale scaled. Fortarsomere 1–3 with apical, pale white scales, tarsomere 1 with apical pale ring, tarsomere 2 with apical 0.4 pale and tarsomere 3 with apical 0.5 pale; fortarsomeres 4,5 totally dark–scaled; midtarsomeres 1–3 with apical, pale white scales dorsally, midtarsomere 1 with a small spot, 2 with apical 0.4 pale, 3 with few pale scales not visible ventrally; midtarsomeres 4,5 totally dark–scaled. Hindtarsomere 1 with an

apical ring of white scales; hindtarsomere 2 dark–scaled at approximatelly 0.5 and white–scaled apically; hindtarsomeres 3–5 white–scaled. *Abdomen*: integument black; terga I–VII without scales, covered with dark setae; sterna with basolateral pale spots; sternum VII with pale spatulate scales; cerci with dark scales and few pale scales at apex.

Male: Similar to female except for sexual differences. Maxillary palpus dark–scaled; scales semierect on palpomere 1 and on basal 0.5 of palpomere 2, decumbent on palpomeres 3, 4 and 5; palpomere 2 with pale scales at apex, palpomere 4 with long, setae along border of ventral surface and pale scales at apex and base; palpomere 5 dark–scaled with white scale along dorsal surface. Wing with less quantity of scales in comparison to female; length smaller than females; wing spots measurements in Table 5, humeral dark, presector pale, presector dark, sector pale and proximal sector dark forming a single spot with length of 0.70–0.75 mm (mean = 0.73 ± 0.01) (n = 10); vein R_{4+5} variable, pale spots bigger than in females, sometimes mostly pale–scaled than dark–scaled.

Male genitalia. Segment VIII: Tergum and sternum narrow, without scales and with long and short setae. *Segment IX*: Sternum rectangular, anteromedial and posteromedial border shallow emarginated without apodeme. *Proctiger:* membranous mesally, strongly sclerotized laterally, spiculose dorsally; apex narrow. *Gonocoxite*: length about 0.30–0.31 (mean = 0.30 ± 0.00) (n = 3); tergal surface laterally covered with large, obovate scales, with 4 long and 3 short tergomedial setae, 2 apicolateral setae and one apicomesal setae immediately based of gonostylus; parabasal seta retrorsely hooked; dorsomedial rim long, strongly developed; accessory setae moderately long, broad, tapering to apex, ending in a

narrow sharply pointed apex, dorsal seta curved posteriorly, about 0.43–0.46 (mean = 0.45 ± 0.01) (n = 3) length of gonocoxite, ventral seta straight about 0.86–0.89 (mean = 0.87 ± 0.01) (n = 3) length of dorsal setae; internal setae slender, about 0.63–0.82 (mean = 0.76 ± 0.07) (n = 3) length of ventral accessory seta, curved anteriorly. Sternal surface covered with white scales. *Gonostylus*: curved mesally along entire length, broad at base, narrow at middle part, broad at apical 0.3; dorsal margin with 13–14 minute setae; seta b slender, single, inserted basolateral to gonostylar claw; gonostylar claw short, moderately sclerotized, slender. *Dorsal Claspette*: paired, short, pedicel about 0.28–0.30 (mean = 0.28 ± 0.01 (n = 3) length of gonocoxite; pedicel moderately broad rounded at base, broad and sclerotized at apex. Apex with 2 ventral setae at apex and 1 dorsal seta arising subapically, setae curved mesally, dorsal seta without basomesal projection, the most lateral ventral seta with weakly developed basomesal projection, the mesal seta strongly developed, swollen at middle length, tapering to apex, without developed basomesal projection. *Ventral Claspette* (Fig. 3A): about 0.3 length of gonocoxite, somewhat trapezoidal in shape, without spicules, apex narrow, straight; dorsal surface mostly plain, with two small subapical lobes arising mesally; ventral surface with distinct, swollen lobes, projecting ventrally, basally developed as a pair of large basal lobules connected by a membrane, mesal border of each lobe close together bordering mesal cleft; mesal cleft long, extending ventrally from apex to base of ventral claspette, more sclerotized at posterior 0.5; median sulcus in shape of V. *Phallosome*: extending posteriorly beyond ventral claspette, composed of a central aedeagus, a pair of parameres, a pair of basal pieces; aedeagus long, narrow, strongly sclerotized laterally; aedeagal subapical leaflets present, well developed, curved mesally,

parallel to the longitudinal axis, strongly sclerotized, strongly serrated along dorsal and lateral surfaces; apex of aedeagus somewhat triangular in shape, moderately sclerotized, outer border of apex at level of insertion of leaflets hyaline; ventromesal subtriangular projection subapical in position, mesally fused forming a collar positioned approximately at middle length of insertion and apex of leaflets (Fig. 3B).

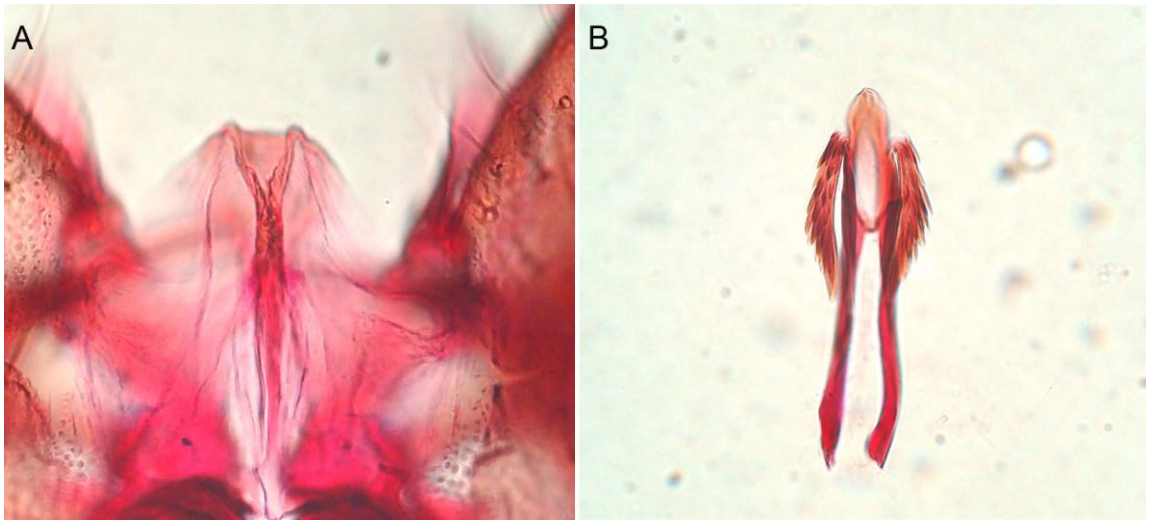

Fig. 3. Microphotos of male genitalia of *Anopheles (Nys.) guarani*, showing details of ventral claspette (A) in ventral view; and aedeagus (B).

Fourth–instar larva. Range and modes of branches in Table 6. Measurements from 10 specimens unless otherwise indicated. *Head*: length 0.64– 0.74 mm (mean = 0.70 ± 0.03) (n = 10); width 0.63–0.72 mm (mean = 0.68 ± 0.03) (n = 10); integument weakly sclerotized, somewhat pigmented yellowish with dark spots on posterior region of dorsal apotema; dorsomentum strongly sclerotized, blackish, median tooth broad, pointed, stronger than lateral teeth. Seta 2–C single**,** 1.08–1.54 (mean = 1.36 ± 0.15) (n = 10) length of 3–C; 0.04 mm (n = 10) distance between bases of 2–C; 3–C aciculate (brushlike), 0.75–1.06 (mean = 0.90 ± 0.07) (n = 10) length of 2–C; clypeal

index (distance between bases of 2–C and 3–C one side / distance between bases of 2–C) 1.10–1.63 (mean = 1.39 ± 0.11) (n = 10). Seta 4–C with 2–4 branches, short; seta 7–C, 5–C and 6–C with the apex in the same height (Fig. 4A). Collar dark brown, strongly pigmented. *Antenna*: 0.22–0.24 mm (mean = 0.23 ± 0.01) (n = 10), with spicules on mesal margin and ventral surface with longer spicules; 1–A with 4–6 long branches, inserted 0.04–0.05 mm (mean = 0.05 ± 0.00) (n = 10) distance from base. *Thorax*: granules under integument, seta 1,2–P not sharing a common tubercle, 1–P with 3–8 narrow, pointed leaflets, 2–P with 8–15 branches; 14–P with 4–7 long branches; 1–M strongly plumose, 20–33 branches; 3–T with 4–11 somewhat transparent leaflets; 11–T single. *Abdomen*: integument with minute spicules on ventral surface of segments II–VIII; seta 0–II–VII minute; 1–I with 3–6 branches, 1–III–V palmate, narrow, with hyaline leaflets (Fig. 4C); 2–II with 7–11 large branches, 2–III with 6–11 braches, 2–IV with 6–11 branches; 5–I with 3–5 branches, inserted on lateral margin of abdomen, 13–IV with 3–5 large branches, 13–V with 3–4 branches larger than 13–IV. Seta 6–IV–V with 3–6 branches, 6–VI with 5–12 branches (Fig 4D). Spiracular apparatus (Fig. 4B.), pecten plate with 8–10 long spines, 5–7 short spines. Segment X: covered with fine spicules dorsally, spicules longer ventrally; seta 1– X as long as saddle, inserted on ventral margin of saddle.

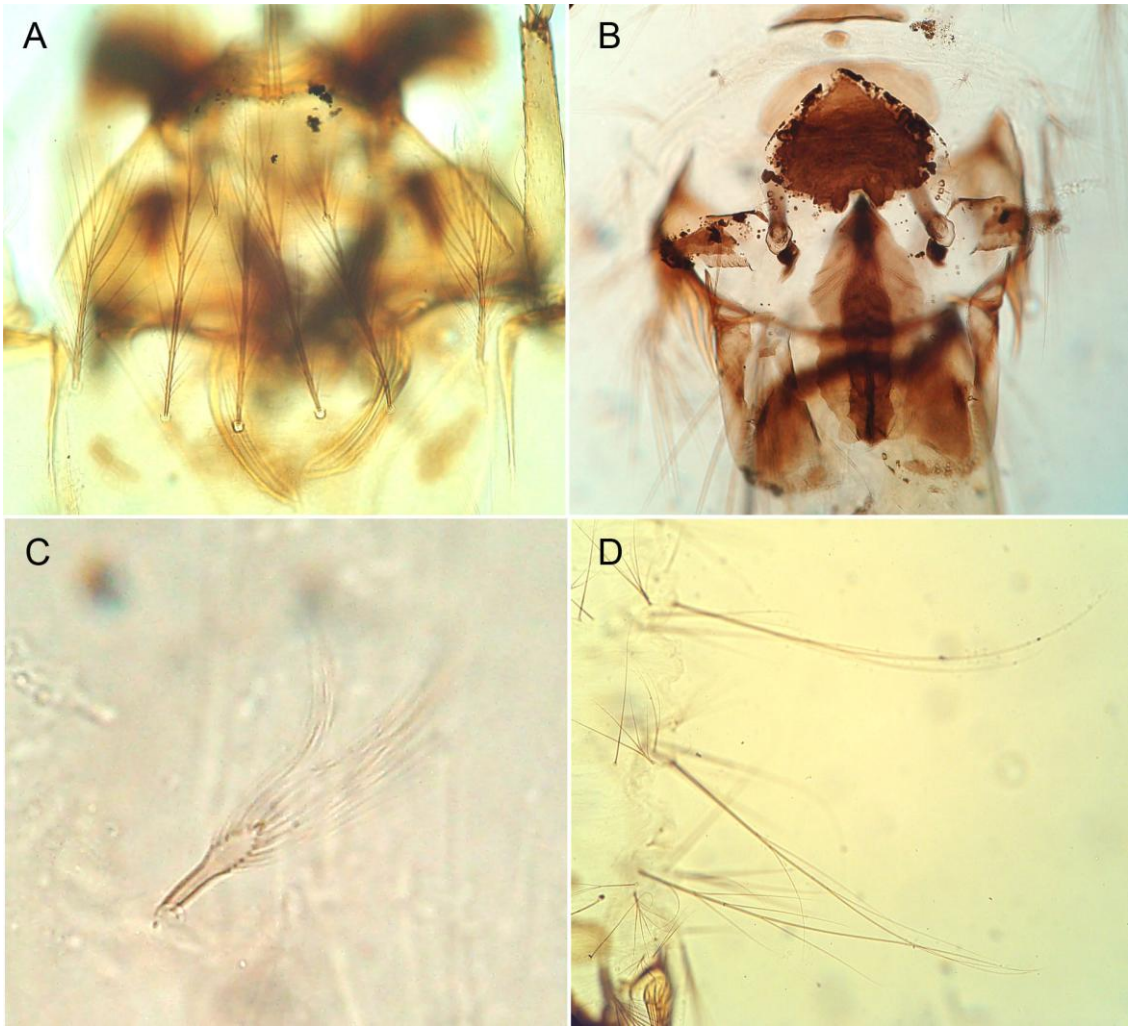

Fig. 4. Microphotos of larva of *Anopheles (Nys.) guarani*, showing details of setae 5,6,7–C (A); spiracular apparatus (B); abdominal seta 1–III–V (C); seta 6 of segments IV–VI.

Table 6. Number and range (mode of setal branches of the fourth–instar larva of *Anopheles (Nys.) guarani* (n = 10). (n.c. = not counted).

Seta	Head	Thorax		
No	C	P	M	T
0	0	1	–	–
1	1	3 – 8 (4)	20 – 33 (27)	1
2	1 – 2 (1)	8 – 15 (10)	1 – 3 (2)	1
3	1	1 – 2 (1)	1 – 2 (1)	4 – 11 (4)
4	2 – 4 (3)	19 – 33 (31)	2 – 4 (3)	3 – 5 (4)
5	11 – 17 (16)	23 – 35 (30)	1	30 – 39 (37)
6	13 – 18 (16)	1	2 – 4 (3)	1 – 3 (2)
7	13 – 20 (15)	31 – 36 (31)	3 – 4 (3)	21 – 31 (30)
8	3 – 7 (5)	25 – 34 (30)	14 – 21 (21)	20 – 34 (27)
9	3 – 9 (7)	1	1 – 4 (1)	1 – 3 (2)
10	2 – 5 (4)	1 – 2 (1)	1 – 2 (1)	1 – 2 (2)
11	0	2 – 3 (3)	1	1
12	4 – 7 (6)	4 – 8 (5)	1 – 2 (2)	1 – 4 (2)
13	2 – 5 (3)	3 – 7 (5)	2 – 6 (3)	3 – 5 (4)
14	1	4 – 7 (6)	5 – 8 (6)	–
15	1 – 3 (1)	–	–	–

Continued

Seta	Abdominal segments								
No	I	II	III	IV	V	VI	VII	VIII	X
0	–	1	1	1	1	1	1	1	–
1	3 – 6 (3)	6 – 9 (8)	0	0	0	5 – 9 (8)	6 – 9 (6)	1 – 2 (2)	1
2	2 – 4 (3)	7 – 11 (9)	6 – 11 (7)	1	5 – 7 (7)	5 – 8 (6)	4 – 5 (5)	6 – 10 (6)	0
3	2 – 3 (3)	1 – 3 (1)	1 – 2 (1)	2 – 4 (3)	1	1 – 2 (2)	2 – 3 (2)	9	3
4	3 – 7 (5)	4 – 7 (4)	2 – 4 (4)	2 – 4 (3)	2 – 5 (3)	1 – 2 (1)	1 – 2 (2)	1 – 2 (1)	8
5	3 – 5 (4)	4 – 8 (6)	5 – 10 (7)	4 – 7 (5)	4 – 8 (7)	5 – 10 (10)	10 – 11 (11)	3	–
6	21 – 30 (28)	23 – 32 (29)	23 – 34 (25)	3 – 6 (3)	3 – 6 (5)	5 – 12 (9)	5 – 6 (5)	1–S	5 – 7 (6)
7	21 – 28 (26)	23 – 33 (30)	3 – 5 (4)	3 – 5 (4)	2 – 5 (4)	2 – 4 (3)	4	2–S	3 – 5 (3)
8	–	2 – 3 (2)	1 – 3 (2)	1 – 3 (2)	2 – 4 (3)	2 – 4 (3)	3 – 5 (4)	6–S	1 – 2 (1)
9	4 – 7 (5)	4 – 8 (6)	4 – 7 (5)	3 – 6 (5)	4 – 7 (5)	5 – 9 (6)	4 – 5 (4)	7–S	1 – 2 (1)
10	1 – 3 (2)	1 – 3 (2)	1 – 2 (2)	1 – 2 (2)	1 – 3 (2)	1 – 3 (3)	4	8–S	1 – 4 (3)
11	4 – 6 (5)	2 – 3 (2)	1 – 3 (2)	1 – 2 (2)	2 – 3 (2)	1 – 3 (2)	1 – 4 (3)	9–S	1 – 3 (2)
12	2 – 6 (3)	3 – 4 (3)	1 – 3 (2)	2 – 3 (2)	1 – 3 (2)	1 – 3 (1)	1 – 2 (2)	–	–
13	3 – 4 (4)	4 – 7 (5)	3 – 5 (4)	3 – 5 (4)	3 – 4 (3)	3 – 6 (4)	2 – 3 (3)	–	–
14	–	–	–	1	1	1	1	1	–

Pupa. Range, number and mode of branches in Table 7. All measurements from 10 specimens, unless otherwise indicated. *Cephalothorax*: integument weakly pigmented, lightly yellowish with dark areas in legs cases and dorsal part, without a pattern of dark areas; trumpet length 0.38–0.45 mm (mean = 0.42 ± 0.02) (n = 10), pinna moderately to heavily pigmented, difficult to measure, trumpet appearing truncate and flared apically in lateral aspect; seta 1–3–CT short, 10–CT usually single (1–3), longer than 11–CT, 12–CT mostly single. *Abdomen*: integument weakly pigmented, pale yellow; abdomen length 2.61–3.07 mm (mean = 2.76 ± 0.15) (n = 10); 0–II–VIII minute; 1–II,III with median branches; 1–IV with 3–7 branches, 1–VI,VII mostly single and long; 2–I with 2–9 branches; 3–V normally double (1–3 branches); 5–VI–VII normally single and as long as seta 1 from the same segments; 6–I –VII mostly single; 7–I with 1–4 branches, 7–III–V short, mostly with 2 branches, 7–VI, VII single and long; 14–IV–VIII minute. 9–II–IV small and unpigmented 0.01–0.03 (mean = 0.02 ± 0.01) (n = 10), 9–IV 0.93–1.59 (mean = 1.21 ± 0.22) (n = 10) length of 9–III, 9–V strong, 3.11–5.46 (mean = 4.50 ± 0.91) (n = 10) length of 9–IV, 9– VI strong 0.84–1.60 (mean = 1.34 ± 0.22) (n = 10) length of 9–V, 9–VII strong, weakly curved, 1.09–1.22 (mean = 1.16 ± 0.05) (n = 10) length of 9–VI, 9–VIII 0.80–1.00 (mean = 0.89 ± 0.07) (n = 10) length of 9–VII. Paddle longer than wide, length 0.74– 0.79 mm (mean = 0.76 ± 0.02) (n = 10), width 0.50–0.60 mm (mean = 0.54 ± 0.03) (n = 8), presence of serration externally at midlateral margin; midrib distinct basally, indistinct distally; seta 1–P single, 2–P mostly single.

Eggs (Fig. 5). Broadly boat–shaped on dorsal, lateral, and ventral views. Floats lateral in position, long, extending nearly total length of egg, close to dorsal than ventral surface, well developed (Fig. 5C–D). *Dorsal surface*: Deck in the middle

region of egg wide, tapering toward anterior and posterior parts of egg; frill reduced, present only on posterior end of egg (Fig. 5A–B). Deck covered uniformely with nearly globose tubercles (Fig. 5C). *Ventral surface*: Outer chorionic cell of ventral plastron with indistinct boundary, plastron with several irregularly shaped open areas (Fig. 5D–E); micropylar collar separated from anterior margin of egg by narrow area, plastron around micropyllar collar formed by small, flat nodules interspersed with several pores (Fig. 5F).

Table 7. Number and range (mode) of setal branches of the pupa of *Anopheles (Nys.) guarani* (n = 10) (n.c. – not counted).

Seta	Cephalothorax	Abdominal segments									Paddle
No.	CT	I	II	III	IV	V	VI	VII	VIII	IX	P
0	–				1	1	1	1	1	–	–
1	1 – 3 (2)	n.c.	2 – 5 (3)	3 – 7 (5)	3 – 7 (5)	2 – 5 (2)	1 – 3 (1)	1 – 2 (1)	–	1 – 3 (2)	1
2	1 – 2 (2)	2 – 9 (5)	2 – 5 (4)	2 – 4 (3)	1 – 3 (2)	2 – 3 (2)	2 – 3 (2)	1 – 2 (2)	–	–	1 – 2 (1)
3	1 – 3 (2)	1	1	1 – 2 (1)	1 – 7 (3)	1 – 3 (2)	1 – 3 (2)	1 – 3 (2)	–	–	–
4	1 – 3 (1)	2 – 4 (3)	1 – 3 (2)	1 – 3 (2)	1 – 3 (2)	1 – 4 (2)	1	1 – 2 (1)	1 – 3 (2)	–	–
5	1 – 2 (2)	1	2 – 4 (3)	3 – 7 (5)	1 – 7 (2)	1 – 3 (2)	1 – 2 (1)	1 – 2 (1)	–	–	–
6	1 – 3 (1)	1	1 – 2 (1)	1 – 2 (1)	1	1	1	1 – 4 (1)	–	–	–
7	1 – 2 (1)	1 – 4 (3)	1 – 4 (2)	1 – 3 (2)	1 – 3 (2)	1 – 4 (2)	1	1	–	–	–
8	1	–	–	1 – 3 (2)	1 – 3 (2)	1 – 3 (2)	1 – 3 (2)	1 – 3 (2)	–	–	–
9	1 – 3 (2)	1	1	1	1	1	1	1	1 – 2 (1)	–	–
10	1 – 3 (1)	–	–	1 – 4 (2)	1	1	0	1 – 3 (3)	–	–	–
11	1 – 3 (2)	–	–	1 – 2 (1)	1	1	1	1	–	–	–
12	1 – 3 (2)	–	–	–	–	–	–	–	–	–	–
13	–	–	–	–	–	–	–	–	–	–	–
14	–	–	–	–	1	1	1	1	1	–	–

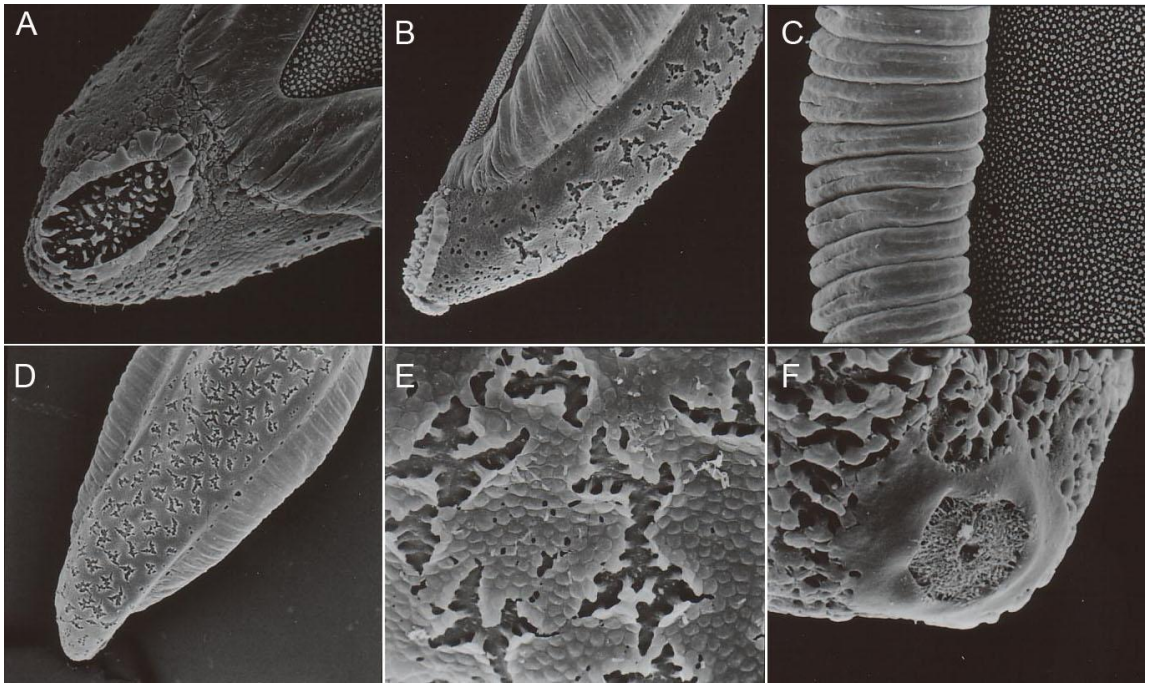

Fig.5. Egg of of *Anopheles (Nys.) guarani*. (A) Posterior end, dorsal view; (B) lateral view of posterior end; (C) deck tubercles and float; (D) posterior end, ventral view; (E) Outer chorion, ventral surface; (F) micropyle.

Distribution

It is plausible to suppose that the geographical distribution of *An. guarani* is larger than the distribution we are reporting. However, because *An. guarani* was in the synonymy of *An. lutzii*, several registers in the literature may be relative to *An. guarani* or *An. lutzii* or perhaps *An. niger*. In this study, we adopted that *An. guarani* may occur in localities in inland Mata Atlantica, in areas of semideciduous forest. However, it is very possible that *An. guarani* and *An. niger* occur in sympatry.

Anopheles guarani (identified as *An. lutzii*) has been registered in Argentina and Brazil. In the former country, the species was found in Province of Misiones, Puerto Iguazú (Duret, 1948), Montecarlo, Eldorado, Arroyo Piray–Guazú, Los Helechos (Bejarano & Duret, 1949). In Brazil, it was reported in Paraná state

(Rachou & Ricciardi, 1951), Iguaçu National Park (Guimarães et al., 2003); São Paulo state, Dourado municipality (Forattini et al. 1998), Sertãosinho (Pinto, 1930), Rifaina (Lane, 1935), Paranapanema basin (Tubaki et al., 1999).

Bionomics

In Foz do Iguaçu, State of Paraná, we collected individuals in April, in Shannon trap from 6.00 to 8.00 p.m., near river Almada. The locality had secondary forest; temperature was approximately 25ºC and altitude 185 meters. In Puerto Iguazú municipality that borders with Foz do Iguaçu, Duret (1948) collected one female in September, at dusk, in a forest located approximately four kilometers from the Hotel Cataratas, in Foz do Iguaçu. Forattini et al (1998) collected females with human bait in Dourado. Pinto (1930) collected adult female biting on horse at the margin of pounds in Sertãosinho, State of São Paulo at 5.30 p.m. However, *An. lutzii* was not found indoors in houses situated in the vicinity of larval habitats, even at night, indicating that they were zoophilic.

Medical importance

Both medical and veterinary importance of *An. guarani* is unknown.

Material Examined

State of São Paulo, Dourado, SP255, Km 124, near Jacaré Pepira river (22º05’22”S 48º26’33”W), Bergo coll., V–1994, det. Bergo 1994: E–10489 [F], E–11803 [F], E–11804 [E], E–11805 [F]; State of Paraná, Foz do Iguaçu municipality (25º28’50”S,

54º35’12”W), Nagaki *et al*., coll., 28–IV–2008, det. Nagaki & Sallum, 2008, progenies from two females collected on Shannon trap: PR29(8)–1 [F], PR29(8)–3 [LePe] PR29(8)–4 [FLePe], PR29(8)–5 [FLePe], PR29(8)–7 [LePe], PR29(8)–8 [LePe], PR29(8)–9 [F], PR29(9)–2 [MLePe], PR29(9)–3 [G], PR29(9)–5 [M], PR29(9)–7 [LePeG], PR29(9)–8 [MLePeG], PR29(9)–10 [FLePe], PR29(9)–11 [M], PR29(9)–17 [LePe], PR29(9)–24 [F], PR29(9)–26 [M]. All the specimens used in this study are deposited in the Entomological Collection of Faculdade de Saúde Pública, Universidade de São Paulo, Brazil.

Anopheles (Nyssorhynchus) niger (Fig. 6)

Anophleles (Nys.) niger (Theobald, 1907: 78). Type: Lectotype female, Cantareira (São Paulo), 9 Nov 1904, A. Lutz (BM). Belkin et al., 1971: 6 (type information); Belkin, 1968: 10 (lectotype designation); Chagas, 1907: 3 (synonymy with *An. lutzii*). RESSURECTED FROM SYNONYMY WITH *AN. LUTZII*.

Male genitalia characterization

Male genitalia: the following description is based on the male genitalia of a single specimen. *Segment IX*: Sternum rectangular, anteromedial and posteromedial border shallow emarginated, without apodeme. *Proctiger*: membranous dorsomesally, with sparse spicules, strongly sclerotized laterally, tapering to apex, basomedial sclerotinization with a patch of spicules. *Gonocoxite*: tergal surface covered with large, obovate scales laterally; parabasal setae retrorsely hooked; dorsomedial rim long, strongly developed; accessory setae moderately long, tapering to apex, ending

in a narrow sharply pointed apex, dorsal seta broad, slightly curved posteriorly, ventral seta less developed than dorsal setae, straight; internal setae slender, curved anteriorly. *Gonostylus*: curved mesally along entire length, broad at base, narrow at middle part, broad at apical 0.3; dorsal margin with 12–15 minute setae; seta b slender, single, inserted basolateral to gonostylar claw; gonostylar claw short, moderately sclerotized, moderately broad. *Dorsal claspette* (Fig. 6B): paired, short; pedicel moderately broad, rounded at base, broad and sclerotized at apex, 2 ventral setae arising from apex and 1 dorsal seta arising subapically approximately 0.3 distant from apex of pedicel. *Ventral claspette* (Fig. 6A): about 0.3 length of gonocoxite, somewhat trapezoidal in shape, apex rounded, with shallow mesal emargination, without spicules, dorsal surface mostly plain; mesal cleft narrow, long, extending ventrally from apex to base, strongly sclerotized at posterior 0.5, median sulcus narrow, tubular, slightly narrowed at middle length; basal lobule poorly developed. *Phallosome*: extending posteriorly beyond ventral claspette, composed of a central aedeagus, a pair of parameres, a pair of basal pieces; aedeagus long, strongly sclerotized laterally; aedeagal subapical leaflets present, well developed, straight, forming an angle of 25º with aedeagus, strongly sclerotized, serrated along dorsal and lateral surfaces; apex of aedeagus longer than broad, more sclerotized laterally, lateral sclerotinization not reaching apex of aedeagus, apex somewhat rounded at distal margin, outer border at level of insertion of leaflets hyaline; ventromesal subtriangular projection positioned approximately at middle length of insertion and apex of leaflets (Fig. 6B).

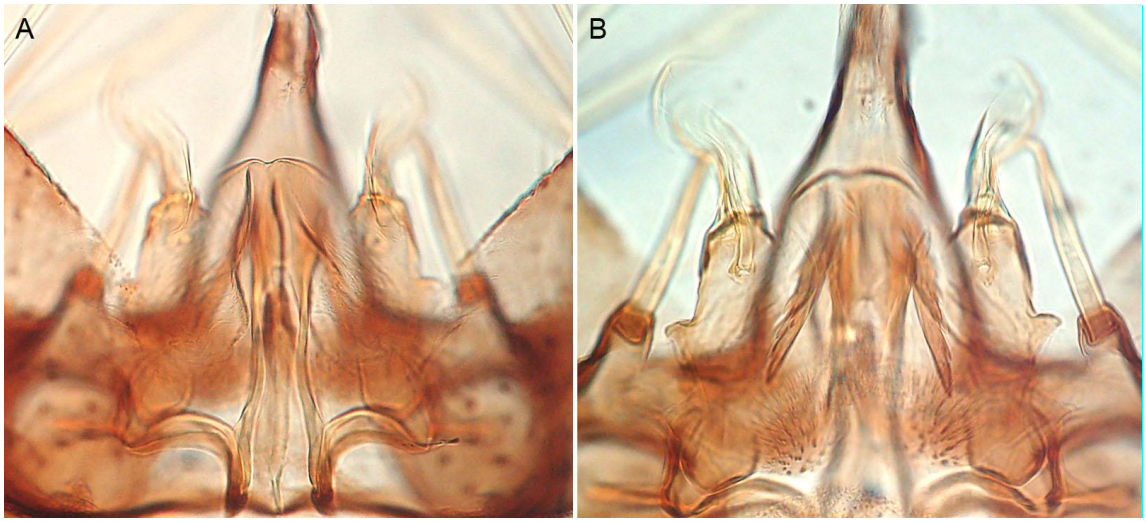

Fig. 6. Microphotos of male genitalia of *Anopheles (Nys.) niger*, showing details of ventral claspette (A); dorsal claspette and aedeagus (B).

Distribution

Geographic distribution of *An. niger* is unknown, however, it was found in Vila do Morro Grande in Cotia municipality (Ramos, 1937), in Horto Florestal da Cantareira (Deane, 1971), in Vila Ema district (Forattini et al. 1973), Perús district, and Represa de Santo Amaro, São Paulo municipalities (Foratinni et al. 1970).

Bionomics

Nothing is known about bionomics of *An. niger*.

Medical Importance

Deane et al. (1971) studying the epidemiology of the transmission of simian malaria in Brazil captured 46 individuals of *An. niger* (as *An. lutzii*) in Horto Florestal da Cantareira, State of São Paulo. These 46 specimens were examined for plasmodium sporozoites, but none was found infected.

Material examined.

All the specimens are from Brazil. State of São Paulo, São Paulo municipality, Vila Ema, Galvão coll., V–1940, det. Galvão, V–1940: E–2045 [FLe], E–2046 [FLePe]; Represa de Santo Amaro, det. Lane, 1937: 1584 [FLe], 1617 [MG]; Perus, det. Lane, 1945: 5046 [F], 5047 [F].

Discussion

Based on morphological observations of specimens from the type locality of *An. niger*, *An. guarani* and the syntypes of *An. lutzii*, we concluded that *An. guarani* and *An. niger* are valid species. Consequently, both taxa are here resurrected from the synonymy. *Anopheles lutzii*, *An. niger* and *An. guarani* can be distinguished by the SEM morphology of the eggs, male genitalia and fourth instar larvae characteristics. Belkin et al. (1971) considered that there was some controversy about the type specimens of *An. lutzii* deposited in IOC collection; consequently, further studies would be necessary to establish the lectotype. While examining the syntypes of *An. lutzii*, it was possible to find some controversy regarding to the type locality. According to Cruz (1901) the material was collected in the "Jardim Botânico – Sarapuhy". However, in both labels with the syntypes and in the collection records, the locality is "Lagoa Rodrigo de Freitas". Moreover, the date on the label on the wing microscope slide is July 1934. Unfortunately, it was not possible to clarify whether that date refers to the collection, mounting, or deposit at Costa Lima collection. Additionally, we observed that it was not mentioned on the specimen label and in the collection records the collector's name (Fig.7). According to Guimarães (1997), Lutz collected and Cruz described the species. The syntypes of

An. lutzii were the first specimens deposited in the entomological collection of IOC (former Manguinhos). In considering that Costa Lima worked in the research institution and that in 1929 he considered *Nyssorhynchus* a genus, and Myzorhynchella a subgenus of *Anopheles*, we therefore believe that he filled the forms of the specimens and elaborated the labels that are fixed in the slide with the wing and in a glass vial containing the syntypes of *An. lutzii*. Based on the importance of the syntypes, we assume that Costa Lima would have mentioned that the adults were types of *An. lutzii*, however in the collection records it is mentioned "da antiga coleção", and also the collector was signed with a question mark (?) (Fig. 7).

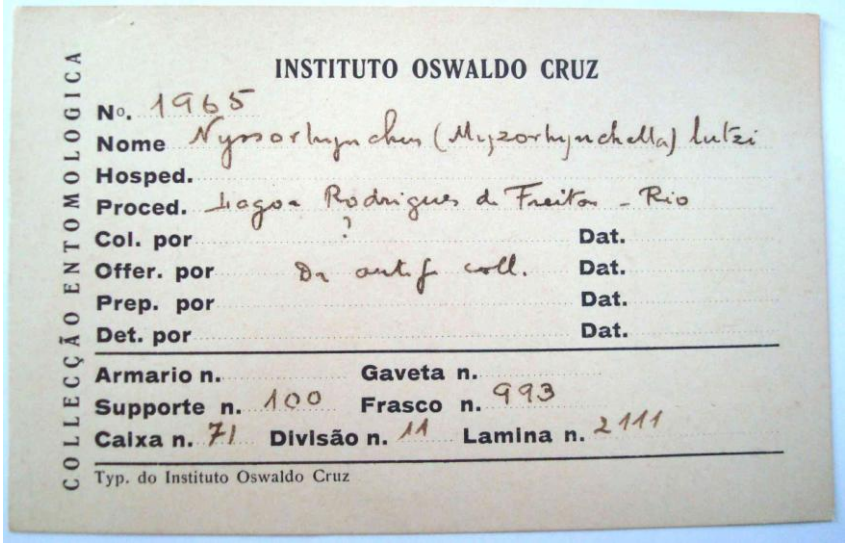

Fig. 7. Label of the *An. lutzii* syntypes specimens deposited in IOC collection.

Furthermore, the hindtarsomere has important features that also show some ambiguity with the syntypes. In the original description, Cruz (1901) described that the hindtarsomere 4 possess a ring of dark scales extending from junction with hindtarsomere 3 to middle portion of the segment, the remaining of segment and

hindtarsomere 5 are entirely white scaled. Contrasting to Cruz's (1901) description, in the syntypes the hindtarsomeres III, IV and V are entirely white. Howard et al. (1917) noted that Cruz (1901) indicated the presence of a dark ring on the hindtarsomere IV, while in subsequent descriptions of *An. lutzii*, the hindtarsomere IV was described as entirely white–scaled. However, the authors feel bound to accept the synonymy of *An. niger*, indicated by the Brazilian observers, as they say it was no doubt based upon ample data. In considering the presence of a dark ring at basal 0.5 of hindtarsomere 4, we assume that either Cruz (1901) made a mistake in the description of hindtarsomere 4 or the specimens deposited in IOC are not from the type series. This needs further investigation in addition to collections in the type locality of *An. lutzii*. Because of these facts mentioned, we agree with Belkin et al. (1971) on that further studies will be necessary to fix the type status of the specimens deposited in IOC.

Based on comparisons among the syntypes, specimens from Vale do Ribeira, southern São Paulo state, and Nova Friburgo, Rio de Janeiro, we concluded that the specimens may belong to *An. lutzii*. Forattini et al. (1998) described the eggs of *An. lutzii* using SEM microphotos of specimens from Vale do Ribeira, and compared them with eggs from Dourado, inland São Paulo. Results of morphological comparisons showed that Dourado and Vale do Ribeira populations could belong to distinct species that were misidentified as *An. lutzii*. Detailed morphological studies of additional specimens from Vale do Ribeira, consisting of adults linked to the immatures, and specimens from Nova Friburgo, Rio de Janeiro corroborated that population may belong to *An. lutzii*, but distinct from Dourado.

Following specimens from Dourado were compared to specimens from Foz do Iguaçu, the type locality of *An. guarani*. SEM eggs from Dourado (Forattini, 1998) were found to be identical to those from Foz do Iguaçu. Consequently, specimens from Dourado were identified as *An. guarani*, which were resurrected from synonymy with *An. lutzii*.

Specimens of *An. lutzii* and *An. guarani* were contrasted with specimens of *An. lutzii* deposited in the FSP collection. These specimens were employed by Galvão (1941) in the revision of the Myzorhynchella section. Additionally, in the same collections, there were deposited one male genitalia in a microscope slide, one adult female and one microscope slide with the larval exuviae collected by Lane in Represa de Santo Amaro (current Represa de Guarapiranga). It is interesting that these specimens in addition to specimens from Vila Ema, São Paulo may represent topotypes of *An. niger*. By comparing the male genitalia characteristics of *An. lutzii*, *An. guarani* with that specimen from Represa de Santo Amaro, it is evident that they belong to distinct species. Similarly to *An. guarani*, *An. niger* is also resurrected from the synonymy with *An. lutzii*.

Anopheles lutzii, *An. guarani* and *An. niger* can be recognized as species of the Myzorhynchella section in the adult stage by having the abdominal segments I-VIII without scales, these are present only in the genitalia; in the male genitalia, by possessing the dorsal clapette supporting two apical and one subapical setae, the aedeagus with subapical leaflets; in the fourth instar larvae in having abdominal setae 6-IV-VI branched (Galvão 1941)

A character largely used to separate species of the Myzorhynchella section is the pattern of pale and dark scales in R_{4+5} vein (Galvão 1941, Lane 1953, Forattini

1962, 2002, Gorham 1967). However, comparisons carried out in the study, showed that this character can be ambiguous and lead to misidentification specially because some species showed polymorphism (Galvão 1941). This was observed in the new species of the Myzorhynchella section described by Nagaki et al. (unpublished).

Anopheles lutzii, An. niger and *An. guarani* cannot be distinguished by adult female characteristics, considering the polymorphism in R_{4+5} vein. However, the fourth instar larva of *An. lutzii* can be easily distinguished from that of *An. guarani* and *An. niger* by possessing the spiracular apparatus broad, abdominal seta 1 palmate, well developed in segments II-VII, whereas in *An. niger* and *An. guarani* the spiracular apparatus is narrow (Fig. 4B), and seta 1-III-V possess hyalines branches (Fig. 4C). *An. niger* and *An. guarani* cannot be distinguished based on morphological characteristics of the larvae.

In male genitalia, *An. lutzii* can be distinguished from *An. guarani* and *An. niger* by possessing the leaflets of aedeagus arising from the aedeagus forming a 45º angle (Fig. 1B), while in *An. guarani* it is curved mesally, parallel to the longitudinal axis, strongly serrated along dorsal and lateral surfaces (Fig. 3B), and in *An. niger* it is straight forming a 25º angle (Fig. 6B). Beyond that, *An. lutzii* ventromesal subtriangular projection is positioned approximately at apex length of leaflets apex, in *An. guarani* it is positioned approximately at middle length of insertion and apex of leaflets, similar to *An. niger*. *Anopheles niger* also present a patch of spicules at basomedial proctiger, characteristic not found in *An. lutzii* neither *An. guarani*. Ramos (1937) described a similar genitalia of specimen from Cotia, São Paulo state.

Nagaki & Sallum (unpublished) employed sequences of the ITS2 rDNA and COI mtDNA to examine phylogenetic relationships within the Myzorhynchella

section. As result, sequences generated for *An. lutzii* and *An. guarani* were found to cluster together in strongly supported monophyletic groups. Unfortunately, there was no specimen of *An. niger* available to sequence. However, we believe that the morphological evidences are strong and support that *An. niger* is a valid species. Further studies including specimens of *An. niger* will be necessary to validate the hypothesis.

Finally, the Myzorhynchella section current is comprised of the following species: *An. lutzii*, *An. niger*, *An. guarani*, *An. nigritarsis*, *An. antunesi*, *An. antunesi* Form 1 Nagaki & Sallum and *An. parvus*. It is possible that undescribed new species occur in other localities in Brazil.

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4.3. MANUSCRITO 3

"Molecular phylogeny of species of the Myzorhynchella Section of the *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae)"

(em preparação)

Molecular phylogeny of species of the Myzorhynchella Section of the *Anopheles* (*Nyssorhynchus*) (Diptera: Culicidae)

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Running title: Phylogeny of the Myzorhynchella section

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Summary

Phylogenetic relationships among species of the Myzorhynchella section of the Neotropical subgenus *Nyssorhynchus* of *Anopheles* were reconstructed using partial sequences of the mitochondrial cytochrome oxidase I (COI) gene. Phylogenetic analyses were carried out using maximum parsimony, maximum likelihood, and Bayesian methods. The topologies generated for the COI data set supported the monophyly of the Myzorhynchella section, a sister relationship of *An. parvus* and *An. guarani*, *An. antunesi* and *An. lutzii*, and a fifth species in the section which is sympatric to *An. antunesi*, *An. antunesi* Form 1. Results of the ITS2 sequence analysis corroborate the presence of five distinct species groups within the Myzorhynchella.

Key words: ITS2 - COI - species complex – parsimony - likelihood - Bayesian

Introduction

Anopheles Meigen subgenus *Nyssorhynchus* Blanchard includes 33 species (Harbach, 2004). The subgenus is divided into three sections based on morphological characters: Argyritarsis, Albimanus and Myzorhynchella. The latter comprises four nominal species, *An. lutzii* Cruz, *An. parvus* (Chagas), *An. nigritarsis* (Chagas) and *An. antunesi* Galvão & Amaral (Galvão, 1941; Harbach, 2004). Species of this section were defined based on morphological characters, and there is no register on molecular data for any of them. Nagaki et al. (unpublished) described a new species of the Myzorhynchella section from Serra da Mantiqueira. Additionally, Nagaki &

Sallum (unpublished) elevated *An. niger* and *An. guarani* from the synonymy with *An. lutzii*.

In this study, we analyzed sequence data from the rDNA second internal transcribed spacer 2 (ITS2) and a fragment of the cytochrome oxidase subunit I (COI) of the mitochondrial genome. The objectives of this study are: (1) to stablish phylogenetic relationships within the Myzorhynchella, (2) to examine the monophyletism of *An. antunesi* and *An. antunesi* Form 1, and (3) to examine if *An. guarani* is a valid species. The specimens utilized to generate sequences were collected in several localities in the states of Paraná, São Paulo, Rio de Janeiro and Minas Gerais (Brazil), including the type localities of *An. guarani* and *An. antunesi*.

Materials and Methods

Mosquito collection

The species sampled for this study and the sources of specimens are listed in Table 1. Larvae and pupae were either collected from field habitats or obtained from link-reared offspring (egg, larvae, pupae and adults) of blood fed females collected in the field. Immatures collected in the field were maintained in the laboratory to obtain adult males and females associated with larval and pupal exuviae. Freshly emerged mosquito were quickly anesthesiated with ethyl acetate vapors, and kept either separate in minute plastic vials in silica gel or individually frozen at –80°C. Species identification of each specimen was based on either adult male genitalia or fourth-instar larval characteristics.

Table 1. Specimens codes, molecular markers, localities, geographical coordinates and species employed in the study.

Specimens	Marker	Locality (State)	Coordinates	Species
PR28(5)-1	COI	Guaíra (PR)	24º16'17,4"S 54º17'26,0"W	*An. parvus*
PR28(18)-1	COI/ITS2			
PR28(65)-6	COI/ITS2			
MG07(9)-20	COI	Frutal (MG)	20º01'31,0"S 49º04'35,4"W	*An. parvus*
RJ03(6)	COI/ITS2	Itatiaia (RJ)	22º24'58,7"S 44º37'19,7"W	*An. antunesi*
RJ03(11)	COI/ITS2			
RJ03(12)	COI/ITS2			
RJ03(13)	COI/ITS2			
PR29	COI/ITS2	Foz do Iguaçu (PR)	25º28'50"S 54º35'12"W	*An. guarani*
PR29(8)	COI/ITS2			
PR29(9)-6	COI/ITS2			
SP02(9)-2	COI/ITS2	Pariquera-Açu (SP)	24º44.975'S 47º56.944'W	*An. lutzii*
SP02(10)-5	COI/ITS2			
SP02(11)-9	COI/ITS2			
SP02(12)-1	COI/ITS2			
SP02(13)-3	COI/ITS2			
SP02(14)-6	COI/ITS2			
SP02(15)-5	COI/ITS2			
VP11a	COI/ITS2	Pindamonhangaba (SP)	22º45'31,7"S 45º30'55,8"W	*An. antunesi*
VP11d	COI/ITS2			
VP11c	COI			
VP11b	COI/ITS2	Pindamonhangaba (SP)	22º45'31,7"S 45º30'55,8"W	*An. antunesi*
VP19-17	COI/ITS2			Forma 1
SP50a	COI/ITS2	Pindamonhangaba (SP)	22º45'30,5"S 5º30'55,0"W	*An. antunesi*
SP50b	COI/ITS2			Forma 1
SP53-5	COI/ITS2			
SP51-100	COI/ITS2			
SP55(2)	COI/ITS2			
SP55(4)	COI/ITS2			
SP53-100	COI/ITS2			
SP53-101	COI/ITS2			
SP53-4	COI/ITS2			
E-11803	ITS2	Dourado (SP)	22º05'00"S 48º26'33"W	*An. guarani*
E-12370	ITS2	Campos do Jordão (SP)	22º45'50"S 45º30'87"W	*An. antunesi*

DNA Sequences

DNA was extracted following the animal tissue DNA extraction protocol provided by the QIAgen DNeasy® Blood and Tissue Kit (QIAgen Ltd., Crawley, UK). For two specimens, only the abdomen was used. The extraction protocol for this specimen was the same used for fresh specimens except that the DNA was eluted in 50 µl of buffer AE. Since the chance of cross contamination is high, DNA was extracted in a separate room in a flow microbiological safety cabinet.

ITS2 amplification was carried out using the 5.8SF (5' - ATC ACT CGG CTC GTG GAT CG - 3') and 28SR (5' - ATG CTT AAA TTT AGG GGG TAG TC - 3') primers (Djadid et al. 2007). PCR products were amplified in 25 µl reaction mix containing: 10 mM Tris-HCl, pH 8.3; 50 mM KCl; 1.5 mM $MgCl_2$; 2.5 µl DMSO; 5 picomoles of each primer; 200 µM each dNTPs; and 2.5 U New England Biolabs® *Taq* polymerase. One µl of the first elution was used as DNA template in the PCR reactions. PCR protocol consisted of a 2-min denaturation at 94°C, 34 cycles at 94°C, 57°C and 72°C for 30 sec each, followed by a 10 min extension at 72°C. PCR products were visualized in 1.5% TAE agarose gels stained with GelRed^{TM} (Biotium).

COI gene fragments were amplified using LCO1490: 5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3' and HCO2198: 5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3' primers (Folmer et al., 1994). PCR was carried out in a 25 µl reaction mix containing 1 µl of DNA of the second elution, 2.5µl 10x PCR buffer (New England BioLabs® Inc), 200 µM each dNTPs, 5 pmol of each primer; 2.5 U of *Taq* polymerase (New England BioLabs® Inc). PCR amplification protocol consisted of 2 min denaturation at 95ºC followed by 35 cycles at 94ºC for, 55ºC and 72ºC for 1

mim each, followed by a final extension at 72ºC for 7 min. PCR products were electrophoreses in 1.5% TAE agarose gels stained with GelRed™ (Biotium).

Sequencing reactions were carried out in both directions using the PCR primers and the Big Dye Terminator Kit v.3.1 (PE Applied Biosystems, Warrington, England). Sequences were analyzed on an ABI Prism 3130 (Applied Biosystems/Hitachi, Foster City, CA, U.S.A.).

Sequence Analysis

Sequences were edited using Sequencer version 4.9 for Windows (Gene Code Corporation, Ann Arbor, USA), aligned in CLUSTAL X 1.6 (Thompson et al. 1997) and optimized manually in MacClade, version 4.3 (Maddison & Maddison, 2000).

Sequence similarity of the ITS2 sequences generated in this study with that previously available in GenBank was assessed using BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). Intraspecific sequence differentiation was assessed using the mean uncorrected P distance in PAUP (Swofford 2003). Accuracy of nucleotide sequence alignments of COI was examined using amino acid sequence alignment, including sequences from the outgroups, *An. strodei* and *An. nuneztovari.*

Template DNA from this study is retained at -70°C in the Faculdade de Saúde Pública (FSP-USP), São Paulo, Brazil, for future reference.

Phylogenetic analysis

Unweighted parsimony analyses for COI mtDNA were performed using PAUP 4.0b10 (Swofford 2004) by using a heuristic search with tree bisection

reconnection (TBR) branch-swapping and 1000 random taxon additions. Parsimony bootstrapping (Felsenstein 1985) used 1000 pseudoreplicates, with 10 random taxon addition replicates per pseudo-replicate. Parsimony-uninformative characters were excluded from all the analyses.

Maximum likelihood (ML) analyses were performed using PAUP 4.0b10 (Swofford 2004). Optimal evolutionary models were determined for COI gene using the Akaike Information Criterion (AIC) (Posada and Crandall, 1998) in Modeltest (Posada and Crandall, 1998). Support for each clade generated from data sets was assessed by 100 bootstrap replicates. The program MrBayes (Huelsenbeck and Ronquist, 2001) was employed for the Bayesian analyses under the model selected by Modeltest in the Akaike Information Criterion (AIC). For COI, two separate MCMC runs were made, each with four chains in the Metropolis-coupled MCMC. Runs were done for 6,000,000 generations, sampling every 100. A burn-in of 1,000,000 of the samples was used.COI and ITS2 sequence of *An. strodei* (Sallum et al. unpublished) and *An. nuneztovari* (Accession AF368094) were used as outgroups in the phylogenetic analyses.

Results

Internal transcribed spacer 2 sequences

The ITS2 sequences of the 31 specimens showed considerable heterogeneity in size among species of the Myzorhynchella section (Table 2). It varied in length from 448 base pairs (bp) in *An. guarani* to 480 bp in *An. lutzii.* Sequences generated from distinct individuals of a species share 100% similarity, no intraspecific

variation was observed. The CG contents of the ITS2 varied from 57% in *An. parvus* to 64% in *An. guarani* (Table 2).

A FASTA search using the algorithm 'Database: nucleotide collection - Optimize for: Somewhat Similar' revealed that the ITS2 sequences of all specimens with the exception of those from *An. parvus*, shares higher similarity with *An. pictipennis* Phillip (Accession EU433947.1). Sequence similarities obtained in the Blast search are in Table 3.

Table 2. ITS2 length (base pairs) and percentage of GC content for five unique sequences generated for five species of the Myzorhynchella Section of *Anopheles* (*Nyssorhynchus*).

Species	Length (bp)	CG (%)
An. antunesi	472	62
An. antunesi Form1	470	60
An. lutzii	480	59
An. guarani	448	64
An. parvus	463	57

Table 3. ITS2 sequences similarity and query coverage of unique sequences generated for five species of the Myzorhynchella section using the Blast search. Sequence comparisons were performed with that of *An. pictipennis* (GenBank EU433947)

Species	Similarity	Query coverage
An. antunesi	77%	84%
An. antunesi Form 1	81%	81%
An. lutzii	80%	80%
An. guarani	87%	22%
An. parvus	-	-

Cytochrome oxidase subunit I

The COI sequences of the 32 individuals of five species of the Myzorhynchella section consist of 658 base pairs (bp). The sequences revealed 12 unique sequences. The overall base composition was (range in parentheses) A, 28.9% (28.1-29.6%); C, 16.1% (14.9-17.9%); G, 16.3% (15.8-16.8%); T, 38.6% (37.5-39.7%). The number of constant, variable and parsimony informative sites is listed in Table 4.

Phylogenetic analysis

Results of the parsimony analyses performed for 32 COI sequences of five species of the Myzorhynchella section supported both monophyly of the section and of the five species included in the study. However, the basal resolution was recovered as a polytomy, and thus relationships among members of the section remains unresolved. *Anopheles lutzii*, *An. antunesi* and *An. guarani* clustered together in a poorly supported group (56% bootstrap value), with *An. guarani* as sister to the clade formed by *An. antunesi* and *An. lutzii*. *Anopheles antunesi* and *An. lutzii* are sisters; however the split leading to these species is moderately supported (81% bootstrap value). It is interesting that within the clade leading to *An. antunesi*, there are two strongly supported groups. One group consisting of two individuals from Campos do Jordão (100% boostrap value), and the second group formed by four individuals from Itatiaia and two from Campos do Jordão (95% boostrap value). Monophyly of *An. parvus* and *An. antunesi* Form 1 is strongly supported (100% bootstrap value); interesting, within the *An. parvus* clade, the COI sequences from Paraná state clustered together in a strongly supported group sister to the specimen

from Minas Gerais. Basal relationships among the five species are not resolved (Fig. 1).

Table 4. Partitions, number of constant, variable sites and number of parsimony informative sites in each partition of 32 COI sequences of five species of the Myzorhynchella section.

Partition	Sites	Constant	Variable	Parsimony informative
Pos1	219	199	20	18
Pos2	219	219	0	0
Pos3	220	86	134	127

Maximum likelihood and Bayesian analyses were performed for COI data set under the GTR plus G model selected in Modeltest 3.7 (Posada & Crandall 1998). Maximum likelihood (ML) topology is shown in Figure 2, and the Bayesian topology is in Figure 3. ML and Bayesian topologies are identical and similar to the MP strict consensus topology, except for disagreement in the phylogenetic relationship of *An. parvus* and *An. antunesi* Form 1 that were recovered either as sister species in the Bayesian analyses (Fig. 3) or as separate groups within a polytomy in MP (Fig. 1) and ML (Fig. 2) analyses.

The Bayesian topology (Fig. 3) showed five strongly supported clades. Furthermore, sequences generated from distinct individuals of the same species clustered together. The posterior probability for the splits leading to each species was high. Additionally, *An. lutzii* was recovered as sister to An. *antunesi*, and the clade *An. lutzii* plus *An. antunesi* was sister to *An. guarani*. *Anopheles parvus* clustered with *An. antunesi* Form 1, and this group was the sister group of a clade consisting of

An. lutzii, *An. antunesi* and *An. guarani*. The monophyly of *An. parvus* is strongly supported; however, one individual from Minas Gerais was recovered as sister to the lineage leading to individuals from Paraná state. Similarly, within the clade formed by *An. antunesi*, sequences of two individuals formed a strongly supported group that is sister to the remaining individuals of *An. antunesi.*

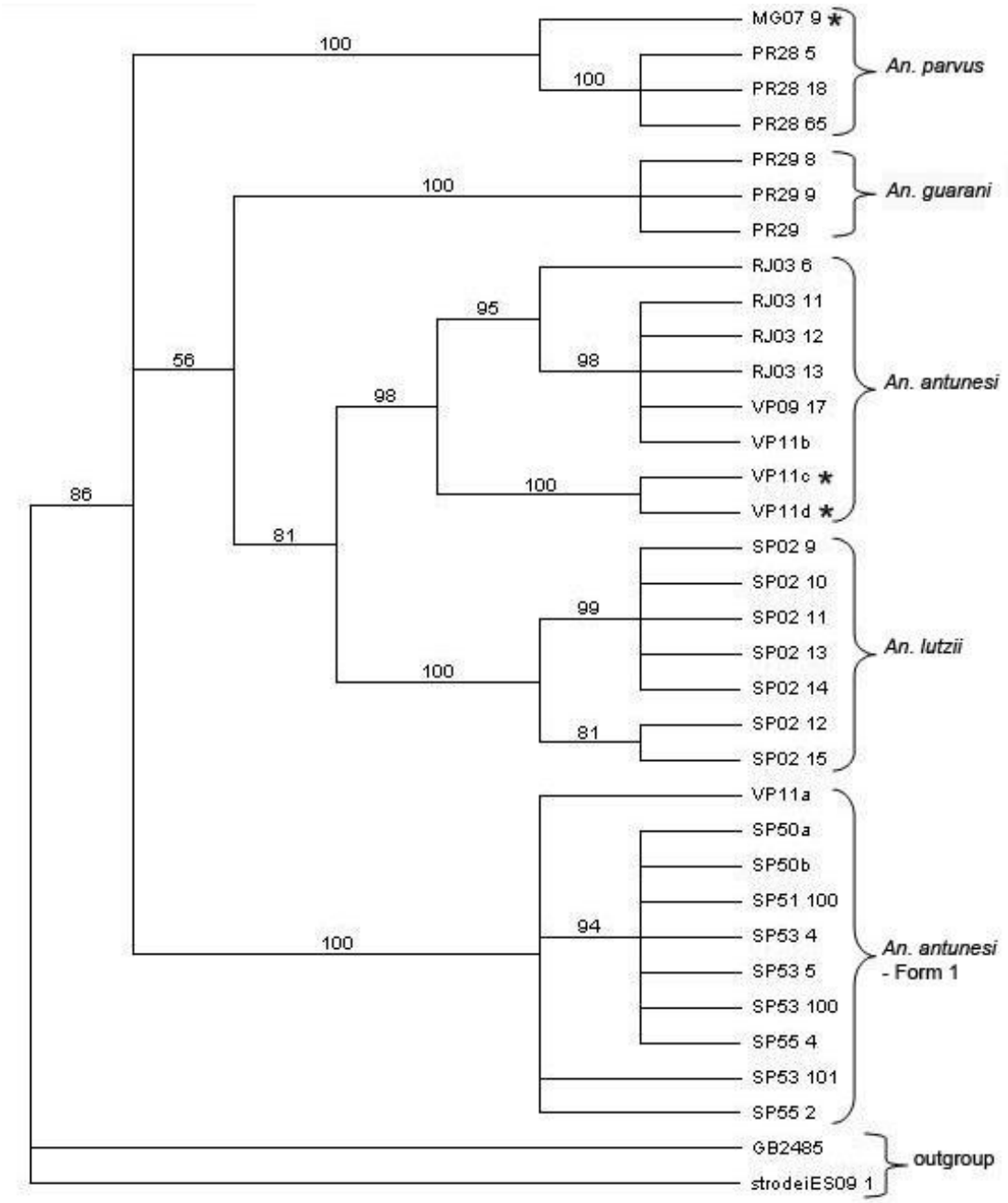

Fig. 1. Strict consensus topology of eight equally parsimonious topologies generated in the maximum parsimony analysis for COI mtDNA for 32 individuals of five species of the Myzorhynchella section. *Anopheles* (*Nyssorhynchus*) *strodei* (strodeiES09_1) and *An*. (*Nyssorhynchus*) *nuneztovari* (GB2485). Characters were equally weighted; parsimony informative sites were excluded from the analyses. Numbers above the branches represent maximum parsimony bootstrap support values for the split. L= 285.

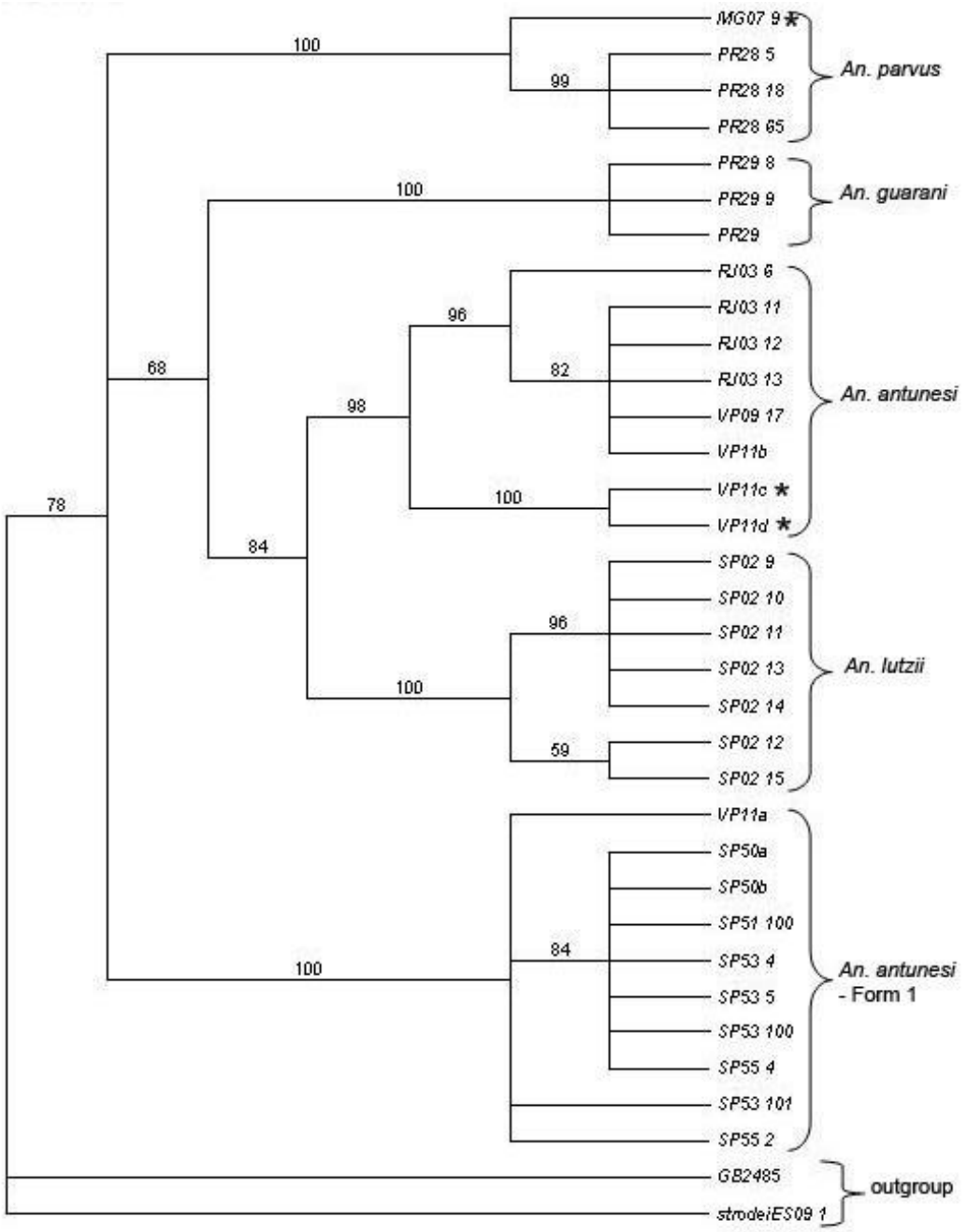

Fig. 2. Fifty percent majority rule consensus topology generated in the maximum likelihood analyses under the GTR+G model for COI mtDNA for 32 individuals of five species of the Myzorhynchella section. *Anopheles* (*Nyssorhynchus*) *strodei* (strodeiES09_1) and *An*. (*Nyssorhynchus*) *nuneztovari* (GB2485) are the outgroups. The GTR+G model was selected in ModelTest 3.7 using AIC. Numbers above the branches are likelihood bootstrap values (100 replicate; multrees = no).

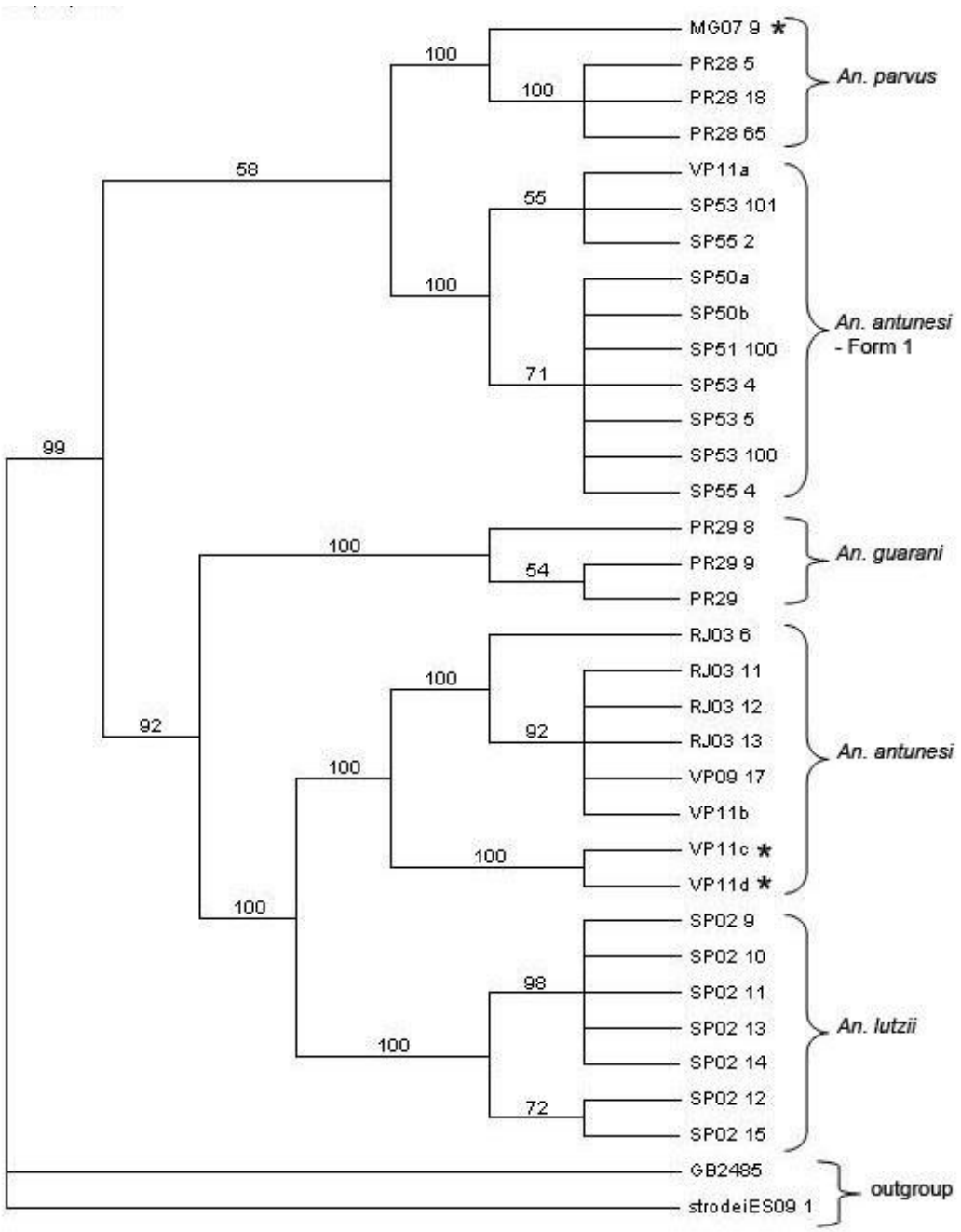

Fig. 3. Bayesian topology generated under the GTR+G model suggested by Modeltest 3.7, using the AIC (Posada & Crandall 1998), for COI mtDNA for 32 individuals of five species of the Myzorhynchella section. *Anopheles* (*Nyssorhynchus*) *strodei* (strodeiES09_1) and *An*. (*Nyssorhynchus*) *nuneztovari* (GB2485) are the outgroups. Numbers above the branches are posterior probability values.

Discussion

Results of MP, ML and Bayesian phylogenetic analyses for COI data set corroborated the monophyly of the Myzorhynchella Section, and the monophyly of *An. guarani*, *An. antunesi* Form1 and *An. lutzii*. The paraphyly within *An. parvus* and *An. antunesi* suggest that these taxa may comprise species complex, however, further studies will be necessary. Additionally, our results revealed that *An. antunesi*-Form 1 and *An. parvus* formed a poorly supported clade (0.58 posterior probability), *An. antunesi* + *An. guarani* + *An. lutzii* clustered together in a moderately supported monophyletic clade, (0.92 posterior probability). Bayesian phylogeny revealed two major clades within the Myzorhynchella. One clade onsisting of (*An. parvus*, *An antunesi*-Form 1), and a second formed by (*An. guarani* (*An. antunesi*, *An. lutzii*)). The maximum likelihood (ML) topology is similar to the Bayesian topology, except for the sister group relationship of *An. parvus* and *An. antunesi*-Form 1, which did not cluster together. In the ML topology, basal relationships among the two major groups within Myzorhynchella are unresolved, because they were recovered as a polytomy.

Although published molecular data is lacking for the Myzorhynchella Section, Sallum et al. (2000) and Harbach and Kitching (2005) morphological analyses of Anophelinae also recovers Myzorhynchella species (*An. lutzii* and *An parvus*) as a sister group to all other *Nyssorhynchus* species. Our analysis strongly supports *An. lutzii*-*An. antunesi* as a natural grouping, and paraphyly in the *An. antunesi* and *An. parvus* species identifies them as a candidate species complex.

In conclusion, results of COI phylogeny within the Myzorhynchella section show a need for further studies to resolve discordance in the phylogenetic placement

of *An. antunesi* Form 1 that was recovered either as sister of *An. parvus* (Fig. 3) or within a polytomy leading to two major clades (Figs. 1, 2). Bayesian topology showed better supported basal resolution, however the sister group relationship between *An. parvus* and *An. antunesi* is poorly supported. Finally, the hypotheses of the presence of species complex in *An. antunesi* and *An. parvus* need to be investigating using a broader sample size and sequences data of other gene.

Hemmerter et al. (2009) demonstrated that COI sequence date for *Culex* mosquitoes from Australasian region may overestimate species diversity. Consequently, COI should be applied cautiously with the support from a nuclear gene. For species of the Myzorhynchella, species diversity revealed by the COI sequence data is corroborated by the ITS2 data from the same individuals and when possible by morphological characteristics of the male genitalia and fourth instar larvae. However, it was not possible to obtain specimens male associated with female and immatures. Species of the Myzorhynchella are rare, the Mata Atlantica bioma has been impacted by human activities that has caused high water pollution and forest devastation causing an extra difficult to collect species of the section.

Finally, we believe that the Myzorhynchella section is more diverse than it was suppose to be. Collecting in diverse localities in Mata Atlantica and areas of the cerrado, where any species has been reported will be of great importance to evaluate the speciosity of the group.

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5. CONSIDERAÇÕES FINAIS

Os resultados obtidos através das análises morfológicas e moleculares com espécies da Seção Myzorhynchella permitem concluir que existem mais de quatro espécies até então denominadas dentro da seção.

Com a caracterização molecular e morfológica, pode-se distinguir *An. antunesi* de outra espécie muito similar e que vive em simpatria com *An. antunesi*. Essa espécie, até o momento, desconhecida pelos entomologistas foi caracterizada e será descrita.

An. lutzii foi redescrita a partir de espécimes do Vale do Ribeira, estado de São Paulo. Como não foi possível obter espécimes na localidade tipo, optou-se por redescrever *An. lutzii* a partir de exemplares coletados no Vale do Ribeira. Os síntipos que estão depositados na Coleção Entomológica do Instituto Oswaldo Cruz – FIOCRUZ foram comparados com indivíduos do Vale do Ribeira. Paralelamente, foram validadas as espécies *An. niger* e *An. guarani*, retirando-as da sinonímia de *An. lutzii*. Assim *An. guarani* também teve a resdescrição feita através de larva, pupa, adultos macho e fêmea, genitália masculina e ovos e *An. niger* a caracterização da genitália masculina.

Os resultados obtidos com os marcadores moleculares ITS2 e COI corroboraram com a hipótese levantada com os dados morfológicos. Nesse sentido os resultados das análises filogenéticas demonstraram a presença de cinco grupos monofiléticos dentro da Seção. Não existem trabalhos moleculares com espécies da Seção Myzorhynchella, portanto espera-se que trabalhos futuros sejam beneficiados com o acesso às sequências que estarão depositadas no banco de genes de domínio público Genbank.

Este trabalho demonstra o quão pouco se conhece sobre a biologia e sistemática das espécies da Seção Myzorhynchella e seria de grande importância coletar nas localidades tipo na tentativa de obter topótipos que poderiam auxiliar nas decisões taxonômicas e de nomenclatura.

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